MATERIALS AND METHODS
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Two dicotyledonous plants are the materials for the present investigation. They are *Ludwigia prostrata* Roxb. (Onagraceae) and *Hibiscus vitifolius* L. (Malvaceae). In *L. prostrata* root, stem, leaf and seed, and in *H. vitifolius* the flower petals are used. The materials were collected from Mukkombu, Tiruchirappalli District, Tamilnadu, India as and when required.

Identification of the plants

The plant materials were identified with the flora of the study area (Gamble, 1921) and verified with the specimens available in the Rapinat Herbarium, St. Joseph's College, Tiruchirappalli, Tamilnadu, India.

Herbarium preparation

Herbarium specimens were prepared for the study materials and deposited in the Sri Pushpam Herbarium of A.V.V.M. Sri Pushpam College, Poondi, Thanjavur District, Tamilnadu, India.

Photography

The study materials were photographed in the habitat and in the laboratory with Konica colour negative film with Yashika FX3 SLR camera. Photographs were prepared with Konica colour papers.
Systematic Position of *Ludwigia prostrata* Roxb. (Bentham and Hooker, 1862-1883)

- **Class**: Dicotyledon
- **Sub class**: Polypetalae
- **Series**: Calyciflorae
- **Order**: Myrtales
- **Family**: Onagraceae

Systematic Position of *Hibiscus vitifolius* L. (Bentham & Hooker, 1862-1883)

- **Class**: Dicotyledon
- **Sub class**: Polypetalae
- **Series**: Thalamiflorae
- **Order**: Malvales
- **Family**: Malvaceae

METHODS

i. MORPHOLOGICAL STUDIES

Morphological characters of various parts of *L. prostrata* and *H. vitifolius* were studied using fresh plants with the help of binocular dissection microscope.

ii. ECOLOGY OF THE PLANT

Ecological parameters such as rain fall, temperature, soil type, and water quality were studied.

iii. PHARMACOGNOSTICAL STUDIES

Fresh plants, *L. prostrata* and *H. vitifolius* were collected from the habitat and free hand sections were taken. Good sections were selected, stained with
safranin and mounted with glycerine for observation. Microphotographs were taken. Materials were preserved in FAA and stored for the future use.

Epidermal peelings were taken using scalpel and mounted in glycerine and temporary slides were prepared for the observation of stomata and epidermal hairs. Microphotographs were taken with Practica camera using Konica colour negative films. Photographs were prepared with Konica colour printing paper.

iv. PHYTOCHEMICAL STUDIES

From the collected *L. prostrata* material, the root, stem, leaf and seed were separated. They were shade dried and powdered using mortar and pestle. The powdered materials were extracted with methanol with a soxhlet apparatus. The root, stem, leaf and seed extracts were preserved in air-tight glass bottles and kept in refrigeration for future phytochemical analyses and identification of their chemical constituents. The chemical constituents were identified by standard phytochemical methods (Harborne, 1973). From the shade dried petals of *H. vitifolius* flavonoid was isolated.

1. Test for alkaloids

A small portion of the solvent free extracts were stirred separately with a few drops of dil. hydrochloric acid and filtered. The filtrate may be tested carefully with various alkaloidal reagents. If alkaloid is present it will give yellow precipitate in Mayer's reagent and Hager's reagent, orange precipitate in Dragendorff's reagent and reddish brown precipitate in Wagner's reagent.
2. Test for carbohydrates and glycosides

A small quantity of extracts were dissolved separately in 5 ml of distilled water and filtered. The filtrate was subjected to Molisch's test to detect the presence of carbohydrates.

1. Molish's Test

The filtrate was treated with 2-3 drops of 1% alcoholic alpha napthol solution and 2 ml of concentrated sulphuric acid was added along the sides of the test tube. Appearance of brown ring at the junction of two liquids shows the presence of carbohydrates.

Another portion of the extract was hydrolysed with hydrochloric acid for few hours on a water bath and the hydrolysate was subjected to Legal's and Borntrager's tests to detect the presence of different glycosides.

a. Legal's Test

To the hydrolysate 1 ml of sodium nitroprusside solution was added and then it was made alkaline with sodium hydroxide solution. Appearance of pink to red colour shows the presence of glycosides and aglycones.

b. Borntrager's Test

Hydrolysate was treated with chloroform and the chloroform layer was separated. To this equal quantity of dilute ammonia solution was added. Ammoniacal layer which acquires rose pink colour shows the presence of glycosides.
3. Test of fixed oils

Small quantity of the extracts were separately pressed between two filter papers. Appearance of oil stain on the paper indicates the presence of fixed oils.

4. Separation and detection of fatty acids

Leaf, stem, root and seed were powdered and homogenized using pestle and mortar. These homogenates were loaded on Whatmann No.1 chromatographic paper in 4 different regions respectively. The chromatographic run on Whatmann No.1 paper were sprayed with bromothymol blue (pH range 6.0-7.2). The spots of leaf and stem developed yellow colour while root and seed did not show colour and development. The development of yellow colour confirms the presence of fatty acids (Mahadevan, 1982).

5. Test for saponins

The extracts were diluted with 20 ml of distilled water and it was agitated on a graduated cylinder for 15 minutes. The formation of 1 cm layer foam shows the presence of saponins.

6. Test for tannins and phenolic compounds

Small quantities of extracts were taken separately in water and tested for the presence of phenolic compounds and tannins. It will give violet colour in dilute ferric chloride solution (5%), white precipitate in 1% sol. of gelatin containing 10% NaCl and 10% lead acetate solution.
7. Test for proteins and free amino acids

Small quantities of extracts were dissolved in a few ml of water and treated with various reagents for identification. If protein and free amino acid are present it will give red colour in Millon's reagent, purple colour in ninhydrin reagent, pink or purple colour in Biuret test (equal volume of 5% NaOH solution and 1% copper sulphate solution).

8. Test for terpenoid

a. 50 per cent acetone extracts of root, stem, leaf and seed of L. prostrata were prepared separately. One drop of the extract was placed on the TLC plate and conc. H₂SO₄ was sprayed. The plate was heated at 100°C for ten minutes. Brown or purple colour appears if terpenoid is present in the extract.

b. When 0.05 per cent fluorescein in water is sprayed on TLC plate and exposed to bromide vapour yellow spots appear on red background if terpenoid is present in the extract.

9. Test for flavonoids

If the petal extract of *H. vitifolius* with aqueous sodium hydroxide solution, appearance of blue to violet colour indicates the presence of anthocyanins, yellow colour flavones and yellow to orange colour flavonones.

If the extract is treated with conc. sulphuric acid the appearance of yellowish orange colour indicates the presence of anthocyanins, yellow to orange colour flavones and orange to crimson colour flavonones.
Shinoda's test: The extract is dissolved in alcohol, to that piece of magnesium followed by conc. hydrochloric acid dropwise are added and heated. Appearance of majenta colour shows the presence of flavonoids.

10. Extraction of flavonoid

Freshly collected and shade dried petals (750 g.) of *H. vitifolius* were extracted with 80% ethanol (4 x 500 ml) under reflux. The alcoholic extract was concentrated in vacuo. The aq. concentrate was fractionated successively with petroleum ether (60-80°C) (4 x 250 ml), peroxide free ether (3 x 500 ml) and ethyl acetate (3 x 500 ml). The petroleum and ether fractions did not yield any crystalline solid.

The residue from ethyl acetate fraction was taken up in a small quantity of acetone and left in an ice-chest for a couple of days. The yellow solid that separated was filtered and recrystallised from aq. methanol and pale yellow flakes were obtained. It was freely soluble in ethyl acetate and methanol, but sparingly in water and insoluble in ether and chloroform. It gave a magenta colour with Mg-HCl; greenish-brown colour with alc Fe³⁺; intense yellow colour with sodium hydroxide, appeared yellow under UV and turned bright fluorescent yellow when fumed with ammonia.

v. PHARMACOLOGICAL STUDIES

The extracts of *L. prostrata* (root, stem, leaf and seed) were evaporated to dryness and dissolved in phosphate buffer (pH 7.4) and used for the pharmacological...
studies. In the same way the flavonoid extracted from the petals of _H. vitifolius_ was also dissolved in phosphate buffer and used for the pharmacological studies.

Swiss albino mice having body weight ranging between 20 and 24 g were selected for the present investigation. The animals were fed with standard laboratory diet and water _ad libitum_. Dalton's Ascitic Lymphoma (DAL) cell line was obtained from Cancer Research Institute, Adayar, Chennai and was maintained by weekly transplantation of $10^6$ cells/mouse, intraperitoneally.

1. **Effect of plant extracts on survival time**

Animals were inoculated (i.p.) with $2 \times 10^5$ cells/mouse on day 0, and treatment with plants extracts started 24 h after inoculation at a dose of 50 mg/kg/day i.p. (Group I), 100 mg/kg/day i.p. (Group II). The control (Group III) was treated with same volume of 0.9% sodium chloride. All the treatments were carried out for nine days. The group IV animals were treated with standard drug 5-fluorouracil (5FU) (20 mg/kg/day i.p. for 9 days) and median survival time (MST) noted for each group. The animals surviving more than 60 days were considered to be cured. Survival time of treated groups were compared with those of group IV (treated with 5FU) using the following formulae (Sur and Ganguly, 1994; Umadevi _et al._, 1994).

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\text{Median Survival Time (MST)} = \frac{\text{Day of 1st death} + \text{Day of last death}}{2}
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\text{Increase in life span (ILS)} = \frac{T/C\%}{100} = \frac{\text{MST of treated group}}{\text{MST of control group}} \times 100
\]
2. **Haematological studies**

To measure the influence of plant extracts on the haematological status of DAL bearing mice, comparisons were made among five groups (n=5) of mice on the 14th day after inoculation. The groups are (1) tumour bearing mice treated with plant extracts (50 mg/kg i.p.) for 9 days, (2) tumour bearing mice treated with plant extracts (100 mg/kg i.p.), (3) control tumour bearing mice untreated, (4) tumour bearing mice treated with 5FU and (5) normal mice. Blood was drawn from the tail vein in the conventional way and the red blood cell count, white blood cell count, haemoglobin, protein and packed cellular volume were determined (Ramnik, 1985; Lowry, 1951). The average of 5 determinations was computed.

**a. Method for estimation of red blood cell count**

The tail of the animal was held in one hand and the pipette was held horizontally with the other hand in the drop of blood. The pipette was not allowed to touch the tail. The blood was drawn up to 0.5 mark and wiped off any excess blood from the tip. Then the pipette was filled to 101 mark with Hayem's red cell diluting fluid (0.5 g mercuric chloride, 5 g sodium sulphate and 1 g sodium chloride were dissolved in 200 ml of distilled water) and mixed thoroughly. The first few drops of dilute blood were blown out and few drops were allowed to place in the counting chamber of haemocytometer. Then a cover slip was placed over the chamber so that the fluid run under the slip, filled the chamber. The counting chamber was placed under the microscope at low power. The cells were counted in the four fields. The average
number of cells in each small square was counted. The total number of cells/mm³ was calculated.

b. **Method for estimation of white blood cell count**

The technique of taking the blood sample was the same as that for the red blood cells count. The blood has been drawn up to 0.5 mark, diluted with white cell diluting fluid (1% acetic acid, 1 ml glacial acetic acid was added to 99 ml distilled water) up to the mark 11 and mixed thoroughly. The first few drops were discarded and the fluid was placed in the counting chamber of haemocytometer. One coverslip was placed over the chamber and counting taken under the microscope at low power. Then the total count of WBC was calculated from number of cells/mm³ of the areas of counting chamber.

c. **Method for estimation of haemoglobin content of whole blood**

Blood sample was drawn from the tail as described previously into the pipette to the mark. The tip of the pipette was wiped off to avoid any excess of blood. Blood of the pipette was transferred to the rectangular cell containing a little amount of N/10 hydrochloric acid which is placed in the haemocytometer. If the color of the solution was high, N/10 HCl was added and mixed with stirrer until a good colour match was obtained. From the cuvette reading Hb in gm/100 ml of blood or its percentage was calculated.

d. **Determination of protein**

Blood was collected by cardiac puncture into the centrifuge tube. Three tubes were taken for each set of experiment blank, standard and experiment respec-
tively. To the experiment tube 0.2 ml of serum, 0.8 ml of water and ml of solution C was added. It was kept at room temperature for 10 minutes. Then 0.5 ml of Folin's reagent was added. Optical density was measured at 660 µm after 30 minutes. Same procedure was repeated for blank and standard by replacing the serum by BSA and water respectively.

**Solution A**

- Sodium carbonate - 20.0 g
- Sodium Hydroxide - 4.0 g
- Sodium potassium tartrate - 0.2 g
- Glass distilled water - 1000 ml

**Solution B**

0.5 % w/v of copper sulphate.

**Solution C**

50 ml of solution A was mixed with 1 ml of solution B immediately before use.

**Folin's reagent**

Commercial Folin and Ciocatten's phenol reagent is diluted with equal volume of water (1:1).

BSA - Bovine Serum Albumin.
\[ e. \] *Method for determination of differential count of blood cells*

The blood film was prepared by spreading a large drop of blood in a clean grease-free slide. The blood film was dried in air. The Leishman's stain was added on the dried blood film and evenly distributed over the entire slide. At the end of one minute, double the quantity of buffer (pH 6.8) was carefully added and mixed with the stain by means of a clean pipette. The film was allowed to stain for seven minutes and the excess stain was removed by washing with the distilled water. The film was dried in air. After the general examination of the blood film, the differential leucocytes were observed.

\[ f. \] *Method for determination of packed cell volume*

Packed cell volume is the amount of packed red blood cells, following centrifugation, expressed as a percentage of the total blood volume. When anticoagulated blood is centrifuged at a standard speed, erythrocytes, which are heavier than white cells, platelets and plasma, sediment at the bottom. This red cell column is called packed red cell volume. The blood is filled in a Wintrobe tube and is subjected to centrifugation for 30 minutes at 3000 rpm. Packed cell volume is noted directly from the wintrobe's tube.

vi. CYTOLOGICAL STUDIES

The cytological studies of cancer cells were made under the microscope by preparing the ascitic smear in microslides. Various changes of mature cancer cells and their mitotic figures were studied in the ascitic fluids of mice which were
collected from the control and drug treated mice. Colour microphotographs were taken at different magnifications using Nikon Optiphot Photomicroscope.

1. **Preparation of smear for ascitic fluid**

   The clean dried and grease free slides called smear slides and spreader slide were taken. A drop of fluid was placed half an inch from one end of the smear slide. At an angle of 45° spreader was drawn back against a drop of ascitic fluid which ran across the end filling the angle between the two slides. Then the spreader slide was gently pushed forward with a quick speed and uniform to the other end of the slide. The smear was dried in air.

2. **Methods of Maygrunwald - Giemsa staining' (MGG)**

   Smear of DAL cell was prepared and fixed with methanol for half an hour and the Maygrunwald solution was mixed with PBS solution (pH 6.8) (1:1 concentration) was added to the slide and placed for five minutes. Then it was flooded with distilled water. Then the Giemsa stain was added to the slide (1:9 dilution with distilled water) and placed for 30 minutes. Then it was washed with distilled water and air dried. The slide was cleared with xylene and fixed with D.P.X. mountant.

**Preparation of Maygrunwald solution**

   Maygrunwald reagent

   (Stock solution kept for 2 weeks)

   Eosin methylene blue - 0.5 gm

   Absolute methanol - 100 ml
Working solution

Maygrunwald reagent - 100 ml

Absolute methanol - 50 ml

Preparation of Giemsa solution

Giemsa stain - 0.5 gm

Neutral glycerol - 33 ml

Methyl alcohol - 33 ml

Giemsa stain powder was dissolved in glycerol by heating 60°C for an hour and then methyl alcohol was added.

Buffer preparation: pH 6.8

0.8 % Sodium hydroxide (Solution A) - 23.7 ml

2.72% Potassium dihydrogen phosphate - 50.0 ml (Solution B)

Preparation of stock solution

23.7 ml of solution - A was mixed with 50 ml of solution - B.

Working Solution

10 ml of stock solution was mixed with 490 ml of distilled water.
vii. **STATISTICAL STUDIES**

S.N.K. multiple range test and one way Analysis of Variance for haematological parameters were done. One way Analysis of Variance has been applied to distinguish between the control and various treatment methods (including the 5-FU drug). To know the degree of effect of each treatment method S.N.K. multirange test was applied (Zar, J.H., 1984).