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Antimicrobial Susceptibility of *Streptomyces rochei* (OM746935) Isolated from Bargur Hills, and their characterization of Bioactive Secondary Metabolites

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ABSTRACT: The pursuit of novel bioactive substances from the natural environment has been increasing urgency for reduction of microbial infections. Actinomycetes were isolated from 10 different regions of soil samples in the Bargur hills, from Erode, Tamil Nadu, India. The most potent bioactive compounds producing *Streptomyces rochei* BF3A strain isolated in the 6th region revealed maximum antibacterial activity against all the tested bacteria *viz.*, gram-positive *Staphylococcus aureus* (ATCC 29737), *Bacillus coagulans* (MTCC 6735) and gram-negative bacteria *Escherichia coli* (ATCC 8739), *Klebsiella pneumoniae* (ATCC 9621), *Pseudomonas aeruginosa* (ATCC 27853). The production of bioactive metabolites was extracted using ethyl acetate and the extract showed promising antibacterial activity. The *Streptomyces rochei* BF3A extract revealed minimum inhibitory concentration (MIC) in all the tested bacteria *Staphylococcus aureus*- 1.25 mg/ml, *Bacillus coagulans* – 2.50 mg/ml, *Escherichia coli* – 2.50 mg/ml, *Klebsiella pneumoniae* – 2.50 mg/ml, *Pseudomonas aeruginosa* – 5 mg/ml. The bioactive compounds were partially purified by Thin layer chromatography (TLC) and column chromatography and characterized by gas chromatography-mass spectrometry (GC-MS) and Fourier transformed infrared (FTIR) studies conformed *Streptomyces rochei* strain active fraction showed predominant bioactive compounds Hexahydro-3-(1-methyl propyl) pyrrolo [1,2-a] pyrazine-1,4-dione, Pyrrolo [1,2-a] pyrazine-1,4-dione hexahydro-3-(2-methyl propyl). These findings demonstrated that the existence of bioactive components in BF3A offered a potential source for the creation of powerful antibacterial drugs that were effective against a variety of bacterial infections. As a result, Actinomycetes must be considered as a unique treatment that requires further research in order to prevent the development of antibiotic-resistant microbes.

Keywords: Bioautography, Fourier Transmission Infra-Red Spectroscopy, Gas Chromatography-Mass Spectrum, Secondary metabolites, *Streptomyces rochei*.

INTRODUCTION

Identification of new antimicrobial substances from natural resources is a big challenge for researchers worldwide. Much of the research work has been accomplished to control infectious diseases. Generally, microbes produce secondary metabolites which are unnecessary for their growth and development but useful in defense mechanisms (Basilio *et al.,* 2003). Actinomycetes are an extensive group of gram-positive bacteria, and filamentous fungi it forms conidial spores so it is also termed ray fungi, commonly found in soil. DNA of actinomycetes contains 55 to 75 percent guanine and cytosine (Rugthaworn *et al*., 2007). The diversity of earthly actinomycetes is of remarkable significance in many areas of science and medicine, especially in pharmaceutical industries which produce antibiotics, vitamins, herbicides, pesticides, and enzymes (Magarvey *et al.,* 2004). The discovery of novel antibiotics from microbial sources, particularly in actinomycete, has yielded a magnificent number of molecules that have been employed in agriculture and human medicine during the past 50 years (Busti *et al.,* 2006). Half of all soil actinomycetes belong to the genus *Streptomyces*, which also generates secondary metabolites such as antibiotics, immunomodulators, antiviral drugs, and cancer treatments (Vining, 1990; Sanglier *et al*., 1993; Berdy, 1995). The genus *Streptomyces* is recognized as the predominant source for the identification of novel antibiotics and 80% of commercially available antibiotics are derived from the Streptomyces group of actinomycetes. Additionally, it generates tens of thousands of secondary metabolites, many of which have been defined in useful research for their bioactivity (Miyadoh, 1993). Actinomycetes are a source of about 10,000 bioactive chemicals, of which 25% are obtained from rare actinomycetes and 75% were synthesized by the *Streptomyces* species (Rong

and Huang 2012; Tiwari and Gupta 2012). There is always a need for new antibiotic discovery to counter pathogens and reduce disease. The discovery of novel antibiotics is constantly in demand to manage diseases caused by microorganisms. Actinomycete-producing bioactive compounds are classified based on their structural forms such as aminoglycosides, annamycin's, anthracyclines, β-lactam, macrolides, and amphotericin, tetracycline, neomycin, erythromycin, gentamycin, vancomycin, chloramphenicol, etc (Shimkets and Brun 2014). Forest soil is healthy and fertile, although 75% of biodiversity were living in forests.

Forest soil actinomycetes produce novel bioactive metabolites against bacteria, viruses, parasites, and many pathogens. This present study highlights the soil actinomycetes isolated from Bargur hills reported for the first time. *Streptomyces rochei* (BF3A) shows good antibacterial activity among 30 soil actinomycetes isolated. Chemical profiling of active fraction of metabolite by FTIR and GC-MS.

MATERIALS AND METHODS

A. Area of sample collections

The soil samples were collected from the Thamaraikarai and Solaganai regions of the Eratti Hills of the Bargur Reserve Forest (Bargur hills) in Tamil Nadu's north-eastern, erode district, roughly 70 kilometers from Erode. It's part of the Western Ghats range. It is surrounded by the villages of Thevarmalai, Bejilety, Oosimalai, Kuttaiyur, Onnakarai, Sundappur, Thamratti, Periyasengulam and Sinnasengulam as well as Koilnatham and Velampatti. Thamaraikarai beat has a total size of 3066.55 hectares, while Eratti hill has an area of 600 hectares. In terms of coordinates, the location is 11°45.963' N, 077°33.58' E, and an elevation of 1054 m above mean sea level. The average temperature of the hill is 25°C in the winter and 32°C in the summer, while the yearly precipitation of this hill ranges from 400 mm to 750 mm. Soil samples are taken at various locations in the Bargur reserve forest. Samples were taken at random from depths of 10 to 25 centimetres at each location. Sterile polytene bags are used for the collection and transportation of soil samples to the laboratory. Soil samples are dried at room temperature for five days to eliminate the bacterial load.

B. Isolation of actinomycetes

Dried soil samples were serially diluted, and plated on Actinomycete isolation Agar (AIA) (M-protein sodium salt- 2g, L-Asparagine-0.100, Sodium propionate- 4g, Dipotassium hydrogen phosphate -0.500g, Magnesium sulfate 0.100g, Ferrous sulfate 0.001g, Agar 15g per 1 L) and incubated at 28°C for 5-7 days (Thawai *et al.*, 2005). The isolates were stored and maintained in ISP2 (International *Streptomyces* project 2) medium which keeps the isolates pure (Shirling and Gottlieb 1966). The ISCC-NBS centroid color charts were used to determine the color of aerial and substrate mycelia (Kenneth and Judd, 1883). The primary antibacterial screening was done by cross-streaking method (Duddu and Guntuku 2016).

C. Bacterial strains

The following organisms were used for antibacterial studies such as *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 8739), *Klebsiella pneumoniae* (ATCC 9621), *Staphylococcus aureus* (ATCC 29737), *Bacillus coagulans* (MTCC 6735). The bacteria were grown for 12 hours at 37°C and diluted using the McFarland standard 0.5 scales.

D. Fermentation

Selected strains are inoculated into 100 ml of Yeast malt broth (International *Streptomyces* Project-2 (ISP-2) and incubated for 7-14 days at 37ºC at 80 rpm. Following incubation, the supernatant was filtered and treated with an equivalent volume of ethyl acetate (Usha and Masilamani 2013). After extraction, the solvents were concentrated using a rotary evaporator.

E. Antibacterial activity of extracts

(i) Agar well diffusion method. The antibacterial activity of crude extracts was tested by the agar well diffusion method (Rajesh *et al*., 2015) against the test bacterial strains. Muller Hinton agar was prepared and seeded with test bacterial culture, 8mm wells were bored aseptically and 100µl ethyl acetate extract was filled in the well. The plates were incubated for 24 hours at 37ºC. The test microbes were also checked for sensitivity against the standard antibiotic Amoxicillin, ethyl acetate was used for negative culture (Prashith Kekuda *et al*., 2012).

(ii) Determination of MIC (Minimum Inhibitory Concentration). MIC of BF3A extracts was tested against bacterial strains and was determined by micro broth dilution (Andrews, 2001; Solanki *et al*., 2008) or the double fold dilution method. The extract's lowest concentration produces no visible bacterial growth and was visualized by the color change of the medium after adding dye, and was compared with the control tube (Girao *et al*., 2019). Amoxicillin was used for the control.

F. Characterization of Active secondary metabolites

(i) Thin layer chromatography followed by bioautography assay. On the precoated TLC plate, the BF3A extract of ethyl acetate was spotted. The TLC plate was put vertically in a beaker containing a 6:4 mixture of hexane and ethyl acetate (Ranjan and Jadeja 2017) in the ratio of 6:4. TLC plates are placed in the iodine crystal chamber for a few minutes and the Rf values are calculated. The fractions were identified by observing the separated components under UV light at 254 nm. Bioautography was used to assess the antibacterial resistance of the isolated compounds. The developed chromatogram was placed on the sterile plate and overlayered the Muller Hinton agar seeded with *Staphylococcus aureus* (10^5cfu/ml) (Taddei *et al*., 2006; Selvameenal *et al*., 2009).

The bioactive fraction was evaluated using bioautography. The sample, as well as the silica gel, were scraped from the TLC plate. The scraped components were reextracted with the same solvent and centrifuged for 10 minutes at 8000 rpm. The pellets were removed, and the supernatant containing purified

bioactive compounds was dried and analysed by FTIR and GC-MS (Kumar *et al*., 2019).

(ii) Fourier Transmission Infrared (FT-IR) spectral analysis. The active fraction of the BF3A sample was analysed using FT-IR spectral in the Shimadzu FT-IR system in the spectrum range of 4000 to 500 cm^{-1}.

(iii) Gas chromatography-mass spectrum (GCMS) analysis. The analysis of active fraction determined the volatile constituents present in active fraction. GC-MS analysis was done in the Shimadzu TQ8040NX system at Chennai.

G. Molecular identification of potent strain

The potential bioactive compound producing *Streptomyces* BF3A was characterized by morphological, physiological, and molecular identification. The spore and filamentous identification by SEM imaging and physiological character detected various biochemical reactions (Shirling and Gottlieb 1966). Molecular identification was done by 16S rRNA gene sequencing, and Genomic DNA isolation was followed (Kumar *et al.,* 2012). Amplified DNA fragment was sequenced on ABI 3730xl Genetic Analyzer. The 16S rRNA gene sequence was performed by various bioinformatic tools such as BLAST and clustal W. MEGA X was used to create a distance matrix and phylogenetic tree. The 16S rRNA sequence was submitted to the GenBank database, NCBI, USA.

RESULTS

A. Isolation of Actinomycetes

In a study, it was highlighted the bargur reserved forest area is less explored, and discovering novel bioactive compounds producing unique actinomycetes are. This research, which includes a thorough literature review, is the first to discuss the isolation and identification of actinomycetes from the Bargur Reserved Forest. All the collected soils were pretreated in a hot air oven for ten minutes at 55°C. During the isolation, pre-treated soil exhibited less bacterial and fungal colonization. The samples were gathered during December. Collected soil samples are slightly wet and the soil pH is 7. Totally 30 morphologically different actinomycetes cultures were isolated using AIA as an isolation medium (Figure1). ISP2 medium was used to sustain these isolates. Colony morphology and cultural characters (Table 1) were noted for further study. The colony appearance, pigment, and growth rates of isolated actinomycetes are observed for identification purposes.

B. Preliminary antibacterial screening by Cross streak method

All 30 isolates were tested for the agar cross streak method against test bacteria. The suppression of bacterial growth around the actinomyces colony was used to select isolates for antibacterial screening (Fig. 2).

Fourteen culture was chosen for the secondary metabolite production based on the distance of inhibition between the actinomyces colony and the bacterial growth. The plate contains an MHA medium, and the reverse side of the plate image shows the inhibition of bacterial culture towards the actinomycetes colony margins.

Fig. 1. Pure strains of isolated soil actinomyces using an AIA medium.

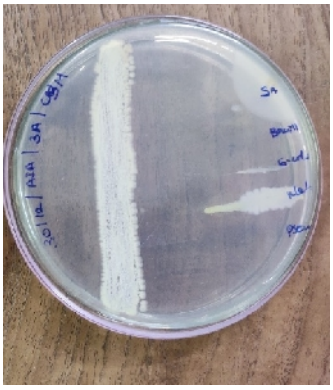


Fig. 2. Primary antibacterial screening by agar cross streak method.

C. Fermentation and extraction of secondary metabolites

Fourteen actinomycetes cultures were selected from primary antibacterial screening by agar cross streak method. These isolates are processed for the synthesis of secondary metabolite, and the ISP2 medium was used for the synthesis of bioactive metabolite. After the incubation period color change was observed in the BF3A strain and the medium was turned from yellow to reddish brown. The fermented medium was extracted with an equal volume of ethyl acetate.

D. Secondary antibacterial screening

Bacterial infections can cause high mortality in humans as well as aquatic animals. Actinomyces are the major group of antibiotic-producing microbe specifically *Streptomyces* sp. The secondary antibacterial screening was confirmed with ethyl acetate extract from isolates which was selected from the primary screening. Agar well diffusion and the minimum inhibitory concentration (MIC) technique were used for this secondary antibacterial screening. The antibacterial activity of the agar well diffusion method exhibited the sensitivity and resistance of the test bacteria. Fourteen isolates were subjected to secondary screening, 5 isolates were active against *Escherichia coli*, 5 isolates

were active against *Klebsiella pneumoniae*, 5 isolates were active against *Pseudomonas aeruginosa*, 7 isolates were active against *Bacillus coagulans*, 7 isolates active against *Staphylococcus aureus*. Based on the inhibition zone BF3A strain was the most antagonistic strain with a zone of inhibition ranging from 10-22 mm (millimetre) against all the test pathogens (Table 2). The ethyl acetate extracted culture supernatants were used for the MIC. The MIC was determined by micro broth dilution technique to reveal the bactericidal/bacteriostatic properties. The lowest concentration of MIC was recorded at 1.25 mg/ml against *Staphylococcus aureus*, 2.5 mg/ml against *Escherichia coli, Klebsiella pneumonia, Bacillus coagulans*, whereas and MIC of *Pseudomonas aeruginosa* is 5 mg/ml (Table 3). These results are comparable to the positive control Amoxicillin at the same concentration. MIC value 0.07 mg/ml against *Escherichia coli*, 0.03 mg/ml against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, 0.15 mg/ml against *Bacillus coagulans*, and *Staphylococcus aureus.* MIC of *Streptomyces rochei* showed the lowest concentration 1.25 mg/ml against the *Staphylococcus aureus*. And highest MIC was indicated at 5mg/ml against *Pseudomonas aeruginosa.*

E. Identification of potent strain BF3A

(i) Colony Morphology. Morphological identification was done by cultural characteristics and growth of BF3A in various mediums. Colony morphology showed color and texture of ariel and substrate mycelium, diffusible pigment was seen on the reverse side of the plate showing reddish-brown pigment.

(ii) Morphology identification by SEM (Scanning Electron Microscope). Microscopic observation in 100X shows the arrangements of spores like spiral arrangement. SEM showed oval-shaped and rough surface spores were seen under a scanning electron microscope (Fig. 3).

1. Gram staining image shows the spiral shape arrangement of the spore and the observation is under a light microscope (100X).
2. Scanning electron microscope photography shows the oval-shaped and rough surface spore.

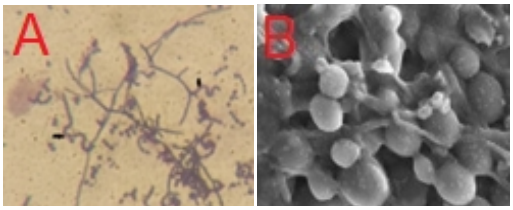


Fig. 3. Scanning electron microscopy photography of isolate BF3A (*Streptomyces rochei*).

F. Biochemical characteristics

The biochemical characteristics (Table 4) show the physiological characteristics of isolated BF3A. These characteristics are similar to the genus of *Streptomyces.*

(i) Species identification by 16S rRNA analysis. Based on the phenotypic character showed the strain BF3A was *Streptomyces* and the species level identification by 16S rRNA. A single distinct 1450 bp of PCR amplicon band was visible when the sample was resolved on an agarose gel. The partial 16S rRNA sequence of isolate BF3A containing 1450 base pair was determined. The sequence was confirmed with BLAST, NCBI tool. The evolutionary method was inferred using the UPGMA method (Sneath and Sokal 1987). The optimal tree is shown, with a branch length sum of 0.00405993. Branch distances are shown in the same units as the evolutionary lengths that were used to construct the phylogenetic tree, which is shown to scale. The evolutionary distances are measured by the Maximum Composite Likelihood method (Tamura, 2014) and are in base substitutions per site unit. There were 11 nucleotide sequences in this study. All ambiguous positions for each sequence pair were removed (pairwise deletion option). PCR amplification yields around 1450 base pairs. The phylogenetic tree represents the strain BF3A was a maximum likelihood and 100% similarity with *Streptomyces rochei* MW527431. MEGA X was used to undertake evolutionary analysis (Fig. 4) (Kumar, 2018). The potent isolate mentioned as *Streptomyces rochei* strain Genbank accession no. OM746935.

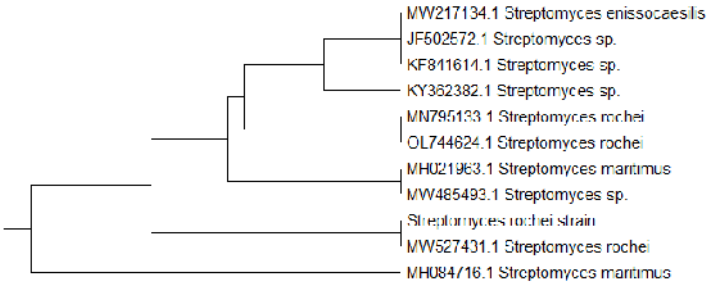


Fig. 4. Phylogenetic tree showing a relationship between closely related neighbors of *Streptomyces species* by UPGMA method.

(ii) TLC followed Bioautography and purification of bioactive compounds. The extract of BF3A was fractionated using thin layer chromatography showed four spots in the TLC sheet, to detect the active fraction of the extract and it was confirmed followed by bioautography. In this study, TLC showed 4 spots when the chromatogram was visualized under a UV lamp at 254 nm. Rf values of BF3A are A-0.94, B-0.86, C-0.6, and D-0.46, Fraction D showed a yellow pigmented spot under the UV lamp.

Bioautography of the extract of BF3A tested against *Staphylococcus aureus* (Fig. 5) confirmed the antibacterial activity. A distinct zone of inhibition is

visible in the inhibition of bacterial growth surrounding fraction D.

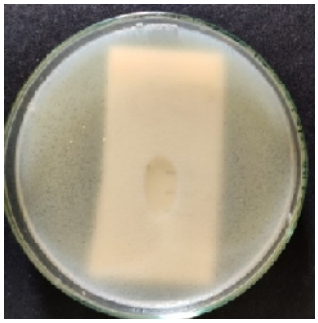


Fig. 5. Zone of inhibition coupled with TLC bioautography in fraction D against *Staphylococcus aureus*.

The active fraction was scraped off and employed for the characterization research after being once more extracted with ethyl acetate. TLC and bioautography of an active fraction are D, and the Rf value is 0.46. Furthermore, the active fraction was purified using chromatographic methods (Selvameenal *et al*., 2009; Oskay, 2004).

G. Characterization of the bioactive compound

(i) Fourier transmission infrared (FT-IR) spectrum analysis. The active fraction of BF3A was characterized by FT-IR spectroscopy. In the FTIR spectrum shown in (Fig. 6), the band 3870.87 cm^{-1} corresponds to alcohols, and the strong band at 3451.38 cm^{-1} corresponds to the N-H stretch of primary amine. The band at 2925.81 cm^{-1}, 2853.49 cm^{-1} correspond to the alkyl group, strong band at 1768.60 is the ketone group. 1633.59 is correspond to the carbonyl group, and the peaks at 1557.41 cm^{-1}, 1458.08 cm^{-1} is C-H bending for the CH_2 group. 1378.04 cm^{-1} corresponds to the C-N stretch of aromatic amine, strong band at 1243.04 cm^{-1}, 1162.03 cm^{-1} is may C-H group of aldehydes. The peak 1105.14 corresponds to P-O-C ester, the strong peak at 1051.13 cm^{-1} has corresponded to aliphatic compounds, and the band 678.90 cm^{-1}, 563.18 cm^{-1} is corresponding to aromatic compounds. These FTIR peaks showed the functional group of the active compounds. FTIR characterization of active groups such as alcohols, amines, ketones, aromatic amines, aldehydes, esters, and aromatic compounds. Peak values show the functional group of the metabolites.

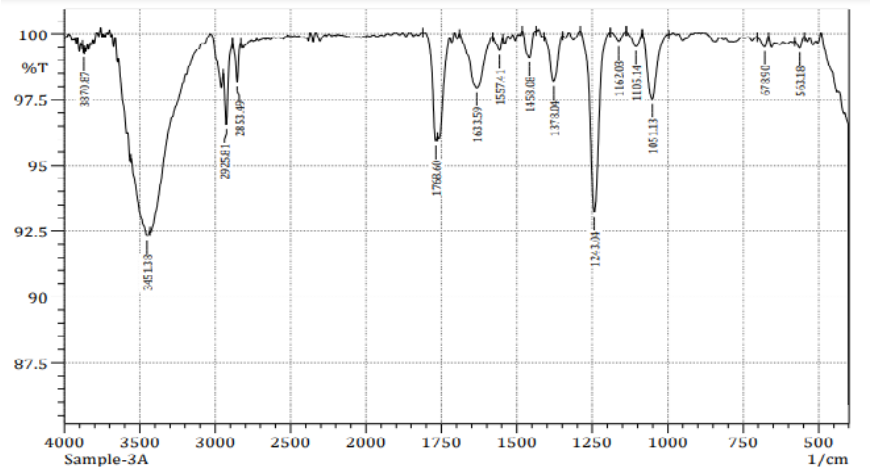
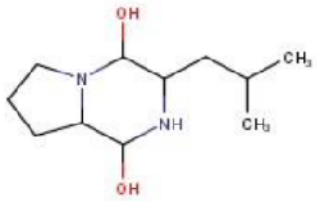
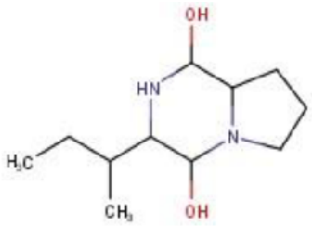


Fig. 6. FTIR analysis of partially purified ethyl acetate extract of BF3A.

(ii) GC-MS analysis. The chromatogram and the signals of retention time are shown in (Fig. 7), and the compound present in the active metabolite (Table 5). The BF3A active fraction showed 30 peaks and contain 75 compounds. The 17 compounds are predominantly presented such as valproic acid, 2,4-Di-tert-butyl phenol, (3S,8aS)-3-Isopropylhexahydropyrrolo[1,2-a] pyrazine-1,4-dione, Hexahydro-3-(1-methyl propyl) pyrrolo[1,2-a]pyrazine-1,4-dione, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methyl propyl), 3,6-Diisopropylpiperazin-2,5-dione, 1,2-Benzenedicarboxylic acid, bis(2-methyl propyl) ester, Phthalic acid, hex-3-yl isobutyl ester, Di-sec-butyl phthalate, 5-Nitroso-2,4,6-triamine pyrimidine, Dibutyl phthalate, Tridecane, 7-cyclohexyl, Cyclopropane carboxylic acid, 1-hydroxy-, (2,6-di-t-butyl-4-methyl phenyl) ester, Ethanone, 1-(5,6,7,8-tetrahydro-2,8,8-trimethyl-4H-cyclohepta[b]furan-5-yl), 2,5-di-tert-Butyl-1,4-benzoquinone, Nonadecyl trifluoroacetate, 3-Benzylhexahydropyrrolo[1,2-a] pyrazine-1,4-dione, Ac derivative, These compounds can control pathogens, some compounds are reported for antibacterial potential. Major compounds were, Hexahydro-3-(1-methylpropyl) pyrrolo[1,2-a] pyrazine-1,4-dione, Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), (3S,8aS)-3-Isopropylhexahydropyrrolo[1,2-a] pyrazine-1,4-dione). These compounds contain similar derivatives and are slightly different in their structure. Di-sec-butyl phthalate and Di-butyl phthalate both are derivatives of the phthalate family. These compounds are produced by various species of *streptomyces* group of actinomycetes. The compound 3-Benzylhexahydropyrrolo[1,2-a] pyrazine-1,4-dione, an Ac derivative also contains the pyrrole group. Many antibacterial compounds have been previously reported in *Streptomyces* sp. BF3A strain produces various antibacterial compounds such as 2,4-Di-tert-butyl phenol, Dibutyl phthalate, and 5-Nitroso-2,4,6-thiamine pyrimidine.

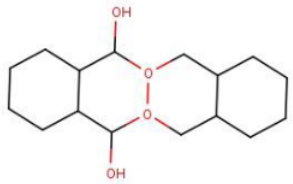


a. Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methyl propyl)

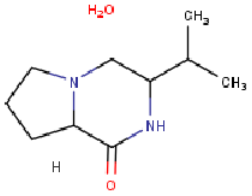


b. Hexahydro-3-(1-methyl propyl) pyrrolo[1,2-a] pyrazine-1,4-dione

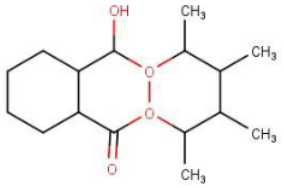
c. 3-Benzylhexahydropyrrolo[1,2-a] pyrazine-1,4-dione



d. Di-butyl phthalate



e. (3S,8aS)-3-Isopropylhexahydropyrrolo [1,2-a] pyrazine-1,4-dione)



f. Di-sec-butyl phthalate

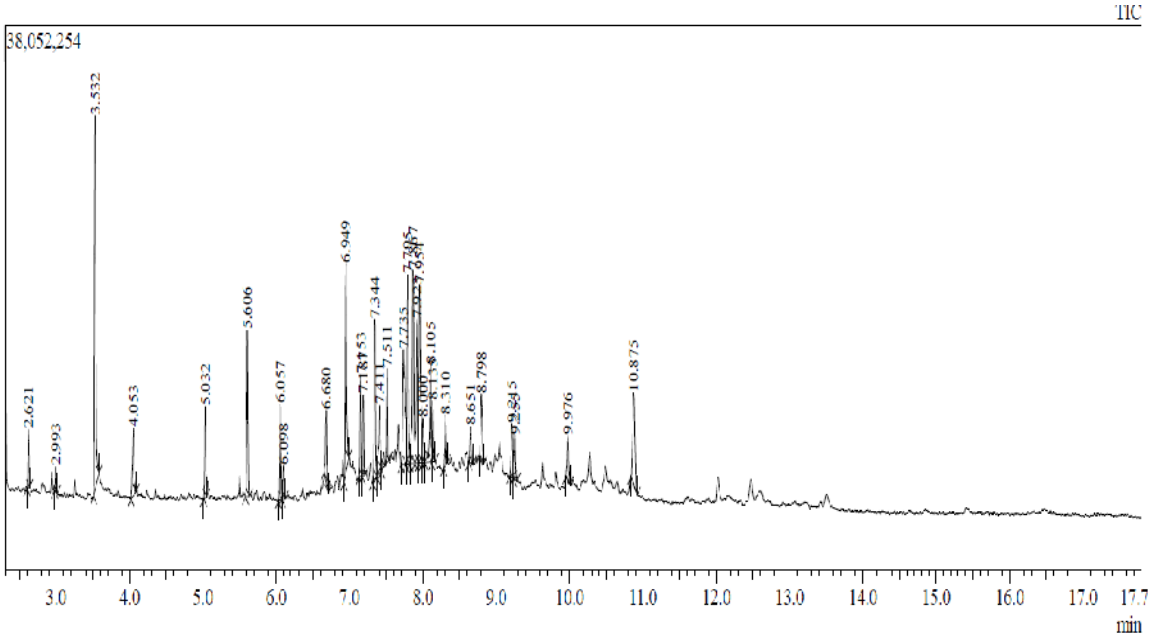


Fig. 7. The GC spectrum of active fraction from BF3A showing 30 peaks.

Table 1: Colony morphology, pigmentation, and growth of isolated actinomycetes from bargur forest soil sample.

Sr. No.	Stain Number	Colony morphology	Pigment	Growth
1.	BF1	Off-white, dry, powdery colonies, slightly white to light green, Irregular colony.	Slight green	+++
2.	BF2	Off-white, irregular, slightly cream	-	++
3.	BF3A	White to light brownish grey colonies, irregular, opaque.	Reddish brown	+++
4.	BF4	Dry, off-white, dark cream dots at centre, regular, smooth.	Brown	+
5.	BF5	Bright white, creamy, smooth colony, initial growth as bacteria, later incubation produces white dry spores.	Light brown	+++
6.	BF6	The white creamy, waxy surface	Black	++
7.	BF7	Off-white, dotted, non-branching.	-	++
8.	BF8	Bright white, dotted colony. Layered like growth	Greenish brown	+++
9.	BF9	Light brown color colony, later incubation white dry spores	-	+
10.	BF10	Dry, rough, white colonies, creamy to greenish,	Greenish brown	+++
11.	BF11	White, dotted, creamy surface	-	+
12.	BF12	Off-white, dry, powdery	-	+++
13.	BF13	Greyish white, branching, cottony surface, rough	-	+++
14.	BF14	White creamy surface	-	+
15.	BF15	Greyish white, branching, cottony surface, rough	-	+++
16.	BF16	White, dry, powdery colonies.	-	+
17.	BF17	Off-white, irregular, non-branching.	-	++
18.	BF18	White to cream, dry powdery colonies.	-	+++
19.	BF19	Dry, dark cream, smooth.	Pale Yellowish	++
20.	BF20	Off-white, creamy colony.	-	+
21.	BF21	Cream color, smooth powdery colony.	-	+++
22.	BF22	Greyish white, cream, irregular, creamy.	-	++
23.	BF23	White to Gray, dotted colony, rough, irregular colonies	Blue	-
24.	BF24	Dull white, dry, dotted colonies.	-	++
25.	BF25	Off-white, irregular, creamy, and waxy surface.	-	+
26.	BF26	White, dotted, creamy surface.	-	+++
27.	BF27	Off-white, dry, and powdery.	-	++
28.	BF28	White, Dry powdery colony.	-	+
29.	BF29	White creamy surface, Greyish white, branching, cottony surface, rough.	-	+++
30.	BF30	Off-white, regular, creamy, and waxy surface, smooth.	-	+

Table 2: Secondary antibacterial screening of ethyl acetate extract of BF3A by agar well diffusion method.

Strain No.	*Escherichia coli (mm)*	*Klebsiella pneumoniae (mm)*	*Pseudomonas aeruginosa (mm)*	*Bacillus coagulans (mm)*	*Staphylococcus aureus (mm)*	*Amoxicillin* (mm)
BF2	0	0	0	12.67±0.58	12.0±0	21.33±0.58
BF3A	17.67±0.58	12.67±0.58	20.33±0.58	16.67±0.58	22.33±0.58	22.0±0
BF6	0	0	0	13.67±0.58	12.0±0	20.0±0
BF9	10.33±0.58	10.0±0	11.33±0.58	0	0	21.33±0.58
BF10	0	0	0	11.67±0.58	13.33±0.58	21.0±033
BF17	11.0±0	10.0±0	11.33±0.58	0	0	21.33±0.58
BF21	0	0	0	11.67±0.58	11.33±0.58	20±0
BF22	10.0±0	11.3±0.58	12.0±0	0	0	21.33±0.58
BF23	0	0	0	12.0±0	10.0±0	22.0±0
BF26	0	0	0	10.0±0	12.0±0	20±0
BF29	12.0±0	11.0±33	10.0±.058	0	0	21.33±0.58

BF3A is active against all the test bacteria, BF2, BF6, BF10, BF21, BF23, and BF26 culture extracts do not inhibit the growth of Gram-negative bacteria. BF9, BF17, BF22, and BF29 culture extracts do not inhibit the gram-positive bacteria. Amoxicillin was used for the positive control.

Table 3: The minimum inhibitory concentration of BF3A shows s lower concentration in *Staphylococcus aureus.*

Sr. No.	Bacterial Culture	Mic values 10mg/ml	BF3A Extract
		Amoxicillin	
1.	*Escherichia coli*	0.07	2.5
2.	*Klebsiella pneumoniae*	0.03	2.5
3.	*Pseudomonas aeruginosa*	0,03	5
4.	*Bacillus coagulans*	0.15	2.5
5.	*Staphylococcus aureus*	0.15	1.25

Table 4: The Phenotypic character of potent strain BF3A.

Sr. No.	Characteristic	Selected isolated BF3A
1.	Spore arrangement	Spiral
2.	Culture consistency	Powdery
3.	Arial mycelium	Brownish Gray color
4.	Substrate mycelium	Cream
5.	Reverse side pigment	Reddish brown
6.	ISP-2	Good
7.	ISP-3	Moderate
8.	ISP4	Moderate
9.	ISP7	Good
10.	Oxidase	+ve
11.	Catalase	+ve
12.	Indole	-ve
13.	Methyl Red	+ve
14.	Voges- proskauer's test	-ve
15.	Citrate	+ve
16.	Nitrate reduction	-ve
17.	Oxidase	+ve
18.	Catalase	+ve
19.	Urease	+ve
20.	Sugar Utilization	Glucose, Sucrose, Maltose.

(+ve denotes Positive, -ve denotes Negative)

Table 5: Major chemical constituents of active fraction in strain BF3A extract analyzed by GC-MS.

Sr. No.	R. time	Area (%)	M.W	Name of the compound	M.F
1.	3.532	11.26	144	Valproic Acid	$C_8H_{16}O_2$
2.	5.605	4.13	206	2,4-Di-tert-butyl phenol	$C_{14}H_{22}O$
3.	7.345	12.58	196	(3S,8aS)-3-Isopropylhexahydropyrrolo[1,2-a] pyrazine-1,4-dione (or) Cyclo (L-prolyl-L-valine)	$C_{10}H_{16}N_2O_2$
4.	7.345	18.23	210	Hexahydro-3-(1-methyl propyl)pyrrolo[1,2-a]pyrazine-1,4-dione	$C_{11}H_{18}N_2O_2$
5.	7.345	12.58	210	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methyl propyl)	$C_{11}H_{18}N_2O_2$
6.	7.41	1.25	198	3,6-Diisopropylpiperazin-2,5-dione	$C_{101}H_{18}N_2O_2$
7.	7.51	2.87	278	1,2-Benzenedicarboxylic acid, bis(2-methyl propyl) ester	$C_{16}H_{22}O_4$
8.	7.51	2.87	306	Phthalic acid, hex-3-yl isobutyl ester	$C_{18}H_{26}O_4$
9.	7.51	9.68	278	Di-sec-butyl phthalate	$C_{16}H_{22}O_4$
10.	7.867	6.51	154	5-Nitroso-2,4,6-triaminopyrimidine	$C_4H_6N_6O$
11.	7.955	6.81	278	Dibutyl phthalate	$C_{16}H_{22}O_4$
12.	8.00	1.29	266	Tridecane, 7-cyclohexyl	$C_{19}H_{38}$
13.	8.31	1.33	304	Cyclopropane carboxylic acid, 1-hydroxy-, (2,6-di-t-butyl-4-methyl phenyl) ester	$C_{19}H_{28}O_3$
14.	8.31	1.33	220	Ethanone, 1-(5,6,7,8-tetrahydro-2,8,8-trimethyl-4H-cyclohepta[b]furan-5-yl)	$C_{14}H_{20}O_2$
15.	8.31	1.33	220	2,5-di-tert-Butyl-1,4-benzoquinone	$C_{14}H_{20}O_2$
16.	9.215	1.6	380	Nonadecyl trifluoroacetate	$C_{21}H_{39}F_3O_2$
17.	10.875	4.35	286	3-Benzylhexahydropyrrolo[1,2-a] pyrazine-1,4-dione, Ac derivative	$C_{16}H_{18}N_2O_3$

DISCUSSION

Bacterial infections have significantly increased in the past decades due to the development of drug resistance to pathogens. The development of a new class of antimicrobial drugs is the need for society with minimal side effects (Gozalbo *et al.*, 2004). The *Streptomyces* strains produce many potential antimicrobial compounds. The worldwide surveys have that soil ecosystems are rich in microbial diversity, especially the mangrove ecosystem (Sengupta *et al*., 2015) reported biologically active actinobacteria were isolated from mangroves. Review articles have reported, *the Streptomyces* group of actinomycetes produced antibiotics (Anderson and Wellington 2001; De Lima *et al.*, 2012). For example, natamycin is a polyene macrolide antibiotic that was produced in submerged fermentation of *Streptomyces natalensis*, *Streptomyces chattanoogensis*, and *Streptomyces lydicus* (De Lima *et al.*, 2012).

In a study, it was highlighted the bargur reserved forest area is less explored, and discovering novel bioactive compounds producing unique actinomycetes are. A substantial literature survey is the first report regarding the isolation and identification of *Streptomyces rochei* from the Bargur reserved forest. All the collected soil samples were pretreated in a hot air oven for ten minutes at 55°C. During the isolation, pre-treated soil exhibited less bacterial and fungal colonization. Totally 30 morphologically different actinomycetes were isolated from soil samples using an AIA medium. Chaudhary *et al*. (2013) reported 31 isolates of actinomycetes from soil samples of different niche habitats of Sheopur district, Madhya Pradesh, India.

Isolation was done by using the AIA medium. Isolated actinomycetes colony morphology, pigmentation, and growth rates are noted for identification purposes. Karuppaiah *et al.* (2013), describe the extraction of red pigment from coral reefs as showing good antibacterial and cytotoxicity effects against various cell lines. Fourteen isolates were selected from primary antibacterial screening by agar cross streak method. (Oskay, 2011), reported *Streptomyces* sp KGG32 strain had different antimicrobial activity against the test organisms. The primary antimicrobial screening assay indicates the KGG32 stain was the most antagonistic *Streptomyces* zone of inhibition ranging from 12-34 mm. ISP2 medium was used for the fermentation medium, and after the incubation period, notable changes occurred. The fermented medium turned from yellow to reddish brown. Selected isolate BF3A produced reddish brown pigment. Kumaran *et al*. (2020) describe *Streptomyces enissocaesilis* produce brown pigment, and extracted pigments were active against fish pathogens. The fermented medium was extracted with ethyl acetate (Parthasarathi *et al*., 2012) reported a maximum yield of bioactive compounds extracted from ethyl acetate (Taechowisan *et al*., 2005; Ilic *et al*., 2005; Thangadurai *et al*., 2004). Agar well diffusion of BF3A shows the zone inhibition ranges from 12-22mm, the maximum zone of inhibition was exhibited by *Staphylococcus aureus.* From (Table 2) gram-negative bacteria are more resistant compared with gram-positive bacteria because the gram-negative bacterium contains lipopolysaccharide in the outer membrane it's made up of a lipid bilayer that acts as a protective barrier, whereas gram-positive bacterium denies lipopolysaccharide in the outer membrane so become sensitive to the active metabolites (Reygaert, 2018). Eber *et al.* (2011) reported the seasonal and temperature-associated increases in gram-negative bacterial bloodstream infections. Oskay *et al*. (2004) reported 50 actinomycetes were isolated from farming soil and screened for antibacterial activity. Results indicate 34% of isolates are active against test pathogens. MIC of *Streptomyces rochei* showed the lowest concentration 1.25 mg/ml against the *Staphylococcus aureus*. And highest MIC was indicated at 5mg/ml against *Pseudomonas aeruginosa.* (Chaudhary *et al.*, 2013) isolated 31 actinomycetes, strain AS7 showed maximum antagonistic activity. The MIC of AS7 was found to be 2.5 mg/ml against *Shigella dysenteriae,* and *Klebsiella pneumoniae*, Vancomycin-resistant *enterococci*, and was 1.25 mg/ml for *Streptococcus pyogenes*, *Staphylococcus saprophyticus*, *Staphylococcus epidermidis*, Methicillin-resistant *Staphylococcus*, *Bacillus cereus*, *Staphylococcus xylosus*, Methicillin-resistant *Staphylococcus aureus*, *Enterococcus faecalis*, and *Staphylococcus aureus*. Radhakrishnan *et al.* (2016) reported in *Streptomyces rochei* and their MIC values range from 7.9-31.2 mg/ml against the multi-drug resistant bacteria and standard bacterial pathogens. Kumar *et al.*, (2019) reported MIC by microdilution method, strain 196 shows inhibition started at 0.0075 mg/ml against *bacillus cereus*, and also positive control of chloramphenicol showed inhibition at 0.0075 mg/ml against the *Bacillus cereus*.

Morphologically *Streptomyces rochei* produced brownish grey colorariel mycelium are a spiral arrangement of spores. Scanning electron microscopic observation showed an oval shape, and rough surface spore. Radhakrishnan *et al.* (2016) reported spores appeared as oval-shaped, smooth surfaced 40–50 numbers of spore arrangement. Based on the phenotypic character showed the strain BF3A was *Streptomyces* and the species level identification by 16S rRNA. PCR amplification of the 16S rRNA gene yield around 1450 base pairs. The Phylogenetic tree represents the strain BF3A was a maximum likelihood and 100% similarity with *Streptomyces rochei* MW527431. TLC and bioautography of active fraction D, and the Rf value is 0.46. (Kumar *et al.,* 2019) reported single active fraction was obtained in *Streptomyces* 196 strain with a Rf value of 0.46 it is similar to the BF3A strain, bioactive compound was identified in bioautography. Furthermore, the active fraction was purified using chromatographic methods (Selvameenal *et al.,* 2006). FTIR characterization of bioactive compounds contains functional groups such as alcohols, amines, ketones, aromatic amines, aldehydes, esters, and aromatic compounds. Peak values show the functional group of the metabolites. The peaks and functional groups in FT-IR were reported it is similar to the study (Kumaran *et al*., 2020; Radhakrishnan *et al*., 2016; Kiran *et al*., 2018). GC-MS analysis showed 17 major compounds which may responsible for the antimicrobial nature. Research workers have reported the antimicrobial property of the compounds. Major compounds are Valproic acid, Hexahydro-3-(1-methyl propyl) pyrrolo[1,2-a] pyrazine-1,4-dione, Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methyl propyl) (Kiran *et al.*, 2018) identified the compound from marine bacteria *Bacillus tequilensis* MSI45, and it is active against multi-drug resistant *Staphylococcus aureus* as well as non-hemolytic and showed antioxidant activity. Kumaran *et al.* (2020) reported Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2- methylpropyl) was identified from marine *Streptomyces enissocaesilis.* (3S,8aS)-3-Isopropylhexahydropyrrolo [1,2-a] pyrazine-1,4-dione, Yang *et al.* (2002) reported the antifungal activity of cyclo (Pro-Val) isolated from *Halobacillus litoralis* YS3106. Di-sec-butyl phthalate and Di butyl phthalate Driche *et al.* (2015) are chemically defined as DBP ester functionally antimetabolite of proline. Roy *et al.* (2006) biosynthesis of DBP from *Streptomyces albidoflavus* and reported for antibacterial metabolite, a glucosidase inhibitor (Lee, 2000). Chawawisit *et al.* (2015) reported 2,4-Di-tert-butyl phenol isolated from *Streptomyces* sp KB active against test pathogens (Varsha *et al.*, 2015). The compound 5-Nitroso-2,4,6-triamine pyrimidine (Khattab *et al*., 2016) was reported as an antibacterial compound. Pyrrolizidine compound is widely produced by several marine *Streptomyces* (Olano *et al.*, 2009; Robertson and Stevens 2014). Pyrrolizidine exhibits a wide range of bioactivities such as antibacterial (Kiran *et al*., 2018), and antioxidant activities (Mohan *et al*., 2014), Pyrrolo is a cyclo derivative, most antimalarial drugs are found in cyclo-

derivatives form (Houl *et al*., 2009; Seebacher *et al*., 2004). However, the BF3A strain produces Tridecane, 7 cyclohexyls it is the form of a weak base, and cyclohexyl is an analogue of the antimalarial drug (Dong *et al.*, 2010). A similar result was observed in another research carried out by Djebbah *et al.* (2022) where they found 23 constituent compounds responsible for the bioactive activity. The GC-MS result showed 17 different compounds these compounds may responsible for the antibacterial activity.

CONCLUSION

Screening of actinomycetes from Bargur forest yielded 30 actinomycetes from 10 different regions, 6th region of the soil sample exhibited *Streptomyces rochei* as potent antibacterial activity against all the tested organisms. Bioactive metabolites were separated by TLC and followed by bioautography. The compounds were characterized by FTIR and GC-MS. The major compound was identified as Hexahydro-3-(1-methyl propyl) pyrrolo [1,2-a] pyrazine-1,4-dione. This compound may responsible for the inhibition of bacterial growth with a lower concentration of 1.25 mg/ml. The purification and structural prediction of compounds will be studied in the future.

FUTURE SOPE

The obtained results are believed to have fulfilled the objective and scope of the study. As a result, there are still numerous chances to find new compounds that are strong antimicrobials. The present study's future aims include developing an in silico docking approach and optimizing microbial target sites for the antibacterial compound.

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