**CHEMICAL ANALYSIS OF BIOACTIVE EXTRACT OF
STREPTOMYCES SP. ISOLATED FROM A FOREST SOIL****K. George Abraham***

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ABSTRACT

Actinomycetes are fungi like bacteria that are well known for their ability to produce secondary metabolites. *Streptomyces* species forms the dominant group of actinomycetes. The present study reports antimicrobial activity of the crude extract prepared from a *Streptomyces* strain isolated from the forest soil. The bioactive extract prepared in ethyl acetate was fractionated using column

chromatography. The active fraction was further analysed chemically by TLC and FTIR.

KEYWORDS: Actinomycetes, *Streptomyces*, soil, chromatography, bioactivity.

INTRODUCTION

Natural products form a fundamental source of new chemicals and therefore are an integral component of today's pharmaceutical industry. There is a renewed interest in the study of microorganisms as a source of structurally unique and pharmacologically active natural products. Actinomycetes are known to be responsible for the production of about half of the discovered bioactive secondary metabolites, notably antibiotics, antitumor agents, immunosuppressive agents and enzymes.^[1]

Streptomyces is the most important and dominant genus of the actinomycetes and is reported to produce a wide range of commercially important products, especially antibiotics.^[2] In view of this, the present investigation was undertaken to isolate a strain of *Streptomyces* from a soil sample collected from a forest in Ratnagiri, Maharashtra state. A crude extract prepared from

this isolate was studied for its bioactivity and further analysed with Thin Layer Chromatography (TLC) and Fourier Transform Infra Red spectroscopy (FTIR).

MATERIALS AND METHODS

Collection of forest soil sample

The upper organic layer of forest soil was collected from Ratnagiri region, Maharashtra state, India.^[3]

Isolation of *Streptomyces* from the soil

1 g of forest soil was suspended in 3 ml of sterile water, thoroughly vortexed for 10 mins and incubated for 1 h at 50°C. The dilutions from 10^{-3} to 10^{-6} were prepared. From these dilutions, three replicates of 100 μ l were plated on ISP2 agar supplemented with Rifampin ($2.5\mu\text{g ml}^{-1}$) as an antibiotic to reduce the growth of eubacteria. This medium was also supplemented with cyclohexamide ($10\mu\text{g ml}^{-1}$) to control the growth of fungi. After incubation for 8 -15 days at 30°C, different types of colonies (morphological appearance) were individually picked up and purified. They were identified by observing under the microscope and using biochemical tests. Purified strains were cultured on ISP2 agar slants & named as FS2, FS7 and FS11. It was noted that all these strains produce same colour, so among these only one *Streptomyces* strain FS7 was taken for further studies in this investigation after mass culturing.

Extraction of culture broth

After incubation, two litre culture was directly added with ethyl acetate and kept for 30 mins and mixing at regular intervals. By using separating flask, the culture broth and EA mixture was allowed to form two layers and the organic layer (EA extract) was separated with care. The separated organic layer was concentrated using rotary evaporator and dried crude extract (12.6 g) was separated and stored at 40°C till further use.

Chromatographic separation of crude extract

The dried ethyl acetate crude extract (1.6417g) of FS7 was partially purified using column chromatography over Sephadex LH 20 with Methanol: chloroform, in the ratio of 1:1. Total 27 fractions were collected and TLC of each fraction was done in suitable solvent system and sprayed with 5% methanol in chloroform. TLC of these fractions was compared and fractions giving similar banding pattern were pooled and concentrated.

Anti-microbial assay

For this assay, Muller Hilton media (MH) with 2% agar (basal layer) and 1% agar (seed layer) were used. The plates were poured with 2% MHA medium (basal layer) and allowed to solidify. The pathogens were sub cultured in tubes each containing 5 ml of Nutrient broth, incubated and stored in refrigerator until use. 25 μ l of each culture were added to 15 ml of 1% MHA (seed layer) medium and were poured onto the basal MHA medium. Wells having a diameter of 6 mm were bored using a cork-borer. The crude ethyl acetate extract as well as its 3 fractions (EA1, EA2 and EA3) were diluted to a concentration of 10 mg/ml. A concentration of 100 μ g of crude extract and its fractions (after dissolving in water) was loaded in the wells. The solvent ethyl acetate was also tested as a positive control. The plates were incubated overnight at 30°C and the zones of inhibition were measured in millimeters.

Thin layer chromatography (TLC)

The crude ethyl acetate extract as well as its fractions were used for Thin Layer chromatographic studies. The TLC plate used was made up of aluminium sheet on to which silica gel was used as an absorbent. The dried ethyl acetate extract as well as its fractions of 2-3 μ l was applied on the TLC plate with the help of capillary tube. The plate was run by using methanol: chloroform system (50:50 vol/vol) as a solvent system. After development, the solvent was evaporated and the dried plates were kept under UV to check UV visible compounds. TLC was developed by spraying 5% H₂SO₄ and ninhydrin for detection of various well separated bands.

Fourier Transform Infra Red spectroscopy (FTIR)

EA3 fraction (5 mg) was taken and mixed with potassium bromide (KBR) and crushed to a fine powder using mortar and pestle. Analysis was done using Shimadzu FTIR system. The scanning was done at frequency wavelength 400-4000 cm^{-1} with resolution of 4 cm^{-1} .

RESULTS

From 5 L of culture broth, 22.38 grams of crude ethyl acetate extract was obtained. This crude extract was fractionated further using column chromatography. Three major fractions viz. EA1, EA2 and EA3 were selected for further studies. Details of these fractions are shown in the Table 1.

Table 1: Column chromatography fractions of *Streptomyces* extract.

Major fractions	Fractions pooled	Final weight
EA1	Frs. 1 - 11	8.6 grams
EA2	Frs. 12 - 19	4.9 grams
EA3	Frs. 20 - 27	6.3 grams

Antimicrobial activity

Crude ethyl acetate extract showed promising antimicrobial activity against the test pathogens. Highest activity was observed against *Shigella flexineri*. Compared to the crude extract, the column fractions EA1 and EA2 failed to show any significant activity. However, the fraction EA3 showed potent activity against six out of the seven test pathogens. No activity was seen against *Vibrio cholerae* (Table 2).

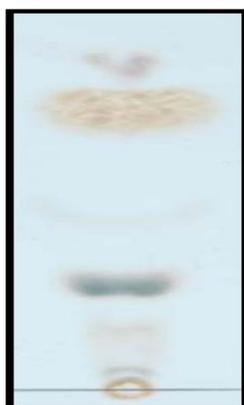
Table 2: Antimicrobial activities of crude *Streptomyces* extract and its fractions against pathogens at 100 µg per disc concentration.

Test pathogens	Crude EA extract	EA1	EA2	EA3
<i>Pseudomonas aeruginosa</i>	6±1	4±1	-	4±1
<i>Staphylococcus aureus</i>	4±	-	-	8±1
<i>Shigella flexineri</i>	12±1	-	3±1	11±1
<i>Salmonella typhii</i>	2±1	-	-	4±1
<i>Vibrio cholerae</i>	-	-	-	-
<i>Klebsiella pneumonia</i>	9±1	-	-	2±1
<i>Bacillus subtilis</i>	6	-	7±1	14±1

Since fraction EA3 exhibited strong antimicrobial activity, it was further analysed by TLC and FTIR.

Thin Layer Chromatography

The bioactive EA3 fraction showed five major compounds on the TLC plate (Fig. 1). The solvent system 50% methanol in chloroform was found to be efficient in separating the compounds on the plate.

**Figure 1: Thin layer chromatography of EA3 bioactive fraction of *Streptomyces* sp.**

Fourier Transform Infra Red spectroscopy

FTIR analysis of EA3 bioactive fraction obtained from crude ethyl acetate extract indicated several peaks (Fig. 2). Some significant absorption frequencies were 3398.57 cm^{-1} which indicated, 1633.71 cm^{-1} indicating presence of the bond C=C having conjugated alkenes as the functional group and 1404.18 cm^{-1} indicating presence of the bond S=O having sulfonyl chloride as the functional group.

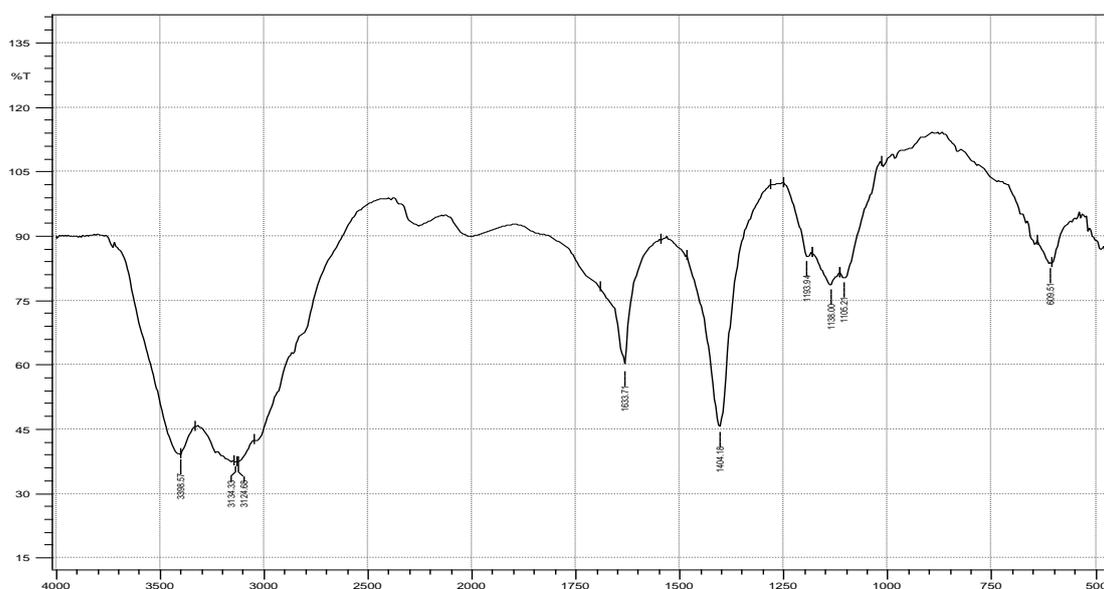


Figure 2: FTIR Spectrum of EA3 bioactive fraction of *Streptomyces* sp.

DISCUSSION

The present study indicates that bioactive actinomycetes belonging to the genus *Streptomyces* can be successfully isolated from the soil sample. Therefore, more studies can be taken up to explore screening of soil samples to isolate novel actinomycetes that could have industrial importance.

Sivakumar *et al.*, (2005) isolated *Streptomyces roseolilacinus* from soil samples collected from the mangrove forest area of Pitchavaram, India which showed inhibitory activity on *Staphylococcus aureus*, *Proteus vulgaris*, *Klebsiella pneumoniae* and *Candida albicans*.^[4] *Streptomyces* sp. from soils of Sundarban mangrove forests of India that inhibited the growth of *B. subtilis*, *Arthobacter protophormiae*, *B. pumilis*, *Lactococcus lactis*, *K. pneumoniae*, *Protues mirabilis*, *Micrococcus lutues* and *E. coli* was reported by Mitra *et al.*, (2008).^[5] Jeffrey (2008) isolated bioactive *Streptomyces* sp. from the farming soil of Agriculture Research Centre Semongok, Sarawak, Malaysia.^[6]

Moses (2009) carried out the isolation of *Streptomyces* sp. from soil samples from Manakkudi mangrove forest ecosystem, Tamil Nadu, India which showed promising activity against human and fish pathogens.^[7] Raghavrao *et al.*, (2012) isolated *Streptomyces* sp. from mangrove forests of Visakhapatnam, India and reported its antibacterial and antifungal activity against *S. aureus*, *B. subtilis*, *B. cereus*, *E. coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *C. albicans*, *Aspergillus niger* and *A. Flavus*.^[8] A novel antimicrobial strain of *Streptomyces* was reported by Deepthi *et al.*, (2012) from the soil samples from Coringa mangrove forest, Andhra Pradesh.^[9]

Based on these reports and observations of the present study, it is suggested that soil samples can be an excellent source to isolate bioactive *Streptomyces*.

The bioactive fraction was successfully extracted using the solvent ethyl acetate and when analysed using TLC and FTIR, showed presence of many compounds mainly having functional groups such as alkenes and sulfonyl chlorides indicating that activity could be attributed to the presence of these compounds which were extracted successfully by the solvent.

CONCLUSION

Streptomyces sp. isolated from the forest soil was found to have a potential for the production of antimicrobial compounds. This crude extract showed activity against six pathogens out of the seven pathogens tested. Further this extract was fractionated in three major fractions by using column chromatography. Out of these three fractions, EA3 showed significant antimicrobial activity against six pathogens. Its activity against *Shigella flexineri* and *Bacillus subtilis* was quite strong. This active fraction was analysed by using TLC and FTIR. TLC of this fraction showed 5 major compounds and FTIR of this EA3 fraction showed presence of alkenes and Sulfonyl chloride. This investigation therefore highlights the importance of the soil derived *Streptomyces* sp. in the production of antimicrobial compounds.

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