Chapter 3

Material and Methods
3.1. ETHNOBOTANY

Ethnic group societies are store houses of acquired experience and knowledge of indigenous vegetation. The focus of the present study is to document the ethnobotanical knowledge with special reference to medicinal plants used by the ethnic groups to cure skin diseases. They represent the pockets of human gene pool and have distinct habits and habitats with ample knowledge on the medicinal properties of their surrounding plants. The Eastern track of Kurnool District, Andhra Pradesh, India is occupied by Nallamalai forest, part of Eastern Ghats, possess different tribal habitations in the form of Gudems or small hamlets.

Among the ethnic groups (Chenchu, Sugali, Yerukala and Yanadi) the Chunches are very ancient and commonly seen adivasis in Nallamalai forest. The yanadis, another seminomadic tribe believed to be derived from the Chenchus of Nallamala (Raghavaiah, 1962). These tribes possess reasonable good knowledge on medicinal properties of plants. The ethnobotanical information on drug yielding plants was documented following standard procedures adopted by Schultes (1960), Jain (1981), Croom (1983), Lipp (1989) and Martin (1995).

Before the commencement of plant exploration an extensive field surveys were carried out in the tribal belts (Thanda or Chenchugudem) and several interviews were conducted to collect the information about ethno medicinal plants used by the ethnic groups to cure skin diseases. Enquiries were made on the food habits, occupation, health practice, medicine, belief, ceremonies, traditions and customs of ethnic groups in the present study.

The traditional healers of ethnic groups were interviewed on the type of medicinal plants used for treating various types of skin diseases, proforma of questionnaire was used to collect the information, specific questions such as plant part used, method of drug preparation and mode of administration such as external (Poulitice, lotion, bath and ointment) and internal (Chewing, ingestion, linking installation) were asked and the information supplied by the traditional healers was documented.
By the nature traditional healers of ethnic groups are conservative and shy and do not like to share the knowledge with outsiders, even with same incentives. The author has even experienced that the traditional healers normally tend to be secretive in the presence of their own tribe, whereas they are fully co-operative when asked in secrecy, therefore they do not like their own tribe to know the traditional knowledge.

3.1.1. Documentation of Ethno-Medicinal Plants

Audio-visual documentation help us in recording the characteristics of the disease symptoms, how to treat that particular ailment with the medicinal plants in tribal people. Documentation on Ethno-medico-botanical information about medicinal plants is recorded by using Handy Camera (Sony). Several tribal people, local traditional healers (Natuvaidhyulu) and vendors are interviewed and information on crude drugs was recorded.

Experimental Medical Ethnobotany

Medicinal plant collection

The Ethnic groups like Chenchu, Sugali, Yerukula and Yanadis follow some principles in collecting the herbal crude drugs.

Preservation

The Ethnic groups like Chenchu, Sugali, Yerukula and Yanadis follow their own methods of drug preservation.

Drug detoxification

The crude drugs which are collected may sometimes contain high amount of toxic, poisonous substances which directly affect the health and cause side effects. The tribal people employ different methods to detoxify such crude drugs.

Herbarium preparation

Before the commencement of plant exploration trips several interviews were conducted in tribal Gudems (villages), (Hamlet) of the representative hotspots in the study region. Mostly the elder people (about 60 years age or above), preferably Grampedda (or) Peddamanishi (Traditional healer), were involved in the interviews.
The information regarding the dosage of crude drugs, mode of preparation and administration was carefully recorded in field note book. Several cross checks were made to evaluate the folklore information by conducting repeated interviews with medicinal / herbal practitioners in different seasons (summer, winter, spring) during my research work.

The voucher specimens for each species was collected in quadruplicates, which are carefully tagged with field numbers after making a critical observation on the habit, habitat, color and odor of flowers, occurrence and other relevant ecological features, which cannot be observed from dried herbarium specimens. Flowering and fruiting periods were accurately recorded for each species. The details were recorded in the field notebook along with vernacular names and relevant information on their utility by the tribal people. The collected specimens were poisoned by dipping the whole twig in ethyl alcohol solution saturated with mercuric chloride. The poisoned specimens were placed between the blotting papers with forceps and these were kept in iron pressers and shade dried. After 12 hrs, the blotting papers were changed and the specimens were spread properly. Some of the leaves were placed facing upwards and some facing down words to show the characters on both surfaces. The spread specimens were once again placed in other blotting papers and tied, and this process was continued until the specimens dried. Flowers and fruits of important and interesting plants were fixed in Formalin: Acetic acid: Alcohol (FAA 5:5:90) in specimen tubes for further studies and reference. The well poisoned, pressed and dried specimens were pasted on herbarium sheets (42" x 28") with the help of natural glue and well stitched according to standard methods (Jain and Rao, 1977). Label containing all relevant information viz., name of the plant, family, locality, altitude, date of collection, notes as in field note book and collector’s name were affixed on the right hand bottom corner of the herbarium sheets. Field accession number was labeled for each collected specimen and the same was provided in the systematic enumeration (followed by vernacular name).

**Identification**

Every specimen, provisional identification was made-with the help of Gamble and Fischer (1957) "Flora of Presidency of Madras", Hooker’s (1879) “Flora of
British India”, “Flora of Kurnool” (Venkataramu and Pullaiah, 1995) as well as other latest floras and monographs. The identification was later confirmed after matching the specimens with the authentic specimens in the S.V. University herbarium (S.V. University), Tirupati. All the specimens were deposited in the herbarium of Botany Department of Sri Venkateswara University, Tirupati.

**Systematic enumeration**

The families, genera within the family and the species within the genus were kept according Bentham and Hooker classification. Nomenclature of each species was checked according to International Code of Botanical Nomenclature (Greuter et al., 1988).

**Citation**

The correct and valid name is given at first, followed by its author name.

**Description**

The usual sequence followed is habit, leaves, shapes, flowers color and fruit type.

**3.1.2. Cross checked with ayurvedic physicians**

Traditional healers of ethnic groups provided information about ethno medicinal plants used to cure skin diseases. The collected information was cross checked with Ayurvedic physicians of Sri Venkateswara Ayurvedic Hospitals, Tirupati, for authentication (Venkatasubbaiah and Savithramma, 2012).

**3.1.3. Statistical analysis**

The data was analyzed statistically using SPSS statistical package for Windows (Version 16.0; SPSS. Inc, Chicago, USA). Chi-square test was carried out to test the association of plant part used to cure skin diseases. Standard error of mean and was carried out to measure the inhibition zones of antimicrobial activity in triplet replications.
3.1.4. Proforma for Collecting Field Data on Medicinal Plants

I. Tribe : Name of the TH:
Gender : Male / Female Age : Below 15 / 15-40 / 40- above
Experience : below 5 / 5-10 /10 above Locality:
Altitude :
Knowledge gained from: Knowledge transferred to:
Occupation :

II. Name of the Diseases :

III. Number of diseases cured :

IV. Data on the Plant :
(a) Scientific name: (b) Vernacular name(s) (specify the dialect):
(c) Family: (d) Habitat: H / S / C / T
(e) In case of tree species: Height and girth and bark nature
(f) Flower color (g) Fruit characteristics (h) smell:
(i) latex present: Yes / No (j) collection and identified: (k) photograph
l) Availability:

V. Description of the Drug:
a) Time of Collection : Morning / Afternoon / Evening / Night
b) Method of preparation of the drug : (1) Natural form (2) Crushed (3) Juice
c) Ingredients Used : single / mixed
d) Mode of Administration :
(1) Internal application (chewing, licking, ingestion, inhalation)
(2) External application (lotion, bath ointment, poultice).
e) Preservation of the drug : Y / N (duration ..............)
f) Plant part used as a medicine: (a) Root (b) Stem (c) Leaf (d) Flower
(e) Fruit (f) Seed (g) Root bark (h) Stem bark (i) Latex (j) Gum
f) Percentage of plant part used for the preparation of drug per 100 gr

VI. Therapeutic indications :

<table>
<thead>
<tr>
<th>Content</th>
<th>Duration</th>
<th>Person</th>
</tr>
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<tr>
<td></td>
<td>Day</td>
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c) diet restriction : Y / N
d) Patent treated with in the Tribe / Other than tribe.
e) Side effects : Y / N / Unknown

VII. Reasons of the Plant for considering as medicine:
(a) Magico – religious belief (b) Traditional (c) Personal experience of healers
d) Strong belief on herbal drugs (e) Tales (f) Proverbs.
b) Satisfaction level on particular drug: satisfied/partially satisfied / not satisfied
c) No.of persons treated :
No.of persons cured :
d) Other Information :
3.2. PHYTOCHEMICAL STUDIES

Preliminary Screening of Secondary Metabolites

The fully matured healthy plant materials collected from Kurnool district of Andhra Pradesh, India during, 2011 different sessions. The roots of Plumbago zeylanica and Withania somnifera; leaves of Eclipta alba, Cadaba fruticosa, Lawsonia inermis and Thespesia populnea; bark of Holarrhena pubescens; seeds of Strychnos nux-vomica; seed oil of Psoralea corylifolia; rhizome of Curcuma longa. To study the cumulative effect of these ten plant parts mixed into equal ratio (2.5 g each) used as an MPE. The materials were washed thoroughly and air dried for 20 days and kept in the hot air oven at 60°C for 24 to 48 hrs and ground to fine powder. This powder extracted with water and preliminary tests were carried out for the detection of secondary metabolites by following preliminary phytochemical screening tests.

3.2.1. Test for Flavonoids (Peach and Tracey, 1956)

The test solution of the extract was dissolved in one ml. of alcohol and then subjected to the following tests:

a. Ferric chloride test: Few drops of neutral ferric chloride solution were added to one ml. each of above alcoholic solution. Formation of blackish red color indicates the presence of flavonoids.

b. Shinoda's test: A small piece of magnesium ribbon or magnesium foil was added to one ml of alcoholic extract and few drops of con. HCl were added. Change in color (from red to pink) shows the presence of flavonoids.

c. Zinc-HCl reduction test: A pinch of zinc dust and few drops of con. HCl were added to alcoholic extract. Magenta color indicates the presence of flavonoids.

d. Lead-acetate test: Few drops of aqueous basic lead acetate solution were added to one ml of alcoholic extract. Reddish brown bulky precipitate indicates the presence of flavonoids.
3.2.2. Test for Steroids (Gibbs, 1974)

The test solution of the extract was dissolved in 5 ml of chloroform separately and was subjected to the following tests:

a. Salkowski test: One ml of con. Sulphuric acid was added to the above solution and allowed to stand for 5 min. after shaking. Lower layer turning into red color indicates the presence of steroids.

b. Liebermann-Burchard test: Few drops of acetic anhydride and one ml. of con. H₂SO₄ was added from the walls of the test tube to one ml. of the extract treated with chloroform and allowed to stand for 5 min. Formation of reddish brown ring at the junction of the two layers and the upper layer turns into green indicate the presence of steroids.

3.2.3. Test for Terpenoids

Fresh plant material was treated with 5 ml of 1% aqueous hydrochloric acid. After 3-6 hrs, the extract was treated with 1 ml of Trim-Hill reagent (10 ml of acetic acid, 1 ml of 0.2% copper sulphate in water and 0.5 ml of concentrated hydrochloric acid) and heated on a water-bath. The appearance of blue colour indicates the presence of diterpenoids while green color indicates the presence of monoterpenoids.

3.2.4. Tests for Tannins (Treare and Evans, 1985)

The test solution of the extract was dissolved in minimum amount of water separately, filtered and filtrates were then subjected to the following tests:

a. Ferric chloride test: Few drops of ferric chloride solution were added to the filtrate. A blackish precipitate indicates the presence of tannins.

b. Gelatin test: Gelatin (Gelatin dissolves in warm water immediately) solution was added to the filtrate. Formation of white precipitate indicates the presence of tannins.

c. Lead acetate test: Few drops of aqueous basic lead acetate solution were added to the filtrate. Reddish brown bulky precipitate indicates the presence of tannins.
3.2.5. Tests for Glycosides (Kokate et al., 1999)

a. Kellar Kiliani test: The test solution of the extract was dissolved in glacial acetic acid and after cooling, 2 drops of ferric chlorides solution was added to it. These contents were transferred to a test tube containing 2 ml of concentrated sulphuric acid. Reddish brown color ring was observed at the junction of two layers.

b. Con. H₂SO₄ test: 1 ml of con. H₂SO₄ was added to 1 ml of test solution and was allowed to stand for 2 min. formation of reddish color indicates the presence of glycosides.

c. Molisch's test: A mixture of Molisch's reagent and con. H₂SO₄ (1:1) was added to the test solution. Formation of reddish-violet colored ring at the junction of two liquids indicates the presence of glycosides.

3.2.6. Tests for Saponins

The test solution of the extract was 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.

3.2.7. Test for Alkaloids (Gibbs, 1974)

The test solution of the extracts was dissolved in chloroform and the solution was extracted with Dil. HCl or Dil. H₂SO₄ and acid layer was taken and tested for presence of alkaloids.

a. Mayer's test: Mayer's reagent (Potassium mercuric iodide solution) was added to the acidic solution. Cream colored precipitate indicates the presence of alkaloids.

b. Wagner's test: Wagner's reagent (Iodine in potassium iodide) was added to the acidic solution. The formation of reddish brown precipitate indicates the presence of alkaloids.

c. Dragendorff's reagent test: 2 ml of Dragendorff's reagent and 2 ml of Dil. HCl were added to the test solution. An orange-red colored precipitate indicates the presence of alkaloids.
3.2.8. Test for Phenols (Gibbs, 1974)

a. Phenol test: 0.5 ml of FeCl₃ (w/v) solution was added to 2 ml of test solution, formation of an intense color indicates the presence of Phenols.

b. Ellagic acid test: The test solution was treated with few drops of 5% (w/v) glacial acetic acid and 5% (w/v) NaNO₂ solution. The solution turns muddy or niger brown colored precipitate in the extract indicates the presence of phenols.

3.2.9. Test for Anthraquinones

0.5 g of the extract was boiled with 10 ml of sulphuric acid (H₂SO₄) and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipette into another test tube and 1 ml of dilute ammonia was added. The resulting solution was observed for color changes.

3.2.10. Tests for Lignin (Gibbs, 1974)

a. Labat test: The test solution was mixed with Gallic acid, it develops olive green color indicating the positive reaction for lignin’s.

b. Lignin test: 2% (w/v) furfuraldehyde was added to the test solution formation of red color indicates the presence of lignin.

3.2.11. Test for Coumarins (Rizk, 1982)

1g of plant powder along with few drops of distilled water is placed in a tube. The tube covered with paper soaked in diluted and boiled NaOH. Yellow fluorescence indicates the presence of coumarins after examination under ultra-violet chamber.

3.2.12. Test for reducing sugars (Fehling’s test)

The aqueous ethanol extract (0.5 g in 5 ml of water) was added to boiling Fehling’s solution (A and B) in attest tube. The solution was observed for a color reaction.
3.3. SYNTHESIS OF SILVER NANOPARTICLES AND ANTIMICROBIAL ACTIVITY

The reason for the selection of ten medicinal plants particularly, 90 plant species mentioned by the traditional healers, 32 plant species are extensively used in the preparation of Ayurvedic medicines by Charaka pharma company (www.charak.com); Ayur pharma company (www.vitiligoherbs.com); Leucoderma drug (www.leucodermatreatment.in); Kerala ayurvedic Ltd; Viadyaratnam Oshadhasala, Kerala; Fours lab, Hyderabad; Imis pharmaceutical company, Vijayawada. Among the 32 plants 5 plant species are extensively used by the pharma companies for the preparation of drugs and five more plants were also selected from 58 plant species which are not using in the preparation of Ayurvedic medicines to cure skin diseases so far. Hence these ten plants and MPE were selected for the preparation of SNPs.

SNPs also preparing chemically, which is cost effective and may have side effects, environmentally pollutant and time consuming, when compare with biological synthesis of SNPs. Moreover the preparing of SNPs from medicinal properties concern plants. Not only is the biological synthesis of SNPs less cost but also these are environmentally safer and less or no side effects.

3.3.1. Collection of plant material

The plant material was collected from the Eastern tract of Kurnool District, Andhra Pradesh, India occupies Nallamalais as a part of Eastern Ghats. The roots of Plumbago zeylanica and Withania somnifera; leaves of Eclipta alba, Cadaba fruticosa, Lawsonia inermis and Thespisia populnea; bark of Holarrhena pubescens; seeds of Strychnos nux-vomica; seed oil of Psoralea corylifolia; rhizome of Curcuma longa. To study the cumulative effect of these ten plant parts mixed into equal ratio (2.5 g each) used as an MPE.

3.3.2. Preparation of extract

Plant materials were washed with tap water and finally distilled water, cleaned and pressed with blotting paper. Then the plant materials were cut into small pieces and shade dried for 45 days and ground to make a fine powders. 5 g of plant powder
was taken into 250 ml conical flask and added 100 ml of sterile distilled water and boiled for 10 min at 100°C on water bath. Then plant material extracts were collected in separate conical flask by standard filtration method and stored at refrigerator for further use.

Soxhlation process

Soxhlation is an extraction process by which the plant material is going to be mixed with the methanol solvent and the plant extract is obtained by maintaining particular temperature. Weigh the plant material. 250 g of weighing powder is taken and it is packed in a filter paper. Place the powder in a soxhlet apparatus. Take 500 ml of solvent in round bottomed flask and placed the downside of the soxhlet, and top side of the soxhlet immerse. The condenser contains the outlet and inlet pipe line for continuous water supply to avoid evaporation of solvents. The total set up have to placed in water bath and allow it to boil for six hours at 64.5 to 68°C temperature. This process was carried out for mixed plants extract only.

3.3.3. Preparation of 1 mM Silver nitrate solution

1 molar silver nitrate stock solution was prepared by 1.7 g of Ag (NO₃)₂ was dissolved in 10 ml distilled water. 1 mM solution was prepared by 1 ml of 1 M solution was made up to 100 ml with 99 ml of distilled water. This solution was stored in amber colored bottle for further use.

3.3.4. Synthesis of silver nanoparticles

5 ml of plant material extracts were taken into conical flasks separately and to this 100 ml of 1 mM AgNO₃ solution was added. The color change of the plant extracts indicated the synthesis of silver nanoparticles. The contents were centrifuged at 10,000 rpm for 15 min. The supernatant was used for characterization of the silver nanoparticles through UV-Vis spectrum and AFM.

3.3.5. UV-Vis spectra analysis

The reduction of pure silver ions was monitored by measuring the UV-Vis spectrum of the reaction medium at 3 hrs. after diluting a small aliquot of the sample
into distilled water. UV-Vis spectral analysis was carried out by using UV-Vis spectrophotometer (Systronics type 118).

3.3.6. AFM analysis

The silver nanoparticles extracted by the above protocol were visualized with an Atomic Force Microscope (AFM). A thin film of the sample was prepared on a glass slide by dropping 100 µl of the sample on the slide and was allowed to dry for 5 min, the slides were then scanned with the AFM (Nano surf ® AG, Switzerland, Product: BTO2089, 3RO). Nanosurf ® Easyscan-2 software was used for the AFM analysis (VIT, Vellore, Tamil Nadu).

Antimicrobial activity of silver nanoparticles

3.3.7. Cultures

The microbial cultures were procured from the Department of Applied microbiology, Sri Padmavathi Mahila Visvavidyalayam (SPMVV) and Sri Venkateswara Institute of Medical Science (SVIMS), Tirupati. Five bacterial strains including Staphylococcus aureus, Salmonella typhi, Escherichia coli, Bacillus subtilis and which were multidrug resistant strains were maintained on nutrient agar slants at 4°C until further use and fungal organisms include Paecilomyces varioti, Penicillium rubrum, Aspergillus flavus, and which were maintained on Potato Dextrose Agar (PDA) slants at 4°C until further use.

3.3.8. Bacterial cultures

Staphylococcus aureus

Staphylococcus aureus is gram-positive bacteria coccid shape, live on the skin and mucous membranes of human about 15-40% of healthy humans are carries of S. aureus. Causes boils, hair loss, etyma, dermatitis and scabies.

Salmonella typhi

Salmonella typhi is a pathogenic gram-negative bacteria predominantly found in the intestinal lumen. Its toxicity is due to an outer membrane consisting largely is lipopolysaccharides (Slauch and James, 2007). Growing temperature minimum 7°C,
maximum 45°C. *S. typhi* causes gastroenteritis in human fever, spots on trunk (Everest and Paul, 2007).

**Escherichia coli**

*E. coli* was discovered by German pediatrician and bacteriologist Theodor Escherich in 1885 (Ryan and Ray, 2004). *E. coli* was Gram negative, non-sporulating, facultative anaerobic, motile and opportunistic colon commensal. Optimal growth of *E. coli* occurs at 37°C (Todar *et al*., 2005). It causes enteric disease, haemorrhage colitis (blood haemorrhage), urinary tract infections, pyogenic infections and septicemia (Todar, 2007 and Riley *et al*., 1983). *E. coli* and certain other food borne illnesses can sometimes trigger serious health problems.

**Bacillus subtilis**

*Bacillus subtilis* was a gram positive, rod shaped, endospore forming catalase-positive bacterium commonly found in soil (Madigan and Mortinko, 2005). *Bacillus subtilis* contaminate food and causes food poisoning (Ryan and Ray, 2004). It causes conjunctivitis followed by iridochoroiditis (Greenwood *et al*., 1997).

### 3.3.9. Fungal cultures

**Paecilomyces varioti**

Paecilomyces is a cosmopolitan filamentous fungus which inhabits the soil, decaying plants and food products. *Paecilomyces varioti* are thermophilic and can grow well at temperatures as high as 50 and possibly 60°C (Dehoog *et al*., 2000 and Sutton *et al*., 2003). *P. varioti* also causes Hyalohyphomycosis in obstetrical patients. It is usually considered as a contaminant but may also cause infections in human and animals (Dehoog *et al*., 2000). Paecilomyces may develop corneal ulcer, keratitis and endophthalmitis due to extended contact lens use or ocular surgery. Paecilomyces is among the emerging causative agents of opportunistic mycoses in immuno compromised hosts (Groll and Walsh, 2001).
**Pencillium rubrum**

*Pencillium rubrum* represent almost a common mycoflora of soil and other organic matter and produces real pigmentation on the substrants on which it grown, the rarity of occurrence of such pigmentation on chrome-tanned hide and impact on other organic matters in surprising and obviously, strict specific involved pigment formation.

**Aspergillus flavus**

*Aspergillus flavus* had a worldwide distribution and normally occurs as a saprophyte in soil and many kinds of decaying organic matter. *A. flavus* was grown by producing thread like branching filaments known as hyphae. Filamentous fungus such as *A. flavus* was sometimes called molds. It can also pathogenic on several plant and animal species, including human and domestic animals. The fungus can infect seeds of corn, peanuts, cotton and nut trees. The fungus can often seen sporulating on injured seeds of maize kernels. Growth of the fungus on a food source often leads to contamination with aflatoxin, a toxic and carcinogenic compound. *A. flavus* was also the second leading cause of aspergillosis in human. Patients infected with *A. flavus* often have reduced or compromised immune systems.

3.3.10. Preparation of the medium

**Nutrient Agar media (pH 7.0)**

For the preparation of 1 liter of Nutrient Agar medium ingredients like 1.5 g of beef extract, 1.5 g of yeast extract, 5 g of peptic digest of animal tissue and 5 g of sodium chloride were weighed and added in 500 ml of distilled water and heated with agitation to dissolve the constituents. Finally, the volumes were made up to 1 liter. Before the addition of agar (15 g) the pH of the medium was adjusted to 7.0 by adding few drops of 0.1 N NaOH or HCl using digital pH meter (Elico Pvt. Ltd., Hyderabad). These were then sterilized by autoclaving at 15 lbs pressure at 120°C for 15 min; cooled to 40°C and approximately 20 ml of medium was poured to each 90 mm sterilized petridishes.
**Preparation of Potato Dextrose Agar medium (PDA)**

For the preparation of PDA, 200 g of potatoes were weighed and cut into small pieces and suspended in 500 ml of distilled water and boiled for 30 minutes or the time till they were easily penetrated by a glass rod and filtered through cheese cloth, squeezing out all the liquid. 20 g of dextrose was added to the potato extract. 15 g of agar was added to 500 ml of heated distilled water in another beaker. Finally the above two were mixed to make 1 liter and sterilized by autoclaving at 15 lbs. pressure at 120\(^0\)C for 15 min cooled to 40\(^0\)C and approximately 20 ml of medium was poured to each 90 mm sterile petridishes. Slants were used to maintain the pure culture to enable the required amount of microorganisms for assay. The master cultures were maintained carefully in sterile conditions during subculture.

3.3.11. Preparation of inoculum

**Bacteria:** 18 hrs old bacterial broth cultures was used as inocula after adjusting its population to 10\(^6\) CFU/ml (Colony Forming Units) using 0.9% (w/v) sterile saline by the method described by Forbes *et al.* (1990).

**Fungi:** 48 hrs fungal cultures grown in Potato Dextrose Broth (PDB) were used for the experimental studies.

3.3.12. Antibacterial and Antifungal Assays by Disc diffusion method

The antibacterial and antifungal activity of isolated plant silver based nanoparticles extract was tested by standard disc diffusion method. The tested bacteria were maintained on nutrient agar (Hi-media) slants. A loopful of culture from the slant was inoculated into the nutrient agar broth and incubated at 37\(^0\)C for 24 hrs and 0.1 ml of this culture evenly spread on the nutrient agar plate. The fungal strains were maintained on Potato Dextrose Agar (PDA) medium (Hi-Media Lab Ltd., Mumbai). A loopful of fungal culture from the slant was inoculated into the Potato Dextrose Agar Broth and incubated at 28\(^0\)C from 48-72 hrs and 0.1 ml of this culture was evenly spread on the nutrient agar plate.

Steriled discs of Whatman No.1 filter paper of about 6 mm diameter were placed on the surface of the medium (Bauer *et al.*, 1966). Using steriled micropipette
20 μl (0.02 mg) of the sample of nano-particle solution or extract was applied on the discs and incubated at 35°C for 24 hrs for bacteria and 48-72 hrs. at 25°C for fungi. After incubation, the plates were observed for zones of inhibition surrounding the disc. Different levels of zones of inhibition were measured using the Hi antibiotic zone scale and recorded. Traditional microbiological plating, were used to study antifungal activities of AgNPs. A zone of inhibition around the disc indicates that the compounds, which diffused into the agar from the disc, inhibited the growth of the organism. The medium without silver nanoparticles served as control. The diameter of inhibition zone around each disc with SNPs of single plant extract (SPE) and SNPs of mixed plants extract (MPEs) is represented and each disc contains of 20 μl of SNPs of individual plant and SNPs of mixed plants extract solution. All experiments were repeated thrice for appropriate results.