3. MATERIALS AND METHODS

For the present study, the medicinal plant *Aegle marmelos* belongs to family Rutaceae was collected from in and around area of Pattukkottai, Tamil Nadu, South India. The plant was identified with the help of flora of presidency, Tamil Nadu and Karnatic flora (Gamble, 1967 and Matthew, 1983) and standard references (Krtikar and Basu, 1935).

3.1 Preparation of leaf powder

The *Aegle marmelos* (Fig. 1) was collected washed, cut into small pieces and dried at room temperature (28±1°C) for two weeks and made into powder by using mixie for further analysis.

3.1.1 Preparation of plant extract

Extraction is a process to separate or isolate the secondary metabolites from plant material. It is basically two types i.e. heat and cold extraction. Heat extraction has some advantage over cold extraction like time consistency and also no contamination by microbes. An apparatus called soxhlet did heat extraction. 100g of the plant leaf powder were packed into the thimble of a soxhlet apparatus. The ratio of the plant powder and solvents were maintained at 1:4.

3.2 Pharmacognostic Evaluation (Wallis, 1985 & Khandelwal, 2002)

3.2.1 Morphological studies

In morphological studies organoleptic characters of the *Aegle marmelos* leaves like shape, size, colour, odour, taste, etc. were studied and details are reported.

3.2.2 Microscopic studies

**Transverse section**

The transverse section of leaf through midrib was prepared by standard method. The results of the study were reported along with microscopic photo.
3.3 Plant Powder analysis

Slides of powdered leaves were prepared by boiling pulverized powder of crude drug with chloral hydrate solution in small quantity. To a little quantity of powder taken onto a microscopic slide, 1–2 drops of 0.1% phloroglucinol solution and a drop of concentrated hydrochloric acid were added, mounted in dilute glycerin, covered with a cover slip and observed under microscope with 10 × 10 magnification. The characteristic structures observed for the powdered leaf was studied and reported.

3.3.1 Physico-Chemical Standardization of Leaves

The various Physico-chemical values of leaves such as ash values, extractive values and loss on drying were determined according to the Pharmacopoeial method.

3.3.2 Determination of individual extractive values (Cold extraction)

The air dried, powdered plant material was extracted with petroleum ether, chloroform, alcohol, acetone-water and water separately in a conical flask at a room temperature. The maximum cold extractive value was noted in ethanol extract.

3.3.3 Determination of individual extractive values (Hot extraction)

The air dried powdered plant material was extracted with Petroleum ether, chloroform, alcohol, acetone-water and water separately in a Soxhlet apparatus. The maximum hot extractive value was noted in ethanol extract.

3.3.4 Fluorescence Analysis (Woltering and Van Doon, 1988)

The air dried plant material was subjected to fluorescence analysis under ultra violet light and day light after giving treatment for 48 hours with various chemical and organic solvents like 1N Sodium hydroxide, Nitric acid, Sulphuric acid, Iodine, Conc. Hydrochloric acid, Ammonia, Ferric chloride, Glacial acetic acid, distilled water, Petroleum ether and Chloroform.
3.4 Determination of Ash Values (Indian Pharmacopoeia, 1996)

3.4.1 Determination of Total Ash Value

Accurately weighed (2 gm) of air-dried leaves powder of *Aegle marmelos* was taken in a silica dish and incinerated at temperature not exceeding 450°C until free from carbon. The resultant ash was cooled and weighed. The percentage of ash was calculated with reference to the air-dried drug.

3.4.2 Determination of Acid Insoluble Ash value

The total ash obtained from 2 gm of leaves powder was boiled for 5 minutes with 25 ml of dilute hydrochloric acid and the insoluble matter was collected on an ashless filter paper. It was washed with hot water, ignited and weighed. The percentage of acid insoluble ash was calculated with reference to the air-dried drug.

3.4.3 Determination of Water Soluble Ash value

The total ash obtained from another 2 gm of leaves powder was boiled for 5 minutes with 25 ml of water; the insoluble matter was collected on an ashless filter paper, washed with hot water, and ignited for 15 min at a temperature not exceeding 450°C. The weight of insoluble matter was subtracted from the weight of the ash, the difference in weight represent the water-soluble ash. The percentage of water soluble ash was calculated with reference to the air-dried drug.

3.4.4 Determination of Sulphated ash value

One gram of plant powder was ignited in an electric furnace until the drug gets charred. The crucible was cooled and the residue was moistened with 1ml of H$_2$SO$_4$, heated gently until the white fumes were no longer evolved and ignited at 800°C ± 25°C until all black particles disappear. The crucible was allowed to cool; few drop of H$_2$SO$_4$ was added and again heated. The ignition was carried as before, allowed to cool and then weighed. This was repeated until the sample reaches a constant weight.
3.5 Extractive Values (Indian Pharmacopoeia, 1996)

3.5.1 Water-soluble extractive value

Accurately weighed (5 gm) leaves powder of *Aegle marmelos* was added to 50ml of boiled water at 80°C in a conical flask. It was then shaken well and allowed to stand for 10 minutes so as to cool it and filtered. 5ml of filtrate was transferred to an evaporating dish, which was 7.5 cm in diameter, the solvent was evaporated on water bath, allowed to dry for 30 minutes, finally dried in an oven for 2 hours at 100°C and residue was weighed. Percentage of water-soluble extractive values was calculated with reference to the air-dried drug.

3.5.2 Acetone-Water (70:30) soluble extractive value

Accurately weighed (5 gm) leaves powder of *Aegle marmelos* was macerated with 100 ml of Acetone-Water (70:30) in a closed flask, shaking frequently during the first 6 hours and allowed to stand for 18 hours. Thereafter, it was filtered rapidly taking precaution against loss of Acetone-Water (70:30). Evaporated 25ml of filtrate to dryness in a tarred flat bottom shallow dish dried at 105°C and weighed. Percentage of Acetone-Water (70:30) soluble extractive was calculated with reference to the air-dried drug.

3.5.3 Alcohol soluble extractive value

Accurately weighed (5 gm) leaves powder of *Aegle marmelos* was macerated with 100 ml of methanol in a closed flask, shaking frequently during the first 6 hours and allowed to stand for 18 hours. Thereafter, it was filtered rapidly taking precaution against loss of Chloroform. Evaporated 25ml of filtrate to dryness in a tarred flat bottom shallow dish dried at 105°C and weighed. Percentage of chloroform soluble extractive was calculated with reference to the air-dried drug.

3.5.4 Chloroform soluble extractive value

Accurately weighed (5 gm) leaves powder of *Aegle marmelos* was macerated with 100 ml of chloroform in a closed flask, shaking frequently during the first 6 hours
and allowed to stand for 18 hours. Thereafter, it was filtered rapidly taking precaution against loss of Chloroform. Evaporated 25ml of filtrate to dryness in a tarred flat bottom shallow dish dried at 105°C and weighed. Percentage of chloroform soluble extractive was calculated with reference to the air-dried drug.

3.5.5 Petroleum ether (40-60°C) soluble extractive value

Accurately weighed (5 gm) leaves powder of Aegle marmelos was macerated with 100 ml of Petroleum ether in a closed flask, shaking frequently during the first 6 hours and allowed to stand for 18 hours. Thereafter, it was filtered rapidly taking precaution against loss of Petroleum ether. Evaporated 25ml of filtrate to dryness in a tarred flat bottom shallow dish dried at 105°C and weighed. Percentage of Petroleum ether soluble extractive was calculated with reference to the bark.

3.5.6 Loss on Drying (Indian Pharmacopoeia, 1996)

Accurately weighed (2 gm) quantity of leaf powder was taken in a tarred glass bottle and initial weight was taken. The sample was heated at 105°C in an oven and weighed. This procedure was repeated until a constant weight was obtained. The moisture content of the sample was calculated with reference to air-dried drug.

\[
\text{% of loss on drying at } 105^\circ\text{C} = \frac{\text{Loss in weight of sample}}{\text{Weight of the sample taken}} \times 100
\]

3.6 Phytochemical screening (Khandelwal, 2002)

The preliminary phytochemical evaluation of leaves was carried on extract prepared by successive extraction method in Soxhlet. The previously dried powdered leaves (50 gm) were extracted in a Soxhlet apparatus with ethanol and water successively. The resultant extracts were evaporated to dryness under vacuum. These extract were subjected to chemical test for different phytoconstituents viz. alkaloids, carbohydrates, phenolics, flavonoids, proteins, amino acids, saponins, mucilage and resins etc.
3.6 Qualitative Phytochemical Analysis

Chemical tests were carried out on the ethanol and aqueous extracts using procedures to identify the phytochemicals as described by Sofowara (1981), Trease and Evans (1989), Harborne (1973) and Kokate (1994). Alkaloids, carbohydrates, tannins and phenols, flavonoides, gums and mucilage, fixed oils and fats and saponins were qualitatively analyzed.

**Alkaloids**

The extracts were dissolved in dilute H₂SO₄ and filtered. The filtrate was treated with Mayer’s, Dragendorff’s, Hager’s and Wagner’s reagents separately. Appearance of cream, orange brown, yellow and reddish brown precipitates in response to the above reagents respectively indicate the presence of alkaloids.

**Carbohydrates**

Three hundred mg of 50% alcoholic extracts were dissolved in water and filtered. The filtrate was treated with concentration H₂SO₄ and then with Molisch’s reagent. Appearance of pink or violet colour indicates the presence of carbohydrates. The filtrate was boiled with Fehling’s and with Benedict solution. Formation of brick red precipitate in Fehling’s and Benedict’s solution is the positive result for reducing sugars and non-reducing sugars respectively.

**Tannins and phenols**

Small quantity of 50% alcoholic extract was dissolved in water and 5% ferric chloride solution or 1% Gelatin solution or 10% lead acetate solution was added. Appearance of blue colour with ferric chloride or precipitation with other reagent indicates the presence of tannins and phenols.

**Flavonoids**

The extract mixed with few ml of alcohol was heated with magnesium and then con. HCl was added under cooling. Appearance of pink colour indicates the
presence of flavonoids. The extract was treated with few ml of aqueous NaOH. Appearance of yellow colour and change to colourless appearance with HCl indicate the presence of flavonoids.

**Gum and mucilage**

About 10ml of the extract was slowly added to 25ml of absolute alcohol under constant stirring. Precipitation indicates the presence of gum and mucilage.

**Fixed oils and fats**

A drop of concentrated extract was pressed in between two filter papers and kept undisturbed. Oil stain on the paper indicates the presence of oils and fats.

**Saponins**

About 1ml of the extract was dissolved in 20ml of water and shook in a graduated cylinder for 15 minutes. Formations of one cm layer of foam indicate the presence of saponins.

**Phytosterol**

The extract was treated with Lieberman Burchard under suitable conditions. Appearance of blue-emerald green indicates the presence of phytosterol and terpenes.

### 3.7 Quantitative Analysis of *Aegle marmelos* leaves extract

Based upon the qualitative analysis the ethanolic and water extract was considered as a potential extract and taken for further phases of the study.

#### 3.7.1 Determination of total phenols

The amount of phenolic compounds in the extracts was determined by the Folin Ciocalteu colourimetric method (McDonald, 2001) and calculated from a calibration curve obtained with Chlrogenic acid as standard. 5ml of Folin Ciocalteu reagent and 4ml of aqueous sodium carbonate were added to 0.5ml of extract. After 15
min. of incubation at room temperature, the absorbance was read at 765nm in a UV-Visible Spectrophotometer.

### 3.7.2 Estimation of Flavanoid

The aluminium chloride colourimetric method was used for flavanoids determination (Chang et al., 2002). Each extracts (1mg/ml) was prepared in 70 : 30% ethanol and 0.5ml of each sample was separately mixed with 5ml of ethanol, 0.1ml of 10% aluminium chloride, 0.1ml of 1M potassium acetate and 2.8ml of distilled water were added and kept at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 415 nm. The calibration curve was prepared using catechin.

### 3.7.3 Total Tannin Determination

Tannin-phenolics were determined by the method of Peri and Pompei (1971). 1ml of sample extracts of concentrations (1mg/ml) was taken in test tubes. The volume was made up to 1ml with distilled water and 1ml of water serves as the blank. To this 0.5ml of folins phenol reagent (1:2) followed by 5ml of 35% sodium carbonate was added and kept at room temperature for 5 minutes. Blue colour was formed. The colour intensity was read at 640nm. A standard graph of tannins (gallic acid conc - 1mg/ml) was plotted, from which the tannin content of the extract was determined.

### 3.7.4 Estimation of Ascorbic Acid (Vitamin – C)

Ascorbic acid (Vitamin – C) was estimated following the procedure of AOAC (Anonymous, 1980). Five gram of dried plant powder (sample) was ground well in a pestle and mortar with oxalic acid. Known volume of (10ml) the above was changed to de-hydroform using the procedure adopted for working standard. Different aliquot (0.2 to 2ml) of de-hydroform of working standard was taken in test tubes and their volume was made to 3ml with water. To each tube was added 1ml of DNPH and 1 to 2 drops of thiourea. The tubes were incubated at 37°C for 3 hours. After incubation
the orange-red oxazone crystals formed was dissolved by adding 7ml of 80% H₂SO₄. The absorbance was measured at 540nm and standard graph was plotted. Dehydroform of sample was taken in aliquots and preceded it for plotting on the standard curve. The absorbance was compared with the standard graph and the percentage of ascorbic acid was calculated.

### 3.7.5 Estimation of β-Sistosterol

Estimation of β-Sistosterol was carried out by using Indian Pharmacopoeia (1996). The absorbance of the samples and standards were noted at different wave lengths from 200 to 700 nm in Shimadzu UV 160 A° spectrophotometer. The absorbance was plotted against the wave length and absorption maxima were determined and the percentage of β-Sistosterol was estimated and dry weight basis.

### 3.7.6 Estimation of Total Terpenoid

Hundred gram of plant powder were taken separately and soaked in alcohol for 24 hours and then filtered. The filtrate was extracted with petroleum ether; the ether extract was treated as total terpenoids (Anonymous, 1959).

### 3.7.7 Estimation of Total Alkaloid

This alcoholic extract of plant sample was treated with 0.1N HCl and aqueous acidified layer thus obtained was partitioned with chloroform in a separating funnel. The chloroform layer is rejected. The aqueous layer was basified with ammonium hydroxide and then partitioned with chloroform. The chloroform layer was concentrated and tested for alkaloids with alkaloid testing reagents (Anonymous, 1959).

### 3.7.8 Isolation of Tannin-Free Total Glycosidal Extract

100g of air-dried powder was extracted with ethanol: water (2:1). The aqueous ethanol extract thus obtained contains tannins which usually interfere with the biological activities. Hence, this should be removed by treating with 5% neutral lead
acetate reagent which precipitates the tannins as lead tannate. The aqueous ethanolic solution was treated with 5% neutral lead acetate solution and the precipitated lead tannate was filtered off. This process is repeated with until no more precipitate was obtained. The clear filtrate now contained the excess un-precipitated lead ions in solution which were removed by passing $\text{H}_2\text{S}$ gas into the solution. This removed the lead ions as insoluble complex black lead sulphide. The black precipitate was filtered and this process was usually repeated until no more black precipitate was formed and the solution strongly smelled of $\text{H}_2\text{S}$. The solution, usually of syrupy consistency, was concentrated over water-bath maintained at $55^0\text{C}$. This procedure also removed the excess of $\text{H}_2\text{S}$ (Indian Pharmacopoeia, 1996).

### 3.8 MINERAL STUDIES

#### 3.8.1 Major Elements (Harbone, 1973 and 1998)

Two gram of dried powder of the sample was taken in 250ml conical flask and 12ml of triple acid mixture (nitric, sulphuric and perchloric acid in the ratio of 1:2:1) was added and the mouth of the flask was covered with a funnel. The contents were digested in the flask over a sand bath till a clear solution was obtained. The solution was filtered through Whatman No. 40 filter paper and the filtrate was collected in 250ml volumetric flasks. The conical flask was washed with small increments of hot water and the water was added to the filter paper. Residue on the filter paper was also washed with hot water till the filtrate runs free of chloride. The volumetric flask was cooled under tap water and made upto 250ml with cold distilled water. This triple acid extract was used for the analysis of mineral constituents. The minerals (P, K, Na and Ca) were estimated using Flame Photometer.

#### 3.8.2 Trace Elements (Anonymous, 1996)

The dried powder was accurately weighed in 0.2g quantity in a dry conical flask and 10ml of di-acid mixture (5:2 of nitric acid and perchloric acid) were added. The contents of conical flask were allowed to stand for a few hours in cold digestion. The conical flask was then kept on a hot plate and the contents were digested by
slowly increasing the temperature. The digestion was continued till the contents become colourless.

The digested material was filtered through Whatman No. 40 filter paper by repeatedly washing the conical flask with a small a volume of distilled water. The filtrate collected was made upto a suitable volume and fed into Perkin Elmer, Optical Emission Spectrophotometer – Optima 2100 Dr.

3.9 PROFILING OF SECONDARY METABOLITES

3.9.1 Thin Layer Chromatography of *Aegle marmelos* Leaf Extract

TLC studies on total alkaloids (TA), total terpenoids (TT), total glycoside (TG), alcohol, water and 50% alcohol extracts of the powdered drugs of all the samples were carried out.

**Procedure**

10 mg per ml of *Aegle marmelos* leaf extract was dissolved in water and ethanol solvent and used for TLC examination. TLC plates were prepared by using Silica Gel-G as adsorbent. 100g silica gel-G was mixed with sufficient quantity of distilled water to make slurry. The slurry was immediately poured into a spreader and plates were prepared by spreading the slurry on glass plates of required size. Plates were allowed to air dry for one hour and layer was fixed by drying at 110°C for two hours. Using a micropipette, about 10µml of extracts were loaded gradually over the plate and air dried. The procedure was carried with solvents like ethyl acetate, acetone and finally chloroform: Methanol 9:1 ratio showed distinct bands. Three major bands were observed in Long UV and Iodine sprayed plates. The chromatograms were observed under visible light and were photographed. The Rf value was obtained by using the following formula.

\[
R_f = \frac{\text{Distance travelled by the substance (cm)}}{\text{Distance travelled by the mobile phase (cm)}}
\]
3.9.2 High Performance Liquid Chromatography of *Aegle marmelos* leaf Extract

Phytochemicals were analysed by HPLC according to the method of Hertog *et al.* (1992) and Gennaro *et al.* (2002). Standards used are Rutin, Quercetin, Kaempherol, Farmarixetin, Isorhamnetin, Marmesin and Ursolic acid.

**Procedure**

HPLC was conducted in a column of C18 (reversed phase column Lichrospher 100 : RP18) length 4.6 mm x 25 cm, equipped with a pump (LC -10AT VP1), SIL-6A automatic injector furnished with a 50 Dl loop, detector (SPD - 10AVP) set at 370 nm and C- R6A chromatography data station software. 10 Dl of the sample was injected into the loop and the temperature was maintained at 40°C. The solvents were used at a constant flow rate of 0.6 ml/min. The solvent system consists of 50 ml of methanol (A), 50 ml of phosphoric acid (B) and 1ml water (C) with a gradient system 50% of A in B. All the solvents used were of HPLC grade. Sample peaks were quantified with the external standard method. The quantities of phytochemicals were expressed as mg/100 g of fresh weight. Alcohol, water, and hydrochloric acid (50:20:8) mixture were used as extraction solvent. Methanol, water, and phosphoric acid (100:100:1) mixture used as mobile phase were used as standards.

20 µL of the standard solutions and *Aegle marmelos* leaf extract were separately injected into the chromatograph; chromatograms were recorded, and the major peaks areas were measured. The percentage of each phytochemical in the sample was calculated.

3.9.3 High Performance Thin Layer Chromatographic analysis of *Aegle marmelos* leaf Extract

The HPTLC system (Camag, Muttenz, Switzerland) consists of

- TLC scanner connected with a PC running WinCATS software under MS Windows NT
- Linomat V Sample applicator
**Spotting of samples**

The chromatographic estimation was performed by streaking the extracts in the form of narrow bands of 6 mm length on the precoated silica gel 60 F 254 TLC plate (5 cm × 10 cm), at a constant application rate of 150 µl/s and gas flow at 10 s/ µl was employed with the help of Camag 100 µl syringe connected to a Nitrogen tank; using a Camag Linomat V (Camag, Muttenz, Switzerland). 15µl of 1% of ethanolic extract was applied. After spotting the plate, it is subjected to linear ascending development up to a distance of about 90 mm in a solvent system of Toluene: Ethyl acetate: Diethylamine: Methanol: Chloroform in the ratio of 10:6:2:2:1 v/v., in a glass chamber, which was saturated with the same solvent system at room temperature just 10 minutes prior to development.

**Scanning of plate**

TLC plate was dried in flowing air at room temperature. Densitometric scanning was carried out using Camag TLC Scanner III (Camag, Muttenz, Switzerland) at wavelength of 200-450 nm with a slit dimension of 6.00 × 0.30 mm, with scanning speed of 20 mm/s, and data resolution was at 100 µm/ step. The chromatograms were integrated and regression analysis and statistical data were generated using WinCATS evaluation software (Version 1.4.2.8121).

**3.9.4 Gas Chromatography- Mass Spectrum analysis (GC-MS)**

GC-MS technique was used in this study to identify the phytocomponents present in the leaves extract of *Aegle marmelos*. GC-MS technique was carried out at IICPT laboratory, Thanjavur, Tamil Nadu. GC-MS analysis of this extract was performed using GC GC–Clarus 500 system and gas chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with Elite-1 fused silica capillary column (Length : 30.0 m, Diameter : 0.25 mm, Film thickness : 0.25 µm Composed of 100% Dimethyl poly siloxane). For GC-MS detection, an electron ionization energy system with ionization energy of 70eV was used. Helium gas (99.8%) was used as the carrier gas at a constant flow rate of 1.1ml/min and an injection volume of 2µl was employed.
(split ratio: 1:25). Injector temperature was 250°C and Ion-source temperature was 250°C. The oven temperature was programmed from 70°C (isothermal for 2 min.), with an increase of 300°C for 10 min. Mass spectra were taken at 70eV; a scan interval of 0.5 seconds with scan range of 40 – 1000 m/z. Total GC running time was 35 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was a GC MS solution ver.2.53.

**Identification of components**

Interpretation of mass spectrum GC-MS was conducted using the database of National Institute Standard and Technique (NIST08s), WILEY8 and FAME having more patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST08s, WILEY8 and FAME library. The Name, Molecular weight, Molecular formula and Structure of the component of the test material were ascertained.

**3.10 Antimicrobial Activity**

**3.10.1 Test Microorganisms**

Six bacterial species were employed as test organism. These include *Streptococcus pyogenes*, *Staphylococcus aureus*, *Salmonella typhi*, *Klebsiella pneumonia*, *Helicobacter pylori* and *Psudomonas auroginosa*. Three fungal species were employed as test organism. These include *Aspergillus niger*, *Candida albicans* and *Trichoderma viride* which were isolated from skin infected person.

**3.10.2 Antibacterial activity**

Antibacterial activity of the extracts and fractions were tested using the agar diffusion method described by Collin and Lyne (1970). The different extracts were tested for the antibacterial activity against the bacteria such as *Streptococcus pyogenes*, *Staphylococcus aureus*, *Salmonella typhi*, *Klebsiella pneumonia*, *Helicobacter pylori* and *Psudomonas auroginosa*. Varying concentrations of the
extracts and fractions were prepared and tested using Nutrient agar medium. The plates were incubated at 37°C for 24 hours and the zones of inhibition measured.

**Composition of nutrient agar medium**

- Peptone: 5 g
- Beef extract: 3 g
- NaCl: 5 g
- Agar: 15 g
- Distilled water: 1000 ml

The pH of the medium was adjusted to 7.

### 3.10.3 Antifungal Activity

Antifungal activity of the extracts and fractions were tested using the agar diffusion method described by Collin and Lyne (1970). The extract was tested with different solvents for the antifungal activity against the fungi such as *Aspergillus niger*, *Candida albicans* and *Trichoderma viride*. Varying concentrations of the extracts and fractions were prepared and incorporated into Potato dextrose agar. The plates were incubated at 25°C for 48 hours and inhibition of growth was noted.

**Composition PDA Medium**

- Potato: 250 g
- Dextrose: 20 g
- Agar: 15 g
- Distilled water: 1000 ml
- Streptomycin sulphate: 100 mg

The pH of the medium was adjusted to 5.6
3.11 PHARMACOLOGICAL METHODS

All Pharmacological experiments involving animals described in the present work were carried out and get approved by Local Animal Ethical Committee of Dept. of Pharmacology, Periyar College of Pharmacy for women, Trichy.

3.11.1 Toxicological Studies

3.11.1.1 Preliminary screening and estimation of LD$_{50}$

The study was carried out in the laboratory of PRILS Institute of Paramedical Science, Pattukkottai. The ethical committee of the institute approved for the study. Twelve groups of rats were selected for the LD$_{50}$ studies. In each group 4 rats were participated (n= 4). Drug Aegle marmelos were given to the animals orally, at the dose of 50mg, 100mg, 150mg, 200mg, 300mg and 500mg. The animals were observed for alertness, gait, posture, tremor and response to touch, pain, sound etc, continuously for first 6 hrs and later at intervals of 24 hours for 3 days. Observed mortality in any group was noted.

3.11.1.2 Acute toxicity studies (Turner, 1965)

In the 14 days sub-acute studies also was carried out in the PRILS institute of Paramedical Science, Pattukkottai. For the sample there was four groups of animals and in each group there were 10 animals (n=10) participated. Control group were fed with normal saline and the other three groups with 50 mg/kg/body wt, 100mg/kg/body weight dose of drugs. Their physical activity, every day body weight, water and food intake and temperature were also recorded for all groups of animals. After 14 days, all the animals were sacrificed and samples of liver, stomach and intestine were taken for study. The necropsy findings were tabulated.

3.12 Anti-Ulcer Activity (Modified Pyloric Ligated (Shay) Rat Model)

The experimental procedures of Shay et al. (1945) methods were used. The rats weighing 150 – 160g were divided into groups of six animals each and were placed in cages with grating floor to avoid coprophagy and fasted for 48 hours.
allowing free access to water. One group received water (1ml/kg) and was served as control. Ranitidine (30 mg/kg) was selected as standard drug and was given to a group, for comparison. For the test group, the animals were grouped into two, receiving the drug at a dose level of 50 & 100mg/kg body weight. Aspirin plus pylorus ligation (PL) model: Ulceration in rats was induced as described by Goel et al. (1985). Aspirin suspension in 1% carboxymethylcellulose in water was administered orally in a dose of 200 mg/kg once daily for three days. On fourth day, pylorus was ligated as per the method of Shay et al. (1945).

Under light ether anesthesia, the abdomen was opened by a small mid-incision below the xiphoid process, pyloric portion of stomach was slightly lifted out and ligated avoiding traction to the pylorus or damage to its blood supply. The stomach was replaced carefully and the abdomen wall closed by interrupted sutures. The test drugs (Aegle marmelos) were administered once daily orally for two weeks. The animals were deprived of both food and water during the post-operative period. Four hours after the ligation, animals were sacrificed. Ulceration in the stomach was assessed by means of a scoring technique whereby macroscopic examination of the stomach was made using a hand lens and ulcers were scored using the method and criteria of Shay et al. (1945). Normal gastric mucosa was scored nill, punctuate haemorrhage, pinpoint ulcer was scored 0.5, one or two small hemorrhages ulcer was scored 1.0 while ulcers greater than 3mm in diameters were scored 2.0 ulcer index and percentage inhibition of ulceration was calculated.

\[
\text{Ulcer index} = \frac{(\text{Mean degree of ulceration} \times \% \text{ group of ulceration})}{100}
\]

\[
\% \text{ inhibition of ulceration} = \frac{(\text{Ulcer index in control} - \text{Ulcer index in test})}{\text{Ulcer index in control}} \times 100
\]
Collection of gastric juice

The stomach was excised carefully keeping the esophagus closed, opened along the greater curvature and the luminal contents were removed as described (Jain and Santhani, 1994). The gastric contents were collected in a beaker and centrifuged at 1000 rpm for 10 minutes as recommended. Gastric juice was collected from the pylorus-ligated rats. The gastric juice thus collected was centrifuged and the volume of gastric juice as well as pH of gastric juice was measured. The sodium (Na\(^+\)) and potassium (K\(^+\)) ion concentration of gastric juice was carried out in flame photometer (Jeffery et al., 1991). Then the gastric juice was subjected to bio-chemical estimation as follows.

3.12.1 Determination of pH

Supernatant of gastric juice solution containing Hydrogen ion concentrations were measured with the help of pH meter. The reading of pH was read in electronic display.

3.12.2 Estimation of gastric content (Vogel, 1992)

Principle

The total gastric content is estimated based on its acid content, which is found by titrating the gastric juice obtained against sodium hydroxide using Topfer’s reagent as indicator.

Reagents

1. 0.01 N sodium hydroxide : Dissolved 0.4g of NaOH in 1000 ml of water
2. Topfer’s reagent

Procedure:

The total volume of gastric content was measured. The gastric content was centrifuged at 1000 rpm for 10 min. One ml of the supernatant liquid was pipetted out and diluted to 10 ml with distilled water. The solution was titrated against 0.01N
NaOH using Topfer’s reagent an indicator, to the endpoint when the solution turned to orange colour. The volume of NaOH needed was taken as corresponding to the free acidity. Titration was further continued till the solution regained pink colour. The volume of NaOH required was noted and was taken as corresponding to the total acidity.

\[
\text{Acidity (mEq/l)} = \frac{\text{Volume of NaOH} \times \text{Normality}}{0.1} \times 100
\]

3.12.3 Estimation of Pepsin (Debnath et al., 1974)

For each determination four tubes (1) and (2) containing 5ml of substrate, (3) and (4) containing 10ml TCA was placed in the water bath at 37°C. The gastric juice was mixed with an equal volume of HCl at pH 2.1, warmed to 37°C and added 1ml of mixture to each tube (1) and (4), incubated for 15 minutes and at the end mixed the contents of tube (1) with tube (3) and allowed to stand in the bath for about 4 minutes. Contents of tube (1) and tube (3) give test and contents of tube (2) and tube (4) gives blank. Both the contents were filtered after 25-30 minutes, 2ml of filtrate was pipetted into 10 ml of NaOH, mixed by gentle rotation, then 1ml of phenol was added and again mixed by gentle rotation. After 30 min, intensity of colour was measured at 680 nm in Systronics UV-VIS spectrophotometer- 180.

The difference between test and blank gives a measure of peptic activity. As standard, 2ml of freshly prepared phenol solution containing 50μg/ml was mixed with 10ml of NaOH and 1ml of phenol reagent was added. After 5-10 minutes, the colour intensity was measured at 680nm.

3.12.4 Estimation of Sodium and Potassium ion concentration (Jeffery et al., 1991)

The estimation for sodium (Na⁺) and potassium (K⁺) ions concentration in gastric juice was carried out using Systronics mediflame 127 – flame photometer.
Preparation of stock solution

- Sodium stock solution was prepared by dissolving 2.542g NaCl in 1 liter of distilled water. It contains 1mg Na per ml (i.e. 1000 ppm). Stock solution was diluted to give four solutions containing 10, 5, 2.5 and 1 ppm of sodium ions.

- Potassium stock solution was prepared by dissolving 1.909g KCl in 1 liter of distilled water. It contains 1mg potassium per ml (i.e. 1000 ppm). Stock solution was diluted to give four solutions containing 20, 10, 5 and 2 ppm of potassium ions.

Procedure

For sodium and potassium, the flame intensity corresponding to the concentration of stock solution was noted using appropriate filters. The results were plotted in a graph. The flame intensity of the gastric juice was noted. The concentration of sodium and potassium ions was calculated from the graph. The results are expressed in terms of mg/l.

3.12.5 Estimation of total proteins (Lowry et al., 1951)

The dissolved protein in gastric juice was estimated in the alcoholic precipitate obtained by adding 90% alcohol with gastric juice in 9:1 ratio. Then 0.1ml of alcoholic precipitate of gastric juice was dissolved in 1 ml of 0.1N NaOH and from this 0.05ml was taken in another test tube, to this 4ml of alkaline mixture was added and kept for 10 min. Then 0.4ml of phenol reagent was added and again 10 min was allowed for colour development. Reading was taken against blank prepared with distilled water at 610nm in Systronics UV-VIS spectrophotometer-180. The protein content was calculated from standard curve prepared with bovine albumin and was expressed in terms of µg/ml of gastric juice.
3.12.6 Estimation of total carbohydrates (Goel et al., 1985)

The dissolved mucosubstance in gastric juice was estimated in the alcoholic precipitate obtained by adding 90% alcohol with gastric juice in 9:1 ratio. Briefly the method consists of taking two aliquots of gastric juice and treated as follows:

To 1ml of gastric juice, 9ml of 90% alcohol was added. The mixture was kept for 10 minutes before it was centrifuged. The supernatant was discarded. The precipitate was dissolved in 0.5ml of 0.1N NaOH. To this 1.8ml of 6N HCl was added. The mixture was hydrolyzed in water bath at 100 °C for 2 hours. The hydrolysate was neutralized by 5N NaOH using phenolphthalein as indicator and the volume was made up to 4.5ml with distilled water and used for the estimation of total hexoses, hexosamine and fucose as described below.

To the other aliquot of 0.5ml gastric juice, 4.5ml of alcohol was added. The mixture was shaken for 10 minutes and centrifuged to obtain precipitate. The precipitate was dissolved in 0.5ml of 0.1N H₂SO₄. This reconstituted solution was transferred to glass-stoppered tubes and then hydrolyzed in a water bath at 100 °C for 1 hour. After hydrolysis, the volume restored to 0.5ml; 0.2ml of this hydrolyzes was used for the estimation of sialic acid.

After obtaining the concentration (µg/ml) of individual carbohydrates namely hexose, hexosamine, fucose and sialic acid, the total carbohydrate content was calculated by adding the concentration of individual carbohydrates. Mucosubstances activity has been expressed as ratio of total carbohydrates to total proteins.

3.12.7 Estimation of hexoses (Winzler, 1958)

To 0.4ml of hydrolysate, 3.4ml of Orcinol reagent was added. The mixture was then heated in the boiling water bath at 60°C for 15 minutes. This was then cooled under running tap water and intensity of the colour was read in Systronics UV-VIS spectrophotometer- 180 at 540nm against the blank by using distilled water
instead of hydrolysate. Total hexoses content was determined from the standard curve of D(+) – galactose-mannose and has been expressed in µg/ml of gastric juice.

3.12.8 Estimation of hexosamine (Disch and Borentreund, 1950)

0.5ml of the hydrolysate fraction was taken. To this 0.5ml of acetyl-acetone reagent was added. The mixture was heated in boiling water bath at 60 °C for 20 minutes, and then cooled under running tap water. 1.5ml of 90% alcohol was added and allowed for 30 minutes. The colour intensity was measured in Systronics UV-VIS spectrophotometer- 180 at 540nm against blank prepared by using distilled water instead of hydrolysate. Hexosamine content was determined from the standard curve prepared by using D(+) – glucosamine hydrochloride and concentration has been expressed in µg/ml of gastric juice.

3.12.9 Estimation of fucose (Dische and Shettles, 1948)

In this method, three test tubes were taken. In one tube 0.4ml of distilled water was taken to serve as control and in each of the other two 0.4ml of hydrolysate was taken. To all three tubes 1.8ml of H$_2$SO$_4$: water (6:1) was added by keeping the test tubes in ice-cold water bath to prevent breakage due to strong exothermic reaction. The mixture was then heated in boiling water bath for exactly 3 min. The tubes were taken out and cooled. To the blank and to one of the hydrolysate containing tube (unknown), 0.1ml of cysteine reagent was added while cysteine regent was not added to the last test tube containing the hydrolysate (unknown blank). It is then allowed for 90 minutes to complete the reaction. The reading was taken in Systronics UV-VIS spectrophotometer- 180 at 396 and 430nm, setting zero with the distilled water. The optical density for the fucose in the hydrolysate was calculated from the differences in the reading obtained at 396 and 430nm and subtracting the values without cysteine. This was read against standard curve prepared with D(+) – fucose content and was expressed in terms of µg/ml of gastric juice.

$$\text{True optical Density} = \frac{(\text{OD}_{396} - \text{OD}_{430})^{\text{unknown}} - (\text{OD}_{396} - \text{OD}_{430})^{\text{unknown blank}}}{(\text{OD}_{396} - \text{OD}_{430})^{\text{water blank}}}$$
3.12.10 Estimation of Sialic acid (Warren, 1959)

To 0.5ml of the hydrolysate in 0.1N H$_2$SO$_4$, 0.2ml of sodium periodate was added and mixed thoroughly by shaking. A time of 20 min was allowed to elapse before addition of 1ml of sodium arsenite solution to this mixture. The brown colour produced disappeared after shaking. Then 3ml of thiorbituric acid was added and the mixture was heated in boiling water bath for 15 minutes. After cooling the tubes, 4.5ml of cyclohexanone was added and thorough shaking was done for 15 seconds till all the colour was taken up by the cyclohexanone supernatant. The mixture was centrifuged to get a clear pink layer of cyclohexanone. This supernatant was pipetted out and intensity of colour was measured in Systronics UV-VIS spectrophotometer-180 at 550nm. The sialic acid content of the sample was determined from the standard curve of sialic acid and has been expressed in terms of µg/ml of gastric juice.

3.13 Antipyretic Activity (Loux et al., 1972 and Lassman et al., 1977)

Hyperpyrexia was induced in rats by subcutaneous injection of 10 ml/kg of a 20% aqueous suspension of dried yeast in the back below the nape of the rat. The animals were then fasted for the duration of the experiment, water being made available ad libitum. Control temperatures were taken 24 hr after the yeast injection to determine the pyretic response to yeast. Temperatures taken for 1 hour prior to drug administration in fevered animals served as the pre-drug control. Extract of plants (50 & 100 mg/kg body weight) was given orally 12 hr after yeast injection. Paracetamol (250 mg/kg) served as the reference drug. The temperatures were recorded and compared.

3.14 Antioxidant Activities

3.14.1 Estimation of lipid peroxidation (Ohkawa et al., 1979)

Rat liver homogenate was used as the source of polyunsaturated fatty acids for determining extends of lipid peroxidation. Liver was collected immediately after the sacrifice of the animals by cervical dislocation under mild ether anesthesia. The liver was homogenized with 40 mM Tris-Hcl buffer (pH 7.0) and centrifuged at 3000 rpm
for 10 min to get a clear supernatant. HAEGG solution of different concentration (25 – 1000 µg/ml) and 100 µl of each of 1.5 M KCl, 15 mM FeSO₄ and 6 mM ascorbic acid was incubated at 37°C for 1 hour. 1ml of 10% TCA was added to the reaction mixer and centrifuged at 3000 rpm for 20 min at 4°C to remove the insoluble proteins. Supernatant was removed and 1 ml of TBA (0.8%) was added to this fraction followed by heating at 90°C for 20 min in a water bath. After cooling, the coloured TBA-MDA complex was extracted with organic solvent (2 ml butanol) and absorbance was measured at 532 nm. Percentage inhibition was calculated using the formula.

\[
\text{Percentage inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}}
\]

3.14.2 Estimation of Reduced Glutathione (GSH)

Reduced glutathione was determined by the method of Ellman (1959). This method is based on the development of yellow colour when 5,5’ dithiobis nitro benzoic acid (DTNB) is added to compound containing sulphydryl group.

Tissue of known weight was homogenized in phosphate buffer (pH 8.0: 0.2M) 0.5ml of tissue homogenate or plasma was pipetted out and precipitated with 2ml of 5% TCA. 1.0ml of the supernatant was taken after centrifugation and 0.5ml of Ellman’s reagent and 3.0ml of phosphate buffer were added to it. The yellow colour developed was read at 412nm. A series of standards were treated in a similar manner along with a blank containing 3.5ml of buffer. The amount of reduced glutathione was expressed as mg/dl.

3.14.3 Estimation of thiobarbituric acid reactive substances (TBARS)

The TBARS was determined by the method of Niehans and Samuelson method (1968). In this method, malondialdehyde (MDA) and other thiobarbituric acid reactive substances (TBARS) were measured by their reactivity with thiobarbituric acid in the acidic condition to generate a pink coloured chromophore, which was read at 535nm.
Lipid peroxidation was estimated as evidence by the formation of thiobarbituric acid reactive substances (TBARS).

1.0 ml of liver homogenate was taken and 2ml of TCA was added and centrifuged it. 2ml of supernatant is taken and 1.5ml of TBA is added. The solution was heated for 20 minutes in boiling water bath. After cooling the absorbance of the sample was read at 532nm against blank in UV spectrophotometer. The TBARS activity was expressed as Nanomoles of MAD/mg of protein.

3.14.4 Determination of Catalase

The catalase (CAT) was determined by the method of Ming and Zigman (1978). In this method dichromate in the acetic acid is converted to perchromic acid then to chromic acetate when heated in the presence of hydrogen peroxide ($\text{H}_2\text{O}_2$). The chromic acetate formed was measured at 620nm.

To 0.9ml of the phosphate buffer taken in each four test tubes, 0.1ml of plasma or hemolysate or tissue homogenate was added. Then 0.4ml of hydrogen peroxide was added to each tube and the reaction was arrested at 15, 30, 45 and 60 seconds by addition of 2.0ml of the dichromate acetic acid reagent. The tubes were kept at boiling for 10 minutes and cooled. Finally, it was read at 530nm. The rate of removal of hydrogen peroxide by catalase was calculated using hydrogen peroxide standards in the concentration range of 20-100 moles and processed similarly along with a reagent blank.

3.14.5 Assay of Superoxide Dismutase (SOD)

The activity of superoxide dismutase was assayed by the method of Kakkar et al., (1984). Superoxide radicals react with nitroblue tetrazolium in the presence of NADH and produce formazan blue. SOD removes the superoxide radicals and inhibits the formation of formazan blue. The intensity of colour is inversely proportional to activity of the enzyme.
0.5ml of tissue homogenate was diluted to 1ml of water. Then 2.5ml of ethanol and 1.5ml chloroform (all reagents chilled) were added. This mixture was shaken for 1 minute at 4°C and then centrifuged. The enzymes activity in the supernatant was determined. The assay mixture consisted 1.2ml of sodium pyrophosphate buffer, 0.1ml of phenazine methosulfate, 0.3ml of nitroblue tetrazolium, 1ml of appropriately diluted enzyme preparation and water in a total volume of 3ml. Then the reaction was initiated by the addition of 0.2ml of reduced nicotinamide adenine dinucleotide (NADH). After incubation at 30°C for 90 seconds, the reaction was arrested by the addition of 1ml of glacial acetic acid. Subsequently, the reaction mixture was stirred and shaken with 4ml of n-butanol and allowed to stand for 10 minutes. Finally, the butanol layer was separated after centrifugation and the colour intensity of the chromogen in butanol layer was measured at 560nm. The system devoid of the enzyme served as control. One unit of activity was taken as the enzyme reaction which gave 50% inhibition of NBT reduction per minute per mg.

3.15 Antispermatogenic activity of *Gryllotalpa africana*

The reasons for choosing the mole cricket *Gryllotalpa africana* as an experimental insect are as follow:

1. It is available in large numbers during rainy season from the month of September to December.
2. Relatively it is easy to rear and maintain the species in the laboratory condition.
3. The size of the insect is suitable for easy handling, dissections and tissue isolation experiments.
4. Information on the reproductive physiology and histopathology of the genus *Gryllotalpa africana* appears to be fragmentary.
5. It is considered as an agricultural pest, because it causes destruction to roots of cultivated plants like paddy and potatoes (Banerjee and Chatterjee, 1955; Muralirangan, 1980).
3.15.1 Rearing techniques

The adult males and females of *Gryllotalpa africana* (Fig. 40) collected under debris and moist places in and around Thiruvettakudi near Karaikal, were reared in tall jars at room temperature of $30 \pm 1^0\text{C}$. One fourth of the rearing jar was filled with moist sand. The opening of the jar was covered with an aluminum wire mesh. The sand in the jar was slightly moistened regularly by sprinkling cold water. The insects were fed with earthworms, vegetables and root tips. The adults and the nymphal instars thrived well in these laboratory conditions. The adult male insects were used as samples for experimental studies. These insects were sacrificed and their testes were isolated for histological studies.

3.15.2 Preparation of different concentrations of plant extract

I. One ml of the stock solution, which is prepared in alcohol base extract, is evaporated in the water bath till the formation of residue in order to avoid the alcoholic effects. Then 100 ml of distilled water is added to this residue to produce a concentration of 0.01 per cent.

II. Likewise, 5 ml of stock solution with 100 ml of distilled water results in 0.05 per cent and

III. 10 ml of extract with 100 ml of distilled water results in 0.1 per cent concentration.

3.15.3 Biochemical Analysis

Carbohydrates, proteins and lipids which are the major biochemical constitutions are found deposited in insect tissues. These biochemicals are used as energy reserves in majority of insects and are essentially required for growth and metabolism of insects. In the present study mole cricket *Gryllotalpa africana* were selected for biochemical analysis. They were sorted out from the mass culture and reared in glass cylindrical jars ten numbers in each jar and provided with fresh tender groundnut leaves as diet. The larvae were divided into 4 groups with one set serving as control.
The leaves were dipped in different experimental concentrations of leaf extracts of *Aegle marmelos* and fed to the insects for 5 days after which the animals were sacrificed by keeping them in oven at a temperature of 60°C. The insects were then taken and ground well and the powdered form was used for biochemical analysis of total carbohydrates, total proteins and total lipids. The following standard procedures were used for biochemical estimation. Experimental data were collected based on three observations.

### 3.15.1 Quantitative estimation of Glycogen

**Principle**

Sulphuric acid in the Anthrone reagent hydrolyses the glycogen into glucose and then dehydrates it into furfural. This compound reacts with Anthrone to produce a complex coloured product, the colour intensity of which is proportional to the amount of glucose present in glycogen.

**Reagents**

**A. Anthrone reagent**

A solution containing 0.05% Anthrone, 1% Thiourea and 72% sulphuric acid was used. To the 100ml of 72% concentrated sulphuric acid 1 gram of Thiourea was added and then 50 mg of Anthrone was added. The solution was yellowish green in colour.

**B. Glucose standard**

100 mg of D-Glucose was dissolved in 100ml of saturated distilled water.

**C. Deproteinizing agent**

5 mg of TCA (Tri Chloro Acetic Acid) was dissolved in 100 ml of distilled water.

**Procedure**

50 mg of the sample tissue was homogenized. The mixture was centrifuged for 5 minutes at 2,500 rpm. To 1 ml of the supernatant 5 ml of absolute ethanol was
added. Then it was kept in refrigerator overnight. The mixture was again centrifuged for 16 minutes at 2500 rpm. The supernatant was discarded. That 1 ml of distilled water was added to dissolve the precipitated glycogen. To 1 ml of the sample, 1 ml of Anthrone reagent was added. Then the test tubes containing samples, glucose standard and blank were kept in water for 15 minutes. Then the test tubes were cooled in a dark place for 30 minutes. The optical densities of the samples and glucose standard were determined at 620 nm.

3.15.3.2 Quantitative estimation of total proteins

Principle

The CONH groups in the protein molecules react with copper sulphate in alkaline medium to give a blue coloured complex product in addition tyrosine and tryptophan residues of protein cause reduction of the Phosphomolybdate and Phosphotungstate components of Folin ciocalteau reagent to give blush products, which contribute towards enhancing the sensitivity of this method.

Reagents

A. 10% TCA
B. 1 N NaOH
C. Alkaline sodium carbonate solution
   Prepared by dissolving 2g of NaCO₃ in 100ml of 0.1 N NaOH
D. Copper sulphate – Sodium potassium tartarate solution
   Prepared freshly every time by mixing 0.5 g CuSO₄ 5H₂O (0.5%) and 1% sodium potassium tartarate solution in 100 ml distilled water.
E. Alkaline copper reagent
   Prepared freshly by mixing 50ml of reagent C and 1 ml of reagent D
F. Folin-Ciocalteu reagent
   Commercially available reagent wad diluted with equal volume of water just prior to use.
G. Standard solution

100 mg of Bovine Serum Albumin was dissolved in 100 ml of 0.1 N NaOH. 1 ml of this solution contains 1 mg of protein and this was kept as stock solution. From this, 10 ml was taken and made up to 100 ml with 0.1N NaOH as working standard.

Procedure

10 ml of sample was treated with reagent A and centrifuged at 10,000 x g for 10 minutes. The resulting pellet was resuspended in reagent B and boiled for 30 minutes; cooled and then recentrifuged to eliminate light scattering materials. The supernatant was made up to a known volume. To 0.1 ml of the supernatant 0.9ml of distilled water and 4.5 ml of reagent E were added and allowed to stand for 10 minutes and finally 0.5 ml of reagent ‘F’ was added. The absorbance was measured after 30 minutes at 620 nm in colourimetry against the reagent as blank. The amount of protein was calculated with a standard graph prepared by using Bovine Serum Albumin.

3.15.3.3 Determination of total lipids (Bragdon colourimetric method)

Principle

The tissue lipids particularly those containing unsaturated fatty acids are heated with concentrated sulphuric acid. They are oxidized to ketones that subsequently give a colour with potassium dichromate. The intensity of the colour is proportional to the amount of lipid present in the sample.

Reagents

A. Chloroform

B. 2% potassium dichromate in con.H$_2$SO$_4$

C. Olive oil used as standard.
D. Stock standard solution

1 gram of pure olive oil was dissolved in 100 ml of absolute ethanol. This solution contains 1000 mg/100ml of lipids (1%). Lower concentrations were prepared by diluting 10, 15, 20 and 25 ml with absolute ethanol.

Procedure

5 gram of the sample was placed in a dry mortar and ground thoroughly with sufficient amount of choloform (2ml). The homogenate was evaporated to try in a water-bath. To the dry test tube 3 ml of 2% potassium dichromate reagent was added. The tube was shaken well and diluted with equal amount of distilled water. Blank was prepared by adding 3 ml of distilled water to the equal amount of reagent in a clean test-tube. The colour developed was read in Spectro-photometer at 580nm.

3.15.4 Histological methods

The insects were dissected out in insect ringer solution (Ephrussi and Beadle, 1936) using the binocular microscope and the testes were removed. The tissues were fixed in aqueous Bouin’s fixative. After 24 hours of fixation the tissues were dehydrated using ascending grades of alcohol (Gurr, 1958). Tissues were gross stained in 70 per cent aqueous eosin to facilitate orientation of the tissues during embedding. The tissues dehydrated in absolute alcohol and acetone were cleared in xylol and embedded in paraffin wax (58 – 62°C). Sections cut at 6 µm thickness were deparaffinized using descending grades of alcohol and stained with Heidenhain’s haematoxylin and counter stained with aqueous eosin for microscopical observations.

3.15.5 Morphometric analysis of testicular follicle

Morphometric measurements of testicular follicle were taken from histological section with the help of the stage micrometer.