MATERIALS AND METHODS

3.1 SURVEY AND COLLECTION OF MEDICINAL PLANTS:

Data has been obtained from native informants who are healers, Hakims (Ayurvedic medical practiceners), Priests, Sanyasis (Hermit) and common people who have knowledge of therapeutic value of plants. Because of the fact that majority of population in rural area are illiterate, oral interviews were held and derived information has been recorded. Information's were documented with the help of questionnaire containing questions on various utility of plants specially therapeutic uses, local and common name of the plant, economic importance, uses in animal and human diseases, plant parts used, method of preparation of drug its application, doses and duration etc. In majority of the cases prescription are known to all people of the region particularly for common ailments, like cuts, fever, headache etc. Locating knowledgeable informant was not easy, in most villages normally there were one or two elders, who are familiar with the traditional medicines. These medicine man were very resourceful and are called "Vaid ji" or "Syana". Information has also been collected from Sapera tribe (Snake charmer) of Jhajjar district of the state. This community is engaged with the treatment of cases of snake bite of the area. During discussion, the informants were given a leading role and what they said was not contraindicated.

Local people have their own names for all the plants. Some time through local names of the plants the rural people distinguish even closely allied species. Extensive and frequent field survey in all the districts of the state were conducted during Nov, 2006 to July, 2008. The information's obtained from knowledgeable persons were recorded in the pre-designed data collection performa. The knowledgeable persons were requested to accompany in the fields and forest areas for identification of the plants. Plants were collected in the plastic polythene bags brought to the laboratory. They are washed thoroughly to remove dust and are subjected to dry in shade.

Table -1.
Data Collection Performa

Data acquisition questionnaire for utilization of medicinal plants in Haryana, India.

Questionnaire

PART 1: Informants details.

Name.....Sex....M/F. Age.....Years.

Occupation.....

Level of education.....

Location/Residence.....

Efficacy Data

Type of Disease treated .....

Plant name in local language .....

Medicine Preparation method(s).....

Part(s) of plant used .....

Methods of application.....

Response of Patient Good.....Fair..... Poor.....

PART 2: Researcher Declaration

1. The following research will be undertaken with respect to the indigenous knowledge and intellectual property of the traditional healers of Haryana.
2. We will at no given time initiate or conduct practices that are deemed to obtain information from the respondents by intimidation, coercion or false pretence.
3. The respondents will be informed of the intended project elaborately prior to questionnaire administration and in confidential to eliminate any degree of conspiracy.
4. We will be no under any obligation to edit or tamper the information provided by the respondents.
5. The information collected will be used for the described research purpose and not any undisclosed intentions.

Signatory Researchers: Anand Singh Dangi

All medicinal plants collected were identified at Forest Research Institute (FRI), Dehradun and from the herbarium of the department of Genetics, M.D.University, Rohtak. Different medicinal plants collected includes *Achyranthus aspera* (Apamarg), Holoptelea integrifolia (papri), *Cassia fistula* (Amaltas), *Kigellia pinnata* (Balam khira), *Cuscuta* re*flexa* (Amarbel), *Tridex procumbens* (Sadahari), *Coccinia indica* (Ram kachari), *Phyllanthus niruri* (Hajar dana), *Heliotropium zeylanicum* (Hast sundi), *Euphorbia prostrata* (chhoti dudhi), *Tagetus erecta* (Genda*), Asphodelus tunifolius* (pyaji), *Leucas cephalotus* (Goma), *Euphorbia hirta* (dudhi bel), *Canabis sativa* (Bhang), *Solanum xanthocarpum* (Katehli*), Solanum nigrum* (makoi) and *Physalias minama* (Palpotan).

3.2. PREPARATION OF HERBARIUM

Plants for preparation of herbarium were collected from the field area in triplicate. In case of larger plants such as trees and shrubs a suitable branch of appropriate size with at least 2-3 leaves and flowering portion were collected. Other key identification or economically important parts, such as fruits, seeds etc were also collected and pasted separately on the same sheet or put in to museum jars.

Specimens were trimmed, cleaned and labeled in the field and collected in the polythene bags and latter placed in between news paper sheets ensuring that leaves were properly spread out with no overlap of plant parts. Paper containing specimen, were pressed between two parts of herbarium press. The sheets were changed with in 24 hrs and there after every day for ten days until completely dry.

Specimens were poisoned as early as possible. The poisoning was done by dipping the whole plant in a 2% solution of mercuric chloride in ethyl alcohol. The plants were again put in dryer and pressed till completely dried. Mercuric chloride is corrosive for metals and so enamel trays were used. Disposable gloves were used while poisoning because mercuric chloride is deadly poisonous chemical and its effect on human being is cumulative.

The specimens were affixed on a mounting sheets from heavy long lasting white card sheets of size 28 x 42 cm. Specimens were pasted on the sheets with Fevicol.

Herbarium label of size 8 x12 cm containing information about collection number, date, family, genus, species, habitat, distribution, description, vernacular name and uses photo data and collector's name were fixed on the bottom right hand corner of mounting sheet were fixed with paste and glue. Specimens were arranged according to Bentham & Hooker's genera plantarum. Voucher specimens have been deposited in the department of Genetics, Maharshi Dayanand University, Rohtak (Haryana).

Table : 2

List of medicinal plant collected for Antimicrobial activity

S.N.	Botanical name	Vernacular name.	Name of plant part used.	Quantity of dry plant material used.
1.	*Achyranthus aspera* (Linn.)	Apamarg	Whole plant	250 gms
2.	*Holoptelea integrifolia* (Roxb.)	Papri	Leaves	220 gms
3.	*Cassia fistula* (Linn)	Amaltas	Pods	350 gms
4.	*Kigellia pinnata* (DC)	Balamkhira	Flowes	280 gms
5.	*Cuscuta reflexa* (Roxb)	Amar Bel	Whole plant	250 gms
6.	*Tridex procumbens* (Linn)	Sadahari	Whole plant	300 gms
7.	*Coccinia indica* (Wt &Arn)	Ram kachari	Fruits	350 gms
8.	*Phyllanthus niruri* (Hook.f.)	Hazar dana	Whole plant	220 gms
9.	*Heliotropium zeylanicum* (Cl)	Hastsundi	Whole plant	280 gms
10.	*Euphorbia prostrata* (Ait)	Chhoti dudhi	Whole plant	280 gms
11.	*Tagetus erecta (Linn.)*	Genda	Flower	220 gms
12.	*Asphodelus tunifolius* A (Cav.)	Pyaji	Fruits	320 gms
13.	*Leucas cepholotus* (Spreng.)	Goma	Whole plant	360 gms
14.	*Euphorbia hirta* (Linn.)	Dudhibel	Whole plant	280 gms
15.	*Canabis sativa* (Linn.)	Bhang	Whole plant	260 gms
16.	*Solanum xanthocarpum* (Schard.)	Chhoti kateli	Whole plant	320gms
17.	*Solanum nigrum* (Linn.)	Makoi	Whole plant	300 gms
18.	*Physalis minima* (Linn)	Palpotan	Whole plant	350gms

3.3. PREPARATION OF PLANT'S EXTRACTS :

Soxhlet Extraction:- Soxhlet apparatus is first to make use of siphon to effect automatic intermittent infusion. A soxhlet extractor is a piece of laboratory apparatus invented in 1879 by Franz Van Soxhlet. It was originally designed for extraction. A glass soxhlet apparatus is a purification technique particularly useful in cases when pure compound is partially soluble in a solvent and impurity is not soluble in that solvent and vice versa. The coarsely powdered plant material is put in to a paper thimble which is then placed in to the main chamber of soxhlet extractor. Extraction is carried out in a suitable solvent kept at elevated temperature (below the boiling point of the solvent) in the heating mantle for hot extraction, just up to the bead mark in the siphon tube. The amount of coarsely powdered plant material used for extraction has been given in table - 2. Solvent dissolve the colored ingredients. As siphon action begin a continuous flow of extract start in the receiver flask at the bottom. Soon whole of the extract is collected in the flask. The vapors of the solvent formed at elevated temperature condense and increase the solvent level to the bead mark to start siphon action. The process is repeated till all coloring material is extracted and light colored solution is obtained. All collected solution were mixed. The extraction of plant material were made in different solvents viz petroleum ether, ethanol, methanol and water sequentially according to their increasing polarity. Solvents were then recovered from rotary evaporator at 40ºC. Dried extracts were lyophilized in lyophilizer. The lyophilized extracted materials from all the 18 plants were kept in separate screw caped vials and stored in the refrigerator. All vials were labeled and weight accurately to calculate their yield.

3.4. MICROBIAL STRAINS USED.

Following nine bacterial strains and two fungal strains ATCC type(American type culture collection) or MTCC (Microbial type culture collection) has been selected for the present study. The bacterial strains includes, *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus mirabilis, Salmonella typhi, Shigella flexineri, Vibrio cholera* (ogawa) and *Serratia mercesense*. Two fungal strains includes *Candida albicans* and *Aspergillus niger*.

BACTERIAL STRAINS

***Staphylococus aureus* (ATCC 259323)**

They are spherical cocci about 0.8 -1.0 µm in diameter and are arranged in grape like clusters. Cluster formation is due to cell division occurring in more than one plane with daughter cells remaining close together. They are non motile, non sporing. *Staphylococcus aureus* is catalase positive and oxidase negative. It is indole negative and shows positive reactions for Voges Proskeur test, urease, and can reduce nitrogen. Pathogenicity includes cutaneous infection, wound and burn infection, pustules, boils and carbuncles, styes, impetigo and pemphigus. Deep infection includes osteomyelitis, tonsillitis, empyema, bronchopneumonia, breast abscess and septicemia etc. Laboratory diagnosis includes pus culture. Gram staining of pus reveals pus cells and gram positive cocci in clusters.

***Escherichia coli* (ATCC 25922)**

Escherichia coli was isolated in 1885 from faeces of an infant by a microbiologist Escherich**.** It is gram negative, non capsulated bacillus. 80 % strains are motile. It can grow on ordinary media like nutrient agar at 37ºC. Most strains ferment lactose rapidly. The colonies on Macconkey agar medium are pink in colour. *Escherichia coli* are Indole and methyl red reaction positive and is negative for Voges- Proskuer reaction, citrate, urease and hydrogen sulphide production and ferment sugar. *Escherichia coli* forms a part of normal intestinal flora of man and animal. It can cause four types of clinical syndrome i.e. urinary tract infection, diarrhea and dysentery, pyogenic infection and septicaemia.

***Klebsiella pneumoniae* (ATCC-700603)**

Klebsiella are gram negative, non sporing, non motile bacilli. The name *Klebsiella pneumoniae* is used for species as a whole. *Klebsiellae* do not have flagella but most strains posses fimbriae. *Klebsiella* are indole negative but it shows positive methyl red test, citrate and urease test. *Klebsiellae* are widely distributed in nature and in the

gastro-intestinal tract of human and animal. They are found in the oropharynx of 1-6 % of normal healthy individuals. However, a prevalence as high as 20% may be seen in hospitalized patient. It has also been found in urinary tract infection, wounds and burn infection and as a secondary invader in other respiratory infections. In fact they are most frequently encountered gram negative pathogen causing nosocomial infection of lower respiratory tract and are second only to *Escherichia coli* as a cause of primary bacteriemia by gram negative organisms. They may also cause meningitis and diarrhea. The latter may be due to production of heat labile and heat stable enterotoxin by these organisms (Arora *et al*; 1983).

***Pseudomonas aeruginosa* (ATCC 27853)**

It is a slender, gram negative bacillus. It is non sporing and non capsulated. It is strict aerobe and grows well on ordinary media. It gives negative indole test, methyl red, Vogus Proskeur and hydrogen sulphide test. But it shows oxidase positive with in 30 seconds and gives catalase positive reaction. It is very resistant to chemical disinfectant and can even grow in certain type of quaternary ammonium compounds. It has ability to persist and multiply in moist environment of hospital wards, bathrooms, kitchens and even in disinfectant solutions. It also causes urinary tract infection. It is able to multiply on respiratory ventilator and deliver large number of organisms directely in to the lungs. Septicemia may develop in patients with immunosuppressive drugs. It may cause wound and burn infection and can also cause chronic otitis media & externa, eyes infection etc. Specimens like urine, pus, cerebrospinal fluid, blood etc can be inoculated on Macconkey agar and blood agar.

***Proteus mirabilis* (ATCC 43071)**

Proteus are gram negative coccobacilli. They are actively motile by peritrichous flagella. They can grow on ordinary media like nutrient agar and culture emits putrefactive (fishy or seminal) odour. *Proteus mirabilis* and *Proteus vulgaris* posses the ability to swarm (Spread) on solid media. The swarming growth on a plate may eventually appear either as uniform film of growth extending over the whole plate. Swarming of the *Proteus* appear due to vigorous motility of the organism although exact

cause is not yet established. *Proteus mirabilis* shows negative indole test and positive methyl red test, urease and H_2S production test. It is most important species (70-90%) recovered from human particularly as a causative organism of urinary tract and wound infections. It may also cause bed sores, osteomyelitis and in neonates it may cause infection of umbilical stump.

Salmonella typhi (ATCC 13311)

Genus Salmonella has been named after American microbiologist , D.E. Salmon. These are gram negative, non sporing, non acid fast. They are indole test negative, methyl red reaction positive and Voges proskauer test negative. Man acquires infection by ingestion of contaminated food. *Salmonella* causes enteric fever and septicemia. Laboratory diagnosis of enteric fever can be made by isolation and identification from a specimen of blood culture and stool culture. Rose spot and circulating antigen and demonstration of circulating antibodies in patient's serum. *Salmonella* are shed in the faeces throughout the course of disease. Widal test is the only indirect evidence of infection. *Salmonella* food poisoning is generally a zoonotic disease. It is caused by ingestion of contaminated food. Chloremphenicol has been the treatment of choice for typhoid fever since its discovery in 1947. But due to the spread of plasmid mediated chloremphenicol resistant *Salmonella typhi* throughout the world newer antibiotics with good in vivo activity are needed.

***Shigella flexneri* (ATCC 12022)**

Shigella are non motile non flagellate, non sporing, non capsulated gram negative bacilli. They are methyl red positive and shows negative reaction for Voges- Proskauer, citrate and melonate production. Members of the genus *Shigella* produce a serious illness known as bacillary dysentery. It is an acute diarrhoeal disease characterized by more severe infection by presence of blood and mucus in the stool. Human appear to be only natural host for *Shigellae.* They become infected by ingestion of contaminated food and water. Bacillary dysentery has a global distribution. It is commoner in countries with warm and temperate climate and in poor than affluent countries. It is mostly associated in the crowding and bad hygienic conditions encountered in times of wars and other

disasters and in jails and mental institutions. In India *Shigella flexineri* has always been the predominant species (50 -85 %). Stool culture is the main laboratory diagnosis.

Vibrio cholera (Ogawa) (ATCC 39315)

These are gram negative, curved rods that are actively motile by a single polar flagellum. They are catalase positive, oxidase positive and non sporing. S form or spiral may be seen due to two or more cells lying end to end. They show vigorous darting motility. They are gram negative, non sporing, non capsulated and non acid fast. Venkatraman- Ramakrishan medium and Cary-Blair medium are transport medium for *Vibrio.cholerae.* Plating media used commonly is bile salt agar. *Vibrio cholerae* causes an acute diarrhoeal disease known as cholera. It occurs only in man.

Serratia marcescens (ATCC 27137)

These are motile, gram negative coccobacilli. They posses peritrichous flagella and type 1 and 3 fimbriae. *Serrtia mercescens* the most important member of the genus. They are motile and gives negative indole and methyle red test and positive Voges Proskeur and citrate test. The colonies on nutrient agar are usually homogenous and they may develop a convex, red pigmented and relatively opaque centre. The red pigment, prodigiosin is insoluble in water and does not diffuse away from the colony. Therefore the colonies are red or pink. *Serratia* is a saprophyte found in water, soil and food. However it can led to serious nosocomial infection particularly in newborn, debilitated or patients receiving immunosuppressive drugs. These includes infections of wound, urinary and respiratory tract, meningitis, endocarditis, septicaemia and endotoxic shock.

TWO FUNGAL STRAINS INCLUDES

Candida albicans.(ATCC 10231)

This organism occurs both in the form of oval yeast like bodies and as a thick septate psedo-hyphae. It occurs as a normal inhabitant of the mouth and intestinal tract. It is the cause of thrush, a condition at one time common in children, in which white patches containing fungus are found in the mucous membrane of mouth. It may also

cause infection of vaginal and vulval mucosa. On Sabouraud medium the colonies are cream coloured and pasty and on prolonged incubation develop a honeycombed appearance as a result of glucose fermentation. *Candida albicans* ferment glucose and maltose producing acid and gas and sucrose producing acid but no gas; it does not ferment lactose.

Aspergillus niger (ATCC 16404)

Aspergillus species are occasionally responsible for otomycosis, a superficial scaly infection of the skin of external auditory meatus. They also appear to be capable of invading the lungs; this, however is a very uncommon type of infection occurring as a rule only in patients with established pulmonary disease. Since *Aspergilli* are common contaminants a diagnosis of Aspergillosis should be made only when the organisms have been repeatedly isolated and when in addition, it has not been possible to demonstrate any other pathogen. *Aspergillus niger* showed black colored colonies on Sabouraud's dextrose agar.

3.5. COMPOSITION OF VARIOUS CULTURES AND SENSITIVITY MEDIA.

Peptone water

Peptone	= 10 gm
Sodium chloride	=5 gm
Distilled water	= 1000 ml
pH	= 7.4

Boil the contents in distilled water, cool, filter and adjust the pH with the help of Lovibond comparator using phenol red indicator and 1N Hydrochloric acid and 1N Sodium hydroxide. Autoclave at 15 lbs pressure (121º C) for 15 minutes.

Nutrient agar: Nutrient broth is the basis of most of the media. This is the best medium for the growth of all types of bacteria. Nutrient agar is the best medium used for sensitivity purpose as all bacteria can readily grow on it and also used for the preparation of other media like blood agar, salt agar, bile salt agar and MacConkey agar etc with the

slide modifications. But in the present study Muller Hinton agar has been used for sensitivity of all test organisms.

Nutrient agar.

Peptone	= 10 gm
Sodium chloride	= 5gm
Beaf extract	= 10gm
Agar agar	= 20 gm
Distilled water	= 1000ml
pH	= 7.4

After autoclave at 15lbs pressure (121º C) for 15 minutes cool at 40º C and pour in sterile petri plates.

Blood agar.

Peptone	= 10 gm
Sodium chloride	= 5 gm
Beef extract	= 10gm
Agar-Agar	= 20 gm
Distilled water	= 1000 ml
pH	= 7.4

After autoclaving at 15 lbs pressure (121ºC) for 15 minutes. When the medium is cooled at 55º C then added 5-10 % of whole sheep blood, mix well and pour in sterile pteri plates.

Salt agar.

Peptone	= 10 gm
Sodium chloride	= 80 gm
Beaf extract	= 10 gm
Agar-Agar	= 20 gm
pH	= 7.4

Autoclave at 15 lbs pressure (121ºC) for 15 minutes. Cool at 40º C and pour in sterile petri plates. Salt agar is used for the growth of *Staphylococcus aureus* with out any contamination.

Bile salt Agar.

Peptone	= 10 gm
Sodium chloride	= 5 gm
Sodium taurcholate	= 5gm
Beaf extract	=10 gm
Agar-Agar	= 20 gm
pH	= 8.2

Bile salt agar is the medium of choice for the growth of *Vibrio cholerae* isolated from stool culture collected in alkaline peptone water or Vanket Raman fluid (V.R. Fluid). V.R. fluid is the transporting medium for stool samples.

Mac-Conkey agar.

Peptone	= 20gm
Sodium chloride	= 5gm
Sodium taurcholate	= 5 gm
Lactose	= 10 gm
Neutral red	= 3.5ml
(2% in 50% ethanol)	
Distilled water	= 1000 ml

Dissolve the contents in distilled water, boil, cool and filter then adjust pH and add 2% neutral red solution and also add 20 gm of agar agar powder. Autoclave at 15 lbs pressure (121ºC) for 15minutes. Cool at 40ºC and pour in sterile Petri plates. MacConkey agar medium is used for the growth of lactose fermenting (Pink colored colony) and non lactose fermenting bacilli (Non pink colored colony). Most of the bacteria included in this study can grow on this medium except *Staphylococcus aureus*.

Muller Hinton agar media:- This medium is available in dehydrated form (from Hi-Media Pvt Ltd., Mumbai). Suspend 38.0 gm of Muller Hinton agar powder in 1000 ml of distilled water and boil to dissolve the medium completely, adjust pH 7.4 and autoclave at 15 lbs pressure (121º C) for 15 minutes. Dispense the medium in sterile Petri-plates.

Preparation of Mueller –Hinton Agar.

Mueller-Hinton agar preparation includes the following steps.

1. Mueller –Hinton agar should be prepared from a commercially available dehydrated base according to the manufacturers instructions.
2. Immediately after autoclaving, allow it to cool in a 45 to 50º C.
3. Pour the freshly prepared and cooled medium in to sterilized glass or plastic flat bottom Petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 25-30 ml for plates with diameter of 100 mm and 60-70ml of medium for plates with diameter of 150 mm.
4. The agar medium should be allowed to cool to room temperature and the plate can be used same day. Store in a refrigerator (2 to 8º C).
5. Plate should be used with in seven days after preparation.
6. A representative sample of each batch of plate should be examined for sterility by incubating at 30-35º C for 24 hours or longer.

3.6 BIOCHEMICAL TESTS FOR THE IDENTIFICATION OF BACTERIA

A large number of biochemical tests can be employed for the identification of different bacteria. These includes, Indole test, Methyl red, Voges- Proskauer, citrate utilization, urease production, Hydrogen sulphide production, Mannitol motility, Sugar fermentation, catalase production and oxidase test.

1. **Indole test** :- Certain bacteria which posses enzyme tryptophanase, degrade amino acid tryptophan to indole, pyruvic acid and ammonia. Indole can be

detected by inoculating test organisms in to peptone water incubated at 37°C. for 48-96 hours. Now add 0.5ml Kovac's reagent and shake gently. A red color ring indicates a positive reaction. Negative reaction will show yellow color. *Escherichia coli* gives positive and *Klebsiella pneuminiae* gives negative indole test.

2. **Methyl red test**:-This test detects the production of sufficient acid by fermentation of glucose so that pH of the medium fall and it is maintained below 4.5. Incubate the test organism in glucose phosphate broth and incubate at 37º C for 2-5 days. Add 5 drops of 0.04 % solution of methyl red, mix well and read the result immediately. Positive test shows bright red color (indicating low pH). Negative shows yellow color. Escherichia gives positive methyl red test.

3. **Voges- Proskauer test** (for acetoin production): - Many bacteria ferment carbohydrates with the production of acetyle methyl carbinol (acetoin). In the presence of KOH and atmospheric oxygen, acetoin is converted in to diacetyle and alpha –naphthol serve as a catalyst to form red complex. This test is usually done in conjugation with methyl red test. An organism of family enterobacteriaceae is usually either methyl red test positive or Voges Proskaur test positive. Inoculate test organism in glucose phosphate broth and incubate at 37ºC for 48 hours. Add 1ml KOH and 5% solution of alpha-naphthol in absolute alcohol. A positive reaction is indicated by development of pink color in 2-5 minutes and crimson in 30 minutes.

4. **Citrate utilization test:** - This test is done to study the ability of an organism to utilize citrate as a sole source of carbon for the growth. Solid (Simmen's) medium containing citrate as a source of carbon can be used. A part of colony is picked up and inoculated on to the solid medium. Solid medium also contain bromothymole blue as indicator. Appearance of growth and blue color is the positive where as no growth and original green color is negative.

5. **Urease test**: - This test detects the ability of organism to produce urease enzyme. The test organism is inoculated on the entire surface of Christensen's medium

which contain urea and phenol red indicator in addition to other constituents including agar. It is incubated at 37ºC. After 4 hours and after overnight incubation, development of purple pink color indicates production of urease. The latter in presence of water turn urea in to ammonia make the medium alkaline and phenol red indicator changes to purple pink in color.

6. **Hydrogen sulphide test**: - Some organisms produce hydrogen sulphide from sulphur containing amino acid. It may be detected by suspending a strip of filter paper impregnated with 10% lead acetate between cotton plug and tube. In positive test the paper strip will turn black.

7. **Manitol motility test**: - Manitol motility medium containing agar concentration of 0.4 % or less to allow free spread of organism. Inoculation is done by single stab in to the medium. After overnight incubation movement away from the stab line or hazy in appearance through out the medium indicate a motile organism.

8. **Sugar fermentation:** - The ability of an organism to ferment various sugars is tested by inoculation of test organism in different sugar media containing Andrade's indicator. Production of acid indicated by change of color of medium to red or pink. Gas if produced will collect in the durham's tube.

9. **Catalase production**: - Pick up few colonies of test organism with platinum loop from nutrient agar plate and dip it in to a drop of 10% Hydrogen peroxide on a clean glass slide. The production of gas bubble from culture indicates positive reaction.

10. **Oxidase test**:- This test depends on the presence in bacteria of certain oxidase that catalyze the oxidation of reduced tetramethyl-p-Phenylenediamine dihydrochloride (oxidase reagent) by molecular oxygen. Put a drop of freshly prepared 1% solution of oxidase reagent on a piece of filter paper, then rub few colonies of test organism on it. Deep purple color shows positive test and if no color develop then the test is negative. Various species of *Neisseria*, *Psedomonas*,

Vibrio and *campylobacter* are oxidase positive. All members of enterobacteriaceae are oxidase negative.

3.7 ASSAY FOR ANTIMICROBIAL TESTING

Ditch plate method of agar diffusion used by Alexander Fleming was the forerunner of a variety of agar diffusion method devised by worker in this field. With the introduction of variety of antimicrobials it becomes necessary to perform the antimicrobial susceptibility test as a routine. For this, the antimicrobial contained in a reservoir was allowed to diffuse out in to the medium and interact in a plate freshly seeded with test organisms. A variety of antimicrobial containing reservoirs are used but antimicrobial impregnated absorbent paper disc is by far the commonest type used. The disc diffusion method of antimicrobial susceptibility testing is most practical method and is still the method of choice for average laboratory.

Antimicrobial susceptibility testing methods are divided in to types based on principle applied in each system. They includes, Diffusion- Stoke's method and Kirby-Bauer method. Dilution- Minimum inhibitory concentration (Broth dilution &Agar dilution). In the present study Kirby-Bauer method has been used to test the antimicrobial activity. The details of this method is given below.

DISC DIFFUSION METHOD

Kirby-Bauer method being recommended by CLSI (Clinical Laboratory Standard Institution) The accuracy and reproducibility of this test are dependent on maintaining a standard set of procedures as described below.

CLSI is an International, Interdisciplinary, non profit, non-Governmental organization. It promotes accurate antimicrobial susceptibility testing and appropriate reporting by developing standard reference methods, interpretative criteria for results of standard antimicrobial susceptibility test methods, establishing quality control parameter

for standard test methods, provides testing and reporting strategies that are clinically relevant and cost effective.

Interpretative criteria of CLSI are developed based on International Collaborative studies and well correlated with MIC s and results have corroborated with clinical data. Based on study results CLSI interpretative criteria are revised frequently, CLSI is approved by FDA-USA and recommended by W.H.O

Isolated test bacteria were grown on nutrient agar plates and fungi were grown on Saubroud's dextrose agar slants. Bacterial inoculums were prepared from 24 hours old culture in peptone water (HI-Media,Mumbai,India) and turbidity was adjusted equivalent to 0.5 McFarland standard. The microorganisms were inoculated in the peptone water and incubated at 35± 2ºC. for 4 hours. The positive control used for antibacterial activity was Amikacin (30 µg/ml), and for antifungal activity ketononazole (10 µg/ml) was taken. The Dimethyl sulphoxide (DMSO) added discs were taken as negative control to determine possible inhibitory activity of the diluent of extracts. The susceptibility of the isolated pathogens were determined by modified Kirby-Bauer (Bauer *et al*. 1966) disc diffusion method on Mueller-Hinton agar plates. To test the antimicrobial activity all extracts were dissolved in DMSO to make a final concentration of 300 mg/ml. 30 µl of each extract was soaked separately in to sterile discs and discs were dried in hot air oven for 4 hours at 35ºC temperature.

PREPARATION OF DRIED FILTER PAPER DISCS

What man filter paper No -1 is used to prepare discs approximately 6 mm in diameter, which are placed in a petridish and sterilized in hot air oven. The required amount of extract (30 µl) has been soaked on the disc. The discs have been dried under sterilized conditions and stored at 4ºC in refrigerator.

When not in use the dispensing apparatus containing disc should always be refrigerated. The unopened discs containers should be removed from the refrigerator or freezer one or two hour before use so as they may equalize the room temperature before opening. This procedure minimizes the amount of condensation that occur when warm air contact cold discs. Dispenser should be allowed to warm to room temperature before

opening. Excessive moisture should be avoided by replacing the desiccant when indicator changes color.

TURBIDITY STANDARD FOR PREPARATION OF INOCULUMS

To standardize the inoculums density for susceptibility test, a Barium Sulphate turbidity standard, equivalent to 0.5 McFarland standard or its optical equivalent e.g. Latex particle should be used. A Barium Sulphate 0.5 McFarland standard may be prepared as follow.

1. A 0.5 ml aliquot of 0.048 mol / L Barium Chloride (1.175 %W/V $BaCl_2$. $2H_2O$) is added to 99.5 ml of 0.18 mol/L H_2SO_4 (1% v/v) with constant stirring to maintain a suspension.

2. The correct density of turbidity standard should be verified by using a spectrophotometer with a 1-cm light path and matched cuvette to determine absorbance. The absorbance at 625 nm should be 0.008 to 0.10 for the 0.5 Mc-Farland standard.

3. The Barium Sulphate suspension should be transferred in to 4 to 6 ml aliquots in to screw cap tubes of same size as those used in growing or diluting bacterium inoculums.

4. The tube should be stored in dark at room temperature and should be tightly sealed.

5. The Barium Sulphate turbidity standard should be vigorously agitated on a mechanical vortex mixture before each use and inspected for uniformly turbidity appearance. The standard should be replaced if large particles appear.

PROCEDURE FOR PERFORMING THE DISC DIFFUSION TEST INOCULUMS PREPARATION:

GROWTH METHOD

1. At least 3-5 well isolated colonies of same morphological type are selected from agar plate culture. The top of each colony is touched with a sterile loop and

growth is transformed in to a tube containing 3-5 ml suitable broth medium such as peptone water broth.

2. The broth culture is incubated at 37ºC.until to achieve the turbidity of 0.5 McFarland standard or more (2-6 hrs). The turbidity of the actively growing broth culture is adjusted with sterile broth or saline to obtain turbidity optically comparable to that of 0.5 McFarland standards. This result in a suspension containing approximately 1 to 2 × 10^8CFU/ ml for *Escherichia coli* (ATCC 25922). A photometric device can be used to perform this step or it can be done visually. Adequate light is needed to visually compare the inoculums tube and 0.5 McFarland standard.

INOCULATION OF THE TEST PLATES

1. With in 15 minutes after adjusting the turbidity of inoculums suspension sterile cotton swab is dipped in to the suspension. The swab should be rotated several time and pressed firmly on the inside wall of the tube above the broth level. This will remove the excess inoculums from the swab.

2. Dried surface of the Mueller-Hinton agar plate is inoculated by streaking the swab over the entire sterile agar surface. This procedure is repeated by streaking two more times, rotate the plate at approximately 60 degree each time to ensure an even distribution of inoculums. Heavy inoculums must be avoided.

   The discs impregnated already with (30µl) solution of the known concentration (300mg/ml) of the plants extracts is dispensed on to the surface of inoculated agar plate. Each disc must be pressed down to ensure complete contact with the agar surface. Whether discs are placed individually or with a dispensing apparatus, they must be distributed evenly so that they are not closure than 24 mm from centre to centre. Ordinarily no more than 12 discs should be placed on one 150 mm plate or more than 5 discs on a 100mm plate. Because some of the drug diffuses instantaneously, a disc should not be relocated once it has come in contact with agar surface.

3. The plate are inverted and placed in an incubator set at 37°C with in 15 minutes after discs are applied.

MINIMUM INHIBITORY CONCENTRATION (MIC) AND MINIMUM FUNGICIDAL CONCENTRATION (MFC). THE MIC AND MFC HAS BEEN DETERMINED BY TWO METHODS.

The MICs of the all extract for the bacterial and fungal strains were determined by the macro broth dilution method as descried by the NCCLS (1993). Two fold serial dilutions of all extracts (64 to 0.025 mg/ml) were prepared in tubes containing DMSO as dilutent. Bacterial suspensions were adjusted to the 0.5 McFarland standards (10^8 CFU/ml) and 10^4 spore/ ml for fungi. Final inoculates of bacterial suspension, different dilutions of plant extract and Muller Hinton broth were added to all tubes and they were incubated at 37^oC for 24 h for bacteria and for 24 - 48 hour in B.O.D incubator for fungi. The MIC (mg/ml) was the lowest concentration of the extract at which there was no visible growth of the organism.

3.8 MIC AND MFC BY MICRO BROTH DILUTION METHOD

The minimal inhibitory concentration (MIC) values of extracts were determined based on a microdilution method in 96 multi-well microtitre plates (Sarker et al., 2007) with slight modifications. The crude plants extracts were first diluted to the highest concentration, 40000 to 625 µg/ml), to be tested, and 50µl of normal saline was distributed from the second to the ninth well. A volume of 50µl from each extracts was pipetted into the first test well of each microtitre line which act as sterility control, and then 50µl of scalar dilution of plant extract was transferred from the second to the ninth well. To each well 10µl of resazurin indicator solution was added (prepared by dissolving a 270mg tablet in 40ml of sterile distilled water). Using a pipette 30µl of Muller Hinton broth was added to each well to ensure that the final volume was of single strength of the normal saline. Finally, 10µl of the bacterial suspensions were added to each well. In each plate, a column with a broad-spectrum antibiotic was used as the

positive control (streptomycine in serial dilution 40000 to 625 µg/ml). The plates were wrapped loosely with cling film to ensure that bacteria did not become dehydrated, and were prepared in triplicate. Subsequently, they were placed in an incubator at 37°C for 24 hours. The color change was then assessed visually. Any color change from purple to pink or to colorless was recorded as positive. The lowest concentration at which the color change occurred was taken as the MIC value. The average of three values was calculated and that was the MIC for the test material

PHYTOCHEMICAL SCREENING TECHNIQUE OF HERBAL DRUGS

The traditional or Ayurvedic system of medicine employ a holistic approach in therapy and used crude drugs and their extracts which contain major constituents. These includes Swarsa (fresh juices), Hema (cold infusion), Kawatha (decoction), Kalka (wet pillar paste), Churna (powder), Guti and vati(pills), Avaleha (confection), Arishthas (medicated fermented wine), Bhasams (minerals and metals) extracts etc.

In allopathic system of medicine, chemical entities of known structure and biological active parts derived from plants are used in the form of tablets capsule, solution, suspension, emulsion, injection, ointment, creams, paste and powder (external and internal). Removal of phytochemical constituents from plant material is known as extraction. The residue left over on removal of solvent of extraction is known as extract. For the purpose of chemical entities, molecules and biologically active constituents, it becomes essential to prepare extract for phytochemical screening.

3.9 TEST OF EXTRACTED MATERIAL FROM DIFFERENT PART OF PLANT

PRELIMINARY PHYTOCHEMICAL ANALYSIS

The qualitative preliminary phytochemical analysis of the crude extracts of all plants part isolated from different solvents were performed by following standard

methods (Fong *et al.*, 1974; Harborn, 1980; Ajaiyeoba, 2000; Jain, 2005; Adewale *et al.*, 2007; Kumar et al., 2009). The preliminary phytochemical estimation of medicinal has been shown in table 1.

Table 2: Preliminary phytochemical estimation of medicinal plants.

Sr. No.	Phytochemical	Tests	Methodology	References
1.	Alkaloids	Dragendorff's Test	Few mg of the alcoholic or aqueous extract of the plant was dissolved in 5ml of distill water than 2M hydrochloric acid was added until an acid reaction occurs, dragendorff's reagent (1 ml) was added and an orange red precipitate was produced immediately.	Adewale et.al, 2007
		Wagner's Test	The alcoholic extract of the drug (2 gm) was acidified with 1.5% v/v of hydrochloric acid and a few drops of Wagner's reagent was added. A yellow or brown precipitate was formed	
		Hager's Test	To alcoholic extract of the drug (2 gm.) was taken in a test tube, a few drops of Hager's reagent added. Formation of yellow precipitate confirmed the presence of alkaloids	
2.	Flavanoids	Shimoda test	To dry extract (15 mg), ethanol (1ml.) was added and dropped small piece of Magnesium ribbon. The drop wise addition of conc. HCl leads to the development of color ranging from orange to red was confirmatory for flavanoids.	Kumar et.al, 2009
3.	Phenols	Ferric Chloride test	The extract (10 mg) was added in 1ml of 1% ferric Chloride solution, a purple or red color indicated the presence of phenols.	Jain, 2005
4.	Saponins	Hemolytic test	The extract (0.5 gm) was boiled with water for 2 minutes in a test tube. After cooling the mixture	Fong et.al, 1974

			was vigorously shaken and lefted for 3 minutes. The amount of honeycomb frothing may be classified as: No froth – negative Froth less than 1cm – weakly positive Froth greater than 1 cm – highly positive Froth greater than 2cm – strongly positive	
5.	Sterols/ Terpenes	Hoss's Reaction	In this test, the extract was taken in chloroform (2ml) and concentrated sulphuric acid was poured from side of the test tube. The color of the ring at the junction of the two layers was noted. violet green color indicated the presence of cholesterol, sitosterol. A red colour ring showed the presence of sterol / terpenes.	Jain, 2005
		Lieberman Burchard Reaction	To 1ml of extract, 2 ml of acetic anhydride solution, 2ml of concentrated sulphuric acid was added. The change in the color from red to blue was a test for sterols/terpenes.	
		Moleschott's reaction	To 1 gm of the extract was mixed with 5ml of distilled water, 2ml of conc. Sulphuric acid was poured from the side of the tube and the color was noted. Red color changed to violet showed their presence.	
6.	Tannins	Ferric chloride test	To 1 to 2 ml of an aqueous extract, a few drops of 5% aqueous ferric chloride solution were added. A bluish black color was produced which disappears on addition of few ml of dilute sulphuric acid followed by the formation of a yellowish- brown precipitate.	Adewale et.al, 2007

		Lead acetate test	In 5ml of an aqueous extract, few drops of 1% solution of lead acetate were added. A yellow red precipitate was formed.	
7.	Amino acids	Ninhydrin test	To 10 mg of ethanolic extract, few droplet of ninhydrin reagent were added. A purple color showed the presence of amino acids.	Kumar *et.al*, 2009
8.	Anthocyanins	Anthocyanins test	Anthocyanins were identified by adding 1 ml of boiling water, 0.5 ml of 37% HCl to 10 mg of dry extract.The solution was heated at 100^0C, cooled and added 0.4 ml of amylic alcohol.	Jain, 2005
9.	Anthraquinones	Anthraquinones test	Plant extract was boiled with 10%HCl for a few minutes, filtered and allowed to cool. This was partitioned against equal volumes of Chloroform. Formation of a rose-pink color in the aqueous layer on addition of 10% ammonia solution indicated the presence of combined anthraquinones.	Ajaiyeoba, 2000; Kumar et.al, 2009
10.	Cardiac glycosides	Lead acetate test	Sample was extracted with 10 ml of 80% methanol for 5 minutes on a steam bath, filtered and diluted with equal volumes of distilled water. A few drops of Lead acetate were added, shaken and filtered after a while. Filtrate was then extracted with methylene chloride (two times) and was evaporated to dryness on a steam bath. Then, about 1 ml of 2% 3,5-dinitro benzoic acid in ethanol was added to the residue and the solution was made alkaline with 5% NaOH. The formation of a brownish purple color was indicative of the presence of unsaturated lactones.	Ajaiyeoba, 2000; Kumar et.al, 2009

3.10 BASICS STATISTICAL METHOD USED WERE:

Mean ($\bar{x}$) = Sum of all the observations in a sample divided by their number.

$$\bar{X} = \sum x/N$$

Standard Deviation =root mean square of the deviations from the arithmetic mean.

$$S.D\ (\sigma) = \sqrt{(\sum d2\ /N)}$$

where d2 =sum of the squares of the deviations of individual items from the arithmetic mean and N= number of items.

Standard Error =measure of mean difference b/w sample estimate of mean and the population parameter i.e. it is the measure of uncontrolled variation present in a sample.

$$SE = \sqrt{SD\ (\sigma)/N}$$

Statistical Method used for antimicrobial activity:

$$\%\ of\ Extracted\ Value\ = \frac{Extract\ Obtained\ \times 100}{Weight\ of\ powdered sample}$$

Quantitative evaluation of antimicrobial activity

Percent activity: The percent activity demonstrates the total anti- microbial potency of particular extract. It shows number of microbes found susceptible to one particular extract (Bonjar, 2004).

$$Activity(\%)\ = \frac{100 \times no.\ of\ susceptible\ strains\ to\ a\ specific\ extract}{total\ no.\ of\ tested\ microbial\ strains}$$