# ZOOLOGY DEPARTMENT

## REPORT

**FOR**

# MINI BIOFERTILIZER PRODUCTION

# PREPARED BY

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## Introduction:

In past 50 years history, the chemical fertilizers have played a crucial role in boosting the agricultural production, however they have a short history in modern agriculture. Their immediate action and low cost managed to bring them rapidly in to the center of attention. Their toxic effects on environment, plant, animal and human life diverted the focus on eco-friendly plant protection.

Indiscriminate use of chemical biofertilizer contributed in loss of soil productivity along with addition of salts to the soil. To revive the soil health and living on alternate source has become essential concept of biofertilizer came forward, which can be a good supplement for a chemical fertilizers, Biofertilizers are nutrient availability systems in which biological process are involved.

The term biofertilizers includes selective micro-organism like bacteria, fungi and algae. Which are capable of fixing atmospheric nitrogen or convert soluble phosphate and potash in the soil into forms available to the plants. Biofertilizer is a cost effective, eco-friendly & renewable source of land nutrient they play a vital role in maintaining a long term soil fertility & sustainability.

The biofertilizer with nitrogen fixer & phosphate solubilizer fixes 20-40 Kg of nitrogen per acre. The biofertilizer maintain the soil fertility cost by using in the yield is assured with biofertilizer & continuous use of biofertilizer makes the soil very fertile for good yield. The biofertilizer can be manufacture in soil form or in liquid form for spraying on the plants.

Biofertilizer is a need of modern agriculture since demand for safe and residue free food is increasing. Therefore, to cater the need, it is necessary to promote the efforts for production of biofertilizers in the state in private sector to encourage the entrepreneurs.

**Objective of the project:**

1. The objective of this project is to install a manufacturing facility of microbial fertilizer which can play a vital role in maintaining a long term soil fertility & sustainability.
2. Secondly now a day's temperature in India is raising upto $48^{o}$ C in summer in which decrease the viability of the biofertilizer by decreasing the shelf life of the nitrogen fixing microorganism, so temperature tolerant strains were isolated for the same.
3. And to increase the shelf life of the biofertilizer there is a need to change the conventional carrier into a new formulation which is nutritive and moisture quantity is more and give a optimum condition to multiply.

### 1. Nitrogen fixing organisms:

A. ***Azotobacter*** *(A. chroococcum, A. vinelindii, A. Lipoferum):*

Azotobacter is a free-living bacterium that can fix atmospheric nitrogen into the soil, being a great source to obtain a natural biofertilizer that can be used in the cultivation of most crops. It is a great source of nitrogen to meet the needs of crops because also has the capabilities to cause a rejuvenation of soil microbiology to tap out the biological fixation of nitrogen.

**Characteristics of Azotobacter:**

The genus *Azotobacter* is comprised of bacteria that require the presence of oxygen to grow and reproduce, and which are inhabitants of the soil. There are six species of *Azotobacter*.

***Azotobacter chroccocum***

The genus *Azotobacter* is comprised of bacteria that require the presence of oxygen to grow and reproduce, and which are inhabitants of the soil. There are six species of *Azotobacter*.

The bacteria are rod-shaped and stain negative in the Gram staining procedure. Some species are capable of directed movement, by means of a flagellum positioned at one end of the bacterium. Furthermore, some species produce pigments, which lend a yellow-green, red-violet, or brownish-black hue to the soil where they are located. Relative to other bacteria, *Azotobacter* is very large. A bacterium can be almost the same size as a yeast cell, which is a eukaryotic single-celled microorganism.

*Azotobacter* has several features that allow it to survive in the sometimes harsh environment of the soil. The bacteria can round up and thicken their cell walls, to produce what is termed a cyst. A cyst is not dormant, like a

B. ***Rhizobium*** *(R. Japonicum):*

*Rhizobium* is a genus of Gram-negative soil bacteria that fix nitrogen. Rhizobium forms an endo-symbiotic nitrogen fixing association with roots of legumes. The bacteria colonize plant cells within root nodules; here the bacteria converts atmospheric nitrogen to ammonia and then provides organic nitrogenous compounds such as glutamine to the plant. The plant provides the bacteria organic compounds made by photosynthesis.

Survival of *Rhizobium & Bradyrhizobium japonicum* at high temperature is a challenging problem especially in tropical regions of extreme temperature range. Some attention had given to the effect of high temperature on *Rhizobium* . Wilison and Trang 1980, Bushby and Marshall 1977). Temperature is the prime environmental factor, which can limit the survival, density and capacity of inoculums (Shukla 1984). Survival is only possible in narrow range of temperature called Bio-kinetic temperature . Jones and Tisdale (1921) studied the effect of different temperature on nodulation and found that $24^0$C was the optimum temperature for maximum nodulation. Temperature has been shown a marked influence on nodulation, nitrogen fixate on ability and longevity of *Rhizobium.*

Critical temperature for nitrogen fixation is ranges between 35 and 40$^0$C for soybean. Nodulation of soybean was markedly inhibited at 42 and 45$^0$C (Zahran, 1984). The optimum temperature for its growth varies from 28 to 35$^0$C. Kretovich *et . al* (1981) and Jain and Rewari (1983) observe no serious mortality upto 40$^0$C. High soil temperature have been found to have a detrimental effect on the growth and survival of *Rhizobium* (Alexender, 1982).

Liquid bio-fertilizer formulation could be considered as one potential strategy for improving the shelf-life of bio-fertilizer. Further, solid carrier based bio-fertilizers are less thermo-tolerant where as liquid formulations can tolerate the temperature as high as 50°C . Hence, improved shelf-life could be achieved by the application of a liquid bio-fertilizer formulation. Process cost of liquid bio-fertilizer is significantly higher than a solid formulation. Thus, successful commercialization of less expensive liquid bio-fertilizer is a challenge and shelf-life of such products is still a concern.

**C.** I. **Mass production of Biofertilizers:**

**Media Preparation and Starter Culture**

Bacteria require different nutrients for their growth. These include:

a) Organic carbons source,

b) Nitrogen source and

c) A variety of other elements dissolved in water. Blue Green Algae (BGA), that can fix atmospheric carbon dioxide, does not require any carbon source and the nitrogen-fixing bacteria, which can fix atmospheric nitrogen, do not require any nitrogen source. A medium is an aquatic solution of a variety of organic and inorganic compounds that can supplement the above requirements for the growth of different microorganisms.

Generally, media are of two types: a) General media and b) Specific media. General media is constituted for the growth of most of the microorganisms. Such a medium contains all the ingredients required for the growth of any microorganism. A specific medium is constituted for the growth of specific or specific group of microorganisms. During constitution of such medium, one or few additional components are added and /or one or few components are deleted from the general media depending upon the requirement of the specific microorganism.

Again, according to the physical appearance, media are of two types: a) Liquid media and b) Solid media. The liquid medium is solidified by the addition of solidifying agent- agar-agar. Liquid medium can harbor bacterial growth suspended in the media, whereas solid medium harbors microbial growth on the surface. Solid media may be prepared as slant or plate.

**Sterilization of medium in autoclave:** The media is then autoclaved at 121$^0$C temperature and 15LB pressure. The process is as follows

- Check the water level of the autoclave to note whether the heating coil is completely

immersed in water, add water, if necessary.

- Put the conical flasks, Petri-dishes and the beaker with test tubes inside the basket of the autoclave.
- Set the lid, tie the screws of the autoclave and switch on the power supply.
- Keep the outlet of the steam open and wait until the inside air is completely released from the autoclave and only steam is coming out.
- When the inside air is completely released close the outlet and observe the meter indicating the inside pressure.
- When the pressure rises to 15 lbs, adjust the regulating valve to keep the pressure constant.
- Keep this pressure for 30 min and then close the regulating valve and switch off the power supply
- When the pressure comes down to zero, open the regulatory valve to release the steam and then open the lid and take out the contents.

**Prepare slants and plates:** The slants are necessary for culture storage. The slant preparation process is as follows-

- Put the test tubes containing medium in slanting position on the table with the help of a wooden blade and allow cooling down. The medium will be solid after cooling down and thus slants are prepared.
- Take the conical flask containing molten media with agar-agar and the Petri-dishes in the laminar flow cabinet and allow the medium to cool down to 50$^{o}$C.
- Open each Petri-dish by slightly lifting the upper lid, pour 15-20 ml of medium and close the lid.
- Keep to cool down and solidify and thus plates are prepared.

**Preparation of Starter Culture:**

The starter culture is a little amount of bacterial suspension, which is added to the medium to start the growth of that bacterium. Twin flask is a pair of flasks of identical size joined together by a latex tube.

For the preparation of starter culture, this type of flask is used. Each flask contains a side arm below the neck position. The latex tube joining the two flasks is held together by this glass tube. The benefit of the use of the twin flask is that contamination can be avoided.

**Fermentation:**

A Fermentor is a device in which the optimum conditions for the microbial growth and activity is established artificially. This device may be used for the production of microbial metabolites such as antibiotics or enzymes; it may also be used for the growth of microorganisms i.e. production of microorganism itself.

A low cost production unit has been developed for the production of microbial inoculants to be used as biofertilizers such as *Rhizobium*, *Azotobacter*, and Phosphate Solubilising Bacteria. This device can be prepared by investing small amount of money as compared to the scientific Fermentor used in laboratories for research purposes

**Sterilization of the Fermentor:**

Fermentor is a metallic vessel for moist sterilization of any article. The principle of moist sterilization lies in the fact that when water is boiled in a closed system, the water vapor produced due to boiling accumulates within the vessel and increases the inside pressure. Thus the boiling point of water increases beyond 100$^{o}$C, which is the boiling point of water in normal atmospheric pressure. In this condition, the steam, released from the boiling water is of higher temperature. If any article placed in this vessel in such condition, the high temperature destroys the microorganisms present in or on the article.

**Inoculation, Growth, Quality Testing and Termination of growth:**

Inoculation means addition of starter culture to the medium in the Fermentor. For the production of microbial biofertilizers a small amount of suspension of the desired bacterium in pure form is inoculated to the medium. Care should be taken to maintain the quality of starter culture, as extent of purity (no contaminants should be allowed), size of the starter culture (in terms of culture volume and density of cell) and stage of growth. Greater the size of starter culture, lesser the chance of contamination. If the starter culture is inoculated in its log phase, rapid initial growth will occur. Maintenance of proper physical and chemical environment inside the Fermentor is essential for proper growth of microorganism. Quality testing, in this case, is enumeration of cell density and its purity in the broth. When the cell density reaches the desired level, growth is terminated and the culture is ready for mixing with carrier. The time period required for optimum cell density is thus standardized.

**Carrier Preparation**

Carrier is a medium, which can carry the microorganisms in sufficient quantities and keep them viable under specified conditions and easy to supply to the farmers. A good carrier should have the following qualities:

- Highly absorptive (water holding capacity) and easy to process.
- Non- toxic to microorganisms.
- Easy to sterilize effectively.
- Available in adequate amount and low-cost.
- Provide good adhesion to seed.
- Has good buffering capacity.

Different carriers are available in the market like, Charcoal, Peat, Lignite, Rice husk, gums etc. But considering all the above gum is the most suitable carrier. The two different types of gums are used as carrier to know their efficiency in holding capacity of moisture even at high temperature, gums are nourishing and also having the important

characteristics of adhesion, because it was observed that bio-fertilizer require such carrier which could adhere with the seed properly but lignite don't have the characteristics to adhere with the seeds in that condition This is due to-

- It has high water holding capacity (360%).
- It has good pH buffering capacity.
- It contains nutrient so bacteria can remain viable for a long period.
- It is easily available in this region.

**Formulation:**

- Inoculation of the carrier with the culture broth means the mixing of broth and carrier. This operation must be done in aseptic conditions to avoid any contamination.
- Efficiency of strains were checked in gum liquid inoculums in three modified media in comparison with the carrier lignite taking in yeast extract mannitol broth and in control also.

**Quality control of formulation:**

- The quality of the carrier-based inoculums depends upon the viable cell count and the presence or absence of contaminants. A good culture must contain about $10^7 – 10^8$ viable cells of culture. No contaminants are permissible at $10^{-5} – 10^{-6}$ dilutions. These critical values differ according to the type of biofertilizer. As per I.S.I., in case of *Rhizobium*, the carrier based culture must contain at least $10^7$ cell of culture and no contaminants are permissible below $10^{-6}$ dilution. In case of *Azotobacter*, So, the enumeration of cells density and contaminants are important task in the production of carrier based microbial biofertilizer.

**Bioactivity of Azotobacter:**

Nitrogenase is the enzyme catalyzing the reduction of nitrogen into ammonia. This enzyme can also reduce acetylene into ethylene as well. Acetylene and ethylene can easily be measured by a gas chromatograph. In a closed system, if a portion of gas is substituted by acetylene and acetylene is allowed to be reduced for a certain period, the proportion of acetylene and ethylene can be measured by passing the mixture of gas through the column of gas chromatograph and measuring the peak developed.

**Bioefficacy of Rhizobium (Nodulation):**

The study of nodulation efficiency of *Rhizobium* is an important task in Biofertilizer Technology. The number of viable cells in the inoculums can easily be enumerated by dilution plating method but this technique will not show whether the viable cells have retained their nodulation efficiency during the long term laboratory procedure like preservation, production and storage. This technique will enumerate the number of viable cells, which have retained the nodulation capability, this is well known that this nitrogen-fixing bacteria cannot fix nitrogen unless it is symbiotically harbored in the nodule of leguminous plant.

**Application of Microbial Biofertilizer:**

Application of the microbial biofertilizer is an important step in the Biofertilizer Technology.

If the microbial inoculants is not applied properly, the benefits from the biofertilizer may not be obtained. During application one should always remember that the most of the microbial biofertilizers are heterotropic, i.e. they cannot prepare their won food and depend upon the organic carbon of soil for their energy requirement and growth. So, they either colonies in rhizosphere zone or live symbiotically within the root of higher plants. The bacteria which are colonised in the rhizosphere zone obtain their organic carbon compounds from the root exudes of the higher plants. The symbiotic ones obtain organic carbon directly from the root. So, microbial inoculants must be applied in such a way that the bacteria will be adhered with the root surface. So, in case of transplanting crops, the inoculants are applied through roots, and in case of the crops in which seeds are sown directly in the field, the inoculants are applied through the seeds so that they can colonize in the rhizosphere region when the young roots are emerged after germination of seed.

On the basis of the above principal, the following inoculation methods have been developed:

1. Inoculation of the seeds by slurry inoculating technique .
2. Inoculation of seeds by seed pelleting technique.
3. Inoculation of the seedlings.
4. Inoculation of the soil by solid inoculation technique.
5. Inoculation of soil by liquid inoculation technique .

**Laboratory setting and operations 1. Aseptic techniques:**

Working in absence of contaminants is very important thing. Aseptic technique is essential for all pathology work and must be thoroughly practiced and mastered.

Insect pathogenic fungi or bacterial cultures for insect pathology must be pure. This means that they must be free of any living microbes other than the one required. The presence of unwanted microorganism (fungi or bacteria) is known as CONTAMINATION and the microbes responsible for contamination are referred to as CONTAMINANTS. We use aseptic technique so that we can handle, or manipulate microorganisms without appearance of contaminants into the culture. Aseptic technique also helps to protect the operator from potential infection from pathogenic organisms.

ALWAYS use aseptic technique when handling microorganisms and also when preparing microbiological media in which to grow these organisms.

**Sterilization:**

Elimination of all viable microbes from a material is known as sterilization. Sterilization is nonselective process. It is very important stage for any microbiological work. The success of proper sterilization ensures quality of final product. ALL equipment and media to be used during the handling of the microorganism must be sterile.

**Disinfection:**

Disinfection is a way to reduce the contaminant load. It removes potentially infective microbes, but does not render the object sterile.

Many different methods of sterilization are being used. The sterilization method you use depends on the equipment you have and what it is you are sterilizing. As a general rule, the following methods are most appropriate.

**Microbial growth media**

1. Wet heat sterilization, usually using an autoclave although a domestic pressure cooker will do just as well.
2. Raise the temperature to 121°C and the pressure in the closed chamber to 15 psi for 15-20 minutes.
3. DO NOT over fill vessels containing liquid; leave a large space at the top of all bottles to allow for expansion and boiling of the liquid on heating in the autoclave.
4. Loosen screw caps before autoclaving.

**Laboratory growth media**

Sterilize as above using wet heat sterilization or dry heat sterilization in an oven (see lab techniques) at 160°C for 1-2 hours.

**Small pieces of equipments:**

Sterilize glass rods and metal tools by dipping them in 70% ethanol (alcohol) and then flaming to burn off the alcohol. Sterilize inoculating loops and needles by holding in a flame until red-hot.

**Laboratory benches**

Swab the working surface with 70% alcohol or chemical disinfectant to prevent the introduction of contaminants.

NEVER allow anything, which is sterile to come into

DIRECT contact with the bench.

**Other methods of sterilization are available.**

1. **Ultraviolet radiation:** can be useful for benches and clean rooms.
2. **Gamma radiation:** is used for the sterilization of plastics in industry.
3. **Filtration:** using filters of a maximum pore size of 1 mm generally used to sterilize small quantities of liquids which are unstable at high temperatures. However these methods are not often used in insect pathology.

**Use aseptic technique during all microbial transfer**

Make sure that ALL equipment used is properly sterilized using the most appropriate method

**Sterilization technique**

**Metal-** Sterilize needles, wire loops etc. by heating them in a flame until red-hot.

**Glassware-** Can be sterilized in a hot air oven, a domestic oven will do. Do not pack glassware too tightly.

**Media-**Wet heat sterilize in an autoclave or pressure cooker for forty (40) minutes

**DRY HEAT OR OVEN STERILIZATION**

Using the oven method of sterilization, glassware will be sterile as follows:

**Oven temperatures and time for sterilization**

- 120°C 8 hours
- 140°C 3 hours
- 160°C 1 hour
- 180°C 20 min.

**Autoclave or pressure cooker:**

You can use an autoclave or pressure cooker at 15 psi for 15 minutes, this is the standard recommended for most microbiological media. Under certain circumstances, you may be required to alter the temperature/pressure for sterilization. The table below gives the temperatures which will be achieved at various pressures in pounds per square inch (psi), the time required for sterilization should be stated in the instructions given:

**Autoclave pressures and temperatures:**

- 5 psi 107°C
- 7 psi 110°C
- 10 psi 115°C
- 15 psi 121°C
- 20 psi 126°C

POINTS TO REMEMBER WHEN USING A PRESSURE COOKER OR AUTOCLAVE

- Use distilled water (if available) in pressure cookers and autoclaves.
- Ensure that all bottles and containers are heatproof.

Never fill bottles and containers to the top, always leave a gap between the liquid and the lid to allow the liquid to expand and boil. Loosen the cap of all bottles and containers before placing in a pressure cooker or autoclave, this allows the steam to enter the containers and sterilize the contents.

- **Always read the manufacturer's instructions** before using a pressure cooker or autoclave (the instructions for using pressure cookers and autoclaves below are only meant as guidelines).

*N.B. Never open a pressure cooker or autoclave until the valve has been opened to release the pressure.*

**USING A MANUAL AUTOCLAVE**

1. Put in sufficient water.
2. Load the articles to be sterilized into the autoclave.
3. Screw down the lid.
4. Open the steam valve.
5. Switch on. If there are high and low switches on the autoclave make sure both are switched on.

6. Let steam come out for at least five (5) minutes before closing steam valve. Continue heating until the pressure is up to 15 psi.
7. Adjust pressure and turn the heat down or the high switch off.
8. Leave to steam for the appropriate time then turn off the autoclave.
9. Leave to cool to reduce the pressure to zero.
10. Open the steam valve to release any remaining pressure.
11. Wait five (5) minutes before opening the lid.

Using an automatic autoclave:

1. Open the autoclave and fill with water to the right level.
2. Set the timer and switch on the heat.
3. Set the "power" switch to the "off" position as soon as the alarm sounds, or light flashes.
4. Wait until the pressure falls and the temperature reaches 80°C or less.
5. Open the autoclave.

**Agar**

Agar is used to solidify nutrient media for growing bacteria and fungi, as it becomes liquid at 100°C and sets at 40°C. Agar can be obtained either as pure agar powder for adding to nutrient solutions which are prepared in the laboratory or ready mixed as a nutritive substrate in powder form with nutrients added according to specific recipes. Sabouraud dextrose agar (SDA) and Malt extract agar (MEA) can be bought ready prepared or can easily be made up in the laboratory using pure agar powder plus the raw ingredients.

N.B. - Ready mixed agar products are generally more expensive than purchasing the pure agar powder and the media components separately.

Agar powder will only dissolve in boiling water, once dissolved; the solution will remain liquid until it has cooled to 40°C. It will then solidify into a firm gel. If required, agar can be reheated by steaming or autoclaving and will become liquid again at 100°C. Agar can be used in Petri dishes (plates) or in bottles (slopes). If agar is to be used in Petri dishes, it should

be sterilized in a large bottle/bottles and distributed after sterilization. If it is to be used for making agar slopes, it should be distributed into the small bottles once it has dissolved and sterilized in the individual bottles. Several different recipes based on agar are given below.

**Ready mixed powdered agar products:**

Follow the instructions given by the manufacturer.

**Pouring agar plates:**

1. Autoclave the agar (to melt and sterilize). Cool the agar until hand hot.
2. Lay out newly opened plastic Petri dishes OR sterile glass Petri dishes in a clean area.
3. Pour the agar into the dishes to a depth of 0.5 cm (approximately 15 ml in a 9 cm Petri plate)
4. Allow to cool uncovered in a sterile air cabinet, keeping the lids within the sterile

airflow (do not touch the inside surface of the lids as this will cause contamination).

*N.B. If you do not have a sterile air flow cabinet, replace the lid of each Petri plate immediately after adding the agar and allow the agar to set. Once cool, any condensation, which has collected on the lids of the agar plates can be removed by taking the lid and giving it a short sharp shake. Replace the lids immediately.*

5. Cover with lids.
6. If not needed immediately, store in the refrigerator (5°C) for several weeks.

**4. Safety in the laboratory:**

- NO SMOKING
- NO EATING
- NO DRINKING
- GOOD WASHING FACILITIES
- GOOD DISPOSAL FACILITIES
- RESPONSIBILITIES

**The laboratory supervisor/manager must ensure that:**

1. The laboratory is a safe working environment.
2. Equipment is safe.
3. All technicians are aware of the dangers.
4. All technicians are properly trained to do their work.
5. Be responsible for chemicals.

**Technicians are responsible for:**

1. Keeping benches clean and uncluttered.
2. Keeping floors clean.
3. Cleaning and proper storage of glassware.
4. Storing chemicals in proper places (cupboards, stores).
5. Maintaining equipment.

Bringing any problems or potential problems to the attention of the Supervisor

**Clothing**

Technicians must always wear the right kind of protective clothing.

- Laboratory coats should be worn (if the room temperature is high this may prove to be impractical).
- Facemasks must be worn when dealing with dusts.
- Gloves must be worn when handling chemicals.

**Chemicals:**

ALWAYS read the label on the container.

1. Orange background with black cross: harmful
2. Orange background with black skull and crossbones: toxic.
3. Orange background with black fire: flammable.
4. Orange background with black Bar or hand being eaten away: corrosive.
5. There may be written warnings, but they are often only in one language.

- ALWAYS keep a list of chemicals and the dangers associated with them. NEVER store chemicals in anything other than their original container, with the correct label.
- ALWAYS keep flammable and toxic chemicals in a securely locked cupboard.

**Biological hazards:**

The entomopathogenic fungi used for locust control are very safe, BUT they may cause allergic reactions if they are inhaled. Some common contaminants e.g. *Aspergillus* spp. are harmful. Technicians must learn to recognize and dispose of contaminants safely. ALWAYS keep benches, equipment and protective clothing clean.

**First aid:**

ALWAYS keep a first aid box in a secure place, in full view and check it regularly. Write all injuries in an accident book which should be kept next to the first aid box. It is ESSENTIAL that at least a person in a laboratory is trained in first aid procedures.

KEEP AN ANTIDOTE FOR ANY ESPECIALLY DANGEROUS CHEMICALS

**TYPE OF EQUIPMENT IN THE LABORATORY**

When considering the setting up of a laboratory for fermentation, a number of points should be kept in mind.

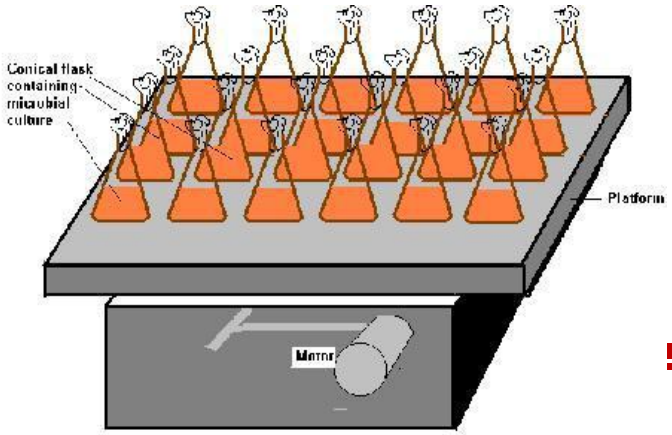
**Nature of fermentation process.**

This really relates to the type of organism to be used; the use of fungi will require equipment quite different in some respects from that employed for "traditional" microbial fermentation. In the description of a fermentation laboratory, which follows, it should be remembered that this describes the "ideal", a lab constructed a finite for the purposes of microbial fermentation. Such an "ideal" is rarely achieved, but is always a useful target.

**Shaker Tables**

Rotary shaker is a platform with many clamps to fit conical flasks. A motor belowit shakes the platform, This shaker is used for the following purposes:

To make a suspension of soil or bacteria. To aerate the liquid bacterial culture.



To prepare a solution which require shaking for a prolonged period.

Shaker tables were designed to assist with oxygen transfer. These tables are designed to run for long periods of time and be free from vibration. The tables are driven by a motor, and normally a rotary shaking action or reciprocating shaking action is produced.

These shakers have to be robust and reliable with no vibration and silent running conditions. One can have a more sophisticated shaker by having an incubator shaking cabinet for shake-flask fermentation in a precisely defined environment. These cabinets can control the temperature, illumination, gaseous levels, and humidity.

Increasing the speed of a shaker can increase the oxygen transfer rate of a particular flask, therefore the optimum speed for that flask and culture has to be found by trial and error.

**Common glassware:**

All the articles used in the laboratory for the measuring of liquid, preparation of solution, etc. are made up of glass. This is because glass is less reactive. The common glasswares are as follows:

**Pipette:**

A pipette is a graduated tube with a pointed end. Liquid of a particular volume can be taken in a pipette by sucking and liquid of desired volume may be released through the pointed tip. Pipette may be of different volumes: 1 mL, 5 mL, 10 mL and 25 mL.

**How to use a pipette:**

In the figure, there is a 10 mL pipette; say, you have to pipette out 6 ml of a solution ‘x’, then,

i) Dip the pointed tip ‘C’ of the pipette in the solution ‘x’.

ii) Suck the solution ‘x’ through the end ‘A’ of the pipette.

iii) After sucking, place the right index finger on the end ‘A’ so that the sucked solution (x) remains in the pipette. Slowly release the finger, if ‘x’ has been sucked above the mark ‘0’ of the pipette, until ‘x’ reaches the ‘0’ level mark.

iv) Place the tip ‘C’ of the pipette on the glassware into which the 6 ml of ‘x’ solution is to be taken.

v) Slowly release the finger as before until ‘x’ reaches the mark '6' of the pipette.

Similarly, you may take solution of any volume from 1 mL to 10 mL, using this pipette.

**Measuring Cylinder:**
Measuring cylinder is a graduated cylinder used to measure the volume of a liquid. While the pipette is used to measure liquid of less volume, measuring cylinder is used to measure liquid of greater volumes like 25 mL, 50 mL, 100 mL, 500 mL, 1000 mL etc. and accordingly, measuring cylinders of different volumes are available.

**Volumetric flasks:**
Volumetric flasks are used to prepare standard solution of a known concentration. It has a narrow neck with an indication mark denoting the exact level of the solvent to be taken to make up the required volume. It also has glass stopper at the mouth. The flask may be of different volumes, 25 mL, 50 mL, 100 mL, 250 mL or 1000 mL.

**Beaker:**
Beaker is a flat-based cylindrical glassware required for the preparation of a solution. Beakers may also be of different volumes viz., 50 mL, 100 mL, 500 mL or 1000 mL.

**Conical flasks:**
As the name indicates, the flask is conical in shape with flat base and gradually narrowing upper part. Conical flask of different volumes are available, 100 mL, 150 mL, 250 mL, 500 mL, 1000 mL, 1.5L, 3L etc. These flasks are used for the culture of plant tissue, microorganisms and also in the preparation of culture media.

**Culture tubes and petridishes:**
Both petridishes and culture tubes are required for culturing plant tissue and microorganisms. Solid medium for plant tissue culture can be prepared as slant within a culture tube. Culture tubes may be of different sizes and volumes. Slants can also be used for culturing microorganisms, especially for germplasm conservation. Petridishes are used for the preparation of solid medium, plates, to develop colonies of microorganisms. A colony is a group of large number of cells developed from a single cell on the surface of solid medium.

**1. Autoclave:**
An autoclave is a device for moist sterilization of any article. The principle of moist sterilization lies in the fact that when water is boiled in a closed system, the water vapor produced due to boiling accumulates within the system and increases the inside pressure. Thus the boiling point of water increases beyond 100$^{o}$C, which is the boiling point of water in normal atmospheric pressure. In this condition the steam, released from the boiling water is of higher temperature. If any article placed in this system in such a condition, the high temperature destroys the microorganisms present in or on the article.

An autoclave is a metal vessel insulated by two metallic walls with a vacuum between the two walls. The upper end is open and can be closed by a lid to be tightened by some screws. At the bottom of the vessel, there is a heating coil, which is kept immersed in water. A metallic basket

is fitted by 3 –4 stands. The articles to be sterilized are placed in this basket so that water does not touch any article. At the top of the autoclave, attached to the lid, there are following devices:

I. A point regulated by a knob to release air or steam.
II. A point for the adjustment of pressure.
III. A meter showing the inside pressure.

**2. Microscope:**

**Laminar flow cabinet**

This is a chamber where microbial inoculation, isolation or any kind of transfer in microbiology or tissue culture is done. Laminar airflow transfer hoods are essential for any commercial operation involving such work.

They provide a sterile atmosphere to work with cultures. Air is forced through a HEPA (high efficiency particulate air) filter, located at the back of the hood that strains out particles as small as 0.3 micrometers. This airflow provides a sterile atmosphere in which the technician works. It is also provided with a UV (ultra-violet) lamp on the ceiling of the hood, which is put on for some time (15 minutes) and then put off before the work is started.

**3. pH meter:**

A pH meter is an instrument that can indicate the pH i.e. acid / base status of a solution. There are several models of pH meter available in the market and each one comes with its own instructional manual.

**pH measurement:**

1. Connect the electrode to the pH meter and switch on the instrument.
2. Prepare 7.0 pH and 4.0 or 9.2 pH buffer solution using buffer tablets.
3. Wash the electrodes with distilled water and dry with a tissue paper.
4. Dip it in 7 pH buffer solution. Keep the temperature knob at $25^0$C. Set the function switch to pH position. The meter will give some reading near to 7.0 pH, say 6.8 . Use STANDARDIZE control to make the reading exactly 7.00.
5. Remove the 7.0 pH buffer solution. Wash and dry the electrode as done before.
6. Dip the electrode in either 4.0 or 9.2 pH solution. If dipped in pH solution 4.0, it will give a reading say,4.3. The error is 0.3 pH. Now use the SLOPE control ( on back side of the instrument)to adjust the pH reading to 4.0. Now the pH meter is standardized and the controls are not to be disturbed.
7. Remove the solution, wash and dry the electrode.
8. Immerse the electrode in the sample solution and directly read its pH value panel.

**Fermentation Glassware :**

The standard 250 ml Erlenmeyer flask is cheap and simple; most of the shaker tables designed to use these flasks although there are tables, which can be adapted to allow other shapes or

bigger flasks.

Baffles have been used in shake flasks to assist in the OTR, as well as preventing vortex formation, but there are only really suitable for low-volume short-term fermentations because of splashing which leads to the cotton-wool plug becoming damp preventing free flow oxygen.

Different plugs can be made of cotton wool, glass wool, polyurethane foam, gauze or synthetic fibrous material. (An aluminum foil cup can sometimes be used in conjunction with these plugs). The plug prevent airborne microorganisms from getting into the medium while at the same time allowing free flow of air into the flask, and for this reason it must not be allowed to become wet.

**Automation and Performance**

Bench-top Fermentor are usually cheaper to purchase than the trolley-mounted or skid mounted Fermentor. This is partly due to the fact that their instrumentation is often not as sophisticated as a laboratory or research Fermentor. This latter have a sophisticated instrument control package for pH, temperature, and agitation, and this obviously costs more.

**Agitation and Aeration**

Most stainless steel Fermentor are designed with bottom drive unit which is belt driven. This has several advantages:

It allows easy access to the top of the vessel and all the moving parts can be isolated and encased underneath the vessel, thus making it a safer piece of apparatus;

With the motor belt driving the agitator any spillage which occur will not fall onto the motor because it is not sited directly below the agitator shaft.

The agitation shaft should have a double mechanical seal which ensures that the medium does not leak out at the shaft housing. The agitation shaft will normally have two or three impellers, each with four or six blades depending upon mixing requirements.

# "Evaluate the antimicrobial activity and phytochemical screening of *Cissus quadrangularis*"

# Research Report

**MUSKAN DHAKAR**

**B.Sc III YEAR**

**2021-2022**

**AND**

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# CONTENTS

# INTRODUCTION AND REVIEW OF LITERATURE

The plant *Cissus quadrangularis*, a climber belonging to the kingdom plantae family vitaceae is the perenial plant of grape family.In the present era of drug development and in discovery of newer drug molecules of many plant products are evaluate on the basis of their traditional uses. One of the many plant which are being evaluated for their therapeutic effectives is Ci*ssus quadrangularis* which is commonly known as Hadjhod (Bengali) and Edible stemmed vine (English). It is an annual or perenial herb, entire leaves, buff colored with greenish ting and requires warm tropical climate and propogated by stem cutting in month of June and July. Although it has many medicinal properties, it is particularly uses to reduce body weight, on thelmintic, muscular pain, asthma, broken bones etc.

It has been used as an osteogenic losteo protective medicine in ayurveda. In India *Cissus quadrangularis* is one of the widely used medicinal plants. The plant grows in the hotter regions of India, Srilanka, Malaysia, Java and west Africa. This plant can heal broken bones and thus commonly known as ‘bone setter’ or Hsthisandhanim Sanskrit and hadjod in Hindi. Phytochemical studies of CQ have been reported the presence of several compounds such as ascorbic acid, carotene, calcium, anabolic steroidal substance, bsitosteral, damyrin, damyrone, flavonoids, triterpinoids, quarcetin, reveratrol, piceatonal, pallidol, per the nocissin and phytosterals.(Sirasanagandla SR,Ranganath Pai,Potu BK ,2014)

## PHYTOCHEMICAL ANALYSIS OF *Cissus quadrangularis*

Plants are used medicinally in different countries and are sources of many potent powerful drugs, *Cissus quandragularis* is more responsive to nutrient group in arid climate and widely distributed in India and other arid area.

Phytochemical studies of *Cissus quandragularis* found several phytochemical constituent such as flavonoids, triterpenoids etc. from the extract from *Cissus quandragularis*. .(Prema Rathinam, K.B.Chandra Sekhar,D Satheesh Sekhar, 2012)

**Phytochemical screening:-**

**Test for tannins:-**

About 0.5 of dried powdered sample was boiled in 20 ml of water in test tube and then filtered now few drops of 0.1% ferric chloride was added brownish green colour observed which confirms tannins. .(Prema Rathinam, K.B.Chandra Sekhar,D Satheesh Sekhar, 2012)

**Test for phlobatannins:-**

In Aqueous extract of plant sample 1% of aqueus HCl was added and then it was boiled. A red ppt was observed which confirms phlobatannins. .(Prema Rathinam, K.B.Chandra Sekhar,D Satheesh Sekhar, 2012)

**Test for saponin:-**

About 2g of the powdered sample was boiled in 20ml of distilled water in a water both and filtered. Filtrate mixed with dist. $H_2O$ was shaken vigorously. A persistent froth was observed. Now 2-3 drops of olive oil was added to froth and mixed. Emulsion was formed which confirms saponin. .(Prema Rathinam, K.B.Chandra Sekhar,D Satheesh Sekhar, 2012)

**Test for flavonoids:-**

To aqueous extract of plant 5 ml of dil. Ammonia solution was added. Now conc. $H_2SO_4$ was added. Yellow colour was observed which confirms havonoids. .(Prema Rathinam, K.B.Chandra Sekhar,D Satheesh Sekhar, 2012)

**Test for steroids:-**

To 0.5gm ethanolic extract of plant 2ml of acetic anhydride was added and then 2ml of $H_2SO_4$ was added. The colour changed from violet to blue or green indicating the presence of steroids.(Prema Rathinam, K.B.Chandra Sekhar,D Satheesh Sekhar, 2012)

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**Test for alkaloid:-**

Take 50ml of extract of plant and add dilute HCl to it and filter it, take 1ml of filtrate and 2ml hager's regent (aqueous solution of picric acid). Yellow ppt was observed which confirms alkaloid. .(Prema Rathinam, K.B.Chandra Sekhar,D Satheesh Sekhar, 2012)

**Flavonoid test:-**

To 25ml of extract add 25ml of distilled water then ad 10% lead acetate solution. A white ppt was observed which confirms flavonoid. .(Prema Rathinam, K.B.Chandra Sekhar,D Satheesh Sekhar, 2012)

**Test for phenolic group:-**

**Gelatin test:-**

50 ml of extract was taken and 5 ml of $H_2O$ was added to it then 2ml of gelatinin was added. White ppt was observed which confirms gelatin.

Screening of phytochemical analysis of *Cissus quandragularis* shows that almost all of the chemical constituents are present in it like tannin, phlobatannins, saponin, flavonoids, steroids, terpenoids, alkaloids are present in its extract.(Prema Rathinam, K.B.Chandra Sekhar,D Satheesh Sekhar, 2012)

**Anti-microbial properties:** *Cissus quadrangularis* possesses significant antimicrobial properties. A study evaluated antimicrobial activity of n-hexane, chloroform, ethyl acetate, methanol and aqueous extracts of the plant against *E. coli, B. subtilis, S. aureus, C. albicans* and *S. cerivisiae*. It was found that n-Hexane, chloroform and ethyl acetate extracts were not effective at all against any bacterial and fungal strain. But aqueous extract was found active against both fungal strains with MIC of 200 µg/ml. Methanol extract was effective against *C. albicans* only (Mishra et al., 2009). Kashikar and George (2006) tested stem extract of *C. quadrangularis* made in ethyl acetate, acetone, petroleum ether, methanol, ethanol and water against *Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus, E. coli, Salmonella typhi and Streptococcus pyogenes* in 96-well sterile microtitre plates. He found that ethyl acetate, acetone, and methanol extracts showed antibacterial activity against *B. subtilis, P. aeruginosa, S. typhi, S. aureus,* and *S. pyogenes*. But none of the extract was found effective in inhibiting *E. coli.* In another research work done for screening the anti microbial activity of leaf extract of the plant against bacterial pathogens such as *Escherichia coli, Klebsiella pneumonia, Staphylococcus aureus* and fungal pathogens such as *Aspergillus flavus, Candidaalbicans* and *Fusarium solani* in diethyl ether, ethanol and water. Out of the three solvents used ethanol extract gave maximum antibacterial as well as anti fungal activity comparable to tetracycline and fluconazole. Ethanol extract gave maximum inhibition against *Candida albicans* (11 mm) followed by *Aspergillus flavus* (10 mm) and *Fusarium* sp. (8 mm) (Merinal and Boi, 2012). In another study, using ethyl acetate extract and methanol extract of both fresh and dry stems for assaying antimicrobial activity against *Bacillus subtilis, Bacillus cereus, Staphylococcus aureus,* and *Streptococcus species.* The results showed that *C. quadrangularis* can as an antibacterial agent (Chidambara *et al*., 2003).

**2.3.2 Anti-oxidant property:** Ethyl acetate and methanol stem extracts of *Cissus quadrangularis* L. were tested for screening its antioxidant activity by beta-carotene linoleic acid model and 1, 1-diphenyl-2-picrylhydrazyl model. The ethyl acetate fraction of both fresh and dry stem extracts showed 64.8% antioxidant activity in the beta-carotene linoleic acid system and 61.6% in the 1, 1-diphenyl-2-picrylhydrazyl system at a concentration of 100 ppm while methanol and aqueous extract didn't show significant activity. Antioxidant activity of the plant is because of the presence of sterols, vitamin C and tannins in the plant extracts (Chidambara et al., 2003). In another study on carbon tetrachloride induced hepatotoxicity in rats, on application of methanol extract of *Cissus quadrangularis* lipid peroxidation was inhibited, free radical production was also reduced and increased the antioxidant enzymes activities, which reveal its antioxidant property. Radical scavenging activity of the plant is responsible for the therapeutic action against tissue damage (Jainu and Devi, 2005).

**2.3.3 Bone repairing property:** *Cissus quadrangularis* is commonly known as the "Bone Setter," and also referred as "Hadjod" and "Asthisamhara" in Hindi because of its ability to join bones. *Cissus quadrangularis* stimulates body metabolism and increases the uptake of minerals such as calcium, sulphur and strontium by the osteoblasts in fracture healing (Udupa et al., 1965). Ethanol extract was evaluated for its antiosteoporotic activity in ovariectomized rat model of osteoporosis at two different doses of 500 and 750 mg/kg per day. The biomechanical, biochemical and histopathological parameters of the rat showed that the ethanol extract has a significant antiosteoporotic effect (Shirwaikar *et al.*, 2003).

**2.3.4 Analgesic property:** The analgesic effect of *C. quadrangularis* exhibited significant analgesic activity comparable to that of Aspirin when tested using Haffner's clip and Eddy's hot plate methods. The duration of analgesic activity was from 2 to 4 hr and optimum effect was observed at 1/20th and 1/10th of LD50 dose (Viswanatha *et al.*, 2006).

***Cissus quadrangularis* L.**

- The antiviral activity of olden system of ayurvedic medicinal plant *Cissus quadrangularis* L. (Vitaceae). Moderately purified methanolic extract of *Cissus quadrangularis* (belonging to Vitaceae member, South Indian medicinal plant) have been estimated for antiviral activity and their phytochemical description. In vitro antiviral activity against HSV type1 and 2, and Vero cells at non-cytotoxic

concentration were detected. HSV1 and HSV2 showed additional sensitivity against the moderately purified compound. Phytochemical investigation showed the existence of the Steroids and Terpenoids. **P. Balasubramanian *et.al***

- The petroleum either extract of *Cissus quadrangularis* (Linn.) increases bone marrow mesenchymal stem cell proliferation and facilitates osteoblastogenesis. To estimate the efficiency of the petroleum ether extract of *Cissus quadrangularis* on the proliferation rate of bone marrow mesenchymal stem cells, the segregation of marrow mesenchymal stem cells into osteoblasts (osteoblastogenesis) and extracellular matrix calcification. This research also aimed to find out the additive effect of osteogenic media and *Cissus quadrangularis* on proliferation, segregation and calcification. The results suggest that *Cissus quadragularis* stimulates osteoblastogenesis and can be used as precautionary/substitute natural medicine for bone diseases such as osteoporosis. **Bhagath Kumar Potu *et.al***
- **T**he antioxidant activity of *Cissus quadrangularis* was carried out to calculate the antioxidant activity of flavonoid rich fraction from *Cissus quadrangularis* Linn on sodium perchlorate induced oxidative stress in rats. Male albino rats were fed with 0.2% sodium perchlorate to induce oxidative stress. The flavonoid rich fraction of the plant (1mg/100 gm, 2mg/100gm) was administered orally along with sodium perchlorate two groups of animals for 30 days, Animals showed improved antioxidant levels in serum, heart, liver, kidney compared with sodium perchlorate treated group. They suggested that a *Cissus quadrangularis* has potent antioxidant property if it posses more flavonoid rich fraction. Therefore, it could be used as a potential antioxidant agent in the healing of variety of diseases. **Sarath Babu K *et.al*.**
- **T**he Pharmacognostical and Phytochemical Evaluation of Stem of *Cissus quadrangularis* L. The current exploration was therefore undertaken to verify the requisite pharmacognostic values for evaluating the plant material. Phytochemical analysis showed the presence of many basis classes of phytoconstituents like alkaloids, flavonoids, cardiac glyscosides and triterpenes. The purpose of these characteristics will aid future investigators in their pharmacological analyses of this species. **Krunal V. *et al*.**
- The anti-osteoporotic activity of the petroleum ether extract of *Cissus quadrangularis Linn.* was done to authenticate the anti-osteoporotic role of the petroleum ether extract

of *Cissus quadrangularis* on ovariectomy-induced osteoporosis in rats. The petroleum ether extract of *Cissus quadrangularis* stem seems to own anti-osteoporotic activity in rats. On the basis of results obtained in this study, They concluded that the petroleum ether extract of*Cissus quadrangularis* stem seems to acquire anti-osteoporotic activity in rats. The results of biomechanical and histomorphometrical analysis of the femur bone seems to support the traditional use of this plant in bone related disorders. **Bhagath Kumar Potu *et al.***

- The studies on the Physico-Phytochemical and Anti-diabeticProperties of *Cissus quadrangularis l.* and *Solanum torvum* The hydroalcoholic extracts of theseplants at dose levels of 200 mg/kg body weight showed promising anti-diabetic activity in theAlloxan- induced model in rats. The overall anti-diabetic activity exhibited by the extracts is found to be low as compared to standard drug Glibenclamide. The *Cissus quandrangularis* rhizome extractexhibited more beneficial anti-diabetic modulating effect on plasma glucose level in Alloxan-induceddiabetic rats than the fruits of *Solanum torvum*. **Vijayakumari P *et al*.**
- The pharmacognostical investigation on *Cissus quadrangularis* Linnrevealed that it consists of β-carotene, c kaempferol and quercetin. The stem consists of two unsymmetric tetracyclic tri terpenoids, 21β-diol and onocer- 7-ene-3β ,onocer-7-ene-3α, 21 α –diol,two steroidal principles I and II, δ-amyrone, δ-amyrin,. The plant is arranged in the prehistoric Ayurvedic literature as a common tonic and analgesic, with definite bone fracture healing properties and the pharmacognostic analysis done on basis of microscopy, macroscopy and physicochemical parameters. **Shah Unnati *et al***
- The current research was intended to assess the antimicrobial activity of diethyl ether, ethanol and aqueous leaf extracts of *Cissus quadrangularis* L. against bacterial pathogens such as *Escherichia coli, Klebsiella pneumonia,* methicillin resistant *Staphylococcus aureus* (MRSA) and fungal pathogens such as *Aspergillus flavus, Candida albicans and Fusarium solani* by *in vitro* agar well diffusion assay. They concluded that the ethanol extract of the plant was found to possess strong antimicrobial activity against tested pathogens. ***Merinal S et al.***
- The preliminary study was undertaken to estimate the effect of methanolic extract of *Cissus quadrangularis* Linn on the curing process of experimentally fracturedradius-ulna of dog. *Cissus quadrangularis* treated animals discovered faster initiation of

healing process than the control animals on histopathological and radiological examination. The treated group also revealed a diminish in serum calcium level to a larger extent than the control group. Curing was almost complete on 21st day of fracture both the groups. ***Deka DK et al.***

- The indian Medicinal Plant *Cissus quadrangularis* Linn. an ethnobotanical and ethnomedicinal assessment. The plant *Cissus quadrangularis* commonly known as'Hadjodi' in Oriya belongs to family Vitaceae. The plant is medicinally important, specially stem which is used to cure various diseases in Indian traditional system of medicine particularly Ayurveda and Unani. All most all parts of the plant are utilized by tribal people. In this review, an attempt has been made to provide utmost information associated with plant *Cissus quadrangularis* to confirm its identity and it has been predicted that this information will be ready to lead a hand for pharmacognostical, phytochemical, pharmacological, toxicological and clinical research in nearfuture. **Ashutosh M *et al***.
- The current research reports some neuropharmacological activitiesof methanolic root extract of *Cissus quadrangularis* in mice. Results showed that the *Cissus quadrangular is* considerably reduced acetic acid induced writhings in mice and enhanced in tail flick24 with drawal response. The dose-dependent inhibition of acetic acid induced writhing indicated aperipheral effect, which was more effective than aspirin29. Tail flick analgesic testing is usually measured appropriate for centrally acting analgesic still clear cut dose response relationship. **Viswanatha Swamy AHM *et al***
- **T**he Sedative and anticonvulsant properties of stems of *Cissus quadrangularis* in mice.The aqueous extract of the stems of *Cissus quadrangularis* powerfully improved the entire sleep time induced by diazepam (50 mg/kg i.p.). It also confined mice against strychnine,pentylenetetrazol, n-methyl-daspartate and maximal electroshock – induced seizures orturning behavior and delayed the onset time of seizures induced by isonicotinic hydrazid acid. The reports lead to the ending that the extract of *Cissus quadrangularis* possesses anticonvulsant andsedative properties in mice and could clarify its use in customary medicine in Africa, in the healing ofinsomnia and epilepsy. **Ngo Bum1 *et al.***
- The efficiency and side effects of *Cissus quadrangularis*L. (Vitaceae) to Daflon (Servier) and placebo in the healing of acute hemorrhoids. To study efficiency and side

effects of *Cissus quadrangularis* L. and micronised purified flavanoid fraction (MPFF) in healing of hemorrhoids. The remedial efficiency of flavanoid mixture, *Cissus quadrangularis* L. and placebo are not dissimilar indicating that they play no task in improving early hemorrhoidal symptoms. Long-term studies should be conducted for property in protective and therapeutic action. **Sukij Panpimanmas MD *et al.***

## HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

To reduce the time of experiment, one can increase the flow rate thereby reducing the retention time of the solute component. High performance liquid chromatography uses an extremely high pressure the flow rate therefore in high and the experimental time is shortened considerably. There is no loss in efficiency the greater advantage of HPLC is that is may employ the principle of adsorption partition, ion exchange, exclusion, and affinity chromatography.

Major component needed to perform HPLC are:-

1. Solvent reservoirs to store the mobile phase.
2. High pressure pump to push the mobile phase through the column.
3. A device to inject the sample into the mobile phase.
4. A column in which the seperation will take place.
5. A detector for detecting the concentration of sample component.
6. A potentiometric recorder to produce a chromatograms.

All solvents to be used in HPCL must be extra pure since even the smallest impurity interferes with the detection system.

**Pumping System:-**

Pumping system can be said to be the heart of HPCL . By producing reproducible high pressure , the pump is a major factor in obtaining resolution high speed analyses and reproducible quantitative analyses

**Sample Injection:-**

Introduction of Sample:-

1. The first method employs a micro syringe designed to withstand pressures with the help of the micro syringe, the sample is introduced either directly or onto an inert material directly about the column.
2. The 2$^{nd}$ , a small volume metal loop which can be filled with the sample

**The column:-** the columns for HPCL are usually made up of stainless stell, glasses, aluminum.

**Detector:-** sensitivity of the detector is sufficiently high and stable.

UV/VIS photometers can be used for HPCL. These detectors are inexpensive, sensitive, insensitive to normal flow and temperature fluctuation. (Upadhyay Nath)

## MATERIALS AND METHODS

**Materials:-**

Chemicals required

Ethanol

Mercuric chloride (For surface sterlization)

**Other Requirement:-**

Distilled water for washing plant. Nutrient agar Media (Freshly Prepared), potato dextrose agar Media (Freshly prepared), antibiotic (tetracycline or any other), whattman paper for making discs.

**Equipment used:-**

Laminar air Flow –

The whole experiment was performed in LAF as to avoid contamination.

Glasswares Used:-

Petriplates

Test tubes

Conical flask

Inoculation loop

## METHODS

### 1. Plant collection and sterilization:-

Fresh healthy *Cissus quadrangularis* plant was collected from any nursery than the plant was taken from the soil and then it was washed properly by distilled water so as to remove soil from the roots then the surface sterilization of plant was done by using 0.1% solution of

mercuric chloride ($HgCl_2$). And then at last the plant was washed three times again by distilled water and wrapped by aluminium foil for further use.

## 2.Preparation of extracts of plant:-

Three type of extracts were prepared from plant stem with various chemical solvents like ethanol, diethyl ether, distilled water. Preparation of different extract are as follows:-

1. **Ethanol extract:-** 2 gm of plans stem were weighed and crushed with 10 ml of ethanol (75%) under LAF and after properly crushing the extract were filter with the help of muslin cloth and the filtrate were used as extract.
2. **Distilled water Extract:-**2 gm plants stem were weighed and crushed with 10 ml distilled water properly under LAF, and then filter with muslin cloth and the filtrate were used as extract.
3. **Diethyl ether Extract:-**2 gm of plants stem were weight and crushed with 10 ml of diethyl ether properly under LAF and then filter with muslin cloth and the filtrate were used as extract.
4. **Antibiotic:-** Pencillin antibiotic drug was diluted properly by mixing with 10ml of distilled water.

## MEDIA PREPARATION

Following media were prepared-

1. **Nutrient Agar Media**
2. **Potato Dextrose Agar Media**

1. **Nutrient agar media:-**

   **Composition:-**

| | | |
|---|---|---|
| Peptone | - | 5g |
| Nacl | - | 5g |
| Agar | - | 15g |
| Beef Extract | - | 3g |
| Distilled water- | - | 1000ml |

All the ingredients were mixed in 1000ml of distilled water and then heated for few min. then pH of NAM was adjusted It should be 7.

2. **Photo Dextrose Agar media:-**

   **Composition:-**

| | | |
|---|---|---|
| Potato | - | 200g |
| Dextrose | - | 20g |
| Agar | - | 15g |
| Distilled water | - | 1000ml |

All the ingredients were mixed in 1000ml of distilled water. Both the media were autoclaved. The pH of media should be 7.

## ANTIMICROBIAL ACTIVIY

- The antibacterial and antifungal activities of *Cissus quandrangularis* were assessed using agar disc diffusion methods.
- In this method replicates of NAM petriplates wear made.
- A sterile paper disk previously soaked in known concentration of extracts was carefully placed at the center of seeded and labeled NAM plates.For each test solution, three replicates were maintained.
- Pure culture of *E. coli* was then taken and then its dilution series was made (series of nine test tube). The culture from the $10^{-7}$ , $10^{-8}$ , $10^{-9}$(1 ml from each) were poured in 3 different NAM plates.
- Then the plates were incubated at inverted position in incubator at $28^0$ C for 48 hours.
- After incubation the plates were observed for formation of clear inhibition zone around the discs placed on the plates, the zone of inhibition was calibrated by measuring the diameter of the zone.
- *Aspergillus niger* was inoculated in PDA plates.
- Then the petriplates were incubated at incubated at $37^0$ C for 48 hour at inverted position.
- Zone of inhibition was observed and calibrated.

# QUALITATIVE PHYTOCHEMICAL ANALYSIS

The sterilized plant was crushed properly under LAF without using any solvent and then it was filtered with muslin cloth and the filtrate was used as plant extract for performing various phytochemical test.

**Detection of alkaloids:-**

Solvent free extract 50 ml was stirred with few ml of dilute hydrochloric acid and filtered. Filtrate was used for test.

**Hagers test:-** To a few ml of filtrate 1 or 2 ml of hagers regent (aqueous solution of picric acid) were added. A prominent yellow precipitate indicated as positive.

**Detection of flavanoids:-**

Lead acetate test:- The extract 50 ml was dissolved in distilled water and 3 ml of 10% lead acetate solution was added. A bulky white lead precipitate indicated the test as positive.

**Detection of tannins:-**

**Ferri chloride test:**The extract 50 ml was dissolved in 5ml of distilled water. To this, few drops of neutral 5% ferric chloride solution were added. A dark green colour indicated the presence of tannins.

**Detection for phenolic compound:-**

**Lead acetate test:-**The extract (50 ml) was dissolved in distilled water and 3 ml of 10% lead acetate solution was added. A bulky white precipitate indicated the test as positive.

**Gelatin test:-**The extract 50 ml was dissolved in 5ml of distilled water and 2ml of gelatin solution containing 10% sodium chloride was added to it. White precipitate indicated the presence of phenolic compounds.

Next level of investigations involving modern instrument like HPLC must be carried out in oreder to isolate and elucidate the active principles in different fractions as an aid to the preliminary phytochemical analysis .

HPLC was performed at SCAN RESEARCH LABORATORY

**Reagents and chemicals**

**Identification of Marker Compound (Quercetin) by HPLC**

**Experimental**

Quercetin was kindly provided by Scan Research Laboratories, Bhopal (India). Methanol and acetonitrile were of HPLC grade and purchased from Merck Ltd, New Delhi, India. Water used was of HPLC grade water from Merck Ltd, New Delhi, India.

**Instrumentation**

A thermospectronic model of Labindia 3000 + UV/VIS Spectrophotometer with 1cm. matched quartz cells was used for determination of λmax. The HPLC system (Waters) consisted of a pump, a U.V.Visible detector, a Thermo C18 (250 X 4.6 mm, 5µm) column, a Data Ace software.

**RP-HPLC Method**

**Chromatographic conditions**

The chromatographic analysis was performed at ambient temperature on a RP-C18 analytical column with a mobile phase composed of Acetonitrile: Methanol (50:50 v/v) and was isocratically eluted at a flow rate of 1 mL min-1. A small sample volume of 20 µL was used for each sample run, being injected into the HPLC system. The chromatogram was monitored with UV detection at a wavelength of 256 nm.

**Preparation of Standard Stock Solution**

10mg of Quercetin was weighed accurately and transferred to a 10ml volumetric flask, and the volume was adjusted to the mark with the methanol to give a stock solution of 1000ppm.

**Preparation of Working Standard Solution**

From stock solutions of Quercetin1 ml was taken and diluted up to 10 ml. from this solution 0.5, 1.0, 1.5, 2.0, 2.5 ml solutions were transferred to 10ml volumetric flasks and make up the volume up to 10 ml with mobile phase, gives standard drug solution of 5, 10, 15, 20, 25µg/ ml concentration.

**Preparation of the Calibration Curve of the Quercetin**

Each of the standard drug solutions were injected 3 times and the mean peak area of drug was calculated and plotted against the concentration of the drug. The regression equation was found out by using this curve.

**Analysis of Extracts**

**Sample Preparation** 10 mg ethanolic Extract was taken in 10 ml of separate volumetric flask and dilute upto the mark with Methanol; resultant solution was filtered through Whatmann filter paper and finally volume made up to mark with same solvent to obtain concentration of 1000 µg/ml. The resulting solution was again filtered using Whatmann filter paper no.41 and then sonicated for 10 min.

A reverse phase C-18 column equilibrated with mobile phase methanol: acetonitrile (50:50, v/v) was used. Mobile phase was filtered through Whatmann filter paper and degassed. Mobile phase flow rate was maintained at 1 ml/min and effluents were monitored at 256 nm. The sample was injected using a 20 µl fixed loop, and the total run time was 10 min. The sample solution was chromatographed and a concentration of Quercetin in Extract samples was found out using regression equation.

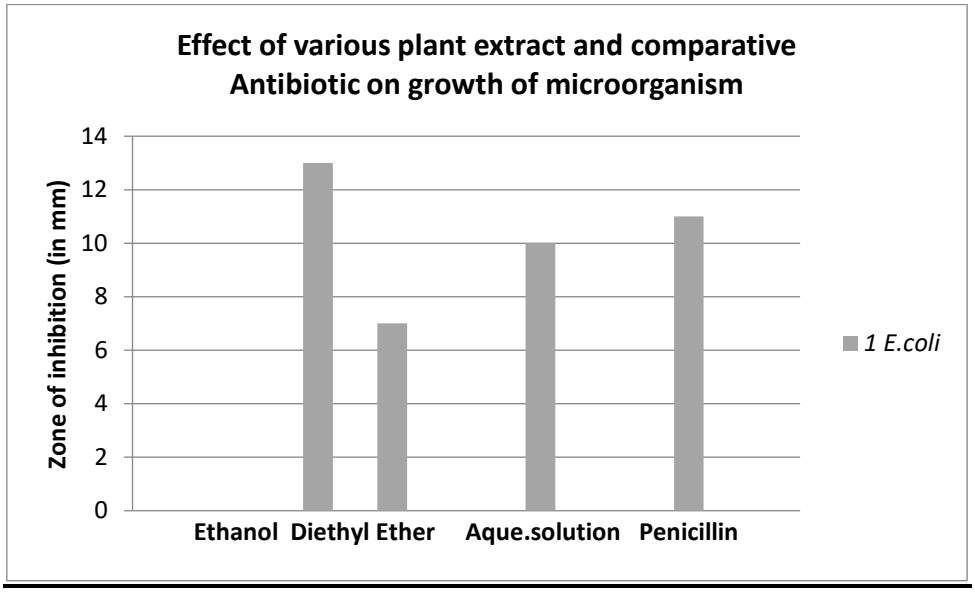
## RESULTS AND OBSERVATION

## PHYTOCHEMICAL ANALYSIS

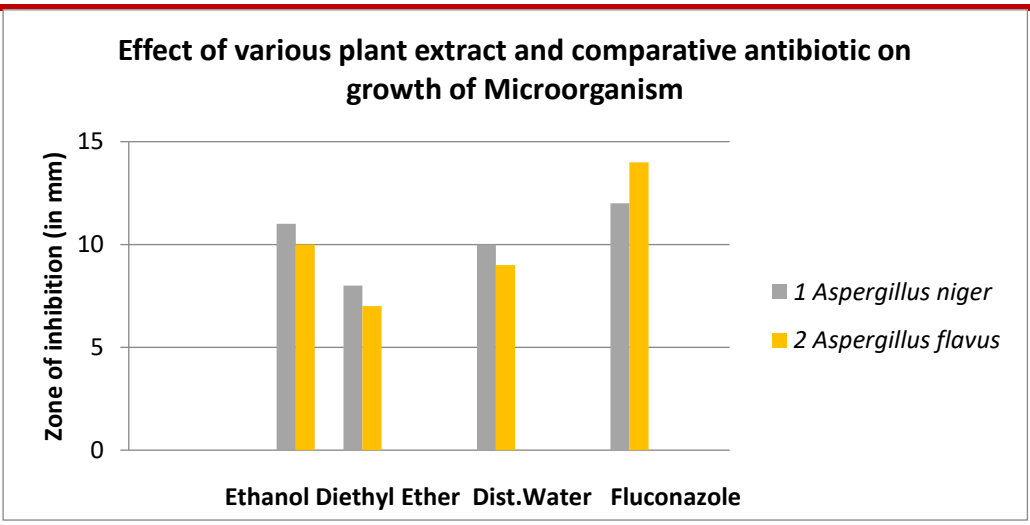
| S.NO | TEST | RESULT |
|---|---|---|
| 1. | ALKOLOID | POSITIVE |
| 2. | FLAVANOID | POSITIVE |
| 3. | TANINS | NEGATIVE |
| 4. | PHENOLIC | POSITIVE |

***E.Coli***

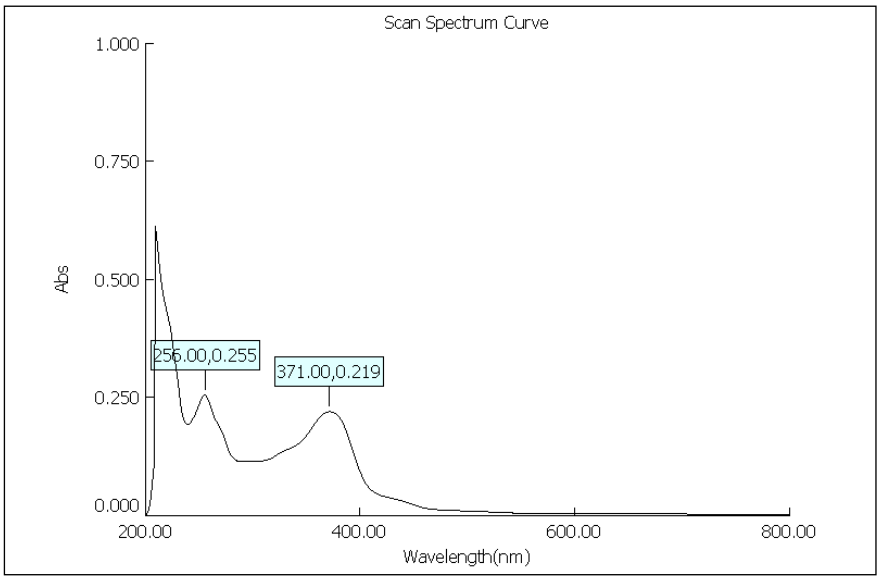
| S.N. | Name of microorganism | Zone of inhibition (in mm) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Ethanol | Diethyl Ether | | Aqueous solution | | Penicillin | |
| 1 | *E.coli* | 13 | 7 | | 10 | | 11 | |




| S.N. | Name of Microorganism | Zone of inhibition(in mm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Ethanol | Diethyl Ether | | Aqueous solution | | Fluconazole | | |
| 1 | *Aspergillus niger* | 11 | 8 | | 10 | | 12 | | |
| 2 | *Aspergillus flavus* | 10 | 7 | | 9 | | 14 | | |

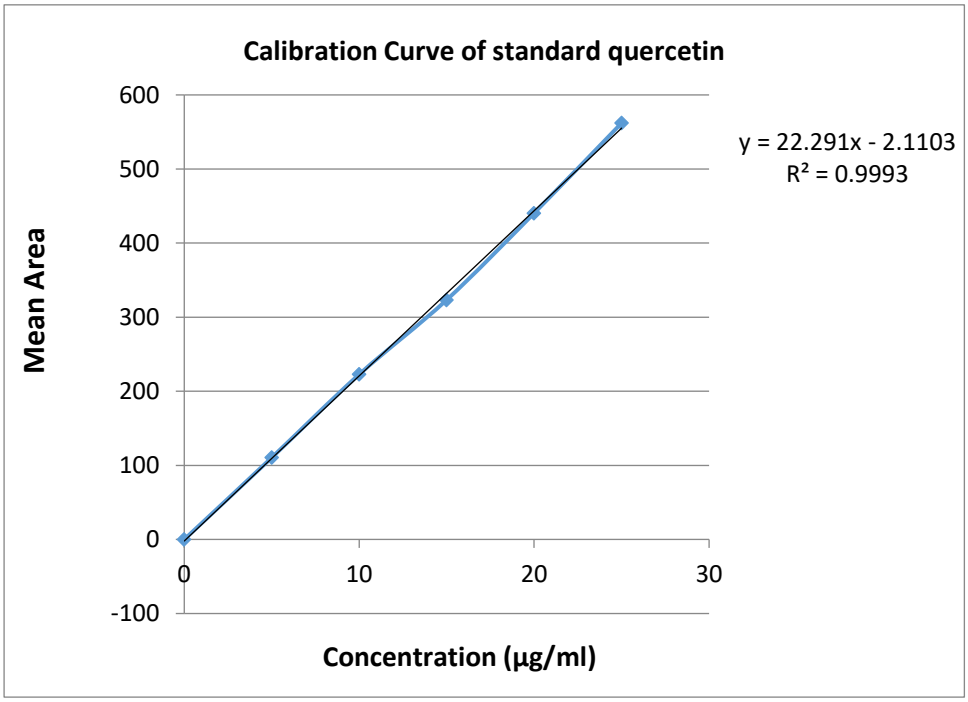



**Determination of lambda max**




**Preparation of Calibration curve**

| S. No. | Conc. | Mean AUC |
|---|---|---|
| 1. | 0 | 0 |
| 2. | 5 | 110.469 |
| 3. | 10 | 222.814 |
| 4. | 15 | 323.334 |
| 5. | 20 | 440.478 |
| 6. | 25 | 562.082 |

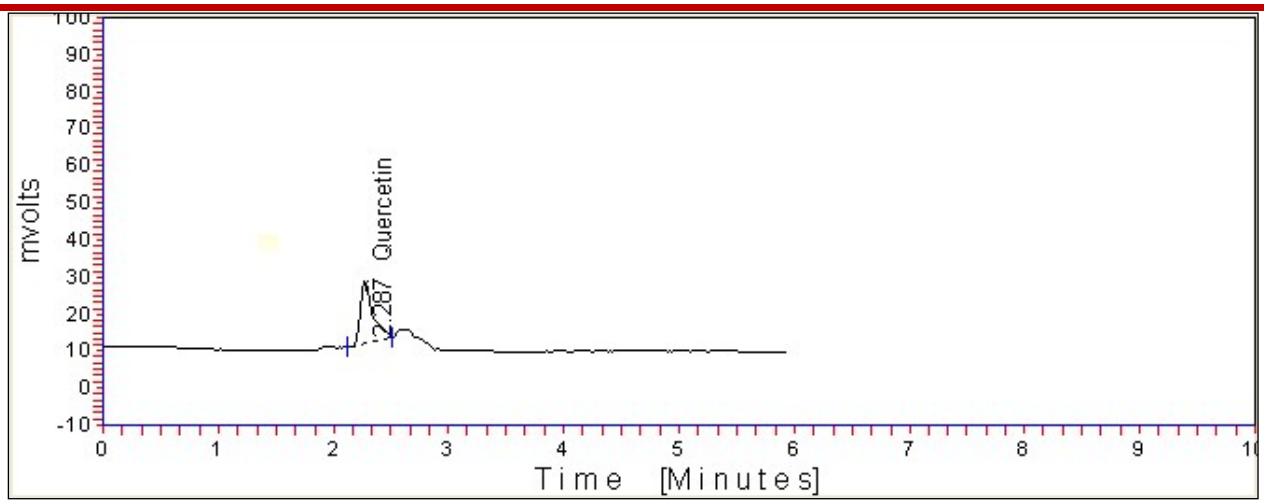



***Characteristics of the analytical method derived from the standard calibration curve***

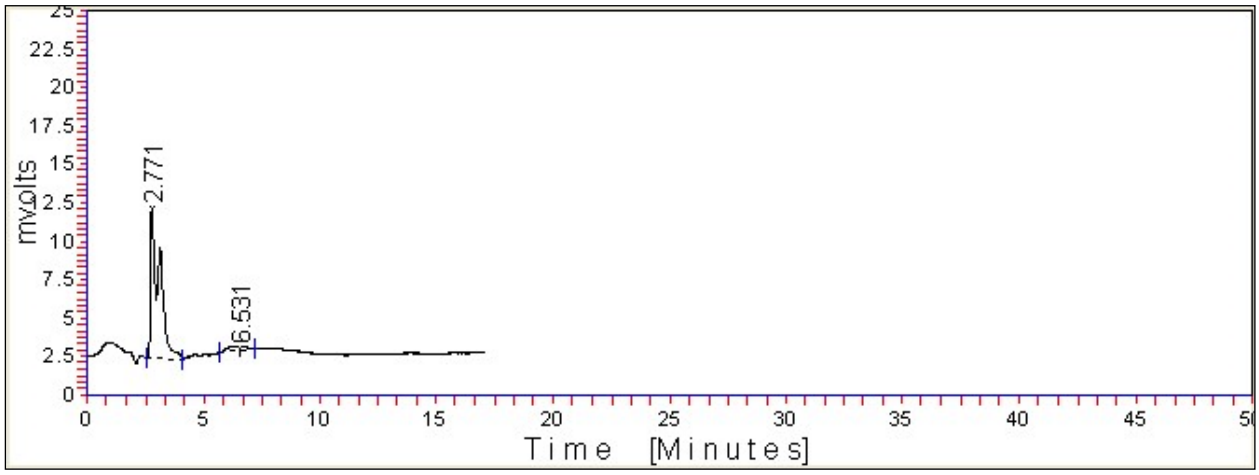
| HPLC | | | | |
|---|---|---|---|---|
| Compound | Linearity range µg/ml | Correlation co-efficient ($r^2$) | slope | intercept |
| Quercetin | 5-25 | 0.999 | 22.29 | -2.110 |

**Standard Chromatogram of Standard Quercetin**

**Standard Quercetin**




**Chromatogram of Ethanolic Extract**




**Result of Assay of Extract formulation**

| S. No. | Extract | Assay (%) |
|---|---|---|
| 1 | Ethanolic | 1.118 |

## SUMMARY AND CONCLUSIONS

This study is to investigate the antimicrobial activity and phytochemical constituents of combined plant extracts. The antimicrobial activity and phytochemical constituents of

ethanolic and ethyl acetate extracts of *Cissus quadrangularis* was investigated, the crude extracts were analysed for the presence of some phytochemical constituents. The crude extracts of plant with various other solvents like ethanol, diethyl ether and distilled water were analysed for the presence of some antimicrobial constituent. Phytochemical study of the crude extracts revealed the presence of flavonoids, alkaloids tannins and phenolic compounds. The extracts were tested for antimicrobial activity against certain micro organisms such as *Escherichia coli, Aspergillus flavus, Aspergillus niger*. They showed significant antimicrobial activity against the above mentioned organisms The zone of inhibition in mm was recorded. Since ancient ages plants have served human beings as a natural source of treatments and therapies amongst them medicinal herbs have gained attention because of its wide use and less side effects.The results lead to the conclusion that the extract of *Cissus quadrangularis* possesses effective antimicrobial activity.

All the work was done in triplicates against the extracts of diethyl ether,aqueous solution,ethanol, and antibiotic and the mean of three was taken according to the present investegation , given three micro organisms shows the maximum zone of inhibition in ***E.Coli*** was 13 mm in ethanol extract and in fungi ***Aspergillus flavus*** it was 14mm in fluconazole and ***Aspergillus niger*** was 12mm in fluconazole.

The test performed for phytochemical screening of crude extract of plant was positive for **flavonoids,alkaloids,phenolic group** except **tannins** for which it was negative.

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**This whole project was done under the guidance of Dr. Rupa Guha Nandi.**

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# Effect of *Aegle marmelos* plant extract on pathogenic microorganisms.

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Abstract

*Aegle marmelos* commonly called as Bael , belongs to family *Rutaceae*, is one of the sacred trees of the Hindus . It possesses great mythological and medicinal significance in ancient system of medicine. Various parts of *Aegles marmelos* have been studied and as a consequence , various classes of compounds viz , Alkaloids , Coumarins , Terpenoids, Fatty acids and Amino acids have been Isolated . Bael leaves exploit its medicinal properties such as anti – depressant , anti diabetic , antifungal , anticancer , anti malarial , antioxidant , anti ulcer , anti inflammatory , anti bacterial . The leaves of this plant exhibit antibacterial property such as inhibiting growth of some pathogens like *Ecoli* , *Staphylococcus aureus etc* . High Flavanoid , Alkaloids and Phenol content in the various extracts of the plant may be one of the reason for their antibacterial activity. Present studies revealed that this plant has a potential to cure many diseases caused by various microbial agents. If this plant extract is used with the combination of other medicinal plants then its efficacy increases to an extent . Even its leaves can be taken orally or in the form of powder which helps in eliminating fever.

Keywords : *Aegles marmelos* , Bael , photochemical constituents , pharmacological activity.

## Introduction

*Aegle marmelos* is one of the sacred trees of the Hindus .Its leaves are offered in prayers to Shiva and Parwati since ancient times[1]. It is also known by various names such as Bilva , Sriphal , Stone Apple. It belongs to Citrus family *Rutaceae* , and was introduced to Europe from India in 1759. It is an indigenous to Indian subcontinent and mainly found in Tropical and Subtropical regions[2]. *Aegle marmelos* is a slow growing sharp tree and medium size , about 12 to 15 m in height with short trunk ,thick and flaking bark[3].

It grows around foothills of Uttarpradesh, Bihar, Chattisgarh, MP, Uttaranchal, Jharkhand and Deccan plateau.. It is believed that the bael fruit is the symbol of lord shiva and its leaf is top of the demand in the season of sawan, as according to hindu mythological belief ,Samudra Manthan also took place in the holy month of sawan and fasting and praying during this month pleases Lord Shiva and Goddess Parvati,who

bless their devotees with a peaceful and prosperous life .The Utility of the bael is mention in the Indian ancient system of medicine , every part of the bael tree such as root , bark , leaf , flower , fruits , seed and even its latex are also important in several traditional system of medicine , that's why it is one of the most important plant in India , and can be used in the treatment of various diseases.

It has been used in Ethno-medicine to exploits its medicinal properties including antidiarroheal, antidysentric, antipyretic and anti inflammatory activities.

Studies have revealed that the compounds purified from bael fruit have biological potential against several diseases like diabetes, gastric ulcer and hyperlipidaemia. It should also be indicated that the therapeutic activities of few isolated constituents have also proven to possess antibacterial, antiviral, antioxidant and radioprotective activities[4].

**Phytochemistry of *Aegle marmelos***

Various chemical constituents like alkaloids, coumarins (marmelosin, marmesin, marmin, imperatorin, scopoletin), steroids, polysaccharides, phenylpropenoids, tannins, flavonoids, carotenoids, saponin, etc have been isolated from various parts of tree such as leaves, fruits, wood, root and bark.

The leaves contain γ-sitosterol, aegelin, skimmianine (tannin), lupeol, rutin, marmesinin, βsitosterol, flavone, glycoside, Limonene and phenylethyl cinnamamides. Fresh leaves of Aegles marmelos consists of alkaloid Shahidine, halfordino, ethylcinnamamide and marmeline. Recently, series of phenylethyl cinnamides (anhydromarmeline, aegelinosides A and B), were isolated from *Aegle marmelos* leaves which are α-glucosidase inhibitors. Rutin flavon, flavon glycosides and flavon3-ols are the major flavonoids of A. marmelos leaves. α-Phellandrene (Terpenoid) was found to be the common constituent of leaves, twigs and fruits.

The fruits of Aegle marmelos consists of tannin (skimmianine also known as 4, 7, 8-trimethoxyfuro-quinoline), phenylpropanoids (hydroxycoumarins, phenylpropenes and lignans), Aegeline, Marmelosin, luvangetin, Aurapten, Psoralen, Marmelide, p-cymene[5].

The seed oil includes palmitic, Stearic, oleic, linoleic and linolenic acid. Some of minerals, viz. phosphorus, magnesium , potassium, calcium and iron. The roots of the tree have also been found to contain psoralen, xathotoxinscoploletin, tembamide, containpsoralen, xathotoxinscoploletin and tembamide[7]. The bark shows the presence of Marmin, Skimmiamine Mature, Fagarine[6].

**MEDICINAL USE**

Diarrhoea and Dysentery

The unripe and half ripe fruit of bael is most effective remedy for the diarrhoea and dysentery. Gastrointestinal infections encompass a wide variety of symptoms and recognized infectious agent. Among the GI infections the diarrhoea is a common symptom of the intestinal disorder and has remained a global threat to human health. It cause morbidity and mortality with over 1000 million episode and over 4 million death annually in children under five years of age[8].

**Antidiabetic Activity**

Diabetes mellitus is a common metabolic disease around the world. A large percentage of the global population is suffering from the same. The modern life style like taking stress and several fast food consumption, and alcohol drinking are the responsible for it. Leaf extract has been used in Ayurvedic system of medicine for diabetes. It enhances the ability to utilize the external glucose load in the body by stimulation of glucose uptake similar to insulin[6].

**Anticancer Activity**

Gastric ulcer resulted from persistent erosion and damage of the stomach wall that might become perforated and develop into peritonitis and massive haemorrhaage as a result of inhibition of synthesis of mucus, bicarbonate and prostaglandins. Bael inhibits in vitro proliferation of human tumors cell lines including the lecukenic K562, T-lymphoid .Most of the potent anti cancer drug are expensive, mutagenesis, and teratogenic. Administration of extract in 400 mg/kg has shown anticancer effect in animal model of Ehrich ascites carcinoma. Researchers evaluated the anticancer potential of folk medicine used in Bangladesh, India. *Aegle marmelos* for cytotoxic action using brine shrimp lethality assay. The fruit extract of bael is also used to improve the immune system that will finally increase the anti cancer activity of the body.

**Cardio Protective Activity**

The leaf extract of *Aegle marmelos* has preventing effects in isoprenaline induced myocardial infarction in rats. The activity of creatine kinase and lactate dehydrogenase was significantly increased in serum and decrease significantly in heart of isoprenaline-treated rats. Use of bael as a cardiac depressant and in palpitation has also been reported [2].

**Constipation**

Constipation is a problem due to the loss of water from the fesses, that's why the person feel difficulty, the ripe fruit of *Aegle marmelos* is a great remedy for the constipation patient, as it is a rich source of fiber, and fiber are essential for the forcing the GIT material towards the excretion. The fiber of bael cleans the intestine. Its regular use up to three months help in the evacuation of even the old accumulated fecal matter from the bowels. In the villages it is mostly consumed as a energy food and in the summer season its ripe pulp is mixed with water or milk to serve as a great drink and as well as a treatment for the constipation suffering person [8].

**Peptic Ulcer**

An ulcer is a result of the defensive failure of mucosal layer of the GIT, it is due to imbalance between defensive and attacking factor like acid. There are several factors which induced peptic ulcer like *Heliobacter.pylori* bacteria, acid secretion, drinking of alcohol, smoking and many more. An infusion of leaves is an effective remedy for peptic ulcer. The leaves are soaked overnight in water and this water is strained and taken in morning, this really works to treat the ulcer and give relief to patients. The ripe fruit of Bael is a rich source of fiber and mucilage that create a protective layer over the stomach and resist the attack of strong acid [3,8,9,10].

**Respiratory Infection**

The oil obtained from the leaves of *Aegle marmelos* is useful in the treatment of the cold and respiratory infection. The juice extracted from leaves is mixed with equal quantity of sesame oil and heated thoroughly; a few seeds of black pepper and half a teaspoonful of black cumin are added to the hot oil and then it is removed from the fire and stored for use in future.[8]

**Antioxidant and Hepatoprotective Activity**

Oxidative stress is produced during normal metabolic process in the body as well as induced by a verity of environmental and chemical factor, which cause a generation of a various reactive free radical and subsequent change in DNA . The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [11,12]. There are two possible mode of work of antioxidant. One is by getting oxidized itself or by creating a protective layer around the active constituents of the material. The antioxidant activity present in the Aegle marmelos confirms the hepatoprotective activity in the same, and it has also been reported [12].

**Antimicrobial activity**

It has been stated that *Aegles marmelos* has traditionally been used to treat a variety of infectious disorders by inhibiting the range of harmful microorganisms .The antibacterial activity of *Aegle marmelos* leaves and fruit extract was investigated.

The inhibitory effect of phytoextracts of leaf, bark and fruits of *A. marmelos* in methanolic, chloroform and aqueous extracts were found to be effective against the bacteria tested. The zone of inhibition of methanolic extracts of the leaf, bark and fruit (6mm, 2mm and 4mm) were found to be less effective when compared to commercial antibiotic. Where as only the fruit extract (4mm) showed inhibition zone against chloroform extract and the aqueous extract did not show any activity against *Klebsiella pneumoniae*.

The results indicates that greater activity resides in methanolic leaf extracts compared to chloroform and aqueous extract. This may be due to the antibacterial principles/chemical constituents which are either polar or non polar and can be effectively extracted only through the organic solvent medium[15]. It was reported that the aqueous, acetone and petroleum ether extract of *A.marmelos* were found to be effective against *B. coagulans, B. subtilis, B. thuringiensis, P.aeruginosa and S.aureus* [12].

**Table 1 : Antibacterial activity of *A. marmelos* against leaf, bark and fruit extracts.**

| Bacteria | Zone of inhibition (mm) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | DMSO | Methanol | | | Chloroform | | | Aqueous | | | Tetracycline (mm) |
| | | Leaf | Bark | Fruit | Leaf | Bark | Fruit | Leaf | Bark | Fruit | |
| *Klebsiella* sps | - | 6 | 2 | 4 | - | - | 4 | - | - | - | 15 |
| *Proteus mirabilis* | - | 13 | 10 | 8 | 10 | 8 | 4 | 4 | 2 | - | 14 |
| *Staphyloccus aureus* | - | 14 | 7 | 11 | 6 | 2 | 5 | 2 | - | 10 | 22 |
| *Salmonella paratyphi A* | - | 24 | 8 | 10 | 16 | 4 | 7 | - | - | - | 20 |
| *Salmonella paratyphi B* | - | 22 | 7 | 18 | 19 | 5 | 14 | 6 | - | - | 20 |
| *Bacillus* sps | - | 6 | 2 | 4 | 2 | 2 | 4 | - | - | - | 11 |
| *E. coli* | - | 10 | 4 | 8 | 8 | 4 | 2 | 4 | - | - | 12 |

**Antipyretic potential**

*A. marmelos* antipyretic movement on Brewer's yeast actuated Pyrexia in pale skinned person rodents. They show that at portions of 200 mg/kg body weight and 400 mg/kg body weight, the ethanolic extricate created a critical (P0.001) decrease in high internal heat level in a dose subordinate way. The concentrates' antipyretic viability was similar to that of paracetamol (100 mg/kg body weight) [20].

**Pharmacological activities of Aegle marmelos**

*Aegle marmelos* is a medicinal plant and researchers scientifically documented the extract of various parts of plant demonstrated the different pharmacological activities such as Antihyperglycemic, Anti-inflammatory, antipyretic, analgesic, Anticonvulsant, Antihistaminic, Anxiolytic, antidepressant, Antioxidant, Hepatoprotective, Antimicrobial, Analgesic, Antifungal, Neuroprotective etc.

Table 1. demonstrated the list of pharmacological activities till date performed on *Aegle marmelos* along with their findings[14].

**Table 1: Reported pharmacological activities of *Aegle marmelos*.**

| Pharmacological activity | Plant part | Results |
|---|---|---|
| Antihyperglycemic | Leaves | Decrease in glucose absorption and inhibition of both α amylase and intestinal disaccharidase enzyme activity due to presence of bioactive components, aegelin 2, scopoletin and sitosterol[10-12]; effective as insulin in restoration of blood glucose.[13] |
| Anti-inflammatory, antipyretic & analgesic | Leaves | Significant inhibit the carragenin-induced paw edema due to presence of lupeol, skimmianine.[14, 15] |
| Anticonvulsant | Leaves | Interfere with GABAergic mechanism to exert their anticonvulsant activity due to presence of flavonoid, Lupeollinoleate, Skimmianine, Eugenol.[16, 17] |
| Antihistaminic | Leaves | Inhibited the histamine release from rat leukemia cell line (RBL-2H3 cell) and also inhibit the histamine release and suppressed $Ca^{2+}$ influx on RBL-2H3 cell line.[18-21] |
| Anxiolytic & antidepressant | Leaves | It enhances anxiolytic and antidepressant activity of imipramine and fluoxetine.[22, 23] |
| Antioxidant | Leaves | It has capability of protecting the cells in oxidative stress due to the presence of flavones, isoflavones, flavonoids, alkaloid, sterpenoids, phenolic content, anthocyanin, coumarin, lignans, |
| | | catechins and isocatechins.[24-26] |
| Hepatoprotective | Leaves, Seed, Fruit | Showed significant decrease in the levels of serum markers, indicating the protection of hepatic cells against ethanol induced hepatocellular injury.[27-30] |
| Antimicrobial | Leaves & Fruit | Inhibit the broad range of pathogenic microorganisms. produced maximum inhibition zone of 11 mm and 9 mm.[31-33] |
| Analgesic | Leaves | Showed significant analgesic activity on acetic acid-induced writhing and tail flick test in mice.[34] |
| Antifungal | Leaves | Interfere with the $Ca^{2+}$-dipicolonic acid metabolism pathway and possibly inhibit the spore formation at concentration of 500 ppm.[35] |
| Neuroprotective | Leaves | Showed acetylcholinesterase (AChE) inhibitory activity in the brain which improves the symptoms of cognitive deficit by elevating the levels of acetylcholine.[36] |
| Anti-ulcer | Fruit | Reduced gastric ulceration and prevent the oxidative stress[37] due to the presence of luvangetin and quercetin which lowers oxidative stress in the gastro duodenal mucosa.[28, 38,39] |
| Antiviral | Fruit | Contain marmilide, which interferes with early events of replicating cycle.[40] |
| Anti-cancer | Leaves, Fruit & Bark | Inhibit the *in vitro* proliferation of human tumor cells, erythroleukemic HEL, melanoma colo38, MDAMB- 231 and breast cancer MCF7 cell lines.[41] Also showed antiproliferative activity against colon, breast carcinoma and leukaemia cell line.[42-44] Due to the presence of lupeol, eugenol, citral, and marmelin skimmianine.[28] |
| Immunomodulatory | Leaves | Stimulate cell mediated and antibody mediated immune responses in rats[45]; also high dose was best effective in humoral immunity.[46] |
| Antithyroid | Leaves | Decreased thyroid hormone level due to presence of scopoletin.[47] |
| cardioprotective effect | Leaves | Exhibited cardioprotective effect against isoproterenol induced myocardial infarction in rats due to the presence of aurapten.[48] |
| antidiarrhoeal | Fruit | Effective remedy for prevention of diarrhea[49] due to presence of tannins and flavonoids.[50-52] |
| Toxicology | Leaves | Chronic administration of leaf powder did not induce any short term toxicity. It have a high margin of drug safety.[53,54] |
| Anthelmintic | Fruit | Showed significant difference in paralysis and death time.[55] |
| Antifertility | Leaves | Showed significant reduction in the weights of testis, epididymes, seminal vesicle, testicular sperm count, epididymal sperm count and motility and abnormal sperm count.[56] |

**Proposed Methodolgy**

Collection of plant material

Extraction using Soxhlet apparatus with chloroform methanol and water separately.

Extraction of plant parts by drying and grinding into fine powder. Then making a solution of it using ethanol, methanol, ethyl acetate and distilled water.

Application of biochemical test of extract for various phytochemicals and antibacterial properties.

**Discussion/ Future aspects**

From the review articles we get to know that the *Aegle marmelos* commonly known as Bael possesses significant pharmacological potential and chemical constituents and traditional healers use this plant to treat a wide spectrum of human ailments . The present findings support the applicability of *A.marmelos* in traditional system for its claimed uses and can be recommended by the scientific community as an accessible alternative to synthetic antibiotics. This study is a preliminary evaluation of antibacterial activity of *A. marmelos* and isolation of the compounds responsible for antibacterial activity would be taken up later.

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# ISOLATION OF MICRO-FLORA FROM HOSPITAL SITES

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## ABSTRACT

**Introduction**: Human body contains normal flora which is helpful in body systems. But some microbes invade human body and cause harm & diseases. These disease-causing organisms can be transmitted from one to other individuals leading to nosocomial and hospital-borne infection. The collection of different microorganisms, mainly bacteria, in a host body is known as flora**.** Micro-flora responsible for harmful diseases is often Allochthonous flora. Microbes are distributed everywhere in the environment surroundings us they are found in the air, water, soil, plant, animal, food product, in the human body and on the surface of the human body. Microorganisms are classified into Archaea bacteria, fungi, algae, virus, protozoa, and multi-cellular organisms. The term Nosocomial infection is used for **Hospital-Acquired Infection and Health care–associated infection** (**HAI** or **HCAI**). Nosocomial infections are caused mainly by airborne pathogen found in healthcare facilities of their surroundings (2015). The aim of this study is to identify bacteria and fungi in a hospital. An infection can be acquired in hospital, nursing home rehabilitation facility, outpatient clinic, diagnostic laboratory or other clinical settings. Infection spreads to the susceptible patient in the clinical setting by various means. Health care staff also spread infection, in addition to contaminated equipment, bed linens, or air droplets. The infection can originate from infected patients, environment & unhygienic staff or in some cases the source of the infection cannot be determined, whereas, sometimes infection spreads from patients own skin microflora this may be due to weak immunity & patients after surgery. The current review explores the studies done on the

nosocomial infections and their impact. **Method**: Electronic search was done to collect relevant studies. The studies were then summarized and analyzed for better understanding of the subject to help in the prevention of this very serious problem faced by hospitals and patients.

**KEYWORDS**: Hospital-acquired infection, hygiene, environment, isolation of microbes, nosocomial infection.

## INTRODUCTION

Now a day's atmospheric pollution is one of the most serious problems and in recent times, it has reached its climax, which poses a great threat to human health that deteriorates well being of the population. Air pollution is the introduction of particulate matter, chemicals and biological materials into the atmosphere that causes discomfort, disease or death to humans, damage to other living organisms including food crops. Exposure to bio-aerosols, containing airborne microorganisms and their by-products, can result in respiratory disorders and other adverse health effects such as infections, hypersensitivity pneumonitis and toxic reactions. Microbes are the basic sources of indoor air contamination. Microbial damage in indoor or outdoor areas is caused most frequently by molds and bacteria. Patients are exposed to greater risk in indoor air environment because confined areas contained aerosols and allow them to develop an infectious level.

Indoor air of hospital contains a variety of microbial population. Nosocomial infection also known as hospital-acquired infection is infection acquired in a hospital environment, which was not present in the patient at the time of admission. Nosocomial infections can cause urinary tract infections, severe pneumonia and infections of other parts of the body. The microorganisms implicated can enter the body through wounds, catheters as well as by inhalation. In the tropics, researchers have identified microorganisms such as *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus* sp., *Klebsiella* sp., *Bacillus* sp, *Penicillium*sp, *Aspergillus* sp and *Candida* sp are some of the most commonly isolated microorganisms from hospital environments Burke *et al.*, (2018).[3]

Hospital-acquired pneumonia, Ventilator-associated pneumonia, Urinary tract infection, Gastroenteritis, Puerperal fever and central line-associated blood stream infections are some common nosocomial infections and causative organisms commonly found (Akbari *et al*, (2015).

The most causative microorganisms responsible for Hospital born infections are *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus*, *Candida albicans*, *Pseudomonas aeruginosa*, *Acinetobacterbaumannii*, *Stenotrophomonasmaltophilia*, *Clostridium difficile*, *Escherichia coli*, Tuberculosis, Vancomycin-resistant *Enterococcus* and Legionnaires' disease (Akbari *et al.*, 2015).

There is large evidence on the hazardous nature of indoor air pollutants, on their sources or conditions leading to the human exposure. As it is reported that 5-10% of all patients who go to hospital for treatment will develop an infection while they are there, this is because the density of pathogen is greater in hospital than most other environment. The air borne route of transmission account for between 10 and 20% of endemic nosocomial infection (Brachman *et al.*, 1970**)** hospital tend to be place where harmful organism are concentrated. Airborne transmission is known to be root of infection for disease. It has also been implicated in nosocomial outbreak of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*. Hospital infection linked with many factors among which are the microbial qualities of the indoor air of the different wards of each hospital, the concentration of bacteria and fungi aerosols in the indoor environment depends. Hospital-acquired infections are caused by viral, bacterial, and fungal pathogens the most common type are bloodstream infection (BSI), pneumonia (e.g., ventilator– associated pneumonia (VAP), urinary tract infection (UTI), and surgical site infection (SSI). Hospital-acquired infection (HAI) is defined as an infection acquired by patients during a short or prolonged hospital stay, (A. Borghesi *et al.*, 2008).

The HAIs are responsible for not only significant morbidity and mortality, but socioeconomic burden to the affected families as well. The development and frequency of HAIs are influenced by several factors which can be categorized under three major factors:-microbial agents, Susceptibility of patient, and environmental factors.

The hospital environment with relatively high counts of airborne microorganisms cannot be misunderstood as pathogenic; they can be non-pathogenic also. However, most studies are done to identify microorganisms that are pathogenic and cause severe problems to patients. Thus, highlighting the need to implement stringent and frequent disinfection procedures, training of healthcare workers on best hygiene practices, well managed surveillance methodology, and installing high efficiency filtration systems to minimize the airborne transmission of infectious pathogens within the hospital. Infectious disease physicians in

collaboration with other departments of hospital must initiate appropriate motivation measures and formulate evidence based policies.

The most sensitive zones of hospital include Operating Theatre and Intensive Care Unit, which showed considerably higher counts of airborne microbes. Identification by molecular means revealed the presence of human pathogens in the hospital air including *Bacillus* sp, *Micrococcus* sp, *Pseudomonas* sp, *Staphylococcuss* sp, *Exiguobacterium* sp, *Enterobacter* sp, *Escherichia* sp, *Sphingomonas*sp, *Massilia*sp, *Kocuria*sp, *Fusarium*sp, and *Aspergillus* sp. Therefore, the implementation of proactive policies and strategies are needed to monitor hospital air quality in sensitive zones as well as other areas of the hospitals (Sivagnanasundaram *et al.*, 2019).

The collection of particles that leaves your mouth every time it is opened is referred to as "aerosols**."** And these aerosols can remain suspended in the air for a few minutes up to several hours. These tiny droplets can travel as far as 6 feet and can spread germs by landing on surface or in another person eyes, nose or mouth. Droplets containing germs are released into the air when a person coughs or sneezes. Air pollution, both indoor and outdoor, is one of the most severe problems of our time. Several airborne diseases have been related to the indoor air quality. Indoor air quality is a significant issue in healthcare. Healthcare facilities have to pay particular care and attention to indoor air concerns due to the presence of air borne microorganism that may cause nosocomial infection People with pre-existing health problems that are going through treatment and those who may have compromised immune systems are very susceptible to indoor as well as outdoor air exposures.

Studies conducted on outdoor & indoor microbial agents showed difference in microflora whereas common bacteria found are:

**Outdoor Microflora:** The air in the atmosphere, which is found outside the buildings is referred to as outside air. The dominant microflora of outside air is fungi. The two common genera of fungi are *cladosporiul* and *sporobolomyces*. *Bacillus*, *clostridium, sarcina*, *mirococcus*, *corynebacterium* and *Achromobacter* are widely found in the outside air, the number and kind of microorganisms may vary from place to place, depending upon the density of human population and environment condition.

**Indoor Microflora:** The air found inside the building is referred to as indoor air. The commonest genera of fungi in indoor air are *penicillium*, *Aspergillus*; the commonest genera

of bacteria found in indoor air are *Staphylococci*, *Bacillus* and *Clostridium*. In case of occupants being infected, the composition shows slight variations with latitude and to a lesser extent with attitude. Indoor micro-flora also varies on the basis of condition and types.[3]

In a developing country like ours, the healthcare facilities face many problems such as overcrowding, improper design and poor ventilation that have an impact on the growth and survival of microorganisms, which is harmful to human health. A study done on the interaction of building designs and microbial load showed that there are high microbial load for those buildings, which does not include vents, proper situation of windows and doors, as well as low head-roof. Moreover, the activity of people and equipment within the indoor environments is thought to be the principal factor contributing to the built-up and spread of airborne microbial contaminations.

In these confined areas, lower respiratory tract and bloodstream infections happen to be the most lethal; however, urinary tract infections are the most common. Infections caused by gram-negative bacteria have features that are of particular concern since these organisms are highly efficient at up-regulating or acquiring genes that code for mechanisms of antibiotic drug resistance, especially in the presence of antibiotic selection pressure. Most common nosocomial pathogens may well survive or persist on surfaces for a long time, what could explain their survival in ICU wards.

Gram-positive bacteria, such as *Enterococcus* spp. (including VRE), *Staphylococcus aureus* (including MRSA), *Clostridium difficile* or *Streptococcus pyogenes*, and many gram-negative bacteria, such as *Acinetobacter* spp., *Klebsiella* spp., *Pseudomonas aeruginosa*, *Serratia marcescens*, *Shigella* spp., may indeed survive for months on dry surfaces. A few others such as *Bordetella pertussis*, *Haemophilus influenzae*, *Proteus vulgaris*, or *Vibrio cholerae*, however, persist only for days. In a study, bacterial diversity in hospital environments including open and crowded environment, the entrance hall of the hospital were studied using modern deep sequencing technologies.[14]

Surgical site infections are attributable to a variety of factors which can be classified into patient-related, procedure-related and others. Other risk factors include the volume of surgeries performed in the department, the season, the working environment in the operation room, and the indications for surgery.

The development of resistant microorganisms can result in increased morbidity, mortality, and costs of care. For example, in one study the presence of MRSA in a surgical incision was associated with a 12-fold increase in 90-day postoperative mortality, compared with uninfected patients. Hospitalization stays for these patients also increased resulting in increased cost compared with uninfected patients. Hospitalized patients with VRE had a 6% increased risk of mortality, 6.2 days of excess hospitalization, and additional hospital costs, compared with uninfected patients. Hospital costs attributable to nosocomial infections caused by resistant bacteria have been conservatively estimated at $1.6 billion per year in the United States.

The effects of relative humidity are complex (Tang, 2009). Studies on airborne Gram-negative bacteria such as *Serratia marcescens, Escherichia coli, Salmonella pullorum, Salmonella derby, Pseudomonas aeruginosa* and *Proteus vulgaris* have found increased death rates at intermediate (approx. 50–70%) to high (approx. 70–90%) relative humidity environments (Webb 1959; Won and Ross, 1966). For some airborne Gram-positive bacteria like *Staphylococcus albus, Streptococcus haemolyticus, Bacillus subtilis* and *Streptococcus pneumoniae* (type 1), death rates were highest at intermediate relative humidity levels (Dunklin and Puck 1948; Webb 1959; Won and Ross 1966). In contrast, aerosolized *Klebsiella pneumoniae* (Gram-negative bacillus) demonstrated relative stability at an intermediate relative humidity of 60% (Bolister *et al.,* 1992).

Bacteria occur in almost every environment particularly in dusty, dirty places inhabited by humans or animals. Most of the species of bacteria isolated from buildings are harmless and frequently include members of the genera, Bacillus species, Micrococcus species and Corynebacterium species. However, the species that have been associated with diseases include *Pseudomonas species especially P. aeruginosa, Flavobacterium species, Staphylococcus aureus, Serratia marcescens and Legionella penumophila* (Binnie, 1991).

Certainly studies have shown links between patient's bacterial flora, environmental flora and the actions of others. Boyce *et al.,* (1997) found that healthcare workers who had no physical contact with a patient would contaminate their gloves with MRSA by touching contaminated surfaces. The study also reported that isolates found near colonized patients and on colonized patients were often the same or a closely related strain. These two findings suggest that environmental surfaces can play an important role in the distribution and transfer of MRSA between patients in close proximity, such as on a hospital ward.[13]

One study examined surfaces before and after routine care by healthcare workers to Vancomycin-resistant Enterococci (VRE) positive patients. It was found that 10.6% of sites that had previously been tested and found to be free of VRE were contaminated after being touched by a HCW who had previously touched a site contaminated with VRE (Duckro *et al.*, 2005). Similar approach to the diverse control measures used routinely by the Dutch to control MRSA was implemented in an Australian hospital to control an outbreak of VRE.[12,13] A high-level group was set up to deal with the problem, emphasising the importance of visible commitment to the program. Additional funding was gained to enhance infection control procedures, which comprised increased screening of patients; staff co-horting; environmental monitoring combined with enhanced cleaning services; medical record analysis; specific arrangements for patients and contacts after discharge from hospital. Targeting relentlessly the avenue by which the bacteria could spread in a zigzag fashion through cross contamination achieved a drop in VRE acquisition from 33.3% to generally below 2%. Verity *et al.*, (2001) examined contamination of side-rooms housing patients suffering from *C. difficile* infection, and found that 25% of sites sampled over a 4 week period were contaminated with *C. difficile*, with the same strain being isolated from both patient and environment. However, 90% of these were the same strain, which limits the discriminatory power of this study in this area.

## CONCLUSION

Nosocomial infection is a serious issue that concerns all personnel working in the hospital and patients equally. The patients are more vulnerable to infections due to their already immune-compromised state. So, it is of utmost importance that hospitals should follow all guidelines of infection control as stringently as possible. Different medical equipment, environmental surfaces, air and hands of health personnel were contaminated with various types of bacterial pathogens. Table surfaces, infusion stands and other movable objects used by health professionals in daily practice can be potential source of nosocomial infections in this hospital. Gram-positive Staphylococci are more frequently isolated from the operating room and wards especially from the environmental samples and health professionals than from the patients with postoperative surgical site infection (SSI). It is imperative that all professionals should take an active role in infection control within their organization and more resources should be provided to encourage good hygienic practice in the hospital.[6]

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# Formulation of herbal hand sanitizer from Indian herbs

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# Formulation of herbal hand sanitizer from Indian herbs

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***Abstract:*** *Hands are the first mode of transmission of microbes and infections. Hand hygiene is a key principle and exercise in the prevention, control and reduction of infections. Due to COVID pandemic the need of hand sanitizer has increased which causes less dryness to hand. Considering the need, we prepared a polyherbal sanitizer using seven plant extracts with other ingredients including isopropyl alcohol, camphor, hydrogen peroxide, glycerol and water. The ingredients were selected on the basis of their antimicrobial property. The ingredients and sanitizer were evaluated for antimicrobial property and showed potent activity against gram positive bacteria S. aureus, whereas mixture of extracts showed potent activity against all the bacterial strains used except B. cereus. The sanitizer also showed potent activity against all the strains used in the ascending order of Klebsiella spp. > B. cereus > S. aureus > S. pyogenes > P. aeruginosa > Dermatophyte > E. coli and gave zone of inhibition of 18 ± 0.05, 12 ± 0.03, 11 ± 0.01, 10 ± 0.01, 9 ± 0.5, 9 ± 0.01 and 7 ± 0.001 mm respectively. The antimicrobial activity was compared with other commercial hand sanitizer and maximum activity was showed against Klebsiella spp. and minimum against E. coli and P. aeruginosa by all the sanitizer used. The efficacy of hand sanitizer was checked on hands of 38 volunteers of laboratory workers, patients and their relatives with written and oral consent. The sanitizer reduced or eliminated the growth of pathogens isolated from hands. Time interval effect was also checked at a time gap of two hours also showed the effect of hand sanitizer for longer time with reduction in bacterial growth. The sanitizer pH was alkaline with good shelf, texture and odor. No turbidity was seen when kept at higher temperature for 3 months and showed no skin dryness with soothing effect after using sanitizer on different volunteers.*

***Keywords:*** *hand sanitizer, infection, skin and microflora*

# I. INTRODUCTION

Hands are the first mode of transmission of microbes and infections. Hand hygiene is a key principle and exercise in the prevention, control and reduction of infections. The bacteria resides on hands are classified in two categories namely resident or transient. The resident flora are resides under the stratum corneum and can be found on surface of skin, namely *Staphylococcus epidermis*, *S. hominis*, *Corynebacteria*, *Propionibacteria*, *Dermobacteria, Micrococci* and fungi *Malassezia* spp.. The resident flora protects skin and has antagonistic functions, but cause infections in sterile body cavities, eyes or on non intact skin. Transient flora colonizes the superficial layers of the skin and gets removed by routine hand hygiene, these flora depends on individual profession, habit and skin moisture and sporadically multiply on skin surface. The hands on healthcare workers get colonize while handling patients include pathogenic flora such as *S. aureus*, *Enterococcus* spp, *Acinetobacter iwoffii*, *Staphylococcus aureus* (MRSA) yeast and many more [1]. These can be source of nosocomial infections if hand hygiene not maintained. These pathogens cannot be removed by simple washing, therefore hand sanitizing is required. Nowadays, in COVID-19 pandemic the need of cleaning hands has become mandatory and people have become aware of cleaning hands. Hand sanitizer or hand antiseptic is an alternative to the hand washing with soap and water [2].

There are different types of hand sanitizer available commercially, liquid, gel or foam form, whereas they differ based on the compositions, alcohol based (contain combination of isopropyl alcohol, ethanol (ethyl alcohol) or n propanol, with versions containing 60% to 95% alcohol) or herb based [3, 4]. Non-alcohol based versions typically contain benzalkonium chloride or triclosan, but are less effective than alcohol based ones. The product is generally used by the doctors, surgeons, pathologists and researchers [5]. Hand sanitizer is less effective at killing certain kinds of germs, such as norovirus and *Clostridium difficile*. The sanitizer may be less effective due to incorrectly wiping out hands before sanitizer dries or if concentrations of alcohol too low in sanitizer. The correct way of using of hand sanitizer is first by applying on palm of the hand and then rub the product over the surfaces of hands and fingers until hands are dry. Compounds such as glycerol may be added to prevent drying of the skin [3]. In some sanitizers fragrances are added, but it is discouraged due to the risk of allergic reactions. The mechanism of action of

alcohol based sanitizer includes membrane damage, and inhibition or uncoupling of mRNA and protein synthesis through effects on ribosome and RNA polymerase, or associated with protein denaturation, but not on spores [6]. The alcohol based sanitizer, especially ethanol acts on viral envelope, derived from host lipid envelopes and protein capsid [7]. The frequent use of hand sanitizer increased due to outbreak of pandemic COVID 19. The excessive use of hand sanitizer leads to number of problems in people having sensitive skins. The drawbacks includes skin dryness, irritation, harsh on skin, ocular irritation [8]. In some cases alcohol may strip the skin of the outer layer of oil, which may have negative effects on barrier function of the skin [9]. The side effects and due to more demand of sanitizer, we emphasized on preparation of polyherbal sanitizer [10]. The sanitizer contains extracts of herbs, ethanol, isopropyl alcohol, hydrogen peroxide, camphor, distilled water and glycerol to provide soothing effect in sanitizer. Plants are rich in vast variety of secondary metabolites such as tannins, terpenoids, alkaloids and flavanoids etc. which have been found to possess in vitro antimicrobial properties, considering this demand, an attempt has been made to screen the classical literature for the herbs with antimicrobial properties. Due to its antimicrobial activities these herbs are used in formulation in number of herb based hand sanitizers.

## MATERIAL AND METHODS

In the present study herbal sanitizer was prepared and its efficacy was checked on different bacterial strains isolated from the hospital premises. The study was carried out in the Department of Research, Jawaharlal Nehru Cancer Hospital & Research Center, Bhopal.

### Collection of leaves of the plant

The plants leaves were collected for the preparation of sanitizer from in and around the campus the hospital premises. The plant selected on the basis of its potent antimicrobial activity reported in research articles (Table -1). The plants used for the study were *Ocimum gratissum* (Van tulsi), *Ocimum sanctum* (Shyama tulsi), *Eucalyptus globules* (Niligiri), *Azadiracta indica* (Neem), *Cuscuta reflexa* (Amerbel), *Aloe barbadensis* (Ghritkumari) and *Menthe arvensis* (Mint). The plants leaves collected were weight, washed, cleaned and shade dried in laboratory. After drying plant extract was prepared in ethanol and used for the preparation of hand sanitizer.

### Preparation of extract for hand sanitizer

The plant extracts was prepared by weighing 50 gm of dried leaves of each plant, powdered mechanically and soaked in 100 ml of ethanol overnight. After 24 hours, the extracts were filtered using funnel and filter paper and used for the preparation of hand sanitizer.

### Bacterial strains

The bacterial strains used for the present study were isolated in Department of Research, Jawaharlal Nehru Cancer Hospital and Research Centre. The strains used were three gram positive bacteria *Staphylococci aureus, Streptococcus pyogenes* and *Bacillus cereus,* Whereas three gram negative strains *Klebsiella*, *Pseudomonas aeruginosa* and *Escherichia coli* and one *Dermatophyte.*

### Preparation of herbal hand sanitizer

The herbal sanitizer was prepared by the following ingredients given below

1. Plant extracts prepared from 50 gram dried plant were added in equal amount
2. Hydrogen peroxide – 2 ml

3. Glycerol – 10 ml
4. Ethanol – 50%
5. Kapoor – 2 Nos
6. Isopropyl alcohol - 70%
7. Distilled water was used to make up to 1000 ml

The pH of the hand sanitizer was checked using pH strips.

**Antimicrobial activity of ingredients of hand sanitizer**

The antimicrobial activity of ingredients of hand sanitizer was evaluated by disc diffusion method. The discs of all ingredients were prepared using Whatmann filter paper and impregnated with all the ingredients. The bacterial strains isolated were inoculated on Mueller Hinton plates using sterile stirrer of high quality stainless steel. After inoculation the discs of all ingredients were placed aseptically using sterile forecep. The plates were incubated at 37℃ for 24 hours in the incubator. The plates were observed next day and zone of inhibition were calculated. The ingredients evaluated were mixture of extracts, isopropyl alcohol, ethanol, camphor, hydrogen peroxide, glycerol and water.

**Antimicrobial activity of hand sanitizer**

The antimicrobial activity of hand sanitizer was evaluated by disc diffusion method using Mueller hinton media. The commercial sanitizers were used for comparative study with the sanitizer prepared in laboratory. The commercial sanitizers used were Sterilium, Savlon, Purest, hand safe, Genius, Lab alcohol (70% isopropyl alcohol as hand disinfectant) and Swach.

**Determination of Efficacy of hand sanitizer**

The modified method of David was used to determine the potency of the hand sanitizers [13]. Thirty eight individuals were selected for the study and verbal and written consent was obtained from all participating subjects prior to the conduct of the experiment. The samples were collected from laboratory employees, including scientist, researcher, technician, sanitary and office workers. As well as samples were also collected from patient and their attendants visiting hospital. The subjects chosen were without clinical evidence of dermatoses, dermal abrasion, trauma and infection. Surface samples were obtained by swabbing each individual hand (areas of 4 $cm^2$) and after using 5 ml of sanitizer respectively. The individuals were asked to rub sanitizer properly, allow it to dry and then sample was taken. The samples obtained was inoculated onto nutrient agar plates, incubated at 37°C for 24 h and examined for growth [11]. The colonies were picked up and identified by gram's staining.

Percentage reduction in the bacterial load was calculated as

$\% R = [(BBW – BAW)/BBW] \times 100$

Where, BAW is bacterial load after sanitizer use and BBW is bacterial load before hand wash.

**Efficacy of hand sanitizer on time intervals**

The experiment was done at three time intervals at the same day and on same individuals. The samples were collected from laboratory staff. The staff includes scientist, researcher, technician, office and sanitary workers. The samples were collected by stabbing fingers on nutrient agar plate before and after applying hand sanitizer. Then second consecutive sample was taken after applying, rubbing hand sanitizer. After the first wash and the first round of inoculation, the same individuals were called up again to repeat procedure. The time interval was 2 hours between first

and second round. Similar way, sample collected from same individuals for the third time after 2 hours again. The media used for the experiment was nutrient agar. After inoculation the plates were incubated at 37 º C for 24 hours and observed next day for the growth of bacterial and fungal colonies. The colonies were counted and picked up and identified by gram's staining.

**Physical stability of hand sanitizer**

The pH of the sanitizer determined after preparation and at a week interval. The physical changes were determined by observing color, odor and pH of sanitizer weekly. The sanitizer were checked for turbidity also weekly. Viscosity and consistency were determined at weeks 0 and 12 of storage (Silvia 13).

**Shelf life of hand sanitizer**

The shelf life of the herbal sanitizer prepared was evaluated at temperature of 4±2°C, 27±2°C and 40±2°C. The hand sanitizer was kept at different temperature and observed every week for homogenicity. Viscosity and consistency were determined at weeks 0 and 12 of storage [14].

**Skin exposure to sanitizer**

Individual exposure will be assessed by questionnaires. The sanitizer prepared was applied 3 ml to different individuals and feedback was taken. The individuals were asked questions regarding sanitizer odor and experience after using. The skin of hand was examined for redness, irritation and dryness. The feedback was collected in consent form filled by individuals.

**Statistical analysis**

All experiments were performed in triplicate and results are expressed as mean ± standard deviation (SD). The mean, standard deviation and percentage reduction values were calculated in MS excel.

**RESULTS**

The polyherbal sanitizer was prepared in the laboratory and its efficacy was checked, its shelf life, pH and other physical parameters were evaluated. The antimicrobial activity of the sanitizer and its ingredients were evaluated on different bacterial strains and its efficacy on different individuals was also evaluated.

The ingredients used for the preparation of sanitizer were isopropyl alcohol, hydrogen peroxide ($H_2O_2$), ethanol, glycerol, camphor, mixture of all plant extracts and distilled water. The plant extract used for the preparation of sanitizer were *Ocimum gratissum* (Van tulsi), *Ocimum sanctum* (Shyama tulsi), *Eucalyptus globules* (Niligiri) and *Azadiracta indica* (Neem), *Cuscuta reflexa* (Amarbel), *Aloe barbadensis (*Ghritkumari) and *Menthe arvensis* (Mint). The plant details with family name are given in tabular (1) form.

**Antimicrobial activity of ingredients of hand sanitizer**

The hand sanitizer was prepared using ethanolic extracts, isopropyl alcohol, ethanol, glycerol, hydrogen peroxide, distilled water, camphor. The sanitizer was prepared by adding all ethanolic extracts in 60% isopropyl alcohol in it ethanol, camphor, distilled water, glycerol and hydrogen peroxide was added. Isopropyl alcohol is reported for its antibacterial property and it damages cell wall of bacteria, whereas glycerol provides soothing effect. Camphor is also known for its antibacterial property. Hydrogen peroxide posses antiseptic properties and distilled water was used in the preparation of hand sanitizer to make up volume. All the ingredients were screened

for antimicrobial activity against all bacterial strains used for the study. The results were done in triplicate and mean value with standard deviation was calculated. The results of antimicrobial activity of all the ingredients used in the sanitizer are given in table 2 and 3.

**Antimicrobial activity of hand sanitizer**

The sanitizer prepared in laboratory was evaluated for antimicrobial activity against all the bacterial strains used above. The results showed potent antimicrobial activity in polyherbal sanitizer prepared by us against all the strains used in the ascending order of *Klebsiella* spp. > *B. cereus* > *S. aureus* > *S. pyogenes* > *P. aeruginosa* > *Dermatophyte* > *E. coli* and gave zone of inhibition of 18 ± 0.05, 12 ± 0.03, 11 ± 0.01, 10 ± 0.01, 9 ± 0.5, 9 ± 0.01 and 7 ± 0.001 mm respectively. A comparative study of antimicrobial activity was done with other commercial sanitizer available in the market. The maximum activity was showed against *Klebsiella* spp. and minimum against *E. coli* and *P. aeruginosa* by all the sanitizer used. The results showed that Savlon showed maximum zone of inhibition against all bacterial strains except *Dermatophyte* as compared to our sanitizer. Comparatively other sanitizers used gave lesser activity than our sanitizer. The results showed that sanitizer made by us has potent antimicrobial activity and greater efficacy as compared to other commercial hand sanitizer (Figure – 2).

**Determination of efficacy of hand sanitizer**

The efficacy of sanitizer was checked on hands. The samples were collected from 38 different volunteers from laboratory staff including scientist, researcher, technician, sanitary workers, as well as from patients and their relatives. The efficacy of hand sanitizer was checked on pathogens residing on their hands because maximum exposure gets on hand surface area. The samples were collected before and after applying hand sanitizer on hand. After applying sanitizer the hands were scrubbed properly and dried and then sample was taken using sterile swab sticks. The number of bacterial and fungal colonies was counted and percentage inhibition was calculated. The results showed reduction in bacterial as well as fungal colony, in most of the cases the number of colonies were eradicated and no growth was observed on culture plates. The results are demonstrated in table 4 and figure 1 shows culture plates. The bacterial colonies found were identified by gram staining. Common bacterial colonies found were *Staphylococci aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococci pyogenes*, *Bacillus* and Micrococcus and fungal colony includes dermatophyte and *Aspergillus*.

**Efficacy of hand sanitizer on time intervals**

The effect of sanitizer for time duration was screened and the results showed decrease in microbial growth. The swab samples from hands were taken by stabbing hands on nutrient agar plate before and after applying sanitizer. The sanitizer was rubbed properly, dried and sample taken. The procedure was repeated again after 2 hours. The number of colonies was reduced after 2 hours. The results showed that sanitizer has effect for few hours also, as well as skin remains healthy also. The results are given in table 5.

**Physical stability of hand sanitizer**

The physical changes were determined by observing color, odor and pH of sanitizer weekly. After sanitizer preparation the pH of the sanitizer was checked. The pH of sanitizer was 8, alkaline. Alkaline pH is good for skin and maintains moisture and does cause dryness of skin.

The sanitizer was prepared in June and its color, pH and odor were checked every week. The sanitizer was found stable for 3 months without change in color, odor and pH.

**Shelf life of hand sanitizer**

The sanitizer was kept at different room temperature and observed for 3 months with no physical change. The sanitizer was kept at high temperature at 40 ± 2°C, room temperature at 27 ± 2°C and low temperature in refrigerator at 4 ± 2°C for three months. The sanitizer was found stable at all the three different temperatures. The physical stability including pH, color and odor was stable. As well as consistency and viscosity was also found stable. The turbidity was checked in sanitizer and no turbidity was also seen. High temperature promotes the growth of bacteria and fungus, but sanitizer made by us did not showed any turbidity or growth in sanitizer.

**Skin exposure to sanitizer**

Skin sensitivity of the sanitizer was checked on different individuals and feedback was collected in consent form. The individuals gave positive response with mesmerizing odor and soothing effect after using sanitizer. The individuals were asked to observe redness, irritation, burning sensation and dryness. But no side effects were seen in any individuals after using sanitizer. Like other commercial sanitizer, our sanitizer gave soothing effect and no dryness was observed.

## DISCUSSION

The skin of human provides nutrients and suitable growth conditions for opportunistic microbes and other pathogens and these resist most of cleaning regimen and contribute to their existence in ecosystem [18]. Hand hygiene is a simple and least expensive means of preventing hospital acquired infections specially derived from environmental surfaces. There are different types of sanitizers available in the market commercially which includes alcohol and non alcoholic based sanitizer. Different sanitizer has different effect of bacterial growth and leads biocidal activity [11]. Nowadays, due to COVID 19 pandemic, there is an urgent need and demand of hand sanitizer not only in medical professionals, but also in common man. Alcohol based sanitizer if used regularly leads dryness of skin and irritation also in sensitive people [15]. So we focused on polyherbal sanitizer. In the present study we selected seven plants and prepared a sanitizer using alcohol, glycerol, hydrogen peroxide and camphor. Every ingredient has a specific role in inhibiting the growth of bacteria and fungus also. Our study focused on antibacterial activity of hand sanitizer and was found potent as compared to other well known commercial sanitizer.

The plants selected for the study were *Ocimum gratissum* (van tulsi), *Ocimum sanctum* (shyama tulsi) , *Eucalyptus globules* (niligiri) and *Azadiracta indica* (Neem), *Cuscuta reflexa* (amerbel) *Aloe barbadensis* (Ghritkumari) and *Menthe arvensis* (Mint). The selection of ingredients was on the basis of its medicinal and antibacterial properties reported. The ingredients were also checked for its antibacterial property against bacterial strains *Peudomonas aeroginosa, Bacillus cereus, Streptococcus pyogenes, Klebsiella* spp., *Escherichia coli, Staphylococcus aureus* and *Dermatophyte* and gave potent activity against all pathogens used for the study.

The use of methanol in hand scrub is not recommended due to its toxic effects and cause severe systemic toxicity, even deaths can occur after oral, pulmonary or skin exposures leads to chronic toxicity (e.g., visual disturbances) if used regularly [15]. Therefore, in place of methanol ethanol, isopropyl alcohol, *n*-propyl alcohol, or their combinations can be used in alcohol based hand rub [16]. Therefore, isopropyl and ethanol was selected in the present study to prepare sanitizer.

Plants have been a valuable source of natural products for maintaining human health and the use of plant secondary metabolites for their antimicrobial activity has gradually increased worldwide. Neem (*Azadirachta indica*) also known as Indian lilac, belongs to Meliaceae family. Nimbidin (the main active antibacterial ingredient), nimbin and nimbinin are stable compounds and found in considerable quantities [17]. Polyphenolic flavanoids purified from fresh leaves of neem were reported for antifungal, antibacterial, [18] antiinflammatory, anti arthritic, antipyretic, hypoglycemic, antigastric ulcer and antitumour activities [19, 20, 21]. The leaves and bark of *A. indica* showed antibacterial activity against antibiotic resistant strains of *Staphylococcus aureus* and *Escherichia coli* [22]. The plant is used in the preparation of hand sanitizer due to its wide spectrum of applications [23, 24].

*Aloe barbadensis*, the pulp of the plant is used in sanitizers to provide soothing effect in hands [10]. The plant is known for its medicinal properties. The plant has various biological and pharmacological activities such as antioxidant, anti-inflammatory, immuno-modulatory, antimicrobial, antiviral, antidiabetic, hepatoprotective, anticancer, skin protective and wound healing. These properties may be due to the presence of bioactive compounds anthraquinones, anthrones, chromones, flavonoids, amino acids, lipids, carbohydrates, vitamins and minerals [25]. The antimicrobial efficacy of *Aloe vera* gel was reported by the presence of zone of inhibition of 24 mm, 21 mm and 24 mm against *Escherichia coli, Enterococcus faecalis* and *Staphylococcus aureus*, respectively [26, 27].

*Cuscuta reflexa* is a perennial, golden yellow, leafless, parasitic herb of the family Convolvulaceae. The plant is rich in number of phytoconstituents includes cuscutin, amarbelin, β-sitosterol, stigmasterol, kaempferol, dulcitol, myricetin, quercetin, coumarin and oleanolic acid [28]. A number of scientists reported antimicrobial activity in *C. reflexa* against gram positive *Bacillus subtilis*, *Staphylococcus aureus* and gram negative bacteria *Escherichia coli*, *Pseudomonas aeruginosa* [29].

*Eucalyptus*, a native plant of Australia, belongs to a diverse genus of flowering trees and shrubs in the myrtle family, called Myrtaceae. The plant is rich source of polyphenols and terpenoids and base composition of the leaves are the Eucalyptol or Cineole [30, 31]. The plant is known for its insecticidal and antimicrobial property and used in preparation of hand sanitizer with combination of rose extract, glycerin [23], *Ocimum sanctum*, alcohol, carbopol, polysorbate, perfume, deionized water, tri ethanol amine and preservative in other. Antibacterial activity was reported in sanitizer against *E. coli, P. aeruginosa, S. aureus, B. subtilis* and Fungi *S. cereviceae* and *C. albicans* by culture sensitivity test [32].

*Mentha arvensis* a medicinally important plant belongs to the Family Lamiaceae, known for cooling effect and traditional medicine for stomach remedies. The plant oil was reported for antibacterial property against pathogen *Staphylococci* and cariogenic pathogens *Streptococcus mutans*, *Streptococcus sangunis*, *Staphylococcus aureus* and *Lactobacillus casei* responsible for dental diseases [34] due to presence of secondary metabolites including alkaloids, tannins, flavonols, steroids, xantones, glycosides, Eucalyptol, Isomethone, Linalool, methnol, 4-Terpineol, oleic acid, tetradecanoic acid, 12-methyl, methyl ester, hexadecanoic acid and (Palmitic acid) methyl ester [33].

*Ocimum sanctum* common Indian aromatic plant, belongs to the family of Lamiaceae. The plant is reported for antimicrobial property against *Actinobacillus actinomycetemcomitans*, a periodontal pathogen responsible for human dental plaque with 22 mm zone of inhibition [34]. The plant used in preparation of hand sanitizer with different ingredients [33] and showed

antimicrobial property against *E. coli, Pseudomonas aeuroginosa, Staphylococcus aureus, Bacillus subtilis* and Fungi-*Sacchromyces cerevisiae* and *Candida albicans* [32].

*Ocimum gratissimum* L. (Labiatae) is widely distributed in tropical and warm temperature regions and commonly used in folk medicine to treat different diseases, e.g. upper respiratory tract infections, diarrhea, headache, ophthalmic, skin diseases, pneumonia, and also as a treatment for cough, fever and conjunctivitis [35, 36]. Previous studies reported antimicrobial activity in plant against *Proteus, Klebsiella, Escherichia, Salmonella, Staphylococcus* and *Shigella* with inhibition zones ranging from 13 to 25 mm [39], due to the presence of volatile oil of this plant contains mostly phenols, particularly thymol [37, 38].

All the above plants used in the present study for the preparation of hand sanitizer and mixture of all extracts showed potent antimicrobial activity against *P. aeruginosa*, *E. coli*, *Klebsiella* spp., *S. aureus*, *S. pyogenes* and *Dermatophyte* with zone of inhibition of 7 ± 0.03, 7 ± 0.03, 8 ± 0.001, 7 ± 0.03, 6 ± 0.02 and 6 ± 0.01 respectively. No zone of inhibition was found against *B. cereus.*

Other ingredients selected in the study includes glycerol. Glycerol is used in number of sanitizers because of its soothing effect. According to World Health Organization (WHO), ethanol based handrub (EBHR) makes the skin dry, therefore its formulation contains 1.45% glycerol as an emollient to protect healthcare workers (HCWs) skin against dryness and dermatitis. However, glycerol seems to negatively affect the antimicrobial efficacy of alcohols [40]. It is also reported by number of researchers that bactericidal efficacy of isopropanol based surgical hand rubs can best be obtained if glycerol is not used in the formulation [41], but due to its soothing effect less amount can be used. In the present study antimicrobial activity was observed against gram negative bacteria *E. coli* and *Pseudomonas aeruginosa* and it can promote other scientist to use glycerol in sanitizer in correct formulation.

Another ingredient used in the preparation of hand sanitizer is Camphor, it is a multipurpose molecule with a most diverse range of applications, ranging from being used to treat medical conditions in humans, as well as a natural poison to kill insects. It is used in ointments and inhalants due to its overwhelmingly distinct aroma, particularly as an adjunct to treat the common cold. Scientifically, numerous biological activities have been attributed to camphor including antibacterial, antifungal, antimutagenic, antitussive and insecticidal properties [42]. Due to its medicinal property the ingredient selected for the preparation of hand sanitizer and gave antimicrobial activity against *Klebsiella*, *S. aureus*, *B. cereus* and *Dermatophyte.*

Hydrogen peroxide is not an active substance for hand antisepsis but it is used in sanitizer to inactivate contaminating bacterial spores in the solution. In the present study hydrogen peroxide showed antibacterial activity against *S. aureus* only and no zone of inhibition found against other strains used for the study. Researchers had reported antibacterial activity in hydrogen peroxide against *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, *Eikenella corrodens* and *Capnocytophaga gingivalis* responsible for causing periodontal disease by causing biocidal action leading to membrane damage and metabolism breakdown in microbes [43].

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**Table 1:** Name of the plant used for the preparation of hand sanitizer with their family and botanical name

| S. No. | Plant Name | Botanical Name | Family |
|---|---|---|---|
| 1 | Van tulsi | *Ocimum gratissum* | Lamiaceae |
| 2 | Shyama tulsi | *Ocimum sanctum* | Lamiaceae |
| 3 | Niligiri | *Eucalyptus globules* | Myrtaceae |
| 4 | Neem | *Azadiracta indica* | Meliaceae |
| 5 | Amarbel | *Cuscuta reflexa* | Convolvulaceae |
| 6 | Ghritkumari | *Aloe barbadensis* | Liliaceae |
| 7 | Mint | *Menthe arvensis* | Lamiaceae |

**Table 2**: Antimicrobial activity of ingredients used in sanitizer against gram negative bacteria. The readings were taken in triplicate, mean and standard deviation was calculated.

| S. No | Name of Ingredients | Zone of Inhibition (mm) | | |
|---|---|---|---|---|
| | | ***P. aeroginosa*** | ***E. coli*** | ***Klebsiella*** |
| 1. | $H_2O_2$ | No zone | No zone | No zone |
| 2. | Isopropyl alcohol | No zone | No zone | 7 ± 0.01 |
| 3. | Camphor | No zone | No zone | 8 ± 0.1 |
| 4. | Ethanol | No zone | No zone | No zone |
| 5. | Distilled water | No zone | No zone | No zone |
| 6. | Glycerol | 12 ± 0.1 | 11 ± 0.01 | No zone |
| 7. | Mixture of all extracts | 7 ± 0.03 | 7 ± 0.01 | 8 ± 0.001 |

**Table 3:** Antimicrobial activity of ingredients used in sanitizer against gram positive bacteria and *Dermatophyte*. The readings were taken in triplicate and mean and standard deviation was calculated.

| S. No. | Name of Ingredients | Zone of Inhibition (mm) | | | |
|---|---|---|---|---|---|
| | | ***S. aureus*** | ***B. cereus*** | ***S. pyogenes*** | ***Dermatophyte*** |
| 1. | $H_2O_2$ | 8 ± 0.1 | No zone | No zone | No zone |
| 2. | Isopropyl alcohol | 7 ± 0.1 | No zone | No zone | No zone |
| 3. | Camphor | 10 ± 0.02 | 6 ± 0.03 | No zone | 6 ± 0.01 |
| 4. | Ethanol | 0.7cm | 0.6cm | No zone | No zone |
| 5. | Distilled water | No zone | No zone | No zone | No zone |
| 6. | Glycerol | No zone | No zone | No zone | No zone |
| 7. | Mixture of all extracts. | 7 ± 0.03 | No zone | 6 ± 0.02 | 6 ± 0.01 |

**Table 4**: Efficacy of hand sanitizer evaluated on different individuals. Total number of colonies counted before and after using sanitizer and percentage reduction value calculated.

| S. N. | Sample Name | Total fungal colonies | | Total bacterial colonies | | % Reduction |
|---|---|---|---|---|---|---|
| | | Before using sanitizer | After using sanitizer | Before using sanitizer | After using sanitizer | |
| 1 | Hemlata Singh | 1 | Sterile | 200 | Sterile | 100 % |
| 2 | Rajendra | 2 | Sterile | No colony | Sterile | 100 % |
| 3 | Nandkishor Kurmi | No colony | Sterile | 200 | 30 | 85 % |
| 4 | Raju Kurmi | 1 | Sterile | 100 | Sterile | 100% |
| 5 | Neha Patel | 2 | Sterile | No colony | Sterile | 100% |
| 6 | Nidhi Kurmi | No colony | Sterile | 200 | Sterile | 100% |
| 7 | Ritu Kurmi | No colony | No colony | 300 | 40 | 86.6 % |
| 8 | Bharti Kurmi | No colony | No colony | 250 | 30 | 88 % |
| 9 | Mamta Kurmi | 2 | 1 | 200 | No colony | 99.5 % |
| 10 | Veshali Kurmi | No colony | Sterile | 250 | Sterile | 100 % |
| 11 | Bhawani Kurmi | No colony | Sterile | 200 | Sterile | 100 % |
| 12 | Hemraj Kurmi | No colony | Sterile | 220 | Sterile | 100 % |
| 13 | Bhavya Kurmi | No colony | Sterile | 150 | Sterile | 100 % |
| 14 | Rajkumar Gupta | No colony | Sterile | 170 | Sterile | 100 % |
| 15 | Pankaj Gupta | No colony | Sterile | 200 | Sterile | 100 % |
| 16 | Rambabu Yadav | No colony | No colony | 280 | 20 | 92.85 % |
| 17 | Ranvir Singh | No colony | No colony | 200 | 10 | 95 % |
| 18 | Shabnam Khan | No colony | Sterile | 280 | Sterile | 100 % |
| 19 | Ranjeet | No colony | No colony | 200 | 50 | 75 % |
| 20 | Giyani | No colony | No colony | 260 | 4 | 98.46 % |
| 21 | Sabia Khan | No colony | No colony | 220 | Sterile | 100 % |
| 22 | Abhishek | No colony | No colony | 300 | Sterile | 100 % |
| 23 | Yasmin Khan | No colony | No colony | 350 | Sterile | 100 % |
| 24 | Saransh Sharma | No colony | No colony | 150 | 50 | 66.66 % |
| 25 | S. Vishwakarma | No colony | No colony | 170 | Sterile | 100% |
| 26 | Brijendra Mishra | No colony | No colony | 380 | 56 | 85.26 % |
| 27 | Pawan | No colony | No colony | 300 | 48 | 83.33 % |
| 28 | Dr. N. Ganesh | No colony | No colony | 640 | 20 | 96.88% |
| 29 | Jyotsana Singh | No colony | No colony | 720 | 0 | 100% |
| 30 | Sameena Akhtar | No colony | No colony | 670 | 0 | 100% |
| 31 | Dr. Shazia Mansoor | No colony | No colony | 800 | 0 | 100% |
| 32 | Dr. Alibha Rawat | No colony | No colony | 860 | 20 | 97.67% |
| 33 | Emrat Lal | No colony | No colony | 990 | 0 | 100% |
| 34 | Meena | No colony | No colony | 910 | 41 | 95.94% |
| 35 | Bharav Lal | No colony | No colony | 940 | 18 | 98.27% |
| 36 | Ruksana Baji | 2 | Sterile | 930 | 72 | 93.01% |
| 37 | Dr. Indu Thakur | 1 | Sterile | 670 | 0 | 100% |
| 38 | Bhawna Kurmi | No colony | No colony | 750 | 0 | 100% |

**Table 5:** Total number of colonies from hands before and after using sanitizer at 2 hours time interval.

| Name of Individual | Time interval (experiment done in duration of 2 hours) Total number of colonies | | | | | |
|---|---|---|---|---|---|---|
| | First round (Time: 10:30 am) | | Second round (Time: 12:30 am) | | Third round (Time: 2:30 am) | |
| | Before | After | Before | After | Before | After |
| Dr. N. Ganesh | 300 | 16 | 170 | 4 | 170 | Sterile |
| Jyotsana Singh | 350 | Sterile | 200 | Sterile | 170 | Sterile |
| Sameena Akhtar | 270 | Sterile | 200 | Sterile | 200 | Sterile |
| Dr. Shazia Mansoor | 350 | Sterile | 250 | Sterile | 200 | Sterile |
| Dr. Alibha Rawat | 370 | 20 | 320 | Sterile | 170 | Sterile |
| Emrat Lal | 400 | Sterile | 370 | Sterile | 320 | Sterile |
| Meena | 450 | 22 | 260 | 15 | 300 | 4 |
| Bharav Lal | 470 | 14 | 300 | 4 | 270 | Sterile |
| Ruksana Baji | 480 | 40 | 320 | 30 | 230 | 2 |
| Dr. Indu Thakur | 250 | Sterile | 230 | Sterile | 200 | Sterile |
| Bhawna Kurmi | 350 | Sterile | 200 | Sterile | 200 | Sterile |

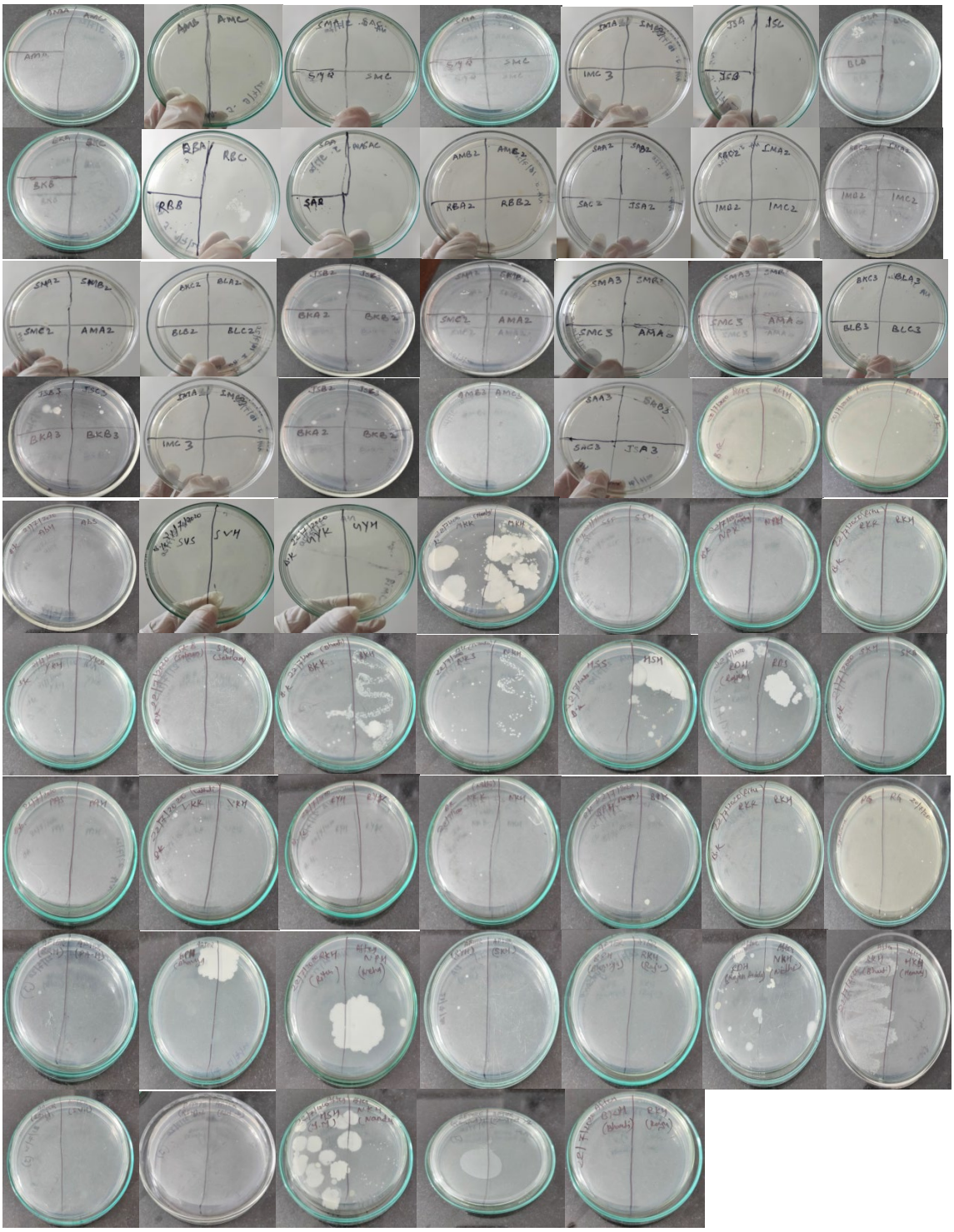

**Fig 1:** Efficacy of polyherbal sanitizer on volunteers hand and bacterial growth. The nutrient agar plates inoculated before and after applying sanitizer

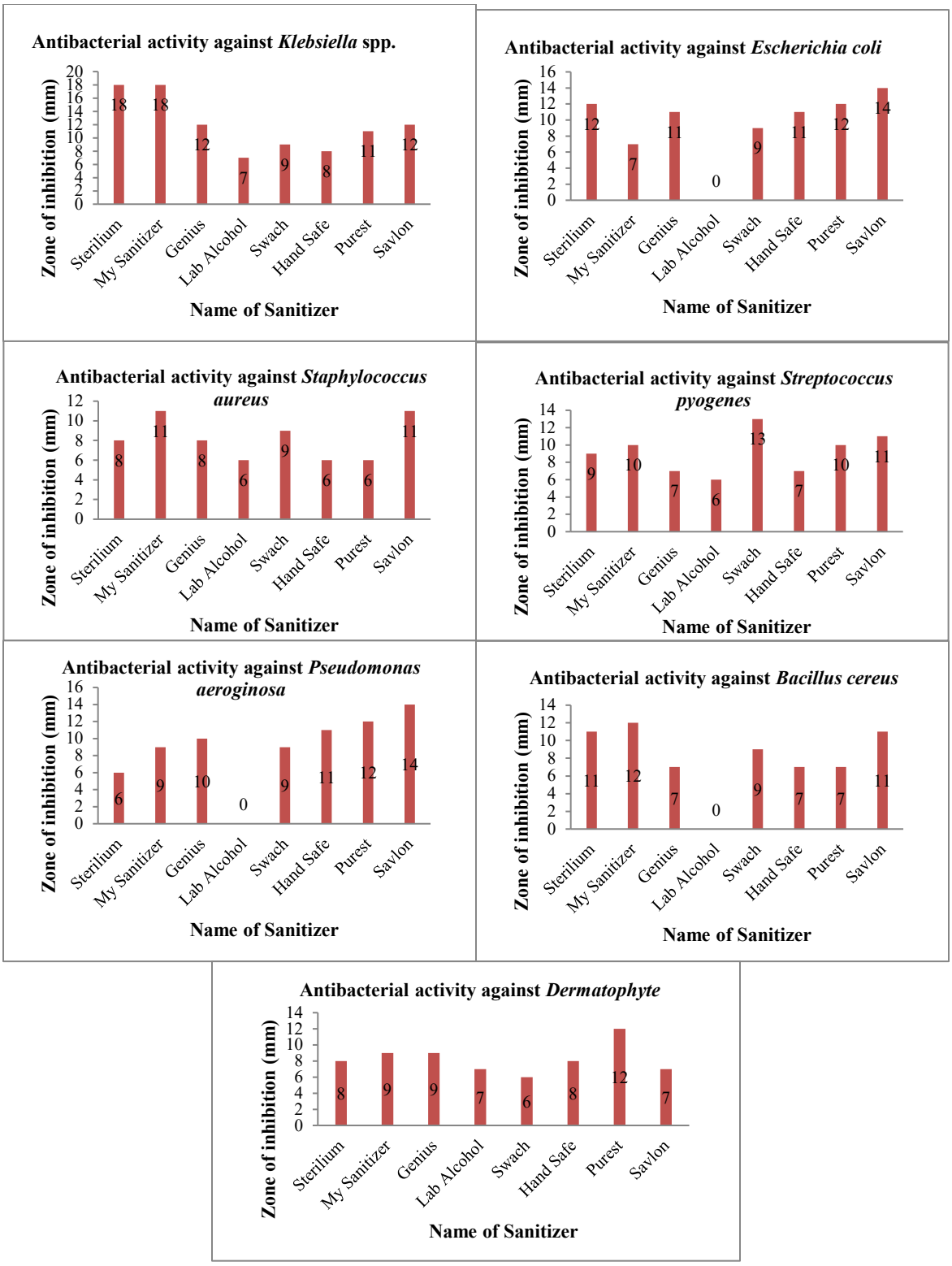


**Fig 2:** Comparative study of different sanitizer with My sanitizer against *Klebsiella* spp., *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Bacillus cereus* and *Dermatophyte*.