4. MATERIALS AND METHODOLOGY

4.1. Collection of water samples

The random samples of water were collected from various sites of Muthupet Mangrove in every season for a period of two years (2010 & 2011). For convenience an year was divided as summer, pre monsoon, monsoon and post monsoon. The microalgae were found as mat in the water, flakes on the dried sediment and different forms. Samples were collected and transported to the laboratory in sterile polythene bags as well as plastic vials containing sterile ASN III medium. The samples were transferred to Erlenmeyer flasks containing the ASN III medium for further identification (Sweeny, 1954).

4.2. Collection & Identification of cyanobacteria from water samples

The collected samples were subjected for isolation of cyanobacteria by using standard microbiological techniques. The isolated cyanobacteria were morphologically examined using conventional methods of both microscopically and macroscopically, identify the isolates were made by using the standard taxonomic manuals of Geitler (1932) and Desikachary (1959). The morphological features of isolates were in different shapes green coloured flat, slimy mats of tangled filaments, filamentous, never branched, usually in fine, smooth, layered (but not leathery) mats, unicellular algal growth, small colonies, elliptical to no definite shape, mucilage boundary etc. The cultures were maintained in ASNIII medium, a standard synthetic medium, under appropriate conditions for further process.

4.2.1. Wet mount method

A small drop of the algal culture was transferred using a fresh pipette onto the centre of a clean grease free glass slide. A cover glass was carefully placed in the drop carefully and a gentle press was made on it. The slide was then observed under 45X objective (Aneja, 2009).
4.2.2. Culture conditions

The isolated microalgal samples were cultured at laboratory scale both in solid and liquid media. Autoclaved ASNIII media were poured into sterile petri dishes, they were inoculated with the isolates and incubated at 30±2°C with an illumination of 4k Lux light intensity. For liquid broth culture two sets were formed. One set was meant for small scale cultivation, for which a 100ml, 250ml and 500ml Erlen Meyer flasks containing autoclaved 70ml, 150ml, 300ml of ASNIII broth respectively were inoculated with isolated microalgae and incubated at 30±2°C (RT) with an appropriate illumination. The second set was diverted into two arrangements; indoor and outdoor setups. For indoor arrangements, 15 Litres (L) of autoclaved ASNIII broth was poured into sterile cylindrical cans of 20L capacity. For outdoor cultivation, autoclaved ASNIII broth was poured into sterile open plastic tubs of 20L capacity. Both the set ups were inoculated with appropriate inoculums of growing algal culture (Pandey and Tiwari, 2010).

The indoor set up was maintained inside the laboratory under aseptic conditions and provided with an illumination of 4 k lux light intensity. Artificial aeration was ensured with the help of an aquarium pump which pumped air at 150 bubbles per minute through a drip set (plastic tubing) fitted with a regulator. The outdoor set up was maintained on the terrace under natural aeration with illumination in the form of direct sunlight. To prevent the effect of photo inhibition and maintaining aseptic conditions, the tubs were tightly covered with sterile sieve cloth of minute pore size. All the broth cultures were regularly agitated daily at periodic intervals to ensure proper mixing and aeration (Pandey and Tiwari, 2010). The algal isolates were similarly cultured in solid and liquid media. The experiments were run in duplicates. All manipulation involving the transfer of cultures in the liquid media or on agar plates were carried out under aseptic conditions in a laminar airflow.

4.3. Growth

4.3.1. Macroscopic Observation

The cultures of algae were observed macroscopically. Their cultural characteristics on ASN III media plates and growth in conical flasks containing broth were observed.
4.3.2. Microscopic Observation

The algal cultures were observed microscopically at 45X objective. The wet mount method was employed for observation.

4.3.3. Growth Measurement

The cultures were grown for a period of 30 days and growth was monitored for each of efficient algal strains, calorimetrically at 560nm. Productivities were calculated from the equation \( P = \left( \frac{X_i - X_0}{t_i} \right) \), where \( P \) = Productivity \((\text{mgL}^{-1})\), \( X_0 \) = initial biomass density \((\text{mgL}^{-1})\), \( X_i \) = biomass density at time \( i \) \((\text{mgL}^{-1})\) and \( t_i \) = time interval \((\text{h})\) between \( X_0 \) and \( X_i \) (Colla et al., 2007). Duplicates were maintained for the experiment.

4.4. Harvesting

Mass cultures of isolates at stationary phase were harvested by using a sterile sieve cloth of minute pore size. Harvesting was initiated after 15 days from the date of inoculation. In interval of two to three days the algae were harvested continuously. Nutrients were added to the medium at regular intervals and this fed batch technique maintains the medium composition during the algal growth.

4.5. Drying

The harvested algal biomass were collected in aseptic plastic bottles and subjected to drying under direct sunlight. After complete drying, the dried algal biomass were placed in hot air oven at 60° C for one hour. Until further use, the algal biomass were stored under refrigerated condition (4° C) (Sarada et al., 1999).

4.6. Preparation of extracts

The dried algal products were mechanically ground using mortar and pestle into fine powders (Plate III). It was further mixed with ethanol 10:1 ratio and placed in a mechanical shaker for 24 hours. The extract was then filtered using Whatmann No.1 filter paper. The extract was further concentrated by using a rotary evaporator for the complete evaporation of the solvent. Within a shortest period, the extract obtained finally was in the form of a dried gum like resin. This was further used for in-vitro and in-vivo analysis (Pandey and Tiwari, 2010).
4.7. Phytochemical analysis

4.7.0. Qualitative analysis of phytonutrients of algal extracts

Qualitative analysis of phytonutrients were done for ethanolic extract of all microalgal isolates (Sofowora, 1993; Evans, 1989).

4.7.1. Test for carbohydrates

A small quantity of the extract was dissolved separately in 5 ml of distilled water and filtered. The filtrate were tested for the presence of carbohydrates.

4.7.1.1 Molisch’s test

To 2ml of algal extract and 2 ml of Molisch’s reagent were added followed by, 2 ml of concentrated sulphuric acid along the sides of the test tubes. Disappearance in colour on the addition of excess solution indicated the presence of carbohydrates.

4.7.1.2. Benedict’s test

To 0.5 ml of extract, 5 ml of Benedict’s reagent was added. The mixture was boiled for 5 minutes and observed for the appearance of a bluish green precipitate indicating the presence of carbohydrates.

4.7.2. Test for Glycosides

To 2ml of algal extract, 1ml of aqueous NaOH solution was added. The appearance of a yellow colour indicated the presence of glycosides.

4.7.3. Test for Proteins and Amino acids

4.7.3.1 Ninhydrin test

A small quantity extract solution was boiled with 0.2% solution of ninhydrin. Purple colour indicated the presence of free amino acids.

4.7.4. Test for Phytosterols and Triterpenoids

4.7.4.1. Salkowski test

To 2 ml of the algal extract, 1 ml of concentrated sulphuric acid was added and chloroform was added along the sides of the test tube. A red colour formation of the
chloroform layer indicated the presence of Phytosterols or yellow colour at the lower layer indicated the presence of triterpenoids.

4.7.5. Test for Flavanoids

4.7.5.1 Zinc hydrochloride reduction test

The extract was treated with a mixture of zinc dust and concentrated hydrochloric acid. Red colour indicated the presence of flavonoids.

4.7.6. Test for Alkaloids

A small portion of the solvent free extract was stirred separately with a few drops of dilute hydrochloric acid and filtered. The filtrate was tested with Mayer’s reagent (Potassium mercuric iodide solution). The cream precipitate indicates the presence of alkaloids.

4.7.7. Test for Tannins

4.7.7.1 Gelatin test

To 5 ml of algal extract, few drops of 1 % lead acetate were added. Absence of a yellow or red precipitate indicated the absence of tannins.

4.7.8. Test for Saponins

To 5 ml of the algal extract, a drop of sodium bicarbonate was added. It was then shaken vigorously and kept undisturbed for 3 minutes. The appearance of a honeycomb like froth indicated the presence of saponins.

4.8. Estimation of In-vitro antioxidant property

In these experiments, two complementary methods of free radical scavenging activity; DPPH assay and FRAP assay were used.

4.8.1. DPPH assay

1 mg of algal extract was dissolved in 1 ml of 50% ethanol solution to obtain 1000 μg/ml sample solution according to the method of Blios (1958). It was further serially diluted into 1 μg/ml, 5 μg/ml, 10 μg/ml, 20 μg/ml, 50 μg/ml, 100 μg/ml, 200 μg/ml, 500 μg/ml, and 1000 μg/ml concentration with 50% ethanol. In each reaction, the solutions were mixed with 1 ml of 0.1 mM 1,1-Diphenyl-2- picrylhydrazyl (DPPH),
0.45 ml of 50mM Tris-HCl buffer (pH 7.4), and 0.05 ml samples at room temperature for 30 min. 50% ethanol solution was used as a control. The reduction of the DPPH free radical was measured by reading the absorbance at 517 nm. DPPH, a purple-coloured, stable free radical is reduced to the yellow-coloured diphenyl picrylhydrazine when antioxidants are added. L-ascorbic acid and (+)-catechin were used as positive controls. The inhibition ratio (percent) was calculated from the following equation: % inhibition = [(absorbance of control – absorbance of test sample) /absorbance of control] x 100. The antioxidant activity of each sample was expressed in terms of IC₅₀ (micromolar concentration required to inhibit DPPH radical formation by 50%), calculated from the inhibition curve.

4.8.2. FRAP assay

The procedure described by Benzie and Strain (1996) was followed. The principle of this method is based on the reduction of a ferric-tripyrroltriazine complex in its ferrous coloured form in the presence of antioxidants. The FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue coloured FeII-tripyridyltriazine compound from the colourless oxidized FeIII form of the action of electron donating antioxidants. The FRAP reagent consist of 300 mM acetate buffer (3.1 g sodium acetate + 16 mL glacial acetic acid, made up to 1 litre with distilled water; pH = 3.6), 10 mM TPTZ in 40 mM HCl and 20 mM FeCl₃·6H₂O in the ratio of 10:1:1. Briefly 50 μL of sample supernatant was added to 1.5 mL freshly prepared and prewarmed (37°C) FRAP reagent in a test tube and incubated at 37°C for 10 min. The absorbance of the blue coloured complex was read against reagent blank (1.5 ml FRAP reagent+50 μl distilled water) at 593 nm. For construction of the calibration curve, five concentrations of FeSO₄ 7H₂O (1000, 750, 500, 250 and 125 μmol l⁻¹) were used and the absorbences were measured as sample solution. The data were expressed as mole ferric ions reduced to the ferrous form per litre (FRAP value).

4.9. Isolation and characterization of active constituents of potential strain
(S. platensis)

Among the algal isolates, S. platensis shown significant antioxidant property and it was subjected for the partial purification and characterisation of bioactive principles.
S. platensis ethanol extract was obtained from its powder (5kg) under reflex and vacuum conditions twice for 2hrs. The obtained soft extract (135gm) was resuspended in petroleum ether, CHCl₃, C₄H₈O₂ and 90% CH₃OH sequentially and kept in the reflex for 2 hours. The final extract was added with 25ml of acetone drop by drop, filtered and the precipitate was collected and weighed (Obdoni and Ochuko, 2001). The purification process was repeated with n-butanol and washed twice with 10ml of 5% aqueous sodium chloride and the remaining solution was evaporated under vacuum. The samples were dried in the oven to a constant weight, where the saponin content was calculated in percentage. TLC analysis of the fraction was carried out to visualize the presence of the spots with reddish fluorescence indicating saponin with an Rf value of 0.67 with the mobile phase Butanol: Glacial acetic acid: water (10:2:0.3) visualized under a UV chamber (Edeoga et al., 2005). The sequential solvent extract was fractionated by using silica gel in column chromatography by using different proportions of solvents (CHCl₃: MeOH: H₂O, 3:4:3 ratio). The chemical structure of active compounds in saponin were determined by Fourier transformed infrared spectroscopy (FTIR) SHIMAZU EQUINOX 55 FTIR spectrometer coupled with a DTGS detector in the range of 4000-400/cm with 64 scan and a resolution of 4 cm⁻¹. Volatile compounds in SFS were identified using Gas chromatography and mass spectroscopy. SFS were confirmed with standard saponin in HPLC (Ballabeni et al., 2004).

4.10. Cell lines and culture medium

Vero (cloned cell line derived from the kidney of an African green monkey) cell lines were obtained from NCCS (National Centre for Cell Science), Pune, India and grown at 28°C as monolayers in MEM (Minimum Essential Medium), supplemented with 10% tryptose phosphate broth, 1% penicillin/streptomycin (Gigco-BRL, USA) (50 μg/mL), 1% amphotericin B (250 μg/mL) (Gibco-BRL, USA), and 10% fetal bovine serum (FBS, Sigma-Aldrich, USA).

4.10.1 Evaluation of cytotoxicity and CC₅₀

The Vero cell viability was detected by using the MTT method (Sigma-Aldrich, USA) using 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide) (Sieuwerts et al., 1995). Vero cell cultures were prepared in 96-well plates at 2 x 10⁵ cells/well and
incubated at 28°C for 24 hrs, further the culture medium was removed and the cells were exposed to different concentration of the SFS (Saponin fractions of *S. platensis*) by adopting four wells per concentration with the volume of 200-μL culture medium per well and controls having 200 μL of media culture and subsequently incubated for seven days. The medium was removed and 50 μL of MTT solution (5 mg/ml) was added followed by re-incubation for four hours and the removal MTT solution; addition of DMSO (100 μL) for dissolving formazan crystals completely was done by shaking gently. The absorbance was read by ELISA equipment (SHIMAZU) at 495 nm. The 50% cytotoxic concentration (CC50) was defined as the sample concentration that reduced cell viability 50% compared to untreated controls.

### 4.11. Invivo study (Animal and management)

Experiments were conducted using Wistar albino rats (male, 150-200 g) and Swiss albino mice (male, 25-30g), procured from the Small Animal Breeding Station (SABS), College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala. The animals were housed in groups for a minimum of 7 days prior to pharmacological experiments. Animal quarters were maintained at a temperature of 22 ± 2ºC and with 12-h light/12-h dark cycle. The animals had free access to commercial food pellets and clean drinking water. The study received approval from the Institutes Animal Ethics Committee (IAEC) for the Committee (CPCSEA) for the Purpose of Control and Supervision of Experiments on Animals (IAEC ref no: IAEC/ KMCRET/Ph.D/6/2011).

#### 4.11.1. Invivo Acute toxicity studies

Albino mice weighing 25-30 g selected by using random sampling technique were used in the study. Acute oral toxicity was performed as per OECD- 423 guidelines (acute class method) (Ecobichon, 1997). The animals were fasted overnight, provided the only water after which extract was administered to the groups orally at the dose level of 5 mg/kg body weight by gastric intubation and the groups were observed for 14 days. If mortality was observed in 2 or 3 animals among 6 animals then the dose administered was assigned as a toxic dose. If mortality was observed in one animal, then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the
procedure was repeated for further higher doses such as 50, 300 and 2,000 mg/kg body weight. The animals were observed for toxic symptoms such as behavioural changes, locomotion, convulsions and mortality for 72 hours.

4.11.2. Antihyperlipidemic study by using High Fat Diet

Male Wister rats, 150-200g, were procured from KMCH (Kovai Medical Centre and Hospital) college of pharmacy, Coimbatore, India. The rats were housed in polypropylene cages, at constant temperature of 22±2°C, with a 12 hour light-dark cycle. The rats were fed with commercial pellet diet and allowed free access for water ad libitum. Overnight fast rats were divided into five groups of five rats each. (1) Normal control group (NC): fed with normal diet and 5% CMC by oral gavage; (2) Hyperlipidemic group (HL) fed with high fat diet (normal diet supplemented with 5% cholesterol from Sigma-Aldrich (St. Louis, MO, USA), 0.5% pig bile salt (Sigma) and 0.2% thimecil (Sigma) and intra-gastrical administration with 5% CMC; (3) Hyperlipidemic and SFS group (HL + SFS (50mg/kg), the animals were fed with high fat diet followed by intragastrical administration of SFS (50mg/kg) with 5% CMC; (4) Hyperlipidemic and SFS group (HL + SFS (100mg/kg) rats were fed with high fat diet plus high concentration of SFS (100mg/kg) with 5% CMC by intra-gastrical administration. (5) Hyperlipidemic and Lovastatin group (HL + LS) rats were fed with high fat diet plus Lovastatin (10mg/kg) with 5% CMC by intra-gastrical administration. During the study period (28 days), rats were administrated with extracts and lovastatin everyday from the period of the 15th day to 28th day. After 28 days of treatment, the animals were sacrificed and blood was taken from their retro orbital sinus vein and liver homogenate for enzymatic assays (Tiejie Wang et al., 2012).

4.11.3. An Antihyperlipidemia study by using Triton

Hyperlipidaemia was induced in Wistar albino rats by single intraperitoneal injection of a freshly prepared solution of Triton-X-100 (100 mg/kg) in physiological saline solution after overnight fasting for 18 h (Hicham et al., 2007). The animals were divided into four groups of five rats each. The first group was given a standard pellet diet, water and orally administered with 5% CMC. The second group was given a single dose of triton administered at a dose of 100mg/kg, i.p. After 72 hours of triton injection, this
group received a daily dose of 5% CMC (p.o) for 7 days. The third group was administered a daily dose of *Spirulina platensis* 0.5g/day suspended in 5% of CMC, p.o., for 7 days, after induced hyperlipidaemia. The fourth group was administered with the standard Fenofibrate 65mg/kg, p.o. for 7 days after induced hyperlipidaemia. Upon completion of the experiment the rats were sacrificed for studying the lipid profile (Muramatsu *et al.*, 1986).

**4.11.3.1. Biochemical analysis of plasma**

The lipid profile measurement of triglyceride, total cholesterol, HDL and LDL in blood plasma were determined by enzymatic colorimetric methods using standard protocols (Tiejie Wang *et al.*, 2012).

**4.11.4. Estimation of HMG CoA reductase Activity**

Rat liver was homogenized with ice-cold 0.25M sucrose solution (4 ml/g tissue wet weight of the liver) and centrifuged at 11, 500 RPM for 20 min. Supernatant was added to 0.1 ml of 88 mM CaCl per ml and placed on ice for 5 min with occasional shaking. The content was again centrifuged at 13,500 RPM for 35 mins. Final pellets were resuspended in 10ml of 0.1M Tris buffer, at pH 7.4 by homogenization, and stored at - 20°C until further use. HMG CoA reductase was measured using previously described method (Vanitha reddy *et al.*, 2012).

**4.12. Studies on inhibition of cardiotoxicity by *Spirulina platensis***

Female Swiss albino mice (weight 30–40 g) were housed under conditions of controlled temperature and a 12 h lighting cycle and fed with standard diet ad libitum. The animals were divided into four groups of 20 animals each. The control animals received only normal saline once in a week for 4 weeks. The second group received four equal injections (each containing 4 mg/kg bw DOX) intraperitoneally, once weekly for 4 weeks (cumulative dose 16 mg/kg) and the third group received *Spirulina* (250 mg/kg bw) orally, twice a day for 7 weeks. The fourth group received *Spirulina* (250 mg/kg bw) orally, twice daily for 3 days, then for 7 weeks along with four injections of DOX similar to the second group. All animals were observed 3 weeks after the last injection of DOX.
for changes in body weight, general appearance and mortality. The surviving animals were killed and the heart tissues were evaluated for antioxidant enzymes, and morphological appearance (Mahmood et al., 2005).

4.12.1. Histopathology of heart

Hearts from mice of all the groups were fixed at 10% buffered formalin and embedded in paraffin. Sections (5 mm) were stained with hematoxylin and eosin and were examined under a light microscope (Mahmood et al., 2005).

4.13. Preparation of virus stock for antiviral assay

Type-1 dengue virus (DENV-1), isolated in Chennai was obtained from the Arbo Virology Laboratory, King Institute of Preventive Medicine & Research, Chennai, India. The virus was replicated in Vero cells for seven days at 28°C to generate working stocks. Supernatant of culture was collected and centrifuged at 2,000 RPM for 15 min and stored at –80°C. Virus titre, expressed as plaque-forming units per millilitre (PFU/mL), was determined by standard plaque assay on Vero cells grown in 96-well plates (Wang et al., 2011).

4.13.1. Antiviral Assay

The Antiviral activity of the samples were also evaluated using the MTT method. To determine the inhibitory potential of the sample for DENV-1 replication, 100 µL of virus suspension was added to a 96 well plate and incubated one hour at 28°C. The viral suspension was removed and various concentrations of SFS were added and incubated for seven days. This was followed by IC_{50} determination by using MTT assay. The 50% inhibitory concentration (IC_{50}) was defined as the concentration that inhibited 50% of viral replication compared to virus controls. The selectivity index (SI), an important parameter adopted for evaluating the antiviral activity was calculated from the CC_{50}/IC_{50} ratio. Antiviral activity was determined according to the following formula (Cheng et al., 2004):

\[
\text{Antiviral activity} = \frac{(OD_t) \text{ DENV} - (OD_c) \text{ DENV} \times 100}{(OD_c) \text{ cells} - (OD_c) \text{ DENV}}
\]

Where (OD_t) DENV denotes the measured absorption of the various concentrations of SFS and DENV-infected cells, (OD_c) DENV - Absorption of the untreated control DENV-infected cells. (OD_c) cells is the absorption of untreated control vero cells.

4.14.1. Enumeration and isolation of associated bacteria

Serial dilution was performed for microbial analysis from the Spirulina products and cultures. 1gm of the sample was diluted in 100 ml sterile distilled water in a sterile conical flask and it acts as a stock solution ($10^{-2}$). Using sterile pipettes transferred 1ml of stock solution to the sterile test tubes containing 9 ml distilled water and is labelled as $10^{-3}$. Similar way the remaining tubes were serially diluted up to $10^{-6}$. 0.1 ml of the sample was taken from the dilutions $10^{-2}$, $10^{-3}$ and $10^{-4}$ and the spread plate technique was followed. To get a uniform distribution of the microbial cells the samples were spread on the nutrient agar (Anderson and Heffernan, 1965) plates using a sterile L-rod. The plates were then incubated at 37°C for 24-48 hrs. After incubation, the plates were observed for the microbial load. The colonies on the plates were counted with the help of colony counter and the results were noted. The enumerated colonies were isolated in the pure form of repeated sub culturing on the nutrient agar plates and stored in the refrigerator for further analysis.

4.14.2. Preliminary Characterization

For the characterization of associated microbes both conventional and molecular techniques were employed. Macroscopic observation of the preliminary characterization of the isolates was carried out by carefully recording the cultural characteristics. The colour, odour and texture of the colonies were recorded. Microscopic observation by gram staining and motility behaviour of the organisms were identified microscopically (Aneja, 2009).

4.14.3. Gram staining

A thin smear was prepared on a clean grease free glass slide. Air dried and heat fixed. Flooded the smear with Gram’s crystal violet for 1-2 minutes, washed with running tap water. Then the smear was flooded with Gram’s iodine and allowed for 1-2 minutes and washed. Decolorization was done using acetone or 95% of absolute alcohol and washed with slow running tap water. Counter stained with Safranin for 1-2 minutes and washed. The slide to be air dried and observed under oil immersion objective.
4.14.4. Motility determination (Hanging Drop Method)

On a clean coverslip, transfer one loopful of sterile water and the culture was mixed with it. The petroleum gel was applied to the four corners of the coverslip. Placed the concavity slide on the coverslip and pressed the slide gently to form a seal between the coverslip and slide. Lifted the preparation and quickly turned to the side, so that the drop is in the hanging position. Observed the slide under the low and high power objective and focused the edge of the drop, the motility of the organism was observed.

4.14.5. Biochemical Characterization

Based on the Gram staining results biochemical tests were also performed. It includes carbohydrate fermentation test, triple sugar iron test, IMViC tests, Urease test, Nitrate reduction test, Catalase test, Oxidase test and Coagulase test.

4.14.5.1. Carbohydrate fermentation test

Various sugar fermentation broths (Glucose, Sucrose, Lactose, Maltose and Mannitol) were prepared and the isolates were inoculated. All the tubes were incubated at 37\(^0\) C for 24 hrs. After incubation the tubes were examined for colour change and gas bubble formation.

4.14.5.2. Triple sugar iron test

It is mainly used to find the ability of the organism, to ferment glucose, sucrose, lactose; gas and hydrogen sulphide production. The TSI agar contains lactose and sucrose in 1% and glucose in 0.1% of concentration. The agar medium was sterilized and 5 ml was dispensed into sterile tubes and the slants were made. Inoculated the isolates by stabbing and streaking. Incubated the tubes at 37\(^0\) C for 24 hrs. After incubation the tubes were observed for the colour change and gas production.

4.14.5.3. Indole test

It detects the ability of the microbes to utilize the amino acid tryptophan. The peptone broth was prepared and inoculated with the test isolates. Incubated at 37\(^0\) C for 24 hrs. After specifying incubation period, Kovac’s reagent was added and examined for the cherry red ring formation.
4.14.5.4. Methyl red test

It is used to determine the ability of the microorganisms to oxidize glucose. The test organisms were inoculated in MR- VP broth and incubated at 37°C for 24hrs. After incubation 5-6 drops of methyl red reagent were added and observed for the colour change.

4.14.5.5. Voges- Proskauer test

This is mainly used to differentiate the enteric organisms. MR- VP broth was used for the inoculation of organisms and proper incubation was given. After incubation Barrit’s reagent was added and the tubes were examined for the colour change.

4.14.5.6. Citrate utilization test

This test mainly helps to detect the ability of the organism to ferment citrate as a carbon source. Simmons citrate agar slants were prepared and the isolates were inoculated. Incubated the slants at 37°C for overnight and were observed in the Prussian blue colour formation.

4.14.5.7. Urease test

The ability of the organisms to degrade urea by the enzyme urease was determined. The isolates were inoculated in urea broth and overnight incubation was given at 37°C. The tubes were observed for the colour change.

4.14.5.8. Nitrate Reduction Test

It is a test to detect the ability of the organisms to reduce nitrate into nitrite or beyond the nitrite state. Nitrate broth was prepared with 0.1% potassium nitrate as the nitrate substrate. The organisms were inoculated in this broth and incubated at 37°C overnight. Following incubation sulfanilic acid and α- naphthylamine were added and observed for the colour change, if there is no colour development a pinch of zinc powder should be added.

4.14.5.9. Catalase test

In clean grease free glass slide a drop of 3% of hydrogen peroxide was added and a part of the colony is emulsified in that. It was observed for the releasing of gas bubbles.
4.15.10. Oxidase test

This test was used to detect the presence of cytochrome oxidase. A clean glass slide was taken and the oxidase disc containing tetra methyl para phenylene diamine dihydrochloride was placed. With the help of the sterile glass rod, the test isolates was picked by touching the colony from the plate and immediately pressed on the surface of the disc. The results were examined within 20 seconds for blue colour formation as positive.

4.15.11. Coagulase test

It is mainly used in the case of isolates which are Gram positive cocci in clusters. Add 0.5 ml of diluted plasma to a clean grease free slide and add 0.5 ml of broth culture. Observe the slides for the agglutination.

4.15. Molecular characterization

4.15.1. DNA extraction from the associated microbes

To extract the genomic DNA from the isolates, the samples were incubated in liquid medium at 37°C for 24 h. Next, the samples were centrifuged at 8,000 RPM / min, after which the pellets were suspended in 100 µL sterile deionized water. Cell suspensions were lysed by heating for 8 min at 100°C to release their nucleic acid and immediately centrifuged at 12, 000 RPM/ min for 10 min at 4°C. The lysate supernatant fluid was transferred to a micro centrifuge tube and 2 µL was used as template for PCR amplification immediately (Ausubel et al., 2001).

4.15.2. Primers for genome amplification

Primers- The target DNA fragments were amplified using the following common primer set for the 16S r DNA of bacteria: of 16S ribosomal DNA were separated and amplified by using PCR with universal primer of 533F 5’-GTGCCAGCMGCGCGGTAA-3’ and 1492R 5’GGTTACCTTGTACGACTT-3’ (Sivalingam et al., 2012).

4.15.3. Amplification of DNA by PCR

Reverse and forward primers were used to amplify the extracted DNA of the bacterial isolates. PCR reaction mixture composed of DNA polymerase 1 unit/10µL,
20mM Tris-HCl, 80mM KCl, 4mM MgCl$_2$ enzyme stabilizer, sediment, loading dye, pH 9.0, 0.5mM of each dATP, dCTP, dGTP, dTTP. The following steps were involved in amplifying the DNA. Added 2μl of the extracted DNA. Then the thawed 2x Premix solution was added. 10μl of the above solution was added to the PCR tubes that contain template DNA. Along with this added 0.4μl of both forward and reverse primers. The total content was 20μl in the PCR tubes which was made by adding with sterile distilled water. The PCR tubes were kept in PCR wells and run the PCR machine for amplifying the DNA.

Polymerase chain reaction is built in 20-40 repeated cycles where the temperature changes in each cycle. The cycling starts with a single temperature step (called hold) at a high temperature (>90 degree Centigrade), and followed by one hold at the end for final product extension or for brief storage. The various steps of PCR were:

**4.15.3.1. Initialization step**

It is the first step of the cycle which consists of raising the temperature of the reaction to 94–96 °C or 98 °C if extremely thermostable polymerases were used, which is held for 1–9 minutes. This process activates the DNA polymerase used in the reaction.

**4.15.3.2. Denaturation step**

It consists of heating the reaction to 94-98 degree centigrade for 20-30 seconds. This helps in breaking of the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

**4.15.3.3. Annealing step**

The mixture is now cooled to a temperature of 50–65 degree centigrade for 20-40 seconds which helps in annealing of the primers to the single-stranded DNA template. Stable DNA-DNA hydrogen bonds were only formed when the primer sequence matches closely with the template sequence that permits annealing of the primer to the complementary sequences in the DNA. As a rule, these sequences are located at the 3’-end of the two strands of the segment to be amplified. The duration of annealing step is usually 1 min during the first as well as the subsequent cycles of PCR. Since the primer concentration is kept very high relative to that of the template DNA, primer-template hybrid formation is greatly favoured over re-annealing of the template strands.
4.15.3.4. Extension/elongation step

It is a DNA polymerase dependent process. Taq polymerase has its optimum activity temperature at 75-78 degree centigrade. The temperature at this step depends on the DNA polymerase used, the complementary strands synthesis takes place by utilizing the 3'-OH of the primer. The primers were extended towards each other so that the DNA segment lying between the two primers is copied; this is ensured by employing primers complementary to the 3'-ends of the segment to be amplified. The duration of primer extension is usually 2 min at 72°C. Taq polymerase usually amplifies DNA fragments of up to 2 Kb; special reaction conditions are necessary for the amplification of longer segments. As a thumb rule, at its optimum temperature, the DNA polymerase were polymerize a thousand bases per minute, leading to exponential (geometric) amplification of the specific DNA fragment.

4.15.3.5. Final elongation

This step is performed at a temperature of 70-74 degree centigrade for 5-15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

4.15.3.6. Final hold

Finally the mixture is allowed to cool to a temperature of 4-15 degree centigrade for short-term storage of the reaction.

4.15.4. Agarose gel electrophoresis

The electrophoresis of DNA is an essential technique in molecular biology, relying on the negative charge of DNA for size separation in a sieving matrix. Traditionally, electrophoresis are performed using agarose (an extract of seaweed) and a Tris buffering solution.

4.15.4.1. Procedure

For a 1% agarose gel, weighed out 1g of agarose into a flask and added 100ml of 1 x TBE. Variety of agarose products are available commercially with different melting properties but for separating simple DNA fragments of 0.1–10 kb, normal grade or low melting temperature 0.5% agarose is adequate. The agarose solution kept in a microwave
or boiling water bath until agarose is completely dissolved. Allowed it to cool in a water bath set at 50 – 55 °C for 10 min. Prepared the gel casting tray by sealing the ends of the gel chamber with tape or appropriate casting system and placed appropriate number of combs in gel tray.

Added 5µl of ethidium bromide to cool gel and poured into gel tray. Allowed it to cool for 15-30 min at room temperature. Gels can also be placed in a cold space and used by the following day. The comb(s) were removed and the gel was placed in electrophoresis chamber and covered with buffer (TBE). Loading buffer was mixed to samples. As a guideline, 1.5 µl has been added to 10x Loading buffer to a 20-25µl DNA solution. The DNA sample and standard (Ladder) were loaded and electrophoresed the gel at 100V for 1 hour. The DNA bands were visualized using a UV light box or gel imaging system.

4.15.5. Gel Dissociation

The agarose gel slice containing relevant fragments was excised and removed the extra agarose if present to minimize the size of the gel slice. The gel slice of about 300mg was transferred into a 1.5ml micro-centrifuge tube. 500µl of DF Buffer was added to the sample and mixed by vortexing. This was incubated at 55-60°C for 10-15 minutes until the gel slice were completely dissolved. During the incubation invert the tubes every 2-3 minutes. The dissolved sample mixture was cooled to room temperature. DF column was placed in a 2ml collection tube. The sample mixture (800µl) from dissolved sample was added into the DF Column and mixture was centrifuged at full speed (13000rpm) for 30 seconds. The flow was discarded and placed the DF column back in the 2ml collection tube. 400µl of wash buffer was added in the DF column and the mixture was centrifuged at full speed (13000rpm) for 30 seconds. The flow was discarded and placed the DF column back in the 2ml collection tube. 600µl of wash buffer (ethanol added buffer) was added into DF column and allowed to stand for 1minute. This mixture was centrifuged at full speed (13000rpm) for 30 seconds and the flow was discarded, the DF column was placed in the 2ml collection tube. Again centrifugation was done for 3 minutes at full speed (13000rpm) to dry the column matrix. The dried DF column was transferred in a new 1.5ml micro centrifuge tube. 20-50µl of elution buffer or TE was added in the centre
of the column matrix and allowed it to stand for 2 minutes until the elution buffer or TE was absorbed by the matrix. The centrifugation was done at 13000 rpm for 2 minutes to elute the purified DNA.

4.15.6. DNA Sequencing

The eluted DNA was sent to *Eurofins Genome India Pvt. Ltd, Bangalore* for sequencing.

4.15.7. Phylogeny construction and gene bank submission

The 16S rDNA sequences of the isolates were compared with sequences available in the Gen Bank database using the NCBI BLAST program. Two or three fragments of each isolate with clear taxonomic status and high similarity to the sequences of target species were selected for the further analysis. The alignment of all the sequences were conducted; the statistics and cluster analysis were performed using the phylogeny by using MEGA 4 package. The genetic distances were calculated and the phylogenetic tree was constructed (Wang Zifeng *et al.*, 2008). 16S rRNA gene sequences of the strain MSK 1, MSK 2, MSK 3 and MSK 4 were aligned with a reference sequence obtained from GenBank using ClustaL X 2.0.11.

4.16. Synthesis of bacterial mediated silver nanoparticles

4.16.1. Preparation of extracellular filtrate from associated microbes

The bacterial cultures were inoculated in the sterile nutrient broth and were incubated at 37°C for 24 hrs. After incubation the cultures were shaken thoroughly and filtered through Whatman No. 1 filter paper under aseptic conditions. The bacterial filtrate was collected and stored (Sivalingam *et al.*, 2012).

4.16.2. Preparation of metal solution

Silver nitrate (Qualigens _99. 8% from SD Fine – CHEM limited, Mumbai, India.) solution at a concentration of 10⁻³ M was prepared separately for each test isolates.

4.16.3. Metal microbe interaction

5 ml of each bacterial extracellular filtrate was taken and added to sterile BOD bottles. To this 95 ml of AgNO3 solution was added (Sivalingam *et al.*, 2012).
4.16.4. Confirmation of interaction

Upon mixing the bacterial filtrate with the silver nitrate solution, the bottles were observed to change in colour and pH for the next 24 hrs. Changes in optical density also indicated the synthesis of silver mediated nanoparticles (Sivalingam et al., 2012).

4.16.4.1. Colour change

The confirmation of the synthesis of metal nanoparticles using micro organisms was identified by a change in colour. The metal processed solution initially appears turbid white in colour and the change to dark brown indicated the synthesis of silver mediated nanoparticle. For comparison, the conical flask containing bacterial filtrate and AgNO3 was incubated under similar experimental condition. The colour change was noted periodically (Sastry et al., 2002).

4.16.4.2. pH

The change in hydrogen ion concentration is an important parameter for the confirmation of nanoparticle synthesis. The change in pH was observed during the microbial synthesis of silver nanoparticle (Revathi and Prabhu, 2009).

4.16.4.3. UV-Vis Spectroscopy

The bioreduction of AgNO3 in the solution was periodically monitored by using UV–vis spectrometric measurements (DMB-PC Based UV Spectrophotometer-Systronics 2202) in the 280–760 nm range. It is an important and easy technique to verify the formation of metal nanoparticle. The OD values of the interacted solution were taken periodically at regular intervals (Rajesh et al., 2009).

4.16.4.4. Concentration techniques

The metal processed bacterial filtrate was centrifuged to separate the compounds. The mixture was transferred to centrifuge tubes under aseptic conditions. Centrifugation was carried out at 2000rpm for 30 mins. Supernatant were discarded and pellets obtained were collected in Eppendorf tubes.
4.16.5. Pelletization

The semisolid pellets of extracellular filtrate from the associated microbes were transferred to dried powdered form. 2-3 drops of petroleum ether were added to each of the crude semi-solid pellet. It was then kept for vaporization for about 15 mins. The powered pellet was stored at 4°C.

4.16.6. Characterization of Silver nanoparticle

The characterization of nanoparticle was done by checking the presence of silver nanoparticles and the size of the nanoparticles produced through SEM-EDAX, X-ray diffraction and FTIR methods respectively using standard protocols.

4.16.6.1. SEM-EDAX

The powdered silver nanoparticles were characterized using SEM-EDAX. The dried powder form of nanoparticles were mixed with acetone and loaded in the sample holder. The loaded samples were then dried under vacuum and subjected to SEM studies. It helps to study the size and the elements present in the sample. The Energy Dispersive Analysis of X-ray (EDAX) was also done along with the SEM analysis. EDAX spectrum recorded in the spot-profile mode from one of the densely populated silver nanoparticle regions on the surface of the film for the element mapping. SEM-EDAX (make- JOEL Model 6390) was carried out at 40 KV (Nithya and Raghunathan, 2009).

4.16.6.2. X-ray diffraction (XRD) patterns

X-ray patterns were carried out to check the presence of silver nanoparticles using a D- Max 3A, Rigaku X-ray diffractometer (Make- Shimadzu Model 6000). The powder form of each sample was coated as a thick smear on a glass slide. Then the sample coated slides were analysed by an X-ray diffractometer in the 2θ range from 30-80 with Cu- Kα radiation at 40 KV and 30 mA current (Govindaraju et al., 2010).

4.16.6.3. FTIR Analysis

The biomolecules of isolates mediated AgNPs were studied by FTIR (FTIR Shimadzu 8400S, Japan) and it was recorded in between the range of 400-4000 cm⁻¹ (Nithya and Raghunathan, 2009).
4.17. Antibacterial assay
4.17.1. Collection and characterization of test organisms

18 hours incubated peptone broth of standard MTCC cultures and Hospital cultures equilibrated to McFarland standard turbidity scale $10^5$-$10^6$ CFU/ml for bacteria, were used for the antibacterial studies.

4.17.1.1. Standard MTCC strains

The cultures *Bacillus subtilis* 2393, *Streptococcus* sp. 389, *Corynebacterium* sp. 3080, *Proteus vulgaris* 426, *Salmonella enterica typhimurium* 98, *Staphylococcus aureus* 3160, *Escherichia coli* 4604, *Vibrio cholerae* 3905 and *Candida albicans* 3017 were obtained from Microbial Type Culture Collection centre, Institute of Microbial Technology, Chandigarh and the lyophilised cultures were revived according to the manufacturer’s instructions.

4.17.1.2. *Bacillus subtilis* 2393

This aerobic organism was inoculated in the growth medium Luria Bertani (LB) broth (Tryptone 10.0g, Yeast extract 5.0g, NaCl 10.0 g in 1000 ml distilled water) and incubated at 37°C for 48 hrs.

4.17.1.3. *Streptococcus* sp. 389

These organisms were grown under the aerobic condition and were inoculated in the Blood agar (Suspend 40.0 g of blood agar base in 1000ml distilled water and heat to boiling to dissolve the medium completely. Sterilize at 15 pounds for 15 minutes and bring the medium to 45-50°C. Add 5% sterile defibrinated blood. Mix well and use). The composition of the blood agar base includes 500.0g of Beef Heart Infusion, 10.0 g of Tryptose, 5.0 g of NaCl and 15.0 g of agar. Incubation was given at 37°C for 12 hrs.

4.17.1.4. *Staphylococcus aureus* 3160

This aerobic organism was inoculated in Nutrient agar plate and incubated at 37°C for 24- 48 hrs.

4.17.1.5 *Corynebacterium* sp. 3080

Nutrient agar medium (Beef extract 1.0g, Yeast extract 2.0 g, Peptone 5.0 g, NaCl 5.0g, Agar 15.0g in 1000 ml distilled water) was used for the inoculation of this aerobic organisms and incubated at 30°C for 24-36 hrs.
4.17.1.6. *Proteus vulgaris* 426

The organisms were inoculated in Nutrient agar and incubated at 37°C for 24 hrs.

4.17.1.7. *Vibrio cholerae* (ELTOR 01) 3905

The lyophilised organism was inoculated in Luria Bertani broth and incubated at 37°C for 9-12 hrs.

4.17.1.8. *Salmonella enterica typhimurium* 98

The organisms grown in aerobic condition and are inoculated in the Nutrient agar plate, incubations were given at 37°C for 48 hrs.

4.17.1.9. *Candida albicans* 3017

Malt Yeast Agar (Malt extract 3.0g, Yeast extract 3.0g, Peptone 5.0 g, Glucose 10.0g, agar 20.0g in 1000ml distilled water) was used for the inoculation of *Candida albicans* and incubated at 25°C for 48hrs.

4.17.1.10. *Escherichia coli* 4604

The organisms were inoculated in Nutrient Broth and incubated at 37°C for 24-48hrs.

4.17.2. Clinical isolates

The clinical samples blood, urine, sputum and vaginal swabs were aseptically collected from the patients and transferred to the laboratory in a sterile manner. 5 ml of blood samples were aseptically collected from the patients and transferred to the Brain Heart Infusion Broth and incubated at 37°C for 24-48 hrs. After incubation, quadrant streaking was performed on Macconkey agar plates (MA), Blood agar (BA) and Chocolate agar plates and again incubated. The mid stream urine samples were inoculated directly to the MA and BA plates and incubated at 37°C for 24 hrs. Vaginal swabs collected from the patients were directly swabbed to the MA and BA plates and incubated. Early morning sputum samples without saliva were collected from the patients and inoculated into the BA, MA and CA plates and incubated. The colonies observed after incubation was confirmed by their morphology, Gram staining and biochemical analysis. The isolated pathogenic strains include *Bacillus* sp., *Proteus* sp., *E. coli*, *Klebsiella* sp., *Candida albicans*, *C. tropicalis* and *C. kruzei* (Aneja, 2009).
4.18. Preparation of MHA plates and seeding of test organisms

Sterile Mueller – Hinton agar plates were prepared. The plates were swabbed with standard MTCC cultures and clinical isolates. Using a sterile cork borer; wells were cut in the Mueller-Hinton agar plates.

4.18.1. Testing of Antibacterial activity of Bacterial mediated Silver nanoparticles

The powdered pellet of bacterial mediated extracellular nanoparticles was diluted with DMSO and 25µl from each was added into the respective wells cut into the MHA plates and incubated appropriately at 37° C for 24 hours. The characterized isolated pathogenic cultures were prepared at the dilution of $10^5$–$10^6$ CFU/ml (using McFarland’s standard). Fresh standardized pathogens were swabbed over Muller-Hinton plates. 6 mm wells were bored aseptically and the wells were filled with 50 µl (50 µg/ml) of AgNPs, the solvent and AgNO3. Then the plates were allowed for appropriate incubation with standard antibiotics discs ciprofloxacin (5 µg), ampicillin (10 µg), vancomycin (10 µg), Cefpodoxime (10 µg) and gentamycin (20 µg) were used as positive controls. Comparison of the growth inhibition was done with that of the standard antibiotics (Aneja, 2009).

4.19. Statistical Analysis

All the mean data were analysed by one-way ANOVA using the Sigma state statistical analysis program. Statistical significance was expressed as * for p<0.05 and ** for p<0.01.