Chapter - 3

MATERIALS AND METHODS

3.1 Materials

The following materials were used for the study.

3.1.1 Equipments

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Equipments</th>
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<tr>
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<td>Olympus</td>
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<td>3</td>
<td>Hot air oven</td>
<td>Satyam</td>
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<td>4</td>
<td>Incubator</td>
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<td>5</td>
<td>Mechanical Grinder</td>
<td>Remi</td>
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<td>Research Centrifuge</td>
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<td>Spectrophotometer V-630</td>
<td>Jasco</td>
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3.1.2 Chemicals

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3.1.3 Plant Material

<table>
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<tr>
<td>1</td>
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<td>Sundarban area, South 24 Parganas, West Bengal, India.</td>
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<tr>
<td></td>
<td>Blume (family-</td>
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<td></td>
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<tr>
<td></td>
<td>Acanthaceae)</td>
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3.1.4 Standard drug

The standard drug, Ethinyl estradiol was obtained from Organon (India) Ltd., kolkatta, West Bengal, India for the evaluation of antifertility activity. The drugs, Diazepam, Pentazocine were obtained from Ranbaxy Laboratories Ltd, Indore, Aspirin
was obtained from Reckitt Benckiser Health care Ltd, Baddi and Albendazole was obtained from Intas Pharmaceutical Ltd, Ahmedabad respectively.

3.2 Animals

Wistar strain, colony-bred virgin female albino rats (150-200gms) were used for antimplantation activity, estrous cycle study and Colony-bred immature female albino rats (Wister strain), 21-23 days old, weighing between 30-45 grams was used for estrogenic activity study. The albino rats (150-200 g) were used for muscle relaxant and antipyretic activity. Swiss albino Mice (weight 25-30 g) were used for analgesic activity. All the animals were provided with rodent diet and water *ad libitum* in animal house. The temperature was 23± 2°C and humidity was 50±5%.

3.2.1 Ethical issues

All animal experimental protocol was approved by Institutional Animal Ethics Committee, School of Pharmaceutical Sciences, Siksha ‘O’ Anusandhan University, Odisha, India. (Registration no-1171/c/08/CPCSEA)

3.3 Preparation of drug

3.3.1 Collection of Plant materials

The fresh plant, *Avicennia alba* Blume (aerial parts) belonging to the family- Acanthaceae was collected from Sundarban area, South 24 Parganas, West Bengal, India in month of October, 2011

3.3.2 Plant Authentication

The plant was identified & authenticated by the taxonomist Dr. K. Karthigeyan, Central National Herbarium, from the botanical survey of India, Botanical Garden, Howrah, West Bengal, India. A voucher specimen of the plant *Avicennia alba* Blume (CNH/128/2011/TECHII/637/DRK-01) has been deposited in the herbarium of the Department of Pharmacognosy, School of Pharmaceutical Sciences, Siksha ‘O’ Anusandhan University, Odisha, India.
3.3.3 Preparation of plant extract

The aerial parts (stems, leaves, fruits and flowers- 4:3:2:1) of plant *Avicennia alba* Blume were air dried, pulverized to a coarse powder in a mechanical grinder and successively extracted in a Soxhlet apparatus with petroleum ether followed by methanol (2.5L) for 18 hours. The petroleum ether was used for removal of fatty materials. The methanolic extract was concentrated to dryness in a Rota Evaporator (Buchi type) under reduced pressure and controlled temperature at 50-55°C. The dry extract was preserved in a refrigerator. A suspension of the dry extract was prepared in distilled water using Tween- 80 (2% w/v) and was taken for different pharmacological studies. Further, the methanolic extracts were subjected for isolation and characterization.

3.3.4 Fractionation of the extract

The extract was dissolved in distilled water and was fractionated by using different polarity based solvent such as n-hexane, chloroform and ethyl acetate successively and these solvent fractions were collected and concentrated using Rota Evaporator. The crude methanolic extract and its various fractions were investigated for antimicrobial study using standard method.

3.4 Acute oral toxicity test

The acute oral toxicity test was carried out as per the guidelines 420, set by Organization for Economic Co-operation and Development (OECD) after approval from Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Govt. of India.

**Principle**

It is a stepwise procedure. Fixed doses of 5, 50, 300 and 2000 mg/kg are considered. The initial dose for the main study is selected on the basis of sighting study.
Description of the method

- **Selection of animal species:** Healthy young wistar albino rats of female weighing between 150-200 grams were used for acute toxicity study of methanolic extracts.

- **Housing and Feeding condition:** The temperature was 23± 2°C and humidity was 50± 5% with 12 hr dark, 12 hr light cycle. The conventional laboratory diet was fed, with drinking water *ad libitum*.

- **Preparation of animals:** These animals were randomly selected, marked to permit individual identification and kept in their cages for five days prior to experiment to allow for acclimatization to the laboratory condition.

- **Preparation of the dose:** The extracts were suspended in 2% Tween-80 in water.

Procedure

Dose administration

First animals are given single dose orally.

Sighting study

One animal is administered one fixed dose and observed for 14 days. After 24 hours of one dose next animal can be administered with higher dose. The outcome of sighting study can be of 3 types (A) Death, (B) Evident toxicity, and (C) No toxicity. The dose which causes evident toxicity is selected for the main study.

Main study

Additional 4 animals of one sex are used in the main study. Now the outcome from 5 animals can be divided into 3 categories (A) ≥ 2Death, (B) ≥ 1 with evident toxicity and/ or <1 Death and (C) No toxicity. If there is no toxicity in all the animals at 2000 mg/kg then another dose of 5000mg/kg may be used. However if all the five
animals do not show any toxicity at 2000mg/kg then the test drug may be classified as class\textsubscript{5}/unclassified.

**Observations**

Observations like behavioral change, change in skin, fur, eyes, mucous membranes, circulatory system, central nervous system, autonomic and somato motor activity and behaviour patterns were reported. Attention was also given to observation of convulsions and tremors.

**3.5 Evaluation of Antifertility Activity**

**3.5.1 Anti-implantation activity study**

Antifertility activity was determined as per method described by Khanna and Chowdhury.\textsuperscript{176} Female albino rats (150 -200 g) of the normal estrous cycle were maintained and provided with food and water \textit{ad libitum}. The females of proven fertility were left overnight with proven fertile male albino rats with 3:1 ratio at early estrous stage of estrous cycle. Then vaginal smear of females were observed for the presence of thick clumps of spermatozoa. Subsequent day was selected as day one of pregnancy. Such pregnant females were grouped into five groups of six animals each for each dose of the plant extract.

**Group I:** Served as positive control, received vehicle (2\% Tween-80 in distilled water) orally for one to seven days of pregnancy.

**Group II:** Received Ethinyl estradiol as standard at the dose of 0.45 mg/kg body weight p.o. for one to seven days of pregnancy.

**Group III:** Received suspension of plant extract 100 mg /kg body weight p.o. for one to seven days of pregnancy.

**Group IV:** Received suspension of plant extract 200 mg /kg body weight p.o. for one to seven days of pregnancy.
**Group V**: Received suspension of plant extract 400 mg/kg body weight p.o. for one to seven days of pregnancy.

On the day 10, laparotomy was performed under light ether anaesthesia using sterile condition. The uteri were examined to determine the number of implantation sites, number of corpora lutea in the ovary and number of resorption sites. The abdominal wound was sutured with sterile sutures in aseptic condition and the rats were allowed to go to term.

The % of antiimplantion and early abortifacient activity were calculated. The summation of antiimplantion and early abortifacient activity gives % of antifertility activity of the tested materials. The calculation formulas are given below.\(^\text{177}\)

\[
\% \text{ of antiimplantion activity} = 100 - \left(\frac{\text{No of implantation}}{\text{No of corpora luteum}}\right) \times 100
\]

\[
\% \text{ abortifacient activity} = \left(\frac{\text{No of resorption}}{\text{No of corpora luteum}}\right) \times 100
\]

\[
\% \text{ Total antifertility activity} = \% \text{ of antiimplantion activity} + \% \text{ abortifacient activity}.
\]

**3.5.2 Estrous cycle study**\(^\text{178,179}\)

Colony bred female albino rats with normal estrous cycles were selected for the estrous cycle study. The vaginal smear and the duration of estrous cycle of various phases were employed. A normal estrous cycle in rats normally occurs 4-5 days. The estrous cycle stages are (i) estrous (cornified epithelial cells), (ii) metaestrus (cornified cells plus leucocytes), (iii) diestrus (leucocytes) and (iv) proestrus (epithelial cells). The selected animals were divided into three groups containing six animals in each group.

**Group I**: Received (2% tween-80 in distilled water) orally for three consecutive estrous cycles.

**Group II**: Received methanolic extract at dose of 200mg/kg body weight orally for three consecutive estrous cycles.
Group II: Received methanolic extract at dose of 400mg /kg body weight orally for three consecutive estrous cycles.

Vaginal smears from the above animals were observed every day morning and the duration of each stages of the cycle noted.

3.5.3 Estrogenic activity study

For estrogenic activity study, the uterine weight and vaginal cornification method were employed.

Immature ovariectomised female albino rats (25-30 days old) will be divided into 4 groups (6 in each) and treated for 7 days by oral administration as follows:

Group I: Received the vehicles (Tween-80, 1%)

Group II: Received a suspension of Ethinyl estradiol at a dose of 0.02mg/kg body weight

Group III: Received the methanol extract at 400 mg/kg body weight

Group IV: Received combination of methanol extract and Ethinyl estradiol (400 mg/kg body weight +0.02mg/kg body weight).

On the 8th day, all the animals were sacrificed and decapitated under light ether anesthesia and the uteri were dissected out, surrounding tissues were removed, filter paper was used for blotting the tissues and weighed quickly on a sensitive balance.

A portion of uterine tissues from control and treated animals were fixed in Bouins fluid for 24 hours, dehydrated with alcohol and then embedded in paraffin. The paraffin block sections were cut at 6μm and stained with haematoxylin-eosin for histological study of uterus. The diameter of uterus, thickness of endometrium and height of the endometrial epithelium were measured by using a calibrated occular micrometer.
3.5.4 Biochemical study

The other portion of uterus was homogenized with ice-cold distilled water in a pre-cooled mortar and pestle that contain 10 mg of tissue/ml. The homogenated fluid was centrifuged at 3000 rpm for 15 minutes and the supernatant was used for the estimation of total protein and cholesterol using standard method.

(a) Total Protein Estimation by Lowry’s Method\textsuperscript{181,182}

Principle:

The phenolic group of tyrosine and trytophan residues which produce a blue purple color with addition of Folin- Ciocalteu reagent at 660 nm wavelength. Thus the intensity of color depends on the aromatic amino acids present in it. Bovin Serum Albumin (BSA) is universally accepted as a standard protein because of high purity, low cost and availability. The pH range adjusted at 9 -10.5.

Reagents Required:

1. Bovin Serum Albumin (BSA) solution (1mg/ml).
2. Folin - Ciocalteu reagent solution (2 ml of commercial reagent + 2 ml distilled water)
3. Analytical reagents
   
   (a) 50 ml of sodium carbonate (2%) mixed with 50 ml of 0.1 N NaOH solutions (0.4 gm in 100 ml distilled water.)
   
   (b) 10 ml of copper sulphate solution (1.56%) mixed with 10 ml of sodium potassium tartarate solution (2.37%).

They were prepared by mixing 100 ml of (a) with 2 ml of (b).

Procedure:

1. BSA solutions are prepared by different dilution with mixing stock BSA solution (1 mg/ ml) and volume made up in each test tube is 5 ml by adding distilled water.
2. 0.2 ml protein solution is pipetted out to different test tubes and 2 ml of alkaline copper sulphate solution is added and they are mixed vigorously.

3. They are incubated for 10 mins at room temperature.

4. After that to each test tube 0.2 ml of reagent Folin-Ciocalteau solution is poured and kept for 30 mins.

5. The absorbance of solution is measured at 660nm.

6. The concentration against absorbance is plotted to get a standard calibration curve.

(b) Determination of Cholesterol by Libermann-Burchard Method

PRINCIPLE:

Cholesterol is the most abundant sterol in animal tissue. This test is based on the fact that the sterols with unsaturation in the ring A or B react with sulphuric acid in the presence of acetic anhydride to give blue colour which immediately changes to green. During the reaction, dehydration, condensation and isomerization take place with the formation of halochromic salt which is green in colour. The exact nature of the chromophore is not known but reaction probably includes esterification of the hydroxyl group at 3- position and sterols become activated and fleeting colours are produced due to shift in double bond.

\[
\text{Cholesterol esters} + \text{H}_2\text{O} \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{FA} \\
\text{Cholesterol} + \text{O}_2 \xrightarrow{\text{Cholesterol Oxidase}} \text{Cholesterol-3-one} + \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 + 4\text{-AAP} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Quinonimine}
\]

Reagents Required:

1. Cholesterol in chloroform (100 microgram/ml).

2. Acetic anhydride-sulphuric acid mixture (30:1, v/v).

3. Chloroform
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Procedure:

1. For preparation of standard curve, solution containing 10-100 microgram Cholesterol is pipette out in different test tubes and the volume is made to 1 ml with chloroform.

2. A suitable volume of the tissue extract in which Cholesterol has to be estimated is taken and its volume is made upto 1ml.

3. 5 ml of acetic anhydride-sulphuric acid mixture is added carefully to each test tube, mixed well and noted the change in colour of the solution. The test tubes are covered and left them in dark for 15 minutes.

4. The absorbance of the solution is measured at 640nm and the standard curve of Cholesterol is drawn.

From the standard curve, the amount of Cholesterol present in 1 ml of the sample preparation is calculated.

3.5.5 Histology study

A portion of ovary tissues from control and treated animals were fixed in Bouins fluid for 24 hours, dehydrated with alcohol and then embedded in paraffin. The paraffin block sections were cut at 6μm and stained with haematoxylin-eosin for histological study of ovary.\textsuperscript{186}

3.6 Analgesic activity study

3.6.1 Tail Flick method

Mice (weight 25-30 g) were divided into 4 groups of 6 each. The tail of the mouse was placed on the nichrome wire of analgesiometer and the time taken by the animal to flick (withdraw) its tail from the hot wire was taken as the basal reaction time. Then they are divided into 4 group’s i.e. Control, standard, test-I and test –II. The control group is administered with normal saline (10 ml /kg). The standard group was administered pentazocine 10mg/kg body weight by i.p. route. The ‘test’ groups are
administered with 100mg/kg body weight and 200mg/kg body weight of methanolic extract of crude drug. The Reaction times were measured in 30 minute intervals for 180 minutes. The latency time for all groups was recorded at 0, 30, 60, 90, 120, 150 and 180 min.  

3.6.2 Tail immersion method

The analgesic activity assessment was done by tail immersion method. Mice (weight 25-30 g) were divided into four groups each of six animals. Water is heated in a beaker by a hot plate up to 55-58°C. Tails of all mice are immersed in the water and the time of the tail removing reflex is noted before the administration of the drug. This is the basal reaction time of the animal. The time in second for tail withdrawal from the water was taken as the reaction time, with a cut-off time of immersion set at 10 second. The animals in the ‘control’ group are administered normal saline (10 ml /kg), the ‘standard’ group animals are administered with standard analgesic drug, pentazocine 10mg/kg body weight by i.p. route. Test animals are administered with methanolic extract of 100 mg/kg body weight and 200 mg/kg body weight respectively. Their reaction times are noted every 30 minute interval for 180 minutes.

3.7 Anti-pyretic Activity

The antipyretic activity was assessed by using brewer’s yeast induced pyrexia method. Pyrexia was induced by injecting 10ml/kg of 20% w/v suspension of brewer’s yeast in normal saline subcutaneously 18 hours before start of the experiment. Only rats whose rectal temperature increased by at least 1.0°C after 18 hours of induced yeast injection were included in this study. The normal body temperature of each rat was measured by using Digital Telethermometer. The experimental rats were divided into four groups containing six animals each. The Group A control was orally administered saline (10 ml /kg) while the Group B standard was given 100mg/kg Aspirin and Group C and Group D were administered 100 mg and 200 mg/kg body weight of methanolic extract of test drug. The rectal temperature was recorded at time intervals of 0, 30, 60, 90 and 120 min after treatment.
3.8 Muscle relaxant activity

Rota-rod test:

Wistar rats (120-150 gm) were placed on a horizontal wooden rod (32 mm diameter) rotating at a speed 20 rpm. The rats capable of remaining on top for 3-5 minutes in three successive trials were selected for this study. The selected animals were divided into 3 groups each group contains 6 animals. Group I served as control and received the vehicles (Tween-80, 1%) orally. Group II received diazepam at a dose of 5mg/kg by i.p. as standard drug and Group III received test drug at a dose 200 mg/kg body weight orally. Each animal was then placed on the rod and the fall off time was noted.¹⁹⁰

3.9 Antioxidant activity

A. DPPH scavenging assay

This assay was carried out as per reported method¹⁹¹ with slight modifications. The DPPH scavenging effect of different concentration of methanolic extract of *Avicenna alba* Blume was compared with standard antioxidant, Ascorbic acid. 1.5ml of 1mM methanolic DPPH solution was mixed with 1.5 ml of various concentrations (20-100 μg/ml) of the extract. The mixture was kept in dark place at room temperature and the absorbance was recorded using a spectrophotometer at 517 nm after 30 min. The experiment was replicated in three independent assays. Ascorbic acid was used as reference standard. The inhibition curve was plotted and IC₅₀ values were calculated.

The % of inhibition was determined by using following formula:

\[ \% \text{ Inhibition} = \left( \frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}} \right) \times 100 \]

B. Superoxide scavenging assay

The superoxide scavenging activity of methanolic extract was done by the reported method.¹⁹² About 1 ml of nitroblue tetrazolium (156 μM NBT in 100 mM phosphate buffer), 1 ml NADH solution (468 μM in 100 mM phosphate buffer) and 0.1
ml of sample solution mixed with 0.1 ml of phenazine methosulphate solution (60 μM PMS in 100 mM phosphate buffer). Then the pH of the assay mixture was adjusted to 7 and was incubated at 25 °C for 5 min. The absorbance of the test sample was measured against blank at 560 nm. Quercetin was taken as standard. Decrease in absorbance of the reaction mixture, indicated increased superoxide anion scavenging activity. The % inhibition was calculated by using following equation:

\[
\% \text{ Inhibition} = \left[ \frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}} \right] \times 100.
\]

**C. Nitric oxide scavenging assay**

Nitric oxide scavenging activity was measured by the standard spectrophotometric method with little modifications. The aqueous solution of sodium nitroprusside generates nitric oxide at its physiological pH and was evaluated by the Griess reagent. The reaction mixture was prepared using 1 ml of sodium nitroprusside (10 mM), 1.5 ml of phosphate buffer (0.2 M, pH 7.4) and 0.5 ml of sample solution, and was incubated at 25 °C for 150 min. After incubation, 1 ml of the reaction mixture was mixed with 1 ml of Griess reagent, which contains 1% sulphanilamide, 2% H₃PO₄ and 0.1% naphthyl ethylenediamine dihydrochloride. Then the absorbance was measured at 546 nm against the blank. Quercetin was used as reference standard. The % inhibition was calculated by using following equation:

\[
\% \text{ Inhibition} = \left[ \frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}} \right] \times 100.
\]

**D. Hydroxyl radical scavenging assay**

The hydroxyl radical scavenging ability was determined using Fenton reaction method. Fenton reaction mixture containing 100 μl 2-deoxyribose (28 mM in 20 mM buffer, pH 7.4), 200 μl ferric chloride (1 mM), 200 μl EDTA (1.04 mM) and, 100 μl ascorbic acid (1 mM) and 100 μl H₂O₂ (1 mM) was added to methanolic extract of *Avicennia alba*. The assay mixture was incubated at 37 °C for 30 min and was added to 1 ml trichloroacetic acid (2.8%) and 1ml thiobarbituric acid (1%). The mixture was kept in water bath for 30 min and allowed to cool. The absorbance was measured at 532
nm against the blank solution. Ascorbic acid was used as standard. The % age of inhibition was calculated by using following equation:

\[
\% \text{ Inhibition} = \left( \frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}} \right) \times 100.
\]

### 3.10 Anthelmintic activity study

**Preparation of extract and standard Drug**

The suspension of methanolic extract of different concentrations (1, 2, 5, 10 and 50 mg/ml) were prepared by using 2% w/v of Tween 80 and final volume was adjusted upto 30 ml for respective concentration. Albendazole (20 mg/ml) was prepared by using suspending agent, CMC (0.5% w/v).

The anthelmintic activity was carried out as per reported method. Adult earthworms (*Pheretima posthuma*) were used to evaluate Anthelmintic activity. They were collected from moist soil and washed with water. The average size of earthworm was 5-7 cm. Earthworms were placed in Petridis containing five different concentrations (1, 2, 5, 10 and 50 mg/ml) of methanolic extract in addition to control and standard drug. Each Petridis containing 6 worms, observed for paralysis or death. The mean time for paralysis of worms was taken when there is no movement was observed except worms, which were shaken vigorously. The time of death of worms (min.) was noted when the worms did not move even after external stimuli. The test result was compared with standard drug.

### 3.11 Antibacterial activity study

The crude methanolic extract and its various fractions were investigated for antimicrobial study using standard method.

**Test microorganisms**

A total of eight bacterial strains were selected for evaluation of antibacterial activity of *Avicennia alba* Blume. Four Gram positive bacteria such as *Enterococcus faecalis* (MTCC-439), *Staphylococcus aureus* (MTCC-7443), *Staphylococcus*
saprophyticus (NA), Streptococcus Mutans (MTCC-890) and four Gram negative bacteria like Acinetobacter baumannii (MTCC-1425), Citrobacter freundii (MTCC1658), Enterobacter aerogenes (MTCC-2990) and Escherichia coli (MTCC-443) were used for this study. All the strains were collected from Department of Microbiology, Institute of Medical Sciences and Sum Hospital, Bhubaneswar, Odisha.

Method

(a) Antibacterial activity assay by agar-well diffusion method

The antibacterial activity of the methanolic extract and its various fractions were determined by agar-well diffusion method as reported earlier. The wells containing 30 min old bacterial lawn, were punched to 6 mm deep, and each well was based with 50 µl molten Muller-Hilton agar. One strain from each bacterial species showing resistance to a maximum number of antibiotics was further used for monitoring antibacterial potentiality of plant extract and its various fractions. Gentamicin (30 µg/ml) was used as standard drug. Further, punched wells were filled with 100 µl aliquots of 30 mg/ml plant extract and various fractions. Plates were incubated at 37°C for 24 h. The microbial growth was determined by measuring the diameter of zone of inhibition. The experiment was carried out three times.

(b) Determinations of MIC and MBC

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of the methanolic extract and its various fractions of Avicennia alba Blume were determined as described by the reported method. Original stock solutions of methanolic extract and its various fractions were prepared with methanol at the concentration of 44.44 mg/ml in 10% DMSO solution. Each stock solution was diluted to obtain final concentrations of 0.29, 0.67, 1.51, 3.41, 4.27, 9.63, 21.67 and 44.44 mg/ml with the DMSO solution. A separate experiment was conducted for methanolic extract and its each fraction. An aliquot of 80 µl of each dilution of extract and fractions was poured to a well of 96-welled (12 x 8) micro-titer plate, along with an aliquot of 100 µl MH broth and 20 µl bacterial inoculate (10⁹ CFU/ml), and 5 µl-aliquot of 0.5 % of 2,3,5-triphenyl tetrazolium chloride (TTC). The micro-plate was
then incubated at 37°C for 18 h. The development of pink colour due to TTC indicated bacterial growth and inhibition of bacterial growth is ascertained when no colour is developed. The first well of the micro plate was taken as control. The MIC value was considered at the well, where no colour was manifested. Further, bacteria from each well of the micro plate were sub-cultured on a nutrient agar plate and the MBC value was noted, where no bacterial growth was observed on the agar plate.

### 3.12 Statistical analysis

The data were analyzed by one-way Analysis of Variance (ANOVA) followed by Dunnet’s t test. All data are expressed as mean ± SD, *p* < 0.05 was considered as statistically significant.

### 3.13 Molecular docking studies

1. **Selection of important Estrogenic receptors**

   The Protein Data Bank was searched for human estrogenic receptors and five structures (1BHS, 1DHT, 1FDT, 1IOL and 3DHE) were reported. These structures were downloaded and analyzed, which revealed that structures 1BHS, 1DHT, 1IOL and 3DHE are similar and mostly complexed with ligand 5-alpha-dihydrotestosterone. Out of these four similar structures 1DHT was selected for docking studies. The active site of the receptor was characterized by selecting the ligand and selecting the residues around 5Å of it.

2. **Selection of suitable compounds**

   Two compounds such as Stigmasterol, Phytol were selected for docking studies in the active site of the estrogenic receptor. The structure files of these compounds were downloaded from Pub Chem in SDF format and energy minimized with Open Babel utility of PyRx in Universal force field. The optimization algorithm conjugate gradient was set for 200 total steps with 1 step for update. Then the compounds were converted to AutoDock ligands and converted to PDBQT format after addition of hydrogens and subsequent charge calculations.
3. Preparation of receptor and grid generation

The estrogenic receptor with PDB ID 1DHT was imported and prepared for docking. First the water molecules present in the structure were removed and both polar and nonpolar hydrogens were added. Then the Gestegier charge calculation was done for the receptor and converted to PDBQT format. A suitable receptor grid was generated by selecting the ligand and expanding the area up to 6Å around it. Thus the grid size was with center_x = 10.62, center_y = 8.80, center_z = -12.33 and size_x = 25.0, size_y = 25.0, size_z = 25.0 respectively.

The molecular docking was simulated by selecting the individual ligands and directing them in the prepared grid site. The ligands were made flexible so as to generate suitable conformations and the exhaustiveness was set 8. The result was saved with 9 conformation poses per ligand. The post docking analysis was done by selecting the best poses of each ligands and then modifying the area around 5Å around them.

3.14 Phytochemical Evaluation

3.14.1 Estimation of total phenolic content (TPC)

TPC of methanolic extract was evaluated by the method as described earlier with little modifications. The phenolic content of the methanolic extract was evaluated from a Gallic acid calibration curve. For the preparation of a calibration curve, 0.5 ml aliquots of 1.0, 2.5, 5.0, 10, 25, 50 and 100 µg/ml Gallic acid in methanol were mixed with 2.5 ml Folin–Ciocalteu reagent and 2.5 ml sodium carbonate (75 g/l). The assay mixture is incubated at 25 °C for 30 min, and the phenolic content was measured at 765 nm against blank by multi plate reader. All determinations were performed in triplicate. Total phenolic content was expressed as milligrams of Gallic acid equivalent (GAE) per gram of extract.

3.14.2 Estimation of total flavonoid content (TFC)

TFC was estimated by using aluminium chloride colorimetric method with suitable modifications. The flavonoid content of methanolic extracts was determined from a Quercetin calibration curve. 0.5 ml of aluminium chloride (2%) was added to
0.5 ml of ethanolic sample. A yellow colour indicates the presence of flavonoids. After incubation at room temperature for 1 hr, the flavonoid estimation was performed at 420 nm against reagent blank by multi plate reader (SynergyH1M, BioTek, USA). All determinations were performed in triplicate. Total flavonoid content was expressed as milligrams of Quercetin equivalent (QE) per gram of the extract.

3.14.3 GC-MS study

The GC–MS analysis of methanolic extract of *Avicennia alba* Blume was carried out using a Clarus 500 Perkin–Gas Chromatograph coupled with a mass detector, Turbo mass gold–Perkin Elmer Turbomass 5.1 spectrometer with 30m x 0.25 mm ID x 0.25μm of capillary column.

Injection temperature was adjusted at 250 °C and Helium flow rate was maintained as 1.5 ml/min with an ion source temperature of 230 °C. Injection volume of the sample was adjusted to 1 μl and instrument was set to an initial temperature of 70°C for 3 min.

The mass spectrum of the isolated compounds in sample was obtained by electron ionization (EI) at 70 eV and scan mode of the detector was operated from 40–700 m/z. The MS start time was 3 min, end time was 34 min with solvent cut time was of 3 min.

**Identification of compounds**

The chemical constituents of the methanolic extract of *Avicennia alba* Blume were recognized by matching their mass spectra with spectra of reference compounds in mass spectral library of National Institute of Standards and Technology (NIST 11). The name, structure and molecular weight of the components of the test materials were confirmed.
3.14.4 Isolation and characterization of compound

The concentrated methanolic extract of *Avicennia alba* Blume was dissolved in little quantity of methanol and adsorbed on silica gel (60–120 mesh) for preparation of slurry. The slurry was then air-dried and chromatographed over a silica gel (60–120 mesh) column. The column was eluted with n-hexane initially, then eluted with n-hexane-ethyl acetate mixtures of increasing polarity (95:5, 90:10, 80:20, 70:30, 60:40 and 50:50). Various fractions were collected separately and matched by TLC to check their homogeneity. The fractions with same $R_f$ values were combined together and crystallized. The compound was then recrystallized with methanol and finally purified by preparative TLC. The isolated compound was subjected to various physical and spectral studies for characterization.

The melting point was determined in open capillary tube (Sisco). The spectra were recorded with the following instruments, IR: Bruker -FTIR-8400S spectrophotometer using KBr powder; NMR: $^1$H NMR and $^{13}$C NMR spectra on Bruker DRX-500 NMR spectrometer using MeOD as the solvent at 500 MHz and 125 MHz respectively; GC-MS: Shimadzu-Mass spectrophotometer; TLC with silica gel GF$_{254}$; column chromatography silica gel (60-120 mesh, Merck) and elemental analysis: Perkin Elmer-2400 Auto system.