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Antibacterial and anti-HIV activity of extracellular pigment from *Streptomyces* sp. S45 isolated from Sabarimala forest soil, India

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Actinobacteria, particularly from under-studied habitats, are often endowed with diverse biological properties. In the present study, about 17 actinobacterial strains were isolated from Sabarimala forest — an understudied ecosystem in Western Ghats, India and screened for their pigment producing potential. Pinkish brown soluble pigment producing *Streptomyces* sp. strain S45 was selected and screened for antibacterial and anti-HIV activity. The bioactivity of ethyl acetate extract of the strain S45 showed maximum zone of inhibition against *Staphylococcus aureus* ATCC 29213 (17.3±0.4 mm) and *Bacillus cereus* (15.6±0.6 mm). Also, it showed anti-HIV activity with the IC50 value of 8.75 µg/mL. The bioactive pigment isolated from the strain S45 was partially purified and characterized using UV absorption. In bio-autography, an antibacterial compound found to be active against *S. aureus* ATCC 29213 and its MIC values ranged between 25-1.56 µg/mL. Variables such as glucose, rhamnose, soybean meal and $CaCl_2$, pH 7 and temperature 30°C were found to influence bioactive pigment production. Potential strain S45 was identified as *Streptomyces* species on the basis of microscopic, cultural, physiological and 16SrRNA analysis. Results suggest that the *Streptomyces* sp. S45 strain explored in this study could be a promising candidate for isolation of antibacterial and anti HIV pigment.

Keywords: Actinobacteria, Antimicrobial, Forest ecosystem, Partial purification, Western Ghats

Infectious diseases are one of the serious problems for human beings causing numerous diseases by lot of microorganisms. In that most of the top antimicrobial resistant (AMR) threats are from bacterial pathogens[1]. Hence, there is deep scope for the screening of novel and effective drugs to combat multi drug resistant pathogens[2]. Actinobacteria is known as a group of microorganisms that are most responsible for the production of crucial bioactive compounds; particularly antibiotics become very important[3]. However, the bioactive compounds produced by actinobacteria especially *Streptomyces* sp. are not limited against bacteria, but also effective as anti-fungal, anti-cancer, and some of them are as anti-virus. *Streptomyces* is also known that one of the largest numbers of bioactive metabolites producing genus in the microbial world and the mangrove associated *Streptomyces* are capable of producing bioactive metabolites with a wide range of activities including antibacterial, antifungal, and anti-HIV[4,5]. Until now, the search of actinobacteria producing new types of bioactive compounds continues. Due to the emergence of multi-resistant microorganisms to almost all available antibiotics, nowadays many researchers are focused on discovering novel antimicrobials from many natural resources produced by actinobacteria especially those isolated from many unexplored environments. Moreover the production of melanin like pigments in tyrosine medium by actinobacteria can enhance the antibiotic synthesis, UV protection, oxidants, enzymatic lysis, killing by alveolar macrophages and chelating metal ions[6].

In this perspective, we studied the multi-potential of *Streptomyces* strain S45 from the forest soil samples of Sabarimalai, Kerala, India against various Gram positive and Gram negative bacterial pathogens as well as screened against HIV-1 virus.

Materials and Methods

Sample collection and pre-treatment

Soil sample was collected from the Sabarimalai forest of Kerala (Lat. 09°25′59″N; Long. 77°04′59″E), India and kept for drying at room temperature (30ºC)

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for two days. Ten gram of soil sample was pre-treated by dry heat in hot air oven at 55°C for 10 min in order to retard the growth of fast growing bacteria and fungi, other than actinobacteria.

Isolation and characterization of actinobacteria

The pre-treated soil sample was serially diluted up to 10^5 dilutions using sterile distilled water blanks. About 100 µL of aliquot from 10^3 to 10^5 dilutions was taken and plated on Starch Casein Agar (SCA) and Kusters Agar (KUA) supplemented with nalidixic acid 50 µg/mL and nystatin 20 µg/mL. Plating was done in triplicate and incubated at 28ºC for one month. Microscopic characteristics such as the presence of aerial mycelium and substrate mycelium were observed by slide culture technique under the bright field microscope at 40X magnification. A cultural characteristic such as growth, colony consistency, colour of aerial mycelium, pigment production was recorded by growing the culture on ISP2 (International Streptomyces Project 2) agar plates at 28ºC for 7-14 days.

Actinobacterial colonies were selected for further studies based on their morphological differences. Purity of all the strains was confirmed by sub-culturing on ISP2 agar plates. All the strains were preserved as slant culture on ISP2 agar slants as well as on 20% glycerol broth.

Preliminary screening for antibacterial activity

Antibacterial activity of actinobacterial strains were tested by adopting agar plug method[7] against *Staphylococcus aureus*-ATCC 29213, *Bacillus cereus*, *Escherichia coli*-ATCC 25922, *Pseudomonas aeruginosa*-ATCC 27853 and *Klebsiella pneumonia*-ATCC 13882. The bacterial strains were grown in nutrient agar medium at 37°C for 18 h. Actinobacterial strains were grown on ISP2 agar plates for 10 days at 28°C. Bacterial pathogens were spread on LB modified agar plate using sterile cotton swab. Agar plug with 5 mm diameter which contains the secreted actinobacterial metabolites were cut from the ISP2 agar medium and placed over LB modified agar plates seeded with test pathogens. All the plates were incubated at 37ºC for 24 h. Zone of inhibition was measured after incubation and expressed in millimetre in diameter[8].

Pigment production from potential strain

Strain S45, an extracellular pigment producing actinobacterial strain, which showed promising antibacterial activity in the preliminary screening was selected as potential strain for further studies. Extracellular pigment production from strain S45 was carried out by both submerged and solid state fermentation using ISP2 medium.

Spores of strain S45 was transferred into each 5 plates (90 mm) of ISP2 agar as well as in each 100 mL of ISP2 broth. The agar plates were incubated at 28°C for 14 days where as the ISP2 broth cultures were kept in rotary shaker with 95 rpm for 14 days at 28°C. The solid and liquid medium was observed regularly for pigment production. For every 24 h, agar plug from ISP2 agar plates and cell free supernatant from ISP2 broth was tested against *S. aureus*-ATCC 29213 by agar plug method and well diffusion method, respectively[9].

Bioactive pigment from actinobacterial strain S45 was extracted from ISP2 agar by solid liquid extraction method using different organic solvents. All the extracts were tested against *S. aureus*-ATCC 29213 by disc diffusion method at 100 µg/disc concentration.

***In vitro* screening for anti-HIV activity**

The anti-HIV activity of ethyl acetate extract was studied against HIV-1 virus. The infectious cells (1×10^4 cells/well) was transferred into the 96-plate well added with 70 µL DMEM (Dulbecco's Modified Eagle) media and incubated in CO_2 incubator at 37°C for 24 h. The working concentrations of the ethyl acetate extract were made from 100 µg/mL to 0.00001 µg/mL. Each 10 µL of extract and virus were added and incubated at 37°C in CO_2 incubator. After three hours of incubation 10 µL of DEAE media was added into each well and incubated for 48 h at 37°C in CO_2 incubator. After incubation, the media was removed and the cells were washed using 1x PBS. Then the cells were lysed with 50 µL of 1X passive lysis buffer (Promega) for 15 min at the room temperature. Then the plate was centrifuged at 2000 rpm for 3 min. Later, 20 µL of the supernatant was transferred to a White Opaque 96-well plates with the 20 µL Luciferase Bright glo™ substrate and measured the relative light unit. The results were analyzed for EC_{50} values using graph pad prism[10].

Partial purification and characterization of active compounds

The crude bioactive pigment from strain S45 was separated by analytical thin-layer chromatography (TLC) (pore size 60Å, mesh size: 230-400, particle size 40 63 µm, Merck) using organic solvents in different ratio.The separated components were visualized under UV light at 254 and 365 nm. The

antibacterial activity of compounds separated on TLC plate was detected by direct bioautography[11]. The developed TLC plates were overlaid with nutrient agar medium supplemented with 0.1% (w/v) 2,3,5-triphenyltetrazolium chloride (tetrazolium red) and test pathogen at a final concentration of 10^7 CFU/mL. The plates were incubated at 37°C for 24 h. Clear zone of inhibition indicated the position of antibacterial compounds on the TLC plate, and the retention factor (Rf) value was calculated. The active fraction was separated from the crude extract by adopting preparative TLC using optimized solvent system. The UV spectrum of purified pigment was recorded at 200-800 nm wave length using Perkin Elmer Lambda-25 UV spectrophotometer. The antibacterial pigment was partially characterized by spraying with chemical reagents such as 10% KOH ethanolic reagent, Millon's reagent, vanillin-HCl reagent, ninhydrin reagents, iodine vapors, 50% ethanolic H_2SO_4 and Dragendorf reagent[11].

Determination of Minimum Inhibitory Concentration (MIC)

MIC of partially purified ethyl acetate extract of strain S45 was tested by adopting micro-broth dilution method concentration ranging from 1.56-250 µg/mL. The bacterial pathogen *S. aureus*-ATCC 29213 was grown up to a final concentration of 1×10^4 CFU/mL. Different concentrations of the partially purified fraction were added into 96-well microtiter plate containing a bacterial culture as test and without bacterial culture as controls. Ampicillin (10 µg/mL) was added in to a separate well along with bacterial culture to serve where as the bacterial culture added with DMSO (10%) was served as negative control. The plates were incubated at 37°C for 36 h and absorbance was taken at 620 nm[12].

Characterization and taxonomy of potential strain S45

Micromorphology of strain S45 was studied under bright field microscope. A cultural characteristic, physiological characteristics such as carbon, nitrogen and minerals utilization and enzyme production was studied by adopting the standard method[13].

Genomic DNA extraction was performed from the strain S45 using GENEI bacterial DNA purification kit. The PCR amplification of 16S rRNA gene was performed by using primers 27F 5′-AGAGTT TGATCMTGGCTCAG-3′ (forward) and 1492R 5′-TACGGYTACCTTGTTACGACTT-3′ (reverse)[14]. The 16S rRNA gene sequence was multiply aligned with selected sequences obtained from the GenBank using the MEGA6 program. The alignment was constructed to phylogenetic tree using neighbor joining[15] in MEGA 6 software. The confidence values for the branches of the phylogenetic tree were determined using bootstrap analyses[16] based on 1000 resembling of the neighbour joining data set. The partial 16S rRNA nucleotide sequence of the potential actinobacterial strain S45 was deposited to GenBank database.

Statistical analysis

The antimicrobial activity was performed in triplicate process and repeated thrice. Readings were taken as the mean ± standard deviation of mean of three replicates calculated using Microsoft Excel XP 2010. One way ANOVA was employed to test the significant differences ($P \leq 0.05$) between antimicrobial activities of different isolates using Agras Agdata.

Results

Isolation and characterization of actinobacterial strains

Seventeen morphologically different actinobacterial strains were isolated from Sabarimalai forest soil, Kerala, India (Table 1). On ISP2 agar medium, about 10 actinobacterial strains were produced powdery growth whereas the remaining seven cultures produced leathery growth. All the cultures showed the presence of aerial and substrate mycelium under bright field microscopic observation.

Preliminary screening for antibacterial activity

In agar plug method, 11 out of 17 actinobacterial strains were found to be active against minimum one of the five bacterial pathogens tested. Notably, strain S45, which produced extracellular pigment (Fig. 1A), was found to be active against both Gram positive and Gram negative bacterial pathogens tested. However, maximum inhibition was observed against Gram positive bacteria such as *S. aureus*-ATCC 29213 (17.3±0.4) and *B. cereus* (15.6±0.6) than Gram negative bacteria. Hence, the strain S45 was selected as potential strain for further studies.

Pigment production from strain S45

Strain S45 showed good growth on both YEME agar as well as in YEME broth medium. Strain S45 showed antibacterial activity against *S. aureus*-ATCC 29213 in agar plug method on 4th day of incubation whereas its cells free supernatant was showed activity against *S. aureus* on 6th day of fermentation. Hence further pigment production from strain S45 was produced by agar surface fermentation (Figs 1B and 2).

Table 1 — Details of actinobacterial cultures used in this study

Strain No	Cultural characteristics						
	Growth	Consistency	AMC	RSP	SP	Aerial mycelium	Substrate mycelium
S33	Good	Powdery	Greyish white	Black	+	+	+
S45	Good	Powdery	White	Pink	+	+	+
S58	Good	Powdery	Rough	Pale brown	-	+	+
S47	Good	Powdery	Black	Greyish black	+	+	+
S38	Good	Powdery	Greyish white	Brown	-	+	+
SACC172	Good	Leathery	Pale yellow	Pale yellow	-	-	+
SACC194	Good	Leathery	Pale yellow	Pale yellow	-	-	+
SACC196	Good	Leathery	Pale yellow	Pale yellow	+	-	+
SACC197	Good	Powdery	Pale yellow	Black	-	-	+
SACC198	Good	Leathery	Pale yellow	Pale black	-	+	+
SACC199	Good	Powdery	Pale yellow	Pale yellow	+	-	+
SACC200	Good	Powdery	Pale Black	Brown	+	+	+
SACC201	Good	Leathery	Black	Black	-	+	+
C5	Good	Powdery	Black	Black	+	+	+
S10A	Good	Powdery	Brown	Red	-	+	+
S1A	Good	Leathery	Greyish white	Greyish white	+	+	+
S6B	Good	Leathery	Greyish white	Greyish white	+	+	+

[AMC, Aerial mycelia colour; RSP, Reverse side pigment; SP, Soluble pigment]

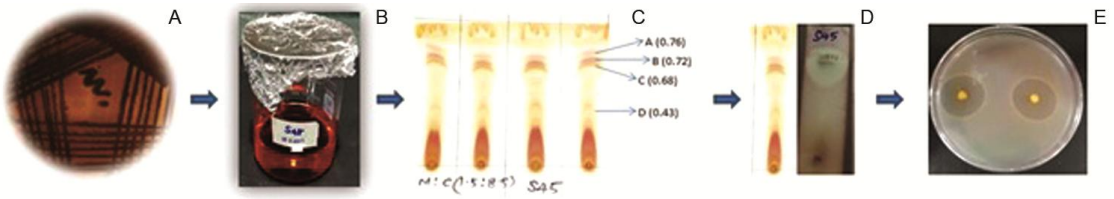

Fig. 1 — Bioprospecting of *Streptomyces* sp. S45, (A) pigment production in ISP2 agar medium, (B) pigment production and extraction using ethyl acetate solvent, (C) compounds separation using TLC, (D) bioautography to identify the active compounds, (E) antibacterial activity.

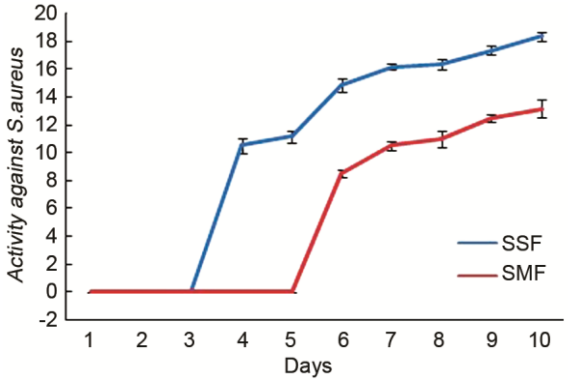

Fig. 2 — Effect of fermentation methods of *Streptomyces* sp. S45 on antibacterial activity against *S. aureus*- ATCC 29213.

Anti-HIV activity

The crude ethyl acetate extract of actinobacterial strain S45 showed anti-HIV activity with the IC50 value of 8.75µg/mL.

Partial purification of bioactive pigment

Among various solvent systems used for TLC chloroform: methanol (8.5:1.5, v/v) was found to be the better solvent system for the separation of antibacterial compounds. Bioassay of TLC extract reveals that the totally four compounds were separated with the Rf value of a-0.78, b-0.70, c-0.68 and d-0.43 (Fig. 1C). The partially purified one of the bioactive compound with Rf value of 0.78 was showed clear zone of inhibition against *S. aureus*- ATCC 29213 and its MIC values were ranged between 25-1.56 µg/mL (Fig. 1 D and E). The chromogenic reactions were showed positive with iodine vapors, vanillin-HCl reagent and Millon's reagent, indicating the presence of conjugated double bond, myrrh constituents and phenol glycosides. The characteristics peak at 290 nm indicates the chances of simple phenols class.

Characterization and taxonomy of strain S45

The phenotypic characteristics of the *Streptomyces* sp. S45 are shown in Table 2. Under bright field microscopic observation, the vegetative substrate mycelium was lengthy and the reproductive aerial mycelium was dark and appeared in recti flexibile (RF) arrangement. It showed good growth on ISP2, ISP4, ISP6 and ISP7 agar medium while moderate growth was observed on ISP1, ISP3 and ISP5 medium. The strain S45 was found to utilize wide

Table 2 — Physiological characteristics and antimicrobial activity against *S. aureus*- ATCC 29213

Characteristics	Variables	S45	Zone of inhibition in mm
Medium	ISP1 medium	+	-
	Yeast extract malt extract agar(ISP 2 medium)	+	14±0.8
	Oat Meal agar (ISP 3 medium)	+	-
	Inorganic salts - starch agar (ISP4 medium)	+	-
	Glycerol asparagine agar (ISP 5 medium)	+	-
	Peptone yeast extract iron agar (ISP6 medium)	+	-
	Tyrosine agar (ISP 7 medium)	+	-
Utilization of Carbon source	Glucose	+	13±0.9
	Lactose	+	12±0.1
	Starch	+	10±1.0
	Mannitol	+	9±0.4
	Inositol	+	10±0.7
	Rhamnose	+	14+1.2
	Raffinose	+	-
Utilization of Nitrogen source	Peptone	+	-
	Yeast extract	+	-
	Malt extract	+	-
	KNO_3	+	10±1.1
	Soybean ($Nano_3$)	+	-
Enzyme production	Asparginase	+	-
	Glutaminase	+	-
	Protease	+	-
	Lipase	+	-
Utilization of Mineral source	NaCl	-	-
	$CaCl_2$	+	12±1.0
	$MnCl_2$	+	-
	$FeSO_4$	+	-
	$MgSO_4$	+	10±1.2

range of carbon, nitrogen and mineral sources on basal medium supplemented with glucose, fructose, sucrose, rhamnose, mannitol and asparagine and able to exhibited aspariginase, glutaminase, protease and lipase activity. Factors which influence the antimicrobial pigment production is given in Table 2.

A 16S rRNA gene sequence of the strain S45 was showed 99.5% similarity with *Streptomyces* sp. and the sequence was submitted in Genbank under the accession number KY689143. A phylogenetic tree was constructed based on 16S rRNA gene sequences to show the comparative relationship between strain S45 and other related *Streptomyces* species (Fig. 3). The comparative analysis of 16SrRNA gene sequence and phylogenetic relationship showed that strain S45 lies in clade with *Streptomyces humi* strain MUSC119T, *Streptomyces* sp., MUSC153T, *Streptomyces* sp. MUSC112, *Streptomyces* sp., DA09205, with which it shares a 16SrRNA gene sequence similarity of 99%.

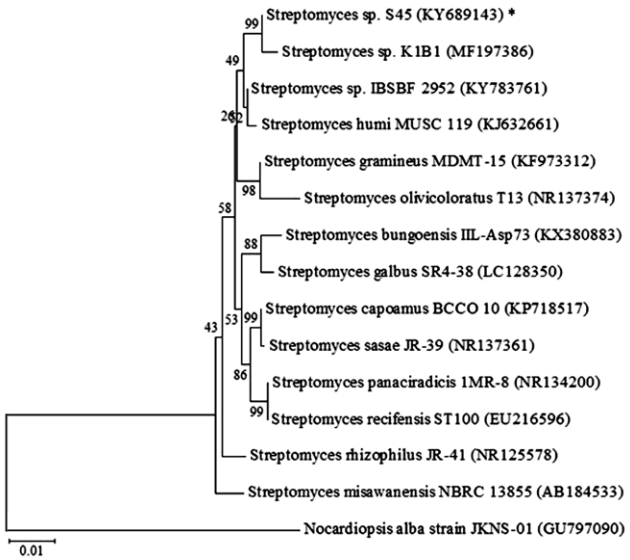

Fig. 3 — The phylogenetic relationship of the potential *Streptomyces* sp. SACC4 based on 16S rRNA gene homology. The tree was constructed using the neighbor-joining method with pairwise-deletion model analyses, which were implemented in the Molecular Evolutionary Genetics Analysis (MEGA), version 6.0 program. The resultant tree topologies were evaluated by bootstrap analysis based on 1000 replicates. *Nocardiopsis alba* JKNS-01 was used as out group. Scale bar indicates the number of substitutions per site.

Discussion

Terrestrial soil is the richest source for actinobacteria notably *Streptomyces*. Using specific media and addition of antibacterial and antifungal agents play an important role in successful isolation

of actinobacteria[17]. In the present study, morphologically different actinobacterial colonies recovered in more numbers from Starch casein agar than from Kusters agar medium. Hence, starch casein agar medium was proved to be the best medium for isolation of actinobacteria from different samples[18,19].

Actinobacteria producing antibiotics like streptomycin and novobiocin firmly covered these chemically prolific bacteria in the center stage of natural products in drug discovery research. In our study the crude pigment producing *Streptomyces* sp. S45 showed significant antibacterial activity against Gram positive and Gram negative pathogens, especially the broad spectrum was observed against *S. aureus*- ATCC 29213 and *B. cereus*. Our research finding is significantly similar to the *Streptomyces* sp. producing crude pigment extract showed significant antibacterial activity against *E. coli*, *L. vulgaris*, *S. aureus*, *P. mirabilis*, *V. cholerae*, *S. typhi*, *S. paratyphi* and *K. oxytoca*[5]. Likewise, other researcher also found that extracted pigment from actinobacteria has 20 mm zone of clearance against *E. coli*[20]. Interestingly, in our study the crude pigment producing *Streptomyces* sp. S45 showed anti-HIV activity with the IC50 value of 8.75 µg/mL. Similarly, *Streptomyces* sp. MA7234 producing Complestatin A and B, isocomplestatin and chloropeptin showed anti-HIV inhibitory activities against HIV-1 virus[21]. Moreover, siamycins, polypeptides isolated from *Streptomyces* were found to inhibit HIV infection *in vitro*[22].

The identification of actinobacteria based on 16S rRNA gene revealed that *Streptomyces* genus was dominated with antimicrobial activity was higher than other actinobacteria genus in terrestrial soil[23]. Our present study also noted that *Streptomyces* strains were more dominated in terrestrial soil of Sabarimala forest, Kerala with having higher antibacterial activity. The result of bio-autography indicates the presence of only one bioactive compound and its character is similar to the others finding suggests that the absence and/or presents of anthraquinones, phenol glycosides, heterocyclic compounds, myrrh constituents, conjugated double bond, cardiac glycoside and free amine groups[24,25]. The characteristics peak at 290 nm indicates chances of simple phenolic class[26]. Phenolic classes of compounds are known for their antimicrobial activities[27].

Nevertheless, the nature of the strain, large geographic variation, different soil types and the difficult ecosystem are also influencing their vast distribution of isolating microorganisms to produce potential and novel antibiotics. The potential *Streptomyces* sp. S45 isolated from Sabarimalai forest ecosystem also deals with the strain SFA5 isolated from the same origin producing antimicrobial[28] and anti-tuberculosis[9] activities. Similarly, our previous studies and other researchers also reported *Streptomyces* as a major actinobacterial population in the terrestrial ecosystem showing considerable antimicrobial, anti-tubercular, anticancer anti-HIV activity[9,29-32].

Only ISP2 media alone showed for biosynthesis of antimicrobial compounds by *Streptomyces* sp. S45 because other ISP media failed to show the inhibition against the pathogen *S. aureus*-ATCC 29213. Rhamnose, KNO_3 and $CaCl_2$ act as the carbon, nitrogen and mineral sources, respectively and showed maximum inhibition against *S. aureus*-ATCC 29213. Similarly, Al-Zahrani[33], reported that most favorable antimicrobial compounds from *Streptomyces* sp. J12 were synthesized in oat meal, starch casein and sabouraud dextrose agar medium. Starch and KNO_3 used as carbon and nitrogen source and showed maximum antimicrobial activity[33]. Also, the *Streptomyces* sp. D25 isolated from terrestrial environment showed maximum antimicrobial activity in the medium influenced by glucose and KNO_3 as carbon and nitrogen sources[34]. Others researchers have also proved that the media and other sources influence the antimicrobial activity[35,36].

The forest ecosystems are rich sources of bioactive metabolites producing actinobacteria, and proper screening of their microbial diversity is important. In the present study, the antibacterial activity of pigment producing *Streptomyces* sp. S45 showed broad range of activity against *S. aureus*-ATCC 29213 and *B. cereus*. Moreover, it is also proved that the strain S45 showed better anti-HIV activity. These searching confirmed that the terrestrial actinobacteria could produce an array of a variety of bioactive metabolites with broad spectrum in nature. Also, these terrestrial ecosystems are protected from human activity that may have contributed to the production of previously unexplored and powerful metabolites.

Conclusion

The results of the present study suggest that forest ecosystems harbour potential bioactive microorganisms, particularly actinobacteria that produce secondary metabolites. The actinobacteria *Streptomyces* strain S45 from the sabarimala forest

soil has been shown to possess a brown extracellular pigment, purified by preparative TLC, exhibited strong antibacterial and anti-HIV activity. It showed activity against *S. aureus*-ATCC 29213 and its MIC value was between 25-1.56 µg/mL. However, further studies will be necessary to determine the structure of the active compound.

References

1 Bloom DE & Cadarette D, Infectious disease threats in the twenty-first century: Strengthening the global response. *Front Immunol*, 10 (2019) 549.

2 Reta A, Bitew Kifilie A & Mengist A, Bacterial infections and their antibiotic resistance pattern in ethiopia: A systematic review. *Adv Prevent Med*, 2019 (2019) Article ID 4380309.

3 Sharma P & Thakur D, Antimicrobial biosynthetic potential and diversity of culturable soil actinobacteria from forest ecosystems of Northeast India. *Sci Rep*, 10 (2020) 4104.

4 Jiang ZK, Tuo L, Huang DL, Osterman IA, Tyurin AP, Liu SW, Lukyanov DA, Sergiev PV, Dontsova OA, Korshun VA, Li FN & Sun CH, Diversity, novelty, and antimicrobial activity of endophytic actinobacteria from mangrove plants in Beilun estuary national nature reserve of Guangxi, China. *Front Microbiol*, 9 (2018) 868.

5 He X, Wang Y, Luo RH, Yang LM, Wang L, Guo D, Yang J, Deng Y, Zheng YT & Huang SZ, Dimeric pyrano-naphthoquinone glycosides with anti-HIV and cytotoxic activities from a soil-derived *Streptomyces. Nat Prod*, 82 (2019) 1813.

6 Sharma P, Singh TA, Bharat B, Bhasin S & Modi HA, Approach towards different fermentative techniques for the production of bioactive actinobacterial melanin. *Beni-Seuf Univ J Appl Sci*, 7 (2018) 695.

7 Radhakrishnan M, Pazhanimurugan R, GopikrishnanV, Vanaja K & Balagurunathan R, *Streptomyces* sp D25 isolated from Thar Desert soil, rajasthan producing pigmented antituberculosis compound only in solid culture. *J Pure Appl Microbiol*, 8 (2014) 333.

8 Eccleston GP, Brooks PR & Kurtböke DI, The occurrence of bioactive *Micromonosporae* in aquatic habitats of the sunshine coast in Australia. *Mar Drugs*, 6 (2008) 243.

9 Radhakrishnan M, Sekar P, Jerrine J & Vanaja K, Antitubercular activity of the pigment from forest soil *Streptomyces* sp. SFA5. *Bangladesh J Pharmacol*, 11 (2016) 138.

10 Puertas MC, Buzon MJ, Ballestero M, Eede P, Van Den, Clotet B & Prado JG. Novel Two-Round Phenotypic Assay for Protease Inhibitor Susceptibility Testing of Recombinant and Primary HIV-1 Isolates. *J Clinical Microbiol*, 50 (2012) 3909.

11 Odakura Y, Kase H, Itoh S, Satoh S & Takasawa S, Biosynthesis of asatromicin 421 and related antibiotics. Biosynthetic studies with blocked mutants of 422 *Micromonospora olivasterospora*. *J Antibiot*, 12 (1984) 670.

12 Zothanpuia, Passari AK, Chandra P, Leo VV, Mishra VK, Kumar B & Singh BP, Production of potent antimicrobial compounds from *Streptomyces cyaneofuscatus* associated with fresh water sediment. *Front Microbiol*, 8 (2017) 68.

13 Shirling EB & Gottlieb D, Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol*, 16 (1966) 313.

14 Gothwal RK, Nigam VK, Mohan MK, Sasmal D & Ghosh P, Extraction of bulk DNA from Thar Desert soils for optimization of PCR-DGGE based microbial community analysis. *Electronic J Biotechnol*, 10 (2007) 400.

15 Saitou N & Nei M, The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol*, 4 (1987) 406.

16 Felsenstein J, Phylogenies and the comparative method. *Am Nat*, 125 (1985) 1.

17 Kumar RR & Jadeja VJ, Isolation of actinomycetes: A complete approach. *Int J Curr Microbiol App Sci*, 5 (2016) 606.

18 Radhika S, Bharathi S, Radhakrishnan M & Balagurunathan R, Bioprospecting of fresh water actinobacteria: isolation, antagonistic potential and characterization of selected isolates. *J Pharm Res*, 4 (2011) 2584.

19 Zothanpuia P, Passari A & Singh BP. Molecular characterization of actinomycetes isolated from Tuichangriver and their biosynthetic potential. *Sci Vis*, 15 (2015) 136.

20 Selvameenal L, Radhakrishnan M, Balagurunathan R. Antibiotic Pigment from Desert Soil Actinomycetes; Biological Activity, Purification and Chemical Screening. *Indian J Pharm Sci*, 71 (2009) 499.

21 Singh SB, Hiranthi J, Gino MS, Deborah LZ, Ali S, Brian H, Keith CS, Russell BL, Olga G, Ana T, Dolores V, Peter F & Daria H, The complestatins as HIV-1 integrase inhibitors. Efficient isolation, structure elucidation and inhibitory activities of isocomplestatin I, new complestatin A and B, and acid hydrolysis products of chloropeptin I. *J Nat Prod*, 64 (2001) 874.

22 Chokekijchai S, Kojima E, Anderson S, Nomizu M, Tanaka M, Machida M, Date T, Toyota K, Ishida S & Watanabe K, NP-06: A novel anti-human immunodeficiency virus polypeptide produced by a *Streptomyces* species. *Antimicrob Agents Chemother*, 39 (1995) 2345.

23 Kitouni M, Boudemagh A, Oulmi L, Reghioua S, Boughachiche F, Zerizer H, Hamdikenb H, Coublea A, Mounieea D, Boulahroufb A & Boiron P, Isolation of actinomycetes producing bioactive substances from water, soil and tree bark samples of the north-east of Algeria. *J Mycol Med*, 15 (2005) 45.

24 Kumar V, Gusain OP, Thakur RL & Bisht GS, Isolation, purification and partial characterization of an anti bacterial agent produced by halo tolerant alkaliphilic *Streptomyces* sp. EWC7(2). *Proc Natl Acad Sci India Sec B Biol Sci*, 83 (2012) 199.

25 Kumar V, BinduN, Omprakash G & Gajraj SB, An actinomycete isolate from solitary wasp mud nest having strong antibacterial activity and kills the *Candida* cells due to the shrinkage and the cytosolic loss. *Front Microbiol*, 5 (2014) 7.

26 Dey PM, Methods in Plant Biochemistry. Volume 1. (Academic Press, USA), 2012, 608.

27 Yogeswari S, Ramalakshmi S, Neelavathy R & Muthumary J, Identification and comparative studies of different volatile fractions from *Monochaetia kansensis* by GCMS. *Global J Pharmacol*, 6 (2012) 66.

28 Sekar P, Kannaiyan SK, Gopikrishnan V, Radhakrishnan M. HPTLC fingerprint profile of antibacterial compound produced from forest soil *Streptomyce*s SFA5. *Bangladesh J Pharmacol*, 11 (2016) 295.

29 Radhakrishnan M, Balaji S, Balagurunathan R. Thermotolerantactinomycetes from the Himalayan Mountain – Antagonistic potential, characterization and identification of selected strains. *Malays Appl Biol*, 36 (2007) 59.

30 Radhakrishnan M, Suganya S, Balagurunathan R & Vanaja K, Preliminary screening for antibacterial and antimycobacterial activity of actinomycetes from less explored ecosystems. *World J Microbiol Biotechnol*, 26 (2010) 561.
31 Mohanraj D, Bharathi S, Radhakrishnan M & Balagurunathan R, Bioprospecting of Actinobacteria from Yelagiri Hills with special reference to antibacterial activity. *J chem pharm res*, 3 (2011) 439.
32 Singh IP, Sandip B. Bharate K & Bhutani K, Anti-HIV natural products. *Curr Sci*, 89 (2005) 269.
33 Al-Zahrani SHM, Studies on the antimicrobial activity of *Streptomyces* sp. isolated from Jazan. *J King Saud Univ Sci*, 19 (2007) 127.
34 Radhakrishnan M, Venugopal G, Ramasamy B & Kumar V, Effect of critical medium components and culture conditions on anti-tubercular pigment production from novel *Streptomyces* sp D25 isolated from Thar desert, Rajasthan. *J App Pharm Sci*, 5 (2015) 15.
35 Hassan MA, El-Naggar MY & Said WY, Physiological factors affecting the production of an antimicrobial substance by *Streptomyces* violatus in batch cultures. *Egyptian J Biology*, 3 (2001) 1.
36 Al-Ansari M, Alkubaisi N, Vijayaragavan P & Murugan K, Antimicrobial potential of *Streptomyces* sp. to the Gram positive and Gram negative pathogens. *J Infect Public Health*, 12 (2019) 861.