5.0. MATERIALS AND METHODS

5.1 Collection and characteristics of chosen halophytes

Five different species of seaweeds such as *Acanthophora specifera*, *Chaetomorpha indica*, *Gracilaria edulis*, *Sargassum wightii* and *Ulva lactuca* were collected from in and around Thondi coastal area and four different mangrove species *Avicennia marina*, *Ceriops decandra*, *Rhizophora mucronata*. One mangrove associate halophytes *Suaeda monoica* collected from Karankadu mangrove ecosystem and one mangrove species *Excoecaria agallocha* was collected from Pitchavaram mangrove ecosystem. The collected seaweeds and mangroves were identified by key reported by Umamaheswara Rao (1987) and keys of identification manual (Kathiressan, 2002).
5.2. Taxonomical position of chosen seaweeds

5.2.1 Acanthophora specifera (Borjesen)

Taxonomic position
Phylum - Rhodophyta
Class - Florideophyceae
Order - Ceramiales
Family - Rhodomelaceae
Genus - Acanthophora
Species - specifera

5.2.2. Chaetomorpha indica (Kutzing.)

Taxonomic position
Phylum - Chlorophyta
Class - Ulvophyceae
Order - Cladophorales
Family - Cladophoraceae
Genus - Chaetopmorpha
Species - indica
5.2.3 *Gracilaria edulis* (Gmelin.)

**Taxonomic position**

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Phylum</th>
<th>Class</th>
<th>Subclass</th>
<th>Order</th>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
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5.2.4 *Sargassum wightii* (C. Agardh)

**Taxonomic position**

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<thead>
<tr>
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<th>Class</th>
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<th>Species</th>
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<td>Fucales</td>
<td>Sargassaceae</td>
<td>Sargassum</td>
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</table>
5.2.5 Ulva lactuca

Taxonomic position

Phylum    -    Chlorophyta
Class     -    Ulvophyceae
Order     -    Ulvales
Family    -    Ulvaceae
Genus     -    Ulva
Species   -    lactuca

(In order to, the above mentioned images were taken from algalbase.com.)

5.3 Taxonomical position of chosen mangroves

5.3.1 Avicennia marina (Forsk.) Vierh.

Taxonomic position

Class     -    Dicotyledonae
Sub class -    Gamopetalae
Series    -    Bicarpellatae
Order     -    Labiales
Family    -    Avicenniaceae
Genus     -    Avicennia
Species   -    marina
5.3.2 *Ceriops decandra* (Griff.) Ding Hou

**Taxonomic position**

Class - Dicotyledonae
Sub class - Polypetalae
Series - Calyciflorae
Order - Myrtales
Family - Rhizophoraceae
Genus - *Ceriops*
Species - *decandra*

5.3.3 *Rhizophora mucronata* Lamk.

**Taxonomic position**

Class - Dicotyledonae
Sub class - Polypetalae
Series - Calyciflorae
Order - Myrtales
Family - Rhizophoraceae
Genus - *Rhizophora*
Species - *mucronata* Lamk.
5.3.4 *Excoecaria agallocha* L.

**Taxonomical Position**

<table>
<thead>
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<tr>
<td>Series</td>
<td>-</td>
<td>Curvembryae</td>
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<tr>
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</tr>
<tr>
<td>Genus</td>
<td>-</td>
<td><em>Excoecaria</em></td>
</tr>
<tr>
<td>Species</td>
<td>-</td>
<td><em>agallocha</em></td>
</tr>
</tbody>
</table>

5.3.5 *Suaeda monoica* *Forsk. Ex Gmel*

**Taxonomic position**

<table>
<thead>
<tr>
<th>Class</th>
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<td>Genus</td>
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<td><em>Suaeda</em></td>
</tr>
<tr>
<td>Species</td>
<td>-</td>
<td><em>monoica L.</em></td>
</tr>
</tbody>
</table>
5.4 Extraction of bioactive compounds from seaweeds and mangroves

Fresh seaweeds viz., _Acanthophora specifera, Chetomorpha indica, Gracillaria edulis, Sargassum wightii_ and _Ulva lactuca_. The mangroves viz., _Avicennia marina, Ceriops decandra, Exoecaria agallocha, Rhizophora mucronata_ and _Suaeda monoica_ were collected from Station 1 (Thondi), Station 2 (Karankadu) and Station 3 (Pichavaram). All the samples were collected by hand picking and without disturbing their own ecosystem and bring out to the laboratory, washed with running tap water to remove the adhering particles and other debris. Finally, it was kept in room temperature and subjected for shade drying. Dried samples were cut in small pieces using scissors. About 500 g of each dried materials were soaked in 500ml of benzene, ethanol and chloroform each separately for 10 days in dark condition in air tight clean glass containers using cold percolation method (Ravikumar _et al._, 2002). After ten days the coloured solvent with bioactive metabolites were filtered with Whatman filter paper (no.1). The filtrate was then subjected to the rotary flash evaporation by using rotary flash evaporator (BUCHI, Japan). The residual extracts were collected, weighed and stored in screw cap bottle. Finally, the percentage of extraction were calculated as follows:

(i) Weight of extract = Empty screw cap bottle weight –

Extract filled screw cap bottle weight

(ii) Percentage of extraction (%) = \[\frac{\text{Weight of the extract (g)}}{\text{Weight of the plant material (g)}} \times 100\]

The residual extracts were stored and further used for the screening of antibacterial and other biological screening.
5.4.1 Cold percolation method

The halophytes were extracted by cold percolation method (Sasikala and Sundaraganapathy, 2017).

5.5 Collection of bacterial pathogens

All the bacterial pathogens were collected from infected patient from post operative, urinary tract and eye infective samples from private clinical laboratories situated in Rajapalayam and Srivilliputtur. Acute bacterial pathogens were identified up to species level by following biochemical, physiological and morphological characteristics (Holt et al., 1994).

5.5.1 Test organisms

5.5.1.1 Post operative Pathogens

Five predominant Post operative bacterial pathogens viz., *S. marcescens*, *Salmonella typhi*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Klebsiella pneumoniae* were isolated and identified.

5.5.1.2 Urinary tract infectious (UTI) pathogens


The post operative pathogens and Urinary tract pathogens were collected from Private clinical Laboratories situated at Srivilliputtur and Rajapalayam, Tamil Nadu and were identified up to species level using a various physiological and biochemical tests.
5.5.1.3 Ophthalmic eye bacterial pathogens

Five different varieties of ophthalmic pathogens namely viz., *Pseudomonas aeruginosa*, *Enterobacter aeruginosa*, *Micrococcus luteus*, *Proteus* sp. and *Acinetobacter* sp. were isolated and identified from ocular infective samples. The samples were collected from Sankara eye hospital, Krishnankoil, Srivilliputtur.

5.6 Screening of antibacterial activity of commercial antibiotics against test bacterial pathogens by disc diffusion method

Mueller-Hinton agar was prepared and poured in to petriplates under aseptic conditions. The pathogenic cultures are swabbed on petriplates. Then the commercially (Himedia) available fluoroquinolone discs were placed on the Mueller-Hinton agar using forceps and the petriplates were incubated overnight without inverting at optimum temperature of 37 °C for 24 hours (Janakidevi et al., 2013).

<table>
<thead>
<tr>
<th>Name of the antibiotics</th>
<th>Symbol</th>
<th>Disc content (concentration in mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cinoxacin</td>
<td>CIN</td>
<td>100 µg</td>
</tr>
<tr>
<td>Lomefloxacin</td>
<td>LOM</td>
<td>10 µg</td>
</tr>
<tr>
<td>Nalidixic acid</td>
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<td>30 µg</td>
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<tr>
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</tr>
<tr>
<td>Gatifloxacin</td>
<td>GAT</td>
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<td>Norfloxacin</td>
<td>NX</td>
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<td>Sparfloxacin</td>
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<td>5 µg</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>OF</td>
<td>5 µg</td>
</tr>
</tbody>
</table>
5.7 Screening of antibacterial assay using chosen halophytes

Three different organic solvent crude extract from marine halophytic plants were tested for antibacterial activity using by agar well diffusion method (Prakash et al., 2016). In brief, overnight test pathogenic culture inoculum were prepared and aseptically spreaded over Mueller Hinton Agar (MHA; Hi-media, India)) plates. Thereafter well of 6 mm diameter were punched in MHA plates using sterile cork borer. The well was loaded with 100μl of DMSO (Dimethyl Sulfoxide, Hi media, India) contain 500μg/ml of each crude extract individually. Then the sample loaded plates were incubated at 37°C for 24 hours were observed. Inhibitory activity in term of zone of inhibition (mm) formed around the wells. The assay was carried out in triplicate.

5.8. Minimum inhibitory concentrations (MIC) and Minimum Bactericidal Concentration (MBC)

Further the Minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) were carried out in crude ethanolic extract of chosen halophytic plants.

5.8.1 Minimal inhibitory concentration (MIC)

Minimal inhibitory concentration (MIC) of the ethanolic extract of chosen marine plants Sargassum wightii and Avicennia marina were checked out by Macro dilution method described by (Das et al., 2010). Before the start up of experiment, 100μl of different extract with different concentration (12.5, 25, 50, 100, 200μg/ml) were prepared by DMSO and 100μl of mentioned concentration was added to culture tube, contain 300μl sterilized broth (Mueller Hinton Broth) and then each pathogenic test bacteria with the cell density of 2.0×10^8cell/ml were added and incubated for
24-36 hours at 37°C. The least concentration of extract, where no visible turbidity (growth of bacteria) was observed and considered as Minimum Inhibitory Concentration (MIC).

5.8.2. Minimal bactericidal concentration (MBC)

The minimum bactericidal concentration (MBC) was determined by sub culturing the MIC dilutions into loopful inoculate into the sterile nutrient agar plates. The lowest concentration of the extracts which inhibits the growth of tested bacteria were observed and tabulated (Ravikumar et al., 2009).

5.9 Antioxidant activity

A substance that reduces damage due to oxygen, such as that caused by free radicals. Well-known antioxidants include enzymes and other substances, such as vitamin C, vitamin E, and beta carotene, which are capable of counteracting the damaging effects of oxidation.

The antioxidant property of chosen ethanolic extracts of *S. wightii* and *A. marina* with fixed test concentration such as 800 mg/ml for *S. wightii* and 500 mg/ml for *A. marina* and 800 mg/ml of Gallic acid as positive control. Altogether three different types of antioxidant assays viz. Total Phenolic Content (TPC), Total Antioxidant Activity (TAA) and Antiradical scavenging activity by DPPH method were performed (Prakash et al., 2016).

5.9.1. Total phenolic content (TPC)

Total phenolic content of the chosen ethanolic extracts of *S.wightii* and *A.marina* was estimated by the Folin-Ciocalteu method (Prakash et al., 2016). In brief, the ethanolic extracts of *S.wightii* and *A.marina* (5 mg) were dissolved
separately in 5ml of ethanol. Then aliquot (800 µl) of each extract were mixed with 2 ml of Folin-Ciocalteu reagent and 1 ml (75 g/lit) of sodium carbonate. The tubes were vortexed for 15 seconds and allowed to stand for 30 min at 40°C in dark condition for colour development. After that, each coloured solution was read at 760 nm using UV–vis Spectrophotometer (PerKin Elmer Lamda 25, Waltham, MA-02451, USA). The total phenolic content was expressed as Gallic acid per gram (GAE/g).

5.9.2 Total antioxidant activity (TAA)

800 µl each of crude ethanolic extracts of chosen individual marine plant were mixed with 3 ml of reagent solution containing 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. The reaction mixture was incubated at 95°C for 90 min in a hot water bath. After that, the absorbance of colour development was measured at 696 nm. Total antioxidant activity was expressed as equivalents of Gallic acid (mg/g).

5.9.3 Anti radical scavenging properties by DPPH

Anti radical scavenging property of ethanolic extracts of marine plants were assessed by the method described by Yen and Chuang., 2000. Briefly, 800 µl of each extracts were added to 2 ml DPPH solution (0.16 mM DPPH prepared in methanol). Then the mixture was vortexed for 1 min. After that, the individual solutions were allowed to stand at room temperature for 30 min in dark condition. Finally, the OD was read at 517 nm. The ability of the extracts to scavenge DPPH radical was calculated by the following formula

\[
\text{Free radical (\%)} = \frac{(A_{OD} - B_{OD})}{B_{OD}} \times 100
\]

\(A_{OD}\): Optical density of control; \(B_{OD}\): Optical density of test samples.
5.10. Anti Hemolytic activity

In the present study, the hemolytic properties of the chosen extracts were screened by two different methods such as agar well blood diffusion method and micro plate assay method as described below.

5.10.1 Agar well diffusion method

The human blood agar plate was prepared by adding 10 ml of human blood in 90 ml of sterile blood agar base medium on sterile petriplates. After solidification, wells were made on the agar plate using a sterile cork borer (6 mm diameter). Each well was loaded with 100 µl DMSO containing 1000µg/ml of individual crude extract of *S. wightii* and *A. marina* and Triton X-100(10µg/ml) as positive control. The plates were incubated for 24 h at room temperature. After the incubation period, the hemolysis was observed by a clear zone of inhibition and it was measured at millimeter level.

5.10.2 Microplate method

Human blood from healthy volunteers was collected in vaccum tubes containing 2.7% EDTA as anticoagulant. The erythrocytes in the blood was separated by centrifugation for 10 min at 2000 rpm and washed three times with phosphate buffered saline (PBS). To the pellet, PBS was added to yield a 10% (v/v) erythrocytes/PBS suspension (0.844 OD). From this 10% suspension was subsequently diluted (1:10) in PBS. 100 ml of this suspension was added in triplicate to 96-well microplates containing 100 ml of different concentrations of individual extract of *S. wightii* and *A. marina* (1 to 5 mg/ml), PBS alone served as negative control and the total hemolysis was achieved with 0.1% Triton X-100 as a positive control. The microplate was incubated for 1 h at 37℃ and then centrifuged for 10 min.
at 2000 rpm. The absorbance of the supernatant was measured by using the ELISA reader (Robonik India Pvt. Ltd., Maharashtra, India) at 540 nm. Furthermore, the results of percentage Hemolysis and Hemolytic index were calculated by ASTM standard practice F 756-00; assessment of hemolytic properties of materials (Prakash et al., 2016).

5.11. *Artemia salina* cytotoxicity assay

*Artemia salina* nauplii cytotoxicity assay is one of the simple screening techniques mainly performed to assess the toxic nature of plant extracts and their derivative compounds. For the toxicity test, varying concentrations of individual plant extracts were transferred in to 24 well flat bottom polystyrene plate, then the extracts were evaporated at room temperature (Yeng, 2012). The live nauplii of *A. salina* (10 nauplii in each well) were inoculated in to the extracts coated plate with 500 ml of filter sterilized seawater. After 24 hours of incubation at 25°C, the live nauplii were counted in each test concentration of the wells. The seawater without extract was used as a negative control. The results were recorded as the percentage of mortality after 24 h and 50% Lethal Concentration (LC50) values were calculated through probit analysis. The assay was screened in three replicates (Prakash et al., 2016).

5.12 Phytochemical analysis of halophyte extracts

To screen the phytochemical component of active fraction no.16 for *S. wightii* and for no.28 for *A. marina* followed by Sofowora (1982) and Kepm (1986). The major pharmaceutically valuable phytochemical compounds investigated in the present study were alkaloids, carboxylic acids, coumarins, flavonoids, phenols, proteins and free amino acids, quinones, saponins, steroids, tannins, xanthoproteins and sugars.
5. 12.1 Detection of alkaloids

A few drops of dilute HCl were separately treated with 500μl of each fraction separately. Then it was filtered and the filtrates were treated with one ml of Dragendorff’s reagent. Formation of reddish orange precipitation indicated the presence of alkaloids.

5. 12.2 Detection of carboxylic acids

500μl each of fraction of chosen halophytes was separately treated with a few ml of saturated solutions of sodium bicarbonate. Observation of effervescence (due to liberation of CO₂) indicated the presence of carboxylic acids.

5. 12.3 Detection of coumarins

500μl each of fraction was treated with alcoholic NaOH solution. Production of dark yellow colour indicated the presence of coumarins.

5. 12.4 Detection of flavonoids

One ml each of the fraction were separately dissolved 1 ml each of alcohol (stock solution) and then subjected of the following tests.

(i) Ferric chloride test

500μl each of fraction was added with a few drops of neutral FeCl₃ solution. Formation of blackish red colour indicated the presence of flavanoids.

(ii) Shinoda’s test

With 500μl each of fraction, a small piece of Mg ribbon or Mg foil was added followed by the addition of a few drops of conc. HCl. Change in colour (red to pink) showed the presence of flavonoids.
5.12.5. Detection of phenols

500μl of the each fraction separately dissolved in 5 ml of alcohol was treated separately with a few drops of neutral FeCl₃ solution. Any change in colour indicated the presence of phenolic compounds.

5.12.6. Detection of protein and free amino acids

One ml each fractions were dissolved in 5 ml of water separately and were subjected to the following tests.

(i) Biuret test

500μl each of the various extracts was warmed gently with 10% NaOH solution and a drop of dilute CuSO₄ solution. Formation of reddish violet colour indicated the presence of proteins and free amino acids.

5.12.7. Detection of Quinones

500μl of the various extracts were separately treated with alcoholic KOH solution. Quinones give coloration ranging from red to blue.

5.12.8. Detection of resins

500μl each of various extracts were subjected to treat with a few drops of concentrated HNO₃ and a few drops of acetic anhydride solution followed by one ml of concentrated H₂SO₄. Resins give coloration ranging from orange to yellow.

5.12.9. Detection of saponins

500μl each of the various extracts were separately mixed with 20 ml of distilled water and then agitated in a graduated cylinder for 15 min. Foam formation indicates the presence of saponins.
5.12.10. Detection of Steroids / Phytosterols / Triterpenoidal sapogenins

One ml each of various extracts were dissolved in 5 ml each of chloroform separately (stock solution) and was subjected to the following tests.

(i) Salkowski test

500µg each of concentrated sulphuric acid was added to the stock solution and allowed to stand for 5 min after shaking. Turning of golden yellow color in the lower layer indicated the presence of sterols, phytosterols and triterpenoidal sapogenins.

(ii) Liebermann – Burchard test

500µl each of the fraction, a few drops of acetic anhydride and one ml of concentrated H₂SO₄ were added from the sides of the test tubes and allowed to stand for 5 min. Formation of brown ring at the junction of the two layers and the upper layer turned green indicated the presence of steroids, phytosterols and triterpenoidal sapogenins.

5.12.11. Detection of tannins

One ml each of the each fraction was dissolved in minimum amount of water separately, filtered and the filtrate were then subjected to the following tests.

(i) Ferric chloride test

To the above filtrate, a few drops of ferric chloride solution were added. The color change indicates the presence of tannins.

(ii) Basic lead acetate test

To the filtrate, a few drops of aqueous basic lead acetate solution were added. Formation of reddish brown precipitate indicated the presence of tannins.
5.12.12. Detection of xanthoproteins

500μl of the each fraction was treated separately with a few drops of concentrated HNO₃ and NH₃ solution. Formation of reddish orange precipitation indicated the presence of xanthoproteins.

5.12.13. Detection of sugars

Five ml each of the various extracts was dissolved separately in distilled water filtered and then subjected to the following test.

(i) Molisch’s test

To the filtrate, a few drops of alcoholic α - naphthol and 2 ml of concentrated H₂SO₄ were added slowly through the sides of the test tube. Formation of reddish brown precipitate indicated the presence of sugars.

5.13. Purification and characterization of bioactive metabolites by Silica column chromatography

The ethanolic crude extract (1.5g) of *S. wightii* and *A. marina* was individually purified using normal phase silica gel column chromatography (60–200 mm mesh size) with a stepwise gradient of solvents, including hexane, benzene, ethyl acetate, water, methanol and ending with warmed ethanol. In total, 30 fractions (each 40 ml) were collected, dried under dark conditions and then all the fractions (200 μg /ml) were screened individually against the chosen test bacterial pathogenic strains through microplate assays. Amongst the fractions, the 16th fraction for *S. wightii* and 28th fraction for *A. marina* showed the biogenic potential properties over the other fractions. Furthermore, the 16th fraction and 28th fraction were purified by thin layer chromatography (TLC). In brief, the each fraction was re-dissolved to the respective solvent system and made up to a volume of 100 mg /ml. From this, 500μl was taken
and spotted on TLC plates (TLC aluminum sheets, 20 × 20 cm, silica gel 60F254, Merck, USA) using the same solvent systems as the mobile phase. Thereafter, the TLC plates were dried at room temperature and observed under UV/vis absorption for detection and marking different spots at different wavelengths of 254 and 365 nm. The Rf values of the TLC plate were then calculated and recorded. Thereafter, the TLC plates were tested against pathogenic bacterial strains through TLC bioautography.

5.14 Antibacterial activity screening of collected column fractions of marine halophytes

To check out the biomedical properties of all collected fractions from S. wightii and A. marina individually. In total, thirty five fractions were collected from crude extract of S. wightii and A. marina using gradient solvent system. Starting from zero polar solvents as hexane to highly polar solvents as ethanol. All the fractions were individually collected and dried in dark condition with room temperature. Each fraction was weighed and used for the antibacterial screening against chosen test pathogens through 96 - micro wells plate micro dilution method (Prakash et al., 2015). In brief, equal volume of all fractions were weighted and dissolved in mother solvent system (100μg in 100μl). Each fraction was loaded in three wells separately. After loading all fraction in the 96 - micro well plate were allowed to evaporate solvent in sterile condition. After the completely dryness of solvent, each well was inoculated with 100μl of volume individual bacterial test pathogens. In separate plates with bacterial density of 2×10^6 cells/ml were incubated at 30°C at 24 hours. After the perfect incubation time, results were noticed for complete suppression of growth of bacteria in term of turbidity formation.
5.15 Chemistry of active fraction in *Sargassum wightii* and *Avicennia marina*

5.15.1 UV Spectral Analysis

Ultraviolet spectrum (λ<sub>max</sub> in nm) was recorded on a UV/VIS spectrophotometer for the active fractions of *S. wightii* and *A. marina* to find out presence of bioactive compounds.

5.15.2 Separation of active fraction by HPLC Method

Data was obtained at 280 nm by using UV-DAD (UV diode array detector). Data consisted of various peaks for each active fraction with their respective retention times. Analytical grade HPLC (High Performance Liquid Chromatography) was performed on a Varian 9021 solvent delivery system equipped with Varian 9065 Polychrom UV-diode array detector (190-367 nm). Data was processed by Star Polychrom version 5.2. The system was maintained in a controlled room temperature at 21±10°C. A flow rate of 1ml/min<sup>−1</sup> and injection volume of 10 µl were used. Sample analysis was performed by gradient elution on a 150 mm x 4.6 mm i.d., 5µM, Luna C-18(2) column (Phenomenex, Australia) with guard column (Phenomenex, Australia). The mobile phases were freshly prepared and degassed under vacuum using Phenomenex nylon 45 µm membranes and sonicated in a sanophon ultrasonic bath (Ultrasonic Industries Pty. Ltd, Sydney, Australia) for 15 minutes prior to HPLC analysis. Solvent A was a mixture of 90:10:1 methanol/acetonitrile/acetic acid (V/V/V) while solvent B was a mixture of 100:1 water/acetic acid (V/V). A six step gradient analysis for a total run time of 60 min was used to get proper separation of peaks. Regression lines for five external standards were used at five different concentrations i.e. 100, 200, 300, 400 and 500 ppm for each standard to get calibration curves. Equation for calibration can be used to get quantity of various
peaks in chromatograms of each active fraction. Quantification of various peaks in chromatograms obtained from *S. wightii* and *A. marina* were quantified in terms of equivalents of five external standards. Data was expressed in terms of mg of standard per gram of crude extract for a specific peak in a chromatogram.

**5.15.3 FT-IR Spectrum Analysis in active fraction**

FT-IR spectral (ν<sub>max</sub> in cm<sup>-1</sup>) analysis was carried out for the active column chromatographic fractions of *S. wightii* and *A. marina* to detect the functional group of the bioactive compounds. It was recorded in KBr on a JASCO FT/IR-460 plus spectrophotometer.

**5.15.4 ¹H-Nuclear magnetic resonance Spectrum Analysis**

The ¹H- Nuclear magnetic resonance (NMR) Spectra of the active column chromatographic fractions of *S. wightii* and *A. marina* was obtained in a Bruker spectrometer 400 MHz. The each active spot was dissolved in dimethyl sulfoxide (DMSO) to provide a field-frequency lock signal and contained 1% tetramethylsilane (TMS) to provide an internal chemical shift standard (Aldrich). One dimensional ¹H-NMR spectra was acquired using a narrow bore probe. Typical experimental conditions included two repetitions between successive acquisition 32768 time domain points and spectral width of 8223.685 Hz. Data processing and ¹H peak integration were done using XWINNMR software (Bruker, Karlsruhe, Germany) running in an R-4000 workstation (Silicon Graphics, Mountain view, CA, USA). This methodology is used to identify the position, type and neighbouring number of protons in the bioactive compounds.
5.15.5 $^{13}$C- Nuclear magnetic resonance Spectrum Analysis

The $^{13}$C- Nuclear magnetic resonance (NMR) Spectra of the active column chromatographic fractions of S. wightii and A. marina was obtained in a Bruker spectrometer 100 MHz. The active spots were dissolved in dimethyl sulfoxide (DMSO) to provide a field-frequency lock signal and contained 1% tetramethyilsilane (TMS) to provide an internal chemical shift standard (Aldrich). One dimensional $^{13}$C-NMR spectra was acquired using a narrow bore probe. Typical experimental conditions included two repetitions between successive acquisition 65536 time domain points and spectral width of 24038.461 Hz. Data processing and $^{13}$C peak integration were done using XWINNMR software (Bruker, Karlsruhe, Germany) running in an R-4000 workstation (Silicon Graphics, Mountain view, CA, USA). This methodology is used to know the carbon skeleton of bioactive compounds.

5.16 Statistical analysis

The data obtained in the present study were subjected to one way and two way ANOVA with Multiple comparison using SPSS.16 (SPSS. Inc. USA). The IC$_{50}$ and LC$_{50}$ values of crude extract purified fraction against antihemolysis, Artemia larvae were calculated by using EPA probit analysis software (Prakash et al., 2016).