MATERIALS AND METHODS

MATERIALS AND INSTRUMENTS USED:

The Chemicals used during work were of different manufacturer like: Nice Chemicals, Hi Media Laboratories Pvt. Ltd., S.D. Fine-Chem. Limited., Fine chemicals etc.

The Instruments used during work were of different manufacturer like: Blue star**,** New-Lab equipment, Genuine, Remi Motors Ltd., Shimadzu etc.

COLLECTION OF SAMPLE: [16,17,18]

The different soil samples nearby different plants were collected from different parts of India like U.P., Kerala, Bihar, M.P. Haryana, Punjab, Rajasthan and Tamilnadu.

The soil samples were collected into sterile plastic bags and stored.
The soil samples were collected from different plants as follows:

There were following soil samples collected -:

1. *Aloe vera* Soil
2. *Atropa belladona* soil
3. *Elettaria cardamomum* soil
4. *Saraca indica* soil
5. *Azadirachta indica* Soil
6. *Citrus sinensis* Soil
7. *Syzygium cumini* soil
8. *Datura stramonium* Soil
9. *Rosa indica* soil
10. *Oscimum sanctum* Soil
11. *Brassica campestris* Soil
12. *Triticum* sps. Soil

13. *Acacia* sps. Soil
14. *Papaver somniferum* Soil
15. *Saccharum arundinaceum* Soil

ISOLATION OF ACTINOMYCETES FROM SOIL SAMPLES: [19, 20,21,53]

Soil samples were serially diluted upto 10^{-6} and 1 ml from each dilution were plated on different isolation media like starch Casein agar, Albumin media, YMA media etc. consisting of antifungal agent Nystatin 50 µg/ ml, by pour plate technique. The ingredients of media were accurately weighed for the each 500 ml of the three type's media i.e.

- Starch Casein Agar Media
- YMA Media
- Albumin Media

Weighed ingredient were dissolved in required quantity of distilled water and sterilized at 121°C (15 lbs) for 15 min by using autoclave. After sterilization the antifungal Nystatin was added (50 µg/ml) then media were poured into Petri dishes under sterile condition (laminar air flow) and allow cooling for sufficient time for the solidification of media.

The diluted soil samples were taken 0.2 ml quantity and spread on the three of the media and kept at 28°C for 1-3 weeks, growth of microbes were observed each day and produced actinomycetes colony were purified on the Petri dishes using streak methods on the same media.

CLASSIFICATION OF ACTINOMYCETES: [19,29,30]

Isolated strains were transferred on different ISP media

Media composition of different ISP media were weighed and dissolved in required quantity of water and sterilized at 121°C (15 lbs) for 15 min by using autoclave. After sterilization the media were poured into Petri-dishes under sterile condition (laminar air flow) and allow cooling for sufficient time for the solidification of media and after

solidification isolated microbes were streaked on solidified media in zigzag fashion and kept for incubation in incubator at 37°C for about 24 hrs.

ISOLATION OF ACTINOMYCETES PRODUCING ANTI-MICROBIAL COMPOUND: [21]

The composition of Mueller hinton agar media weighed and dissolved in required quantity of distilled water and sterilized at 121°C (15 lbs) for 15 min by using autoclave. After sterilization the media were poured into Petri dishes under sterile condition (laminar air flow) and allow cooling for sufficient time for the solidification of media and after solidification the Petri dishes were inoculated with isolated actinomycetes cultures by a single streak of inoculums in the center of the Petri dish and were incubated at 27^{o} -35°C for 4 days. Later, the plates were seeded with test organisms by a single streak at a 90^{0} angle to actinomycetes strains and incubated at 37°C for 24 hrs and observe zone of inhibition. The zones of inhibition producing microbes were isolated purified and sub cultured.

Antagonism was measured by the determination of the size of the inhibition zone.

TEST MICROORGANISMS: [22]

Some bacteria, including Gram positive and Gram negative were used to determine the antimicrobial activity against the isolated actinomycetes.

CHARACTERIZATION OF ISOLATED ACTINOMYCETES FROM SOIL SAMPLES:[23,24,31,32]

Isolated Actinomycetes were characterized by morphological and biochemical methods.

A. Morphological study

The microscopic characterization was performed by cover slip culture method. The mycelium structure, and colour were observed through oil immersion lens.

B. Gram's staining

- The microbes' smears were taken on glass slide.
- The smears were air dried.
- Smears were covered with crystal violet for 30 seconds.
- Covered each smear with Gram's Iodine solution for 60 seconds.
- Washed off Iodine solution with 95% ethyl alcohol, ethyl alcohol was added drop by drop until no more colour flows from the smear.
- The slides were washed with distilled water and drain.
- Safranin was applied to smears for 30 seconds (counter staining).
- The slides were washed with distilled water and blot dried with absorbent paper.
- Let the stained slides air dry.
- The slides were examined under microscope.

C. Biochemical Test[25,26, 27,28,]

Certain biochemical tests were performed for identification of different strains producing antibacterial compound.

- **Melanoid Formation Test**
  - 0.5 gm L-tyrosine was Suspended in 10 ml distilled water in culture tube mixed thoroughly by vortexing and autoclave at 121°C (15 lbs) for 15 min.
  - 100ml base was combined (Beef extract-3 gm, Peptone-5 gm, Agar-15 gm, distilled water-1000 ml), mixed thoroughly by gentle rotation of bottle 2 or 3 times.
  - Aseptically 3-5 ml of media was dispensed into tubes with frequent mixing.
  - Tubes were cooled rapidly to prevent separation of tyrosine.
  - The isolated actinomycetes were inoculated and kept for incubation at 37 °C for 4 days.

- **Test for Nitrate Reduction**
  - The composition of Organic nitrate broth media weighed and dissolved in required quantity of distilled water and sterilized at 121°C (15 lbs) for 15 min by using autoclave. After sterilization the media were poured into Test tubes under sterile condition (laminar air flow) and allow cooling.
  - The isolated actinomycetes were inoculated and kept for incubation at 37°C for 4 days.
  - After incubation, 1 ml of broth was taken and added 2 drops of alpha-naphthalene, 2-3 drops of H_2SO_4 and observed for any change in colour.

- **Test for Acid Production**
  - The composition of Glucose nutrient broth media were weighed and dissolved in required quantity of distilled water and sterilized at 121°C (15 lbs) for 15 min by using autoclave. After sterilization the media were poured into Test tubes under sterile condition (laminar air flow) and allow cooling.
  - The isolated actinomycetes were inoculated and kept for incubation at 37°C for 4 days.
  - The broth was checked daily for any change in colour.

- **Hydrogen Sulphide Production Test**
  - The composition of SIM media were weighed and dissolved in required quantity of distilled water and sterilized at 121°C (15 lbs) for 15 min by using autoclave.
  - After sterilization the media were poured into Test tubes under sterile condition (laminar air flow) and allow to cool.
  - Inoculated the isolated actinomycetes into its appropriately labeled tube by means of stab inoculation.
  - Incubated the inoculated tubes at 35°C for about 48 hrs.

- Examined the tubes for the presence or absence of black coloration along the line of stab inoculation.

Plasmid DNA Isolation from Bacteria

1. Spin 1.5 ml of overnight bacterial culture for 30-60 seconds in a micro-centrifuge.
2. Decant supernatant, leaving 50-100 ul in the tube.
3. Vortex to resuspend the bacteria pellet completely.
4. Add 300 ul of TENS solution.
5. Vortex for 5 seconds to mix.
6. Add 150 ul of the sodium acetate.
7. Vortex for 5 seconds to mix.
8. Spin for 2 minutes in a micro-centrifuge.
9. Transfer supernatant to a fresh tube.
10. Add 0.9 ml of pre-chilled 100 % ethanol.
11. Spin for 5 minutes in a micro-centrifuge.
12. Discard supernatant and add 1 ml of 70 % ethanol.
13. Discard the ethanol and add another 1 ml of 70 % ethanol.
14. Withdraw and discard as much liquid (ethanol) as possible then air-dry the pellet.
15. Resuspend the pellet in 30 ul of distilled water and keep at 4°C or -20 °C

Preparing and Running Standard Agarose DNA Gels

Agarose powder was mixed with electrophoresis buffer to the desired concentration, then heated in a microwave oven until completely melted. Most commonly, ethidium bromide is added to the gel (final concentration 0.5 ug/ml) at this point to facilitate visualization of DNA after electrophoresis. After cooling the solution to about 60°C, it is poured into a casting tray containing a sample comb and allowed to solidify at room temperature .

After the gel has solidified, the comb was removed, using care not to rip the bottom of the wells. The gel, still in its plastic tray, was inserted horizontally into the

electrophoresis chamber and just covered with buffer. Samples containing DNA mixed with loading buffer are then pipeted into the sample wells, the lid and power leads were placed on the apparatus, and a current was applied. We confirmed that current is flowing by observing bubbles coming off the electrodes. DNA migrated towards the positive electrode, which was usually colored red.

When adequate migration has been occurred, DNA fragments are visualized by staining with ethidium bromide. This fluorescent dye intercalates between bases of DNA and RNA. It is often incorporated into the gel so that staining occurs during electrophoresis, but the gel can also be stained after electrophoresis by soaking in a dilute solution of ethidium bromide. To visualize DNA or RNA, the gel was placed on a ultraviolet transilluminator. Be aware that DNA will diffuse within the gel over time, and examination or photography should taken place shortly after cessation of electrophoresis.

PRODUCTION OF ANTI-MICROBIAL COMPOUND USING DIFFERENT MEDIA LIKE STARCH CASEIN BROTH AND L.B. BROTH: [33,34]

The composition of Starch casein broth media and L.B. broth were weighed and dissolved in required quantity of distilled water and sterilized at 121°C (15 lbs) for 15 min by using autoclave and allow to cool. There were 8 Actinomycetes isolated from Rose soil, Ashoka soil, Sugar cane soil and Aloe soil showed zone of inhibition on Mueller Hinton Agar and Bennett Agar. But only two actinomycetes isolated from rose soil and Aloe soil showed good zone of inhibition so these actinomycetes were selected for further study and named as AA1 and AR2. AA1 was isolated from Aloe soil on YMA media and AR2 was isolated from Rose soil on Albumin media.

These were than Inoculated into different media like L.B. broth and Starch Casein Broth and Albumin Broth etc and were kept for incubation at different Temperatures ranging from 28^0C- 40^0C for the production of antibacterial compound upto18 days.

EXTRACTION OF ANTI-MICROBIAL COMPOUND USING DIFFERENT SOLVENTS: [36,37,]

All of three Broth of AA1 and AR2 taken at the end of 7th day and centrifuged at 10,000 rpm for 15 min to separate the mycelial biomass; the supernatant was obtained separated by filtration using Whatman filter paper. Certain solvents used for extraction of antibacterial compound like butanol, n-hexane, ethyl acetate**,** petroleum ether**,** chloroform, ethanol (1:1) ratio. Supernatant mixture was agitated for 50 min. with homogenizer and the solvent was separated from broth by separating funnel, Solvent present in the broth was separated by centrifugation at 5000 rpm for 15 min to remove traces of fermentive broth. All extracts obtained through this method were assayed for antibacterial study against different microbes using respective solvents as control by agar well diffusion method.

ANTI-MICROBIAL SCREENING:[35]

After incubation of 7 days, the L.B.broth and Starch Casein Media showing anti-microbial activity, Then the isolates taken from above media and concentrated up to 10 times at 45°C and anti-microbial activity checked against some gram +ve and some gram –ve bacteria and some fungi using by the standard disc diffusion method and cup plate method. Standard Kanamycin, streptomycin etc. were be used for comparison of the antibacterial activity. The anti-microbial activity checking procedure continued upto 18th day.

Nutrient agar was used as a bacteriological media. Minimum inhibitory concentration (MIC) were calculated.

EXTRACTION OF ANTI-MICROBIAL COMPOUND USING DIFFERENT SOLVENTS: [36,37,38]

There were certain solvents were used for the extraction of antibiotic as follows

- n-butanol
- n-hexane
- Ethyl acetate

- Petroleum ether
- Chloroform
- Ethanol
- Methanol

Above solvents were added to the obtained supernatant in 1:1 ratio. Solvent supernatant mixture were agitated for 45 min. with homogenizer and the solvent was separated from broth by separating funnel, Broth was taken at the end of 7th day and centrifuged at 10,000 rpm for 15 min to separate the mycelial biomass; the supernatant was obtained separated by filtration using Whatman filter paper.

Solvents used for extraction of antibacterial compound in (1:1) ratio. Supernatant mixture was agitated for 50 min. with homogenizer and the solvent was separated from broth by separating funnel, Solvent present in the broth was separated by centrifugation at 5000 rpm for 15 min to remove traces of fermentive broth. All extracts obtained through this method were assayed for antibacterial study against different microbes using respective solvents as control by agar well diffusion method.

The extract showing antibacterial activity was evaporated by subjecting the sample to rotating flash evaporator at 40°C, 50 rpm under vacuum. Until the dark brown gummy substance was obtained. The crude antibiotic was collected and dried in oven at 40°C overnight. Residue obtained was subjected to purification.

PURIFICATION OF ANTI-MICROBIAL COMPOUND:

A. By TLC Method [39,40,41,42]

The plates were prepared using silica gel G and activated at 120°C for half an hour and the extracted compound was spotted using fine capillary and different solvent components were used as mobile phase given as follows

- Ethanol: water: chloroform (40:40:20)
- n-butanol: acetic acid :water (2:1:1)
- Chloroform: methanol (5:95)

- Methanol: chloroform (10:90)

First of all different ratio of mobile phase were prepared and kept in TLC Chamber, after adding mobile phase TLC chamber were closed and allow to saturate for some time.

The plates consisting of stationary phase were kept in the TLC chambercontaining mobile phase and the chamber was closed and allow running the mobile phase for sufficient time until it not reaches upto ¾ of the plate height. After reaching the solvent ¾ height the plates are taken outside and air dried and observed in U.V. chamber. The different spraying reagent were used for the identification of clear spot for the measurement of R_f value and how much component is present in the isolated compounds, necessary for the purification of the antimicrobial compound. by using column chromatography.

B. By Column Chromatography [37]

- Column were taken add a plug of cotton to the bottom of the column.
- Columns were clamped to stand and enough sand was added to fill the curved portion of the column.
- Column was filled 1/4 full with the initial eluent.(n-butanol : acetic acid : water in the ratio of 2:1:1)
- Slurry was prepared using silica gel by pouring dry silica into a beaker of eluent {n-butanol : acetic acid : water (2:1:1)}, silica gel, 20 ml, to approximately eluent, 40 ml.
- Carefully slurry was poured into the column using funnel.
- Outlet was opened to allow solvent to drip into a clean flask, silica was to allowed settle while eluent continues to drip into the flask. Once the silica has settled, carefully sand was added to the top of the column.
- Drain eluent from the column until no solvent remains above the surface of the sand.
- Sample was added to the column.

- Drain eluent from the column until no sample remains above the surface of the sand.
- The flow rate was adjusted one drop per minute.
- Added more eluent as necessary. The eluent collected prior to the elution of sample can be recycled. Analyze the fractions by thin-layer chromatography to determine Pure compound was collected and allowed to vaporized for 5 hrs and dried whole night at 40°C.

ANALYSIS OF THE ISOLATED ANTI-MICROBIAL COMPOUNDS [43,44]

The physical and chemical analysis of the compound was performed as follows:

A. Physical Analysis:

- **Colour of the compound**:
  Colour was observed through naked eye.
- **State**
  State of antibacterial compound was observed through naked eye.
- **Shape of particle:**
  Shape of particle was observed using microscope.
- **M.P. Determination:**

Placed a very small quantity of the solid of interest on a watch glass, and use a stirring rod to grind the solid to a powder. Use a spatula to gather the powder into a small pile. Stick the open end of a melting point capillary into the pile to a depth of about 1 mm, then invert the capillary and tap the sealed end on the bench to encourage the solid to drop to the bottom. The height of solid in the capillary should be no more than 1-2 mm

Turned the Mel-Temp power switch on and choose a voltage setting, using the A voltage setting of 55 was a reasonable choice. While the sample was heating, watched through the magnifying window, while frequently checking the temperature reading of the thermometer. Observed the sample melting and noted down the Melting point

B. Chemical analysis

The sodium fusion extract was prepared by using Sodium fusion tube and the following tests were performed.

- **Presence of hydroxyl group: Lucas test (alcohol)**

  The fusion extract taken in test tube and shake few drops with Cold $ZnCl_2$. (Indication of turbidity at different time)

- **Test for Acidic group**

  The fusion extract taken in test tube and added $FeCl_3$. This shows no colour ppt

- **Test for saturation**

  The fusion extract taken in test tube and 1 ml $KMnO_4$**.** This shows no Discoloration

- **Amide test**

  The fusion extract taken in test tube and heated. NH_3 evolved.

- **Test for Nitrogen**

  The fusion extract taken in test tube and $FeSO_4$ soln. and heated upto boil cool and added H_2SO_4 drop by drop. This shows Green ppt. obtained

SPECTRAL ANALYSIS OF ISOLATED COMPOUNDS29,[36,45,46,47,51,52]

- Calculation of λ max of the isolated compounds
- I.R. SPECTROSCOPY.
- MASS SPECTROSCOPY.
- NMR SPECTROSCOPY

Calculation of λ max of the isolated compounds

The isolated antibacterial compound was taken and diluted upto 5µg/ml using $CHCl_3$ and Spectrum was taken using U.V-Spectrometer for the λ max calculation of the compound.

Using FT-IR Spectroscopy

Procedure:

The spectrum was scanned using KBr pellet technique. The spectra were plotted against Wave number cm^{-1} Vs Transmittance (%) which is shown in results and discussion.

Using NMR Spectroscopy

Procedure:

The NMR spectrum was taken using $CDCl_3$ as a solvent at 300 MHz. The graph was plotted.

Using Mass Spectroscopy

Procedure:

The mass spectrum of antibacterial compound was taken using EIMS at 57 scan and graph was plotted as m/z Vs % of base peak.

***IN-VITRO* MICROBIAL ASSAY OF COMPOUND CHECKING THROUGH CYLINDER PLATE METHOD[48,49,50]**

The antibacterial activity of compound were tested against micro organisms by the standard disc diffusion method and cup plate method. Standard Kanamycin, streptomycin etc. will be used for comparison of the antibacterial activity. Nutrient agar was used as a bacteriological media. The minimum inhibitory concentration (MIC) was calculated.

Minimum Inhibitory Concentration Assay:

Serial dilutions of the antibiotic (representing different concentrations of the antibiotic) were added to a growth medium in separate test tubes. These tubes are then inoculated with the testing bacteria, and decided to test 3 concentrations (10 µg/ml, 1 µg/ml and 0.1 µg/ml) first . Each of these tubes have growth media inoculated with a standard concentration of bacteria and the respective antibiotic concentration.

The tubes were allowed to incubate overnight. Broth tubes that appear turbid are indicative of bacterial growth while tubes that remain clear indicate no growth. The MIC of the antibiotic is the lowest concentration that does NOT show growth, after incubating the tubes overnight, observed the tubes. Repeated the experiment if not showing the proper result.

First of all the MIC of both of the compounds were checked against *E.coli*, *S. aureus etc* and compared with the Streptomycin drug.

The inhibition of microbial growth under standardized condition may be utilized for demonstrating the therapeutic efficacy of antibiotics.

- The compositions of media were weighed and dissolved in required quantity of distilled water and sterilized at 121°C (15 lbs) for 15 min by using autoclave.
- The media was poured into sterilized Petri dishes in the sterilized area to occupy a depth of 3 to 4 mm and plates of media were allowed to solidify the medium.
- The Plates were inoculated with the sensitive bacteria and Bored with borer.
- Solution of different known concentration (0.1 µg, 1 µg, 15µg, 17.5, 20µg etc) were prepared and applied to the surface of the solid medium in sterile cavities prepared in the Nutrient agar Media.
- The Petri dishes are left standing for 3 hours, at 4°C, to diffuse the antibiotic solution.
- They were then incubated for 24 hrs at 37^0C.
- The diameter of the inhibition zones was measured.