Production of Novel Antibiotic From Nocardia Spp And Its Medical Application

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Abstract- *Antibiotics also called antibacterial are a type of antimicrobial drug used in the treatment and prevention of bacterial infections. They either kill or inhibit the growth of bacteria. A limited number of antibiotics also possess antiprotozoal activity. Actinomycetes belongs to the family Actinomycetaceae, is one of the most important sources for the discovery of new antibiotics which is successfully introduced and used today in clinical practice. In the present investigation, a novel antibiotic was produced by using a soil actinomycete Nocardia spp. The synthesised antibiotic was characterized by UV-VIS, TLC and FT-IR. Antibacterial activity was also performed against diabetic wound pathogens, Anti bacterial activity, Minimum Inhibitory Concentration, Autobiography and Antagonistic activity. It was found that the Antibiotic produced by Nocardiopsis flavescens strain RRMVCBNR (MG287120) was active against bacterial pathogens.*

Keywords- Antibiotics, actinomycetes, Nocardia spp and antibacterial activity.

I. INTRODUCTION

Actinomycetes has considered as both bacteria and fungi. Actinomycetes are a gram positive filamentous and its has a rich source of antibiotic. Many species of actinomycetes are occurs in soil and are harmless to animals and higher plants while some are important pathogens, many others are beneficial source of antibiotics. Actinomycetes constitute a diverse group of microorganisms that are widely distributed in terrestrial, fresh water and marine habitats (**Radhika *et al.*, 2011**).

Morphologically the actinomycetes resemble as fungi because of their elongated cells that branch into filaments or hyphae. During the process of composting mainly thermophilic and thermotolerent actinomycetes are responsible for decomposition of the organic solution at elevated temperatures. In the fresh substrate, the actinomycetes are grow and more slowly than other bacteria and fungi. The chitin or the cellulose is the natural substances which degraded the actinomycetes during the composting process.

The actinomycelates characterized with substrate and aerial mycelium growth. They are the most abundant organism, in the soil their filaments structure are like a theard and are responsible for characteristically "earthy" smell of freshly turned healthy soil. Their major role is the cycling of organic matter. It inhibits the growth of several plant pathogens in the rhizosphere and decompose complex mixture of polymer in dead plant, animal and fungal material result in production of many extracellular enzymes which are conductive to crop production.

To the study has focused on the isolation of twenty-three halophilic actinomycetes from two ponds of different salinity and the evaluation of their ability to exert an antimicrobial activity against both their competitors and several other pathogens. From the 23 isolates, 18 strains showed antagonistic activity, while 19 showed activities against one or more of the seven pathogen strains tested.**(Donyez Frikha Dammak et al., 2017).**

The significance and frequency of marine microorganisms as producers of bioactive metabolites-a natural source of drug discovery had varied significantly during the last decades, making marine ecosystem a huge treasure trove of novel isolates and novel compounds. Among the twelve actinomycetes isolated from marine sediment sample (Lat. 17_41096200N, Long. 83_19063300E), amylase, protease, lipase and cellulase activities were exhibited by 8,7,4,3 isolates respectively. Five isolates exhibited L-asparaginase activity, while 5, 6, 2 isolates exhibited antibacterial, antifungal and antimicrobial activities respectivelyIsolation and characterization of each potential actinobacteria having immense industrial and therapeutic value on an unprecedented scale from marine sediments of Visakhapatnam coast will have a burgeoning effect. **(Mobeen Shaik et al., 2017).**

II. MATERIALS AND METHODS

Sub-culturing

In the present study *Nocardiopsis flavescens* RRMVCBNR (MG287120) was obtained from CBNR and used for further studies.

Actinomycetes isolation agar was prepared by the composition of Sodium caseinate 2.00 g/l, L-Asparigine 0.10 g/l , Sodium propionate 4.00 g/l, Dipotassium phosphate 0.50 g/l, Magnesium sulphate 0.10, Ferrous sulphate 0.001, Agar 15.0 and sterilized under autoclave at 121ºC for 15 minutes. After sterilisation *Nocardiopsis flavescens* was streaked and incubated at 29^0C for 5-7 days.

Production of Antibiotic

A well grown agar slant of *Nocardiopsis flavescens* was inoculated into one litre flask containing 500 ml of the formation medium consisting of (g/L). Soluble starch, 20.0; $(NH_4)_2SO_4$, 2.0; K_2HPO_4, 1.0; NaCl, 1.0; $MgSO_4$, 1.0; $CaCO_3$, 2.0; trace salt solution ($FeSO_4.7H_2O$, 0.1; $ZnSO_4.7H_2O$, 0.1 and 1000 ml distilled water), pH value of the medium was adjusted at 7.2 before sterilization. The flask was incubated at 25ºC for 5 days in incubator. Total volume was filtered through Whatman No.1 filter paper, followed by centrifugation at 5000 rpm for 20 minutes. The clear filtrates were tested for their activities against the test organism **(Sathi *et al.*, 2001).**

Screening of the production medium

15 to 20 ml of Muller Hinton agar medium was prepared and poured in to the sterilized Petri plate. After solidification the *E.coli* (80 µl) was evenly swabbed into the agar without disturbing the agar. Using sterile cork borer 3 wells were made (10 mm) and samples were added to the well and the plates were incubated at 37^0C for 24 hour to study the zone of inhibition.

Extraction of compound

Equal volumes of the filtrate and ethyl acetate solvent was mixed thoroughly by shaking them in 250 ml capacity separating funnel and allowed to stand for 30 minutes. Two layers were separated; the aqueous layer and the organic layer, which contained the solvent and the antimicrobial agent. The organic layer was concentrated by evaporation under vacuum to the least volume, after the dehydration with anhydrous Na_2SO_4. The aqueous layer re-extracted and the organic layer added to the above organic layer. Antimicrobial activity was monitored by the agar diffusion method inoculated with the *E.coli* and *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus auerus* as test organism. (A control test for each solvent was also performed). The plates were incubated at 37ºC for 16-24 hour **(Atta *et al.*, 2010).**

Characterization of the compound

UV visible spectroscopy

The extracted sample was observed by examine them under the visible spectrophotometer ELICO SL 159 UV – VIS (250 to 300 nm) (**Brewster *et.al.*, 1977).**

FTIR

The extracted compound was further analysed using FTIR Shimadzu IR-470 plus (700 to 4000 cm^{-1}).**(Faix *et al.*, 1992).**

TLC method

On silica gel TLC plate in (3×3 cm Merck Germany plate) size was used for loaded antimicrobial agent on silica gel slide a capillary tube was touched with one side of the coated slide about 1 cm from the bottom end (not allowing large drop to flow) and then allowed to air dry. After the drop dried it was then ready to be developed. These slides are kept in screwed bottles containing 5 ml of n-butanol – ethanol – water (4:1:5). Solvent in a vertical position. The solvent was allowed to run through silica gel layer until the solvent front reached about 1 cm of the top of the slides then they were removed from the bottles. The solvents were allowed to evaporate from the slides **(Brewster *et al.*, 1977).**

Biomedical application

Antimicrobial activity

Antimicrobial screening and selection of isolates Primary screening was carried out using the modified method of Kirby Bauer antibiotic susceptibility test. The antimicrobial activity was determined by agar well diffusion method. The Muller Hinton agar was prepared and poured into petriplates. After solidification 80 µl of overnight cultures of *E.coli*, *Pseudomonas aeruginosa*, *Staphylococccus aureus* and *Klebsiella pneumoniae* were added separately and uniformly swabbed. After 10 minutes 20mm well were made, to that 20 µl of sample was added. The Plates were incubated at 37ºC for 24 hour. The Zone of inhibition was measured **(Gopinath *et al.*, 2013)**.

Auto biography

The *E.coli* was swabbed onto Mueller-Hinton agar plates for use in contact bio autography technique adopted from the method of Wedge and Nagle with slight modifications. The dried TLC plates with corresponding spots were placed aseptically onto the seeded Mueller-Hinton agar plate overlaid with sterile lens paper. The TLC plate was placed face downward with the silica-coated side in contact evenly with the lens paper and was incubated for 12 to 18 hours at 4 ± 2°C. Then, the TLC plate was removed and the inoculated agar plate was further incubated at 35 ± 2°C for 24 hour in an ambient air incubator. The zone of inhibition was observed and compared with TLC plate value results.

Nocardiopsis flavescens streaked as a straight line and incubated at 30°C for six days. Then the plates were seeded with *E .coli* by a single streak at a 90° angle to the *Nocardiopsis flavescens* and incubated at 37^0C for 24 hours **(Wu *et al.*, 1995).**

Minimum Inhibitory Concentration

MIC of active ethyl acetate crude extract of isolates against test microorganisms was determined by serial dilution method. MIC was determined by enzyme-linked immune sorbent assay (ELISA) in micro-titer plate. The crude extract was twofold serially diluted from 1.25 mg/ml to 10 mg/ml for determining the MIC of active isolates.

Antagonistic effect

The antagonistic activity of soil isolates was evaluated by Cross Streak method **(Rahman *et al.*, 2011).** *Nocardiopsis flavescens* streaked as a straight line and incubated at 30°C for six days. Then the plates were seeded with *E .coli and Staphylococcus aureus* by a single streak at a 90° angle to the *Nocardiopsis flavescens* and incubated at 37ºC for 24 hour. The antagonistic effect of *Nocardiopsis flavescens* isolates on the test organism was analyzed by the determination of size of inhibition zone **(George *et al*., 2010).**

III. RESULT

Sub culturing

The *Nocardiopsis flavescens* was sub cultured an isolation agar used for further studies

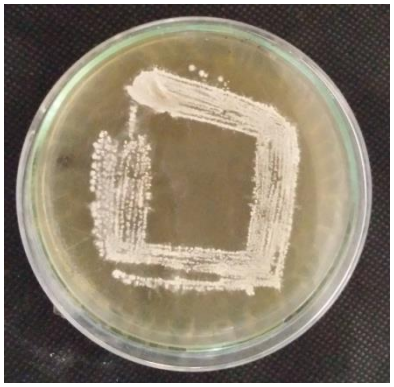


Figure: 1 Sub cultured *Nocardiopsis flavescens* on Actinomycetes isolation agar

Production of antibiotic in production medium

The isolation of actinomycetes broth was prepared, added a 100 µl of culture *Nocardiopsis flavescens.* It was incubated at 37ºC for 7 days. After 7 days the product was recovered by filtering the medium using What man no.1 filter paper and the cell free extract was used for further studies.

Screening of the antibiotic

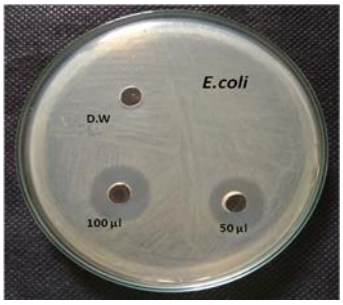


Figure 2: Screening of the extract against *E.coli*

After 24 hour, the zone of inhibition was measured and the presence of antibiotic was confirmed by the inhibition of the activity of organism.

Extraction of the compound

To the production medium, the sodium sulphate was added to extract the compound.

After 30 minutes clear aqueous phase was seperated. To the organic layer ethyl acetate was added. Similar findings were reported by Gopinath and Singara Charya (**Gopinath *et al.*, 2003)** reveals that the antibiotic production of actinomycetes isolated from different etiological conditions were surveyed it was evident that the antimicrobial activity of the eight strains of actinomycetes grown on two different media glycerol-broth and starch-casein broth for 7, 14 and 21 days. Out of thirty seven, 2l isolates were subjected to

submerged culture and 12 (57%) isolates were found to exhibit antimicrobial activity while the other 9 (43%) isolates did not exhibit any activity in broth culture.

Characterization of compound

UV- Spec Analysis

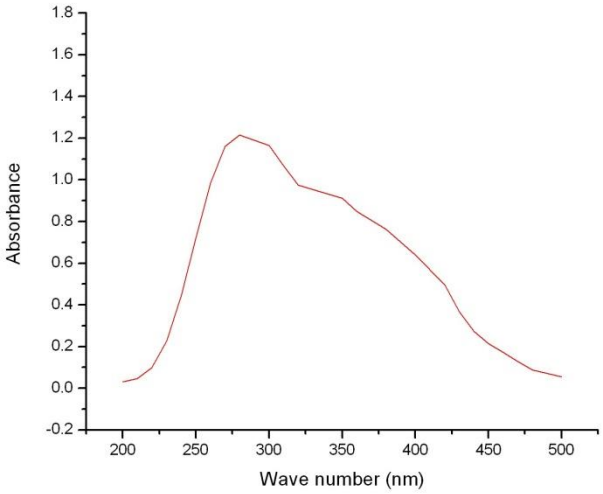


Figure: 3 UV-spec analysis of production medium

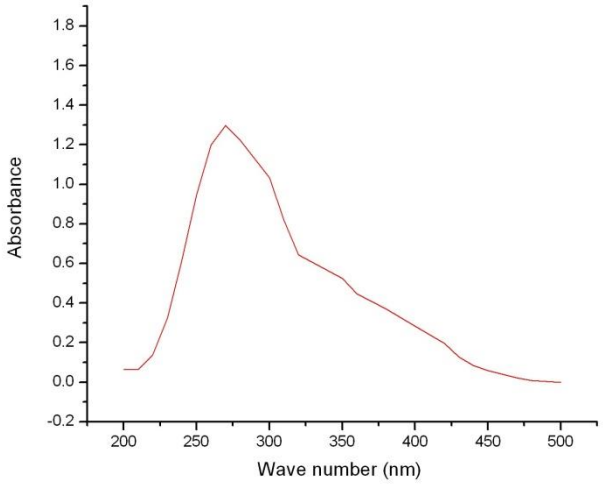


Figure: 4 UV-Spec analysis of ethyl acetate

The peak values are listed under the table. The plasmon peak was observed in the range of 250 nm to 300 nm for all the samples. The UV study helped in the identification of samples which has the best activity. The results were recorded at 0^{th} hour. The observation of the results showed the peak values.

FTIR

FTIR spectrum of each active extract was detected using Shimadzu IR-470 plus. The spectra were also scanned in the 700 to 4000 cm^{-1} range and plotted as intensity versus wave number **(Augustine *et al*., 2005).**

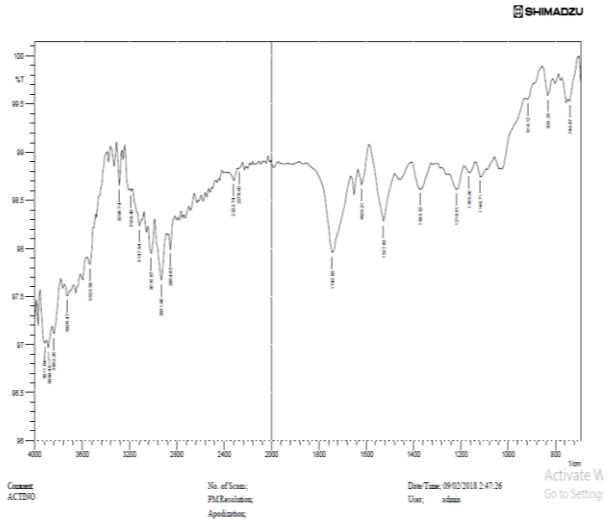


Figure: 5 FTIR result using antibiotic powder

Characterization was done by FTIR to provide the compound ranges. FTIR provides the information about functional groups present in the sample. The FTIR studies showed sharp absorption peaks located at 1383.32 cm^{-1} and 3535.59 cm^{-1}. The peak appeared at 3535.59 cm^{-1} shows that the stretching of bonded N-H stretch. It is the characteristics of N-H stretching in primary/secondary amides. The band seen at 1383.32 cm^{-1} is characteristics of Nitro groups and N=O vibration.

Thin layer chromatography

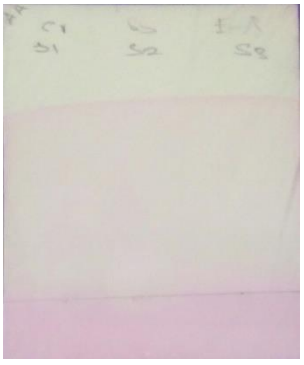
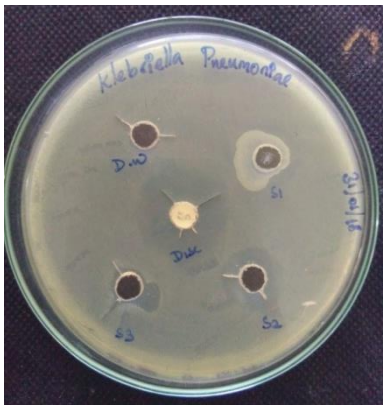


Figure: 6 Thin layer chromatography

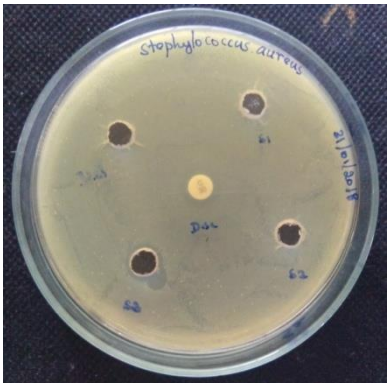
The pink colour indicates the bioactive compound which is presence in our antibiotic sample.

IV. BIOMEDICAL APPLICATIONS

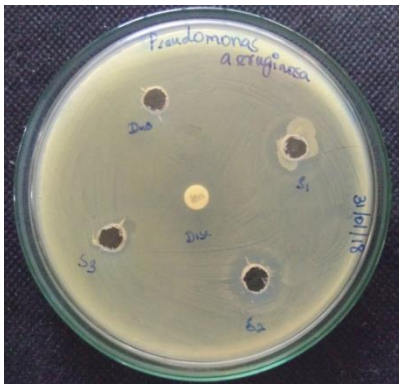
Anti microbial activity



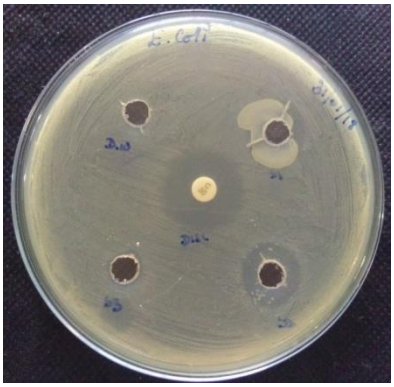
Klebsiella pneumoniae



Stephylococcus aureus



Pseudomonas aeruginosa



Escherichia coli

Figure: 7 the antimicrobial activity using different organism

The antimicrobial activity was studied preliminarily **(Haque *et al.*, 1992)** against bacteria. The test organisms were used are *Klebsiella pneumoniae, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa.* After preliminary testing of the isolates for their antimicrobial activities the most active isolates was selected for further study.

Minimum Inhibitory Concentration

MIC of active ethyl acetate crude extract of isolates against test microorganism was determined by serial dilution method.

The concentration of the samples was mentioned below.

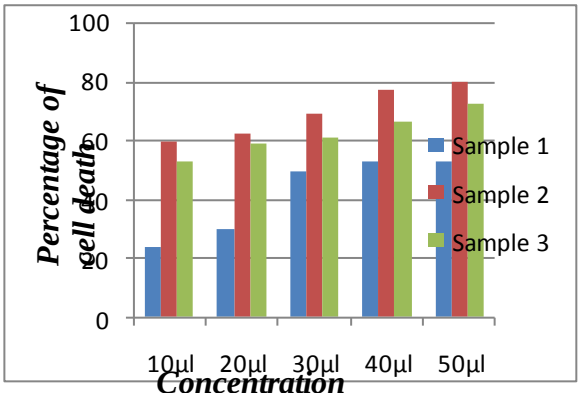


Figure: 8 Percentage of cell death

Percentage of cell death was high in the 50µl of concentration of the sample S2 and S3 when compared with other lower concentrations.

Antagonist activity

The antagonistic activity of soil isolates was evaluated by Cross Streak method **(Rahman *et al*., 2011).**

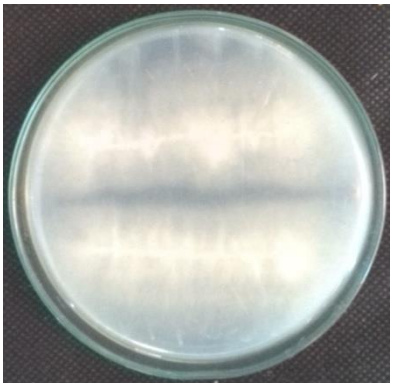


Figure 9 : Antagonist activity

The antagonistic effect of *Nocardiopsis flavescens* isolates on test organism was analyzed by the determination of size of inhibition zone. The death of E.coli cells were noticed at the places were striking took place.

Autobiography

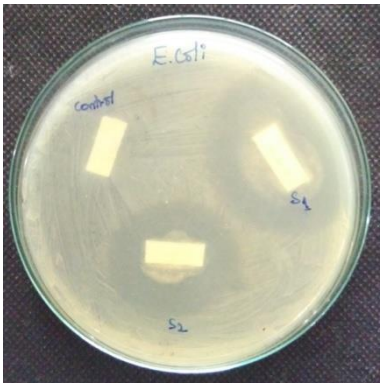


Figure:10 Auto biography

After incubation, zone of inhibition around the spot of antibiotic was observed. The sterile zone on the media proved the presence of active antibacterial compounds.

V. CONCLUSION

In recent year, the increase prevalence of infectious diseases resistant to antibiotics has caused an urgent need to discover new drug against pathogens. In the present investigation, the secondary metabolites (Antibiotics) were produced using *Nocardiopsis flavescens.* The compound was extracted using ethyl acetate followed by Sodium sulphate after production using production medium.

The optimum day was found to be fifth day for compound extraction. The antibacterial screening study was carried out and found that 100µl of the extract moved the maximum activity (8mm). Then further characterized using UV and TLC study. For crude extract the peak was observed at 270 nm and 256 nm for Ethyl acetate. The presence of functional group was also confirmed using FTIR. The amino acid, Amide, Nitrile and Nitro functional groups were confirmed the presence of compounds.

The anti bacterial activity was carried out against various pathogens and found that the compound was active maximum *E.coli* and *Pseudomonas aeruginosa* and *Staphylococcus auereus*. The Autobiography and antagonistic study were also carried out to find out the activity. The present finding revealed that the strain *Nocardiopsis flavescens* produced an antimicrobial compound act against various pathogens to an alternative source.

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