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Evaluation of Anti-mycobacterial Activity of Secondary Metabolites of Actinomycetes from Unexplored Habitats

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Abstract: The aim of the study was to assess the anti-tuberculosis potential of the *Streptomyces* species isolated from unexplored habitats. Six actinomycetes strains were screened for their anti-mycobacterial activity. The crude bioactive compound was prepared and extracted with resin Dianoin HP20. Actinomycete strains EWC 7(2), MN 2(6) and MN 9(V) showed prominent activity, and were further evaluated with BACTEC assay. The anti-mycobacterial activity was observed by detection of inhibition of growth in terms of growth index (GI value). *M. tuberculosis* H_{37}Rv and *M. tuberculosis* H_{37}Ra were found sensitive towards metabolites of *Streptomyces* sp. EWC 7(2), and *Streptomyces* sp. MN 2(6) as observed in the form of decreasing GI values. The findings of present study are promising however, further studies are required to purify and elucidate the structure of metabolites. These isolates may prove to be an important step in development of drug for treating mycobacterial infections.

Key words: Actinomycetes, antimycobacterial activity, BACTEC, growth index.

Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis*, remains the most important infectious cause of mortality in the world, which has recently become apparent as a major opportunist in HIV-infected people in the developed world [1]. The drugs currently used to treat the TB infections are mainly rifampicin, ethambutol, isoniazid and pyrazinamide. The emergence of multiple drug resistant (MDR) strains of *M. tuberculosis* (defined resistance against isoniazid and rifampicin) is now common in number of patients because of uncontrolled application of anti-tuberculosis drugs. At present, the more drug resistant form of tuberculosis XDR-TB (extensively drug resistant tuberculosis) has been reported[2]. Because of rise in the MDR and XDR-TB strains, the urgent need to discover the alternative drug that is effective against all form of infections has occurred.

The discovery of antibiotics and other bioactive metabolites from microbial sources have yielded

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an impressive number of compounds over the past 50 years [3]. Among different microorganisms, actinomycetes are one of the most attractive sources of all types of bioactive metabolites that have important applications in industry, agriculture and human medicines. They have provided many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive substances. Of the 22,500 antibiotics reported from microbial sources, about 10,100 are reported from actinomycetes in which 800-1000 antibiotics are reported as antimycobacterials [4]. From the discovery of streptomycin (the first antibiotic used for anti-TB therapy) from *Streptomyces griseus*, numerous anti-TB antibiotics such as kanamycin and rifampicin have been reported from actinomycetes of terrestrial origin [5].

The fast-growing, acid-fast bacilli *Mycobacterium smegmatis* has been employed as marker in drug screening research for the discovery of anti-tuberculosis compounds [6-7]. The use of this rapidly growing acid-fast bacillus as screening system is an advantage over *M. tuberculosis* because of its simplicity of testing compounds against this organism. Their rapid growth rate within 2-3 days and nonpathogenic nature make the handling of the bacteria easy in normal laboratory conditions. There are number of nonradiometric, microscopic methods available for anti-tuberculosis susceptibility testing. The radiometric BACTEC 460TB system (Becton Dickinson Biosciences, Sparks, MD) has been widely accepted for approximately 20 years for the consistent and rapid testing of the susceptibilities of *M. tuberculosis* [8].

The aim of this study was to assess the anti-tuberculosis potential of the *Streptomyces* species isolated from the previous studies using *M. smegmatis* as a rapid screening model for detection of anti-mycobacterial activity and further to evaluate the active fractions for activity against *M. tuberculosis* using radiometric BACTEC assay.

Materials and methods

Test microorganisms

The two strains of *Mycobacterium smegmatis* used in this study were *M. smegmatis* MTCC 6 and *M. smegmatis* MTCC 994. The culture was maintained on nutrient agar slants at 37°C. The tuberculosis strains were *M. tuberculosis* $H_{37}Rv$ (ATCC 27294) and *M. tuberculosis* $H_{37}Ra$ (ATCC 25177). The TB strains were cultured on Lowenstein-Jansen medium (Difco, MI, USA) slant at 37°C.

Actinomycete strains

Six actinomycete strains used in this study were randomly selected from the Actinomycetes Research Laboratory Department of Microbiology, S.B.S. P.G. Institute of Biomedical Sciences and Research, Balawala, Dehradun, India which were isolated from less explored ecosystems (Table 1) from previous study [9-12] and maintained on yeast extract malt extract agar (ISP2 medium).

Screening of actinomycetes for their activity against *Mycobacterium smegmatis*

All the isolates were preliminary screened for their antibacterial activity by agar plug method [13]. All the isolates were grown on actinomycetes isolation agar and yeast extract malt extract agar at 27°C. After 15 days, three discs (6 mm in diameter) were cut and placed on Mueller-Hinton

Table 1. Details of actinomycetes taken from previous study

Selected isolates	GenBank accession numbers	Most closely related with	Reference
8(1)*	GU064904	*Streptomyces fradie* (AB184776)	Kumar *et al*. 2012a
MN 2(6)	HM991286	*Streptomyces sporocinereus* (AB249933)	Kumar *et al*. 2012a
MN 9(V)	HM991287	*Streptomyces cellulosae* (AB184265)	Kumar *et al*. 2011b
EWC 7(2)	GQ340692	*Streptomyces ghanaensis* (AY999851)	Kumar *et al*. 2012b
DV1S	HM991289	*Streptomyces massasporeus* (NBRC12796)	Kumar *et al*. 2011a
GR9a-5	HM991288	*Nocardia nova* (JCM6044)	Kumar *et al*. 2011a

agar plates seeded with the test organisms and then incubated at 37°C for 24 h. The zone of growth inhibition (in millimetre) of bacteria was observed in case of active metabolite/s produced by the actinomycete.

Fermentation and extraction of metabolite from culture filtrate

The spores of strains were inoculated into a 250 ml flask containing 40 ml of sterile seed medium nutrient broth. The culture was incubated on a rotary shaker (180 rpm) at 27°C for 3 days. The seed culture was transferred (10 %, v/v) into a GS broth (gL^{-1}; 10.0 glucose, 10.0 soybean meal, 10.0 NaCl and 1.0 $CaCO_3$; pH 7.2). The fermentation was carried out at 27°C for 4 days at 210 rpm. The culture filtrates were assayed daily for antimycobacterial activity. The extraction of antibiotic from culture filtrate was performed using resin (Dianion™ HP-20). The 40 ml of filtered fermented broth was taken in 250 ml flask, added 4 gm of resin and was agitated at shaker for 30 min. After shaking the resin was separated from the broth using 10 ml sterile syringe having cotton plug at mouth. The resin was washed several times with sterile double distilled water. Antibiotic was eluted from the resin using methanol (15-20 mL). This process was repeated for 2 L fermented broth. The eluted fractions were concentrated in rota vacuum at 40°C and checked for their antibacterial activity.

Anti-mycobacterial activity of extracted product

The bacterial inoculum was prepared from overnight-grown cultures (24 h) in nutrient broth (Difco) containing tween-80 (0.1 % v D v; Merck), and the turbidity was adjusted equivalent to 0.5 McFarland units (approximately 1.2 X 10^8 CFU/mL). Aliquots (100 µL) of inoculums were spread over the surface of agar plates with a sterile glass spreader. The paper disk (5 mm diameter, Whatman filter paper no. 3; Millipore) was impregnated with 10 µl of test sample and allowed to dry for 30 min and then placed on the premade bacterial lawn. The disks containing solvents served as negative control. The disk containing antibiotic rifampicin (5.0 µg/disk; Sigma, St Louis, MO) was used as positive control. The plates were then incubated for 48 h at 37°C, and the zone of bacterial growth inhibition around disk was measured. The assay was repeated twice, and mean of the three experiments was recorded.

Minimum inhibitory concentration (MIC) determination against *Mycobacterium smegmatis*

The MIC of the test samples was determined by twofold micro-dilution method using sterile flat-bottom 96-well polystyrene micro-titre plates (Axygen, CA, USA). The test samples were diluted serially twofold with nutrient broth containing 0.1 % tween-80 (v D v). The culture-inoculated microtiter plates were incubated at 37°C for 48 h, and the growth was recorded spectrophotometrically at 600 nm. The MIC values were detected using a tetrazolium salt indicator, MTT (3-4 5-dimethylthiazol-2-yl-2, 5-diphenyl tetrazolium bromide; Merck), which is reduced to a blue purple colour by viable bacteria. In the MIC assay, an aliquot (40 µL of a 0.2 mg/mL) of MTT was pipetted into each well, and once growth control wells revealed a purple colour following incubation at 37°C for 1 h, results were noted. The MIC values were read as those concentrations where a marked reduction in colour formation because of bacterial growth inhibition was noted [7,14-15].

BACTEC radiometric susceptibility assay

The anti-tuberculosis activities of crude active extracts against *M. tuberculosis* were detected by BACTEC assay. BACTEC 460 TB system (Becton-Dickinson Diagnostics Instruments System, Sparks, MD, USA) is a comparatively rapid, radiometric, anti-tuberculosis susceptibility assay system for slow-growing *Mycobacterium* species. The cryopreserved *M. tuberculosis* $H_{37}Ra$ (avirulent strain) and *M. tuberculosis* $H_{37}Rv$ (virulent strain) were taken out from) -80°C freezer and cultured on Lowenstein-Jensen medium slant. After 21 days of incubation, bacterial cells were scraped from slants and transferred in 1.0 mL of BACTEC diluting fluid and made complete homogenized suspension by vortexing with glass beads (2 mm diameter). The suspension was allowed to stand for a few minutes to permit sedimentation of the bacterial clumps if any. The turbidity of the homogenous suspension was adjusted

to McFarland standard 1.0 with diluting fluid. A BACTEC 12B vial (Becton-Dickinson) was injected with 0.1 mL of this suspension. This vial was used as primary inoculum after the growth index (GI) reached a value of about 500 (approximately 1.0 X 10^6 CFU/mL). The stock concentrations of test samples were prepared in DMSO. Briefly, 0.1 mL of bacterial suspension from the primary inoculum culture vial (GI 500) was injected into test samples containing vials using 1.0 mL insulin syringe. To comply with 1 % proportion method, 0.1 ml of primary inoculum was added to 9.9 mL BACTEC diluting fluid to obtain 1: 100 dilutions. The 0.1 mL of diluted culture (1: 100) was injected into the 12B vial along with solvent that was considered as control. For recording GI, daily testing schedule was followed. The vials were incubated at 37°C, and the GI was recorded every 24 h. Once the GI of the control vial (1: 100) reached 30, then the GI values of the test (compound containing) vials were compared with that of control vials based on difference in growth (DGI). The result was interpreted as follows: If the difference (called as DGI) in current GI from previous day GI in the case of test samples containing vials is lower than the DGI of control (1: 100) vial for the same period, then the test compound is termed as active against MTB or otherwise inactive [7].

Results and discussion

The antimycobacterial activity was carried out using disc diffusion method against *M. smegmatis*. In the present study, the crude extracts were not evaluated directly by BACTEC assay because of the disposal problem of radioactive medium and the expenses involved. So, the primary screening of extracts for anti-mycobacterial activity was carried out against fast growing species *M. smegmatis*. This acid-fast bacillus selected as screening model for primary evaluation of antimycobacterial activity because of its much similarity in cell wall composition with *M. tuberculosis*. It also shares several clinically important properties with *M. tuberculosis*, including similar resistance to certain macrolide drugs [15]. Actinomycetes strains EWC 7(2), MN 2(6) and MN 9(V) were found to be active against *M. smegmatis*; hence partially purified fractions were further evaluated with BACTEC assay. The anti-mycobacterial activity was observed by detection of inhibition of growth in terms of growth index (GI value) as described in material and method section. *M. tuberculosis* H_{37}Rv and *M. tuberculosis* H_{37}Ra were found sensitive towards metabolites of *Streptomyces* sp. EWC 7(2), and *Streptomyces* sp. MN 2(6) as observed in the form of decreasing GI values (Fig. 1). The MIC values against virulent and avirulent strains of *M. tuberculosis* of isolates are given in Table 2. The strain EWC 7(2) and MN 2(6) showed most promising activity against *M. tuberculosis* H_{37}Rv and *M. tuberculosis* H_{37}Ra. Recently, Culture filtrates and crude extracts were tested against standard strain *Mycobacterium tuberculosis* H_{37}Rv and drug sensitive and drug resistant clinical isolates of *M. tuberculosis* by luciferase reporter phage (LRP) assay [16]. In contrast, Raja and Prabakaran [17] reported four isolates belonging to genus *Streptomyces*, *Micromonospora* and *Micropolyspora* having activity against *Mycobacterium tuberculosis* using well diffusion method. At present, the chemotherapeutic options for TB treatment have been restricted to a handful of compounds introduced 40-50 years ago, which must be administered in combination for extended periods. Finding of new lead compounds with novel antimycobacterial activity is urgent [18]. Early stage drug discovery is a key bottleneck in the pipeline to find novel drugs. Of the 1,556 new chemical entities marketed worldwide between 1975 and 2004, only three were for TB [19]. The emergence of MDR and extensively-drug-resistant (XDR) strains of *Mycobacterium tuberculosis* (MDR-TB and XDR-TB), which are a rising threat in the developing world. MDR-TB treatment requires a two-year course of antibiotics with serious side effects; XDR-TB is even more difficult to cure and often fatal [20]. Cases of MDR-TB and XDR-TB have been reported in the U.S. and other developed countries.

Secondary metabolites of actinomycetes of rare ecosystems are meant to antagonize organisms in their respective environments. These are likely to be novel antimycobacterial compounds as they unknown to human pathogens. It seems that the promising isolates from these unexplored habitats may prove to be an important step in devel-

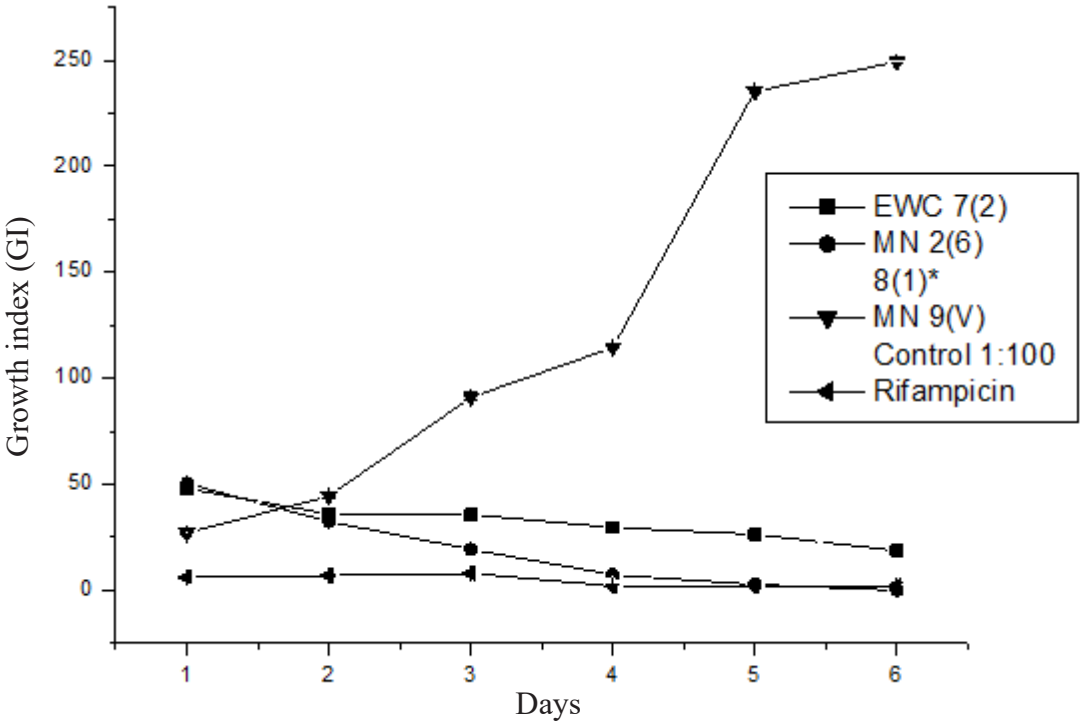

Fig. 1. Inhibition of growth index values of *M. tuberculosis* H_{37}Rv in the presence of metabolites of *Streptomyces* sp. of unexplored habitats

Table 2. Anti-tuberculosis activity (MIC) of purified fractions of selected isolates through BACTEC assay

Purified fraction of isolates	*M. tuberculosis* H_{37}Rv ATCC 27294 (µg/ml)	*M. tuberculosis* H_{37}Ra ATCC 25177 (µg/ml)
EWC 7(2)	12.5	12.5
MN 2(6)	12.5	25.0
MN 9(V)	100.0	100.0
8(1)*	100.0	100.0
GR9a-5	NA	NA
DV1S	NA	NA
Rifampicin	0.25	0.5
Isoniazid	0.1	0.1

NA, not active at a tested concentration of 50 µg/ml
values are mean ± standard deviation of three experiments in replicate

opment of drug for treating mycobacterial infections. In this context, the importance of strain EWC 7(2) and MN 2(6) cannot be avoided therefore merit further studies on isolation, purification and characterization of metabolites.

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