CHAPTER III
MATERIALS AND METHODS

3.1. Plant Collection

Fresh leaves of *Hyptis suaveolens* were collected from South Thamaraikulam village of Agasteeswaram Taluk, Kanyakumari District, Tamilnadu, India. The specimen was confirmed using regional floras and the voucher specimen deposited in the Herbarium of the Department of Botany, Scott Christian College (Autonomous), Nagercoil. The collected leaves were washed repeatedly with tap water and finally with distilled water, dried and powdered with the help of mechanical grinder, sieved and stored for extraction.

3.2. Classification

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<td>Authority</td>
<td>(L.) Poit.</td>
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3.3. Botanical Description

*Hyptis suaveolens* (L.) Poit. is an erect annual or short-lived perennial herb, subshrub or vine commonly growing to about 1.5 m tall, rarely to 3 m. Its stems are green or reddish-green, extremely hairy and square in cross-section. Leaves are oppositely arranged and hairy; the leaf blade is usually ovate (egg-shaped), elliptical (oval) or slightly cordate (heart-shaped) in outline, about 2 to 10 cm long and 1 to 7 cm wide, quite hairy and with shallow teeth along the margins. The pinkish or lavender-blue flowers are about 5 to 7 mm long and are arranged singly or in clusters of up to 5 in the axils of the upper leaves. After the flowers die the corolla is lost but the tubular calyx persists and turns brown and is very distinctive, with each of the five lobes topped by a bristle about 5 mm long. The fruit held within the calyx is a lobed capsule that divides into two nutlets, each of which contains a single seed. The nutlets are dark brown to black in colour with whitish markings at one end and are flattened and shield-shaped and 3 to 4 mm long and 2.5 to 3 mm wide (Parsons and Cuthbertson, 2001) (Plate 3.1).

3.4. Habitat

*Hyptis suaveolens*, a member of the family Lamiaceae, is a common weed growing along roadsides and waste grounds and is native to tropical America. The plant is generally described as an annual or perennial herb or subshrub or vine that occupies roadsides, rail tracks, wastelands, watercourses, pastures and open forests where the soil is well drained. It can form dense thickets in all areas of growth.
Plate 3.1. *Hyptis suaveolens* (L.) Poit. - Habit
3.5. Distribution

*Hyptis suaveolens* is widespread in Australia (Northern Territory and Queensland), China, India, Indonesia, Papua New Guinea, Solomon Islands, French Polynesia, Federated States of Micronesia (Chuuk and Yap Islands), Niue Islands, and Guam and the Hawaiian Islands in the United States of America (USA). It is widely prevalent in West and Central Africa where it is considered an insidious species in some countries. Distribution is now thinning out in the tropical dry deciduous forests of the Vindhyan highlands, which lie in the North Indian River Plain, between the Gangetic plains and Narmada valley in north India, and in Andaman and Nicobar (Sastri, 1959; Yoganarasimhan, 2000) (Plate 3.2).

3.6. Preparation of extracts

The dried powdered sample (100 g) was extracted with 1000 mL of solvents such as water, petroleum ether, chloroform, ethanol and acetone in a Soxhlet apparatus. The resultant filtrate was concentrated to powdered form through complete evaporation of the extraction solvent using rotary evaporator. The solid residue of greenish brown colour obtained was designated as the extract, which was stored in a refrigerator until further analyses.

3.7. Distillation of oil

Hydrodistillation was conducted by using a standard procedure (Clevenger apparatus) with dried *H. suaveolens* leaves which had previously been powdered in a mechanical grinder. The process was carried out continuously on a heating mantle at the temperature 60-80°C until no further oil was extracted. The essential oil was dried over anhydrous Na₂SO₄ and after filtration stored in a dark bottle at 4°C until analysis.
Plate 3.2. Distribution map of *H. suaveolens*

*Source:* accessed through the GBIF data portal
3.8. Preliminary phytochemical screening

Qualitative chemical tests were conducted to gain a general idea regarding the nature of constituents present in the extract. Aqueous, petroleum ether, chloroform, ethanol and acetone extracts were subjected to preliminary phytochemical investigations for detection of specific compounds as per the standard methods prescribed by Harborne (1998).

3.8.1. Tests for Carbohydrates

*Fehling’s Test:* 1 mL of Fehling’s A and 1 mL of Fehling’s B solution were added, mixed and boiled for 1 min. Now equal volume of test solution (aqueous, petroleum ether, chloroform, ethanol and acetone extracts) was added to the above mixture. The solution was heated in a boiling water bath for 5-10 min. A precipitate which was initially yellow turning to brick red colour indicated the presence of carbohydrates.

3.8.2. Tests for Glycosides

*Lugol’s Test:* Concentrated aqueous, petroleum ether, chloroform, ethanol and acetone extracts were treated with a few drops of 10% sodium hydroxide (NaOH) to make it alkaline. Then freshly prepared sodium nitroprusside was added to the solution. Formation of blue coloration indicated the presence of glycosides in the extracts.

3.8.3. Tests for Flavonoids

*Lead Acetate Test:* To a small quantity of extract lead acetate solution was added. Formation of yellow precipitate showed the presence of flavonoids.
3.8.4. Tests for Phenolic compounds

*FeCl₃ Solution Test:* Extracts were treated with 5% FeCl₃ solution. Formation of bluish black colour indicated the presence of phenols.

3.8.5. Tests for Saponins

*Foam Test:* Plant extracts were shaken vigorously with 2 mL of water. If the foam produced persisted for 10 min it indicated the presence of saponins.

3.8.6. Tests for Terpenoids

To the extract chloroform and conc. H₂SO₄ were added. Appearance of red colour indicated the presence of triterpenes.

3.8.7. Test for Steroids

*Salkowski Test:* To 2 mL of extract, 2 mL of chloroform and 2 mL of conc. H₂SO₄ were added. The solution was shaken well. Formation of red chloroform layer and greenish yellow acid layer indicated the presence of steroids.

3.8.8. Test for Alkaloids

The extract (1 mL) was treated with a few drops of diluted HCl and filtered. The filtrate was treated with 1 mL of Dragendorff’s reagent. Formation of reddish brown precipitate indicated the presence of alkaloids.

3.8.9. Test for Quinones

The extract (1 mL) was treated with alcoholic KOH solution. Presence of quinones was indicated by coloration ranging from red to blue.
3.8.10. Test for Coumarins

Alcoholic extract (1 mL) was treated with alcoholic NaOH solution; production of dark yellow colour indicated the presence of coumarins.

3.8.11. Test for Proteins

*Xanthoproteic Test*: To a small quantity of extract 1 mL of conc. H$_2$SO$_4$ was added. This resulted in the formation of white precipitate which on boiling turned yellow. On addition of ammonium hydroxide (NH$_4$OH), the yellow precipitate turned orange.

3.8.12. Test for Phytosterols

The extract was dissolved in 2 mL of acetic anhydride. To this 1 or 2 drops of conc. H$_2$SO$_4$ was added slowly along the sides of the tube and an array of colors was noticed, indicating the presence of phytosterols.

3.9. Quantitative Analysis of Phytochemicals

3.9.1. Estimation of Flavonoids (Evans, 1996)

Total flavonoid content was determined by the aluminium chloride method using gallic acid as the standard; 1mL of test sample and 4 mL of water were added to a volumetric flask (10 mL volume). After 5 min, 0.3 mL of 5% sodium nitrite and 0.3 mL of 10% aluminium chloride were added. After 6 min of incubation at room temperature, 2 mL of 1 M sodium hydroxide (NaOH) was added to the reaction mixture. Immediately the final volume was made up to 10 mL with distilled water. The absorbance of the reaction mixture was measured at 510 nm against a blank spectrophotometrically. Results were expressed as gallic acid equivalent (mg gallic acid/g dried extract).
3.9.2. Estimation of Tannins (Robert, 1971)

One millilitre of the extract was mixed with 5 mL of vanillin hydrochloride reagent a mixture of equal volumes of 8% HCl in methanol and 4% vanillin in methanol). The mixture was allowed to stand for 20 min and the absorbance measured at 500 nm. The standard graph was plotted for working standard catechin solution (0 to 250 µg/µL).

3.10. Fourier-Transform Infrared Spectroscopic Analysis (FT-IR)

Oven-dried leaf samples (60°C) were ground into fine powder using a mortar and pestle. Two milligrams of the sample was mixed with 100 mg KBr (FT-IR grade) and then compressed to prepare a salt-disc (3 mm diameter). The disc was immediately kept in the sample holder and FT-IR spectra were recorded in the absorption range between 400 and 4000 cm⁻¹. All investigations were carried out with a Shimadzu FT-IR spectrometer.

3.11. Gas Chromatography-Mass Spectrometry Analysis (GC-MS)

Ethanolic extract of leaves of *H. suaveolens* were subjected to GC-MS analysis. Extracts were dissolved in high-performance liquid chromatography (HPLC)-grade ethanol and subjected to JEOL GCMATE II GC-MS (Agilent Technologies 6890N Network GC system for gas chromatography). Helium was used as the carrier gas at a flow rate of 1mL/min. The temperature was programmed at 80°C for 5 min then increased to 300°C at the rate of 15°C/min. The temperatures of injector and EI detector (70 eV) were 280 and 300°C, respectively; 2 µL of plant extract was injected with a Hamilton syringe into the GC/MS manually.
3.11.1. Identification of Components

Interpretation of mass spectrum obtained from GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectra of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

3.12. Antioxidant Potential

The antioxidant activity of ethanolic leaf extracts of *H. suaveolens* was determined by *in vitro* methods. 2,2-diphenyl-1-picrylhydrazyl free radical scavenging, hydroxyl radical scavenging, superoxide radical scavenging and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) free scavenging assay methods were employed to assess the antioxidant potential of the extracts. All the assays were carried out in triplicate.

3.12.1. DPPH radical-scavenging activity

The antioxidant activity of the sample was determined in terms of hydrogen-donating or radical-scavenging ability, using the stable radical DPPH, according to the method of Blois (1958). The sample extracts were taken at various concentrations (20-100 µg) and the volume was adjusted to 100 µL with methanol. Five millilitres of 0.1 mM methanolic solution of DPPH was added and allowed to stand for 20 min at 27°C. The absorbance of the sample was measured at 517 nm. Percentage radical-scavenging activity of the sample was calculated as follows:

$$\% \text{ DPPH radical-scavenging activity} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100$$
The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC$_{50}$) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

### 3.12.2. Superoxide radical-scavenging activity

Superoxide radicals were generated by a modified method of Beauchamp and Fridovich (1971). The assay was based on the capacity of the sample to inhibit farmazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system. Each 3 mL reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 20 mg riboflavin, 12 mM EDTA, 0.1 mg NBT and various concentrations (20-100 µg) of sample extracts. Reaction was started by illuminating the reaction mixture with sample extract for 90 s. Immediately after illumination the absorbance was measured at 590 nm. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes with reaction mixture kept under dark condition served as blank. The percentage inhibition of superoxide anion generation was calculated as:

\[
\text{% Inhibition} = \left( \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \right) \times 100
\]

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC$_{50}$) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

### 3.12.3. Hydroxyl radical-scavenging activity

The scavenging activity of the sample on hydroxyl radicals was measured according to the method of Klein et al. (1991). Different concentrations of the extract
(20-100 μg) were added 1 mL of iron-ethylenediamine tetraaceticacid (EDTA) solution (0.13% ferrous ammonium sulphate and 0.26% EDTA), 0.5 mL of EDTA solution (0.018%), and 1 mL of dimethyl sulfoxide (DMSO) (0.85% v/v in 0.1 M phosphate buffer, pH 7.4). The reaction was initiated by adding 0.5 mL of ascorbic acid (0.22%) and incubated at 80-90°C for 15 min in a water bath. After incubation the reaction was terminated by the addition of 1 mL of ice-cold trichloroacetic acid (TCA) (17.5% w/v). Three millilitres of Nash reagent (75.0 g of ammonium acetate, 3 mL of glacial acetic acid, and 2 mL of acetyl acetone were mixed and the volume made up to 1 L with distilled water) was added and the mixture kept undisturbed at room temperature for 15 min. The intensity of the colour formed was measured spectrophotometrically at 412 nm against reagent blank. The % hydroxyl radical-scavenging activity (HRSA) was calculated as follows:

\[
\% \text{ HRSA} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100
\]

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

### 3.12.4. ABTS radical-scavenging activity

The antioxidant activity of the samples was measured by ABTS radical cation decolorization assay according to the method of Re et al. (1999). ABTS was produced by the reaction of 7 mM ABTS aqueous solution with 2.4 mM potassium persulfate under dark condition for 12–16 h at room temperature. Prior to assay, this solution was diluted in ethanol (about 1:89 v/v) and equilibrated at 30°C to give an absorbance
at 734 nm of 0.700±0.02. The stock solutions of the sample extracts were diluted such that after introduction of 10 μL aliquots into the assay, they produced between 20% and 80% inhibition of the blank absorbance. After the addition of 1 mL of diluted ABTS solution to 10 μL of sample (10–100 μg/mL), absorbance was measured at 734 nm exactly 30 min after the initial mixing. Samples were analyzed in triplicate. Percentage radical-scavenging activity of the sample was calculated as follows:

\[
\% \text{ ABTS radical-scavenging activity} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100
\]

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC\text{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

### 3.13. Antihaemolytic Activity (Naim et al., 1976)

Antihaemolytic activity of the sample extract was assessed by the method described by Naim et al. (1976). Blood samples were collected from rats under mild anaesthesia via cardiac puncture method into EDTA-coated tubes. The erythrocytes were separated by centrifugation and washed with 0.2 M phosphate buffer (pH 7.4). The erythrocytes were then diluted with phosphate-buffered saline to give 4% suspension; 250 μg of the sample extract in saline buffer was added to 2 mL of the erythrocyte suspension and the volume was made up to 5 mL with saline buffer. The mixture was incubated for 5 min at room temperature and then 0.5 mL of H\text{2}O\text{2} solution in saline buffer was added to induce the oxidative degradation of the membrane lipids. The concentration of H\text{2}O\text{2} in reaction mixture was adjusted to bring about 90% haemolysis of blood cells after 120 min. After incubation the reaction mixture was centrifuged at 1500 rpm for 10 min and the extent of haemolysis was
determined by measuring the absorbance at 540 nm corresponding to haemoglobin liberation. The analysis was performed in triplicates and results were expressed in terms of percentage activity.

3.14. **In vitro Antidiabetic Activity**

3.14.1. \(\alpha\)-amylase inhibiting activity (Miller, 1959)

The \(\alpha\)-amylase (0.5 mg/mL) was premixed with the extract at various concentrations (100-500 \(\mu\)g/mL) and starch was added as a substrate as 0.5% solution to start the reaction. The reaction was carried out at 37°C for 5 min and terminated by adding 2 mL of 3,5-dinitrosalicylic acid (DNS). The reaction mixture was heated for 15 min at 100°C and diluted with 10 ml of distilled water in an ice bath. \(\alpha\)-amylase activity was determined by measuring spectrum at 540 nm. The % \(\alpha\)-amylase inhibitory activity was calculated by the following formula:

\[
\text{% Inhibition} = \left( \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \right) \times 100
\]

The IC\(_{50}\) value was defined as the concentration of the sample extract to inhibit 50% of \(\alpha\)-amylase activity under assay condition.

3.14.2. \(\alpha\)-glucosidase inhibiting activity (Miller, 1959)

The enzyme \(\alpha\)-glucosidase inhibitory activity was determined by premixing \(\alpha\)-glucosidase (0.07 Units) with 100-500 \(\mu\)g/mL of extract. Then 3 mM \(p\)-nitrophenyl glucopyranoside was added as a substrate. This reaction mixture was incubated at 37°C for 30 min and the reaction was terminated by addition of 2 mL of sodium carbonate. The glucosidase activity was determined by measuring the \(p\)-nitrophenyl
release from p-nitrophenyl glucopyranoside at 400 nm. The % α-glucosidase inhibitory activity is calculated by the following formula:

\[
\% \text{ Inhibition} = \left( \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \right) \times 100
\]

The IC\textsubscript{50} value was defined as the concentration of the sample extract to inhibit 50% of α-glucosidase activity under assay condition.

3.15. Anticancer Activity
3.15.1. In vitro assay for cytotoxicity activity (MTT assay)
3.15.1.1. Cell line

Cell lines were obtained from National Centre for Cell Sciences (NCCS), Pune. The cells were maintained in minimal essential medium (MEM) supplemented with 10% foetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL) in a humidified atmosphere of 50% CO\textsubscript{2} at 37°C.

3.15.1.2. Reagents

MEM, foetal bovine serum (FBS), trypsin, methylthiazolyl diphenyl-tetrazolium bromide (MTT), and MCF 7 Dimethyl sulfoxide (DMSO) were purchased from Hi- Media & Sigma Aldrich, Mumbai.

The anticancer activity of samples on MCF 7 was determined by the MTT assay (Mosmann \textit{et al.}, 1983). Cells (1 × 10\textsuperscript{5}/well) were plated in 0.2 mL of medium/well in 96-well plates. Then the plates were incubated 5% CO\textsubscript{2} incubator for 72 h. Then, various concentrations of the samples were added in 0.1% DMSO for 24 h 5% CO\textsubscript{2} incubator. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 20 μL/well (5 mg/mL) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-
2,5-diphenyl-tetrazolium bromide (MTT) in phosphate-buffered saline solution was added. After 4 h of incubation, 1 mL of DMSO was added. Viable cells were determined by the absorbance at 540 nm. Measurements were performed and the concentration required for a 50% inhibition of viability (IC\textsubscript{50}) was determined graphically. The effect of the samples on the proliferation of MCF7 cells was expressed as the % cell viability, using the following formula:

\[
\% \text{Cell viability} = \frac{A540 \text{of treated cells}}{A540 \text{of control cells}} \times 100\%
\]

3.16. Determination of Antibacterial Activity

3.16.1. Bacterial strains

Four Gram-positive (\textit{Actinomyces howellii} MTCC-3048, \textit{Bacillus circulans} MTCC-9720, \textit{Staphylococcus aureus} MTCC-3160 and \textit{Streptococcus pyogenes} MTCC-1927) and three Gram-negative (\textit{Escherichia coli} MTCC-9721, \textit{Pseudomonas aeruginosa} MTCC-1688 and \textit{Proteus vulgaris} MTCC-7299) bacteria were collected from the Institute of Microbial Technology (IMTECH), Chandigarh, India. The cultures of bacteria were maintained in their appropriate agar slants at 4°C throughout the study and used as stock cultures.

3.16.2. Preparation of standard inoculums, filter paper discs and plates

The microorganisms were inoculated into Muller Hinton broth (MHB) and incubated at 37°C for 24h. The resulting suspension was diluted with MHB to match with 1 McFarland turbidity standard. This level of turbidity is equivalent to approximately 3.0×10\textsuperscript{8} CFU/mL, equivalent to 0.5 McFarland standards. The dried plant extracts were dissolved in 10% aqueous dimethylsulfoxide (DMSO) to a final concentration of 200 mg/mL and sterilized by filtration. Antimicrobial tests were then
carried out by the disc diffusion method using inoculums containing $10^6$ bacterial cells to spread on Muller-Hinton agar plates (1 mL inoculum/plate). The discs (diameter 6 mm) were impregnated with 50 µL of extract (10 mg/disc) at a concentration of 200 mg/mL.

3.16.3. Screening for antimicrobial activities

Antibacterial activities of leaf extracts of *H. suaveolens* were carried out by disc diffusion method using the Kirby-Bauer technique (Bauer et al., 1966). All the bacterial strains were maintained on nutrient agar (NA). Pure culture from the plate were inoculated into Mueller Hinton Agar (MHA) plate and subcultured at 37°C for 24 h. Standardized inoculum was transferred and spread evenly on an MHA plate to yield a lawn culture. Sterile Whatmann No. 1 filter paper discs impregnated with plant extracts (50 µg/disc) were placed on the inoculated MHA plates and allowed to diffuse for 30 min at 4°C and incubated at 37°C for 24 h. Kanamycin (10 µg) served as positive control. The plates were observed for the presence of inhibition of bacterial growth that was indicated by the clear zone around the disc. The size of the zone of inhibition (excluding disc) was measured in millimetres. The absence of zone inhibition was interpreted as the absence of activity. All experiments were carried out in triplicates under strict aseptic conditions and the zone of inhibition around each disc was measured for sensitivity or resistance. The antibacterial activity was expressed as the mean zone of inhibition diameters (mm) ± standard deviation (S.D) produced by the plant extract.
3.16.4. Determination of minimal inhibitory concentration (MIC)

The MIC of the crude extract was determined for each of the test strains in test tubes (Eloff, 2004); 0.5 mL of each of the test isolate was added to different concentrations of the leaf extracts (ranging from 5 to 50 µL/mL) containing 2 mL of nutrient broth. Similar tubes without leaf extract served as control. The cultures were then incubated at 37°C for 24 h. After incubation the tubes were examined for microbial growth by observing the turbidity. The tubes containing the least concentration of extract showing no visible sign of growth was considered as MIC.

3.16.5. Determination of minimum bacterial concentration (MBC)

To determine the MBC, for each of the test isolate 1 mL of the broth was collected from the tubes that showed no growth and inoculated into sterile nutrient agar. The plates were then incubated at 37°C for 24 h. After incubation the concentration that showed no visible growth was considered as the MBC.

3.17. Larvicidal Activity

3.17.1. Collection of eggs and maintenance of larvae

The eggs of *Culex quinquefasciatus* and *Aedes aegypti* were collected from the Centre for Research in Medical Entomology, ICMR, Madurai, Tamilnadu, India. These eggs were brought to the laboratory and transferred to 18×13×4 cm enamel trays containing 500 mL of water for hatching. The mosquito larvae were fed with dog biscuits and yeasts at the ratio of 3:1. The feeding was continued until the larvae transformed into third instar stage.
3.17.2. Larvicidal bioassay

Three trials were carried out against *Aedes aegypti* and *Culex quinquefasciatus* (Tonk *et al.*, 2006). Toxicity assays of the crude extract was conducted separately using the third instar larvae of *Ae. aegypti* and *C. quinquefasciatus*. Stock solution (1000 ppm) was prepared by dissolving 100 mg of crude extract in 1 mL acetone and volume raised to 100 mL with distilled water. From this, different dilutions of 10-100 ppm were prepared in 200 mL deionized water in 250 mL beakers and 20 third instar larvae were released in it and mortality was scored after 24 h. The beakers were kept in room temperature and the larvae exposed to 200 mL water containing 0.1 mL of dimethylsulphoxide (DMSO) served as control. The larvae in each solution were then left for 24 h and the number of dead larvae was counted after, and the percentage mortality was calculated by Abbott’s (1925) formula:

\[
% \text{ mortality} = \frac{\% \text{ test mortality} - \% \text{ control mortality}}{100 - \% \text{ control mortality}} \times 100
\]

3.18. Anatomical Studies

3.18.1. Sample preparation

Fresh healthy *H. suaveolens* plants were collected from South Thamaraikulum, Kanyakumari District, Tamilnadu, India. The required samples of different organs were cut and removed from the plant and fixed in FAA (Formalin-5 mL + Acetic acid-5 mL + 70% Ethyl alcohol-90 mL). After 24 h of fixing, the specimens were dehydrated with graded series of tertiary tutyl alcohol as per Sass (1940). Infiltration of the specimens was carried by gradual addition of paraffin wax (melting point 58-60°C) until TBA solution attained supersaturation. The specimens were cast into paraffin blocks.
3.18.2. Sectioning

The paraffin-embedded specimens were sectioned with the help of a rotary microtome. The thickness of the sections was 10-12 μm. Dewaxing of the sections was by customary procedure (Johansen, 1940). The sections were stained with Toluidine blue as per the method published by O’Brien et al. (1964). Since toluidine blue is a polychromatic stain, the staining results were remarkably good; and some cytochemical reactions were also noticed. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc. Sections were also stained with safranin and Fast-green and IKI (for Starch) wherever necessary.

For studying the stomatal morphology, venation pattern and trichome distribution, paradermal sections (sections taken parallel to the surface of leaf) as well as clearing of leaf with 5% sodium hydroxide or epidermal peeling by partial maceration employing Jeffrey’s maceration fluid (Sass, 1940) were prepared. Glycerine mounted temporary preparations were made for macerated/cleared materials. Powdered materials of different parts were cleared with Naoh and mounted in glycerine medium after staining. Different cell component were studied and measured.

3.18.3. Photomicrographs

Microscopic descriptions of tissues are supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nikon labphoto 2 microscopic Unit. For normal observations bright field was used. For the study of crystals, starch grains and lignified cells, polarized light was employed. Since
these structures have birefringent property, under polarized light they appear bright against dark background. Magnifications of the figures are indicated by the scale-bars. Descriptive terms of the anatomical features are as given in the standard Anatomy books (Easu, 1964).

3.19. Statistical Analysis

The experimental data were expressed as mean ± Standard Deviation (SD). The percent mortality values for the third instar larvae *Aedes aegypti* and *Culex quinquefasciatus* treated with various concentrations (ranging from 10 to 100 ppm) of the leaf extract of *Hyptis suaveolens* was recorded and the percentage mortality was calculated and the data was analyzed using curve expert software for finding the LC$_{50}$, LC$_{90}$ and LC$_{95}$ values. The third degree polynomial fit was used as a suitable mathematic model in the curve expert software.