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ANTI-TUBERCULOSIS ACTIVITY OF CRUDE ETHANOL EXTRACTS OF MANGROVE RHIZOSPHERE FUNGI, TRICHODERMA SPP.

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ABSTRACT

Tuberculosis is a major public health problem in the developing countries including India. Increase in multidrug resistant (MDR) and extensively multi-drug resistant (XDR) strains of *Mycobacterium tuberculosis*, there is an insistent need to search for new alternatives to combat this problem. Marine microbes are a source of novel compounds that can be harnessed for their antibacterial and antimycobacterial activity. The main aim of this study was to find out the anti-mycobacterial activity of crude extract of the rhizosphere soil fungus *Trichoderma spp.* against virulent Mycobacterium *tuberculosis* H37Rv. Totally four *Trichoderma spp.* were processed for crude extraction and screened for their anti-mycobacterial activity by both conventional and radiometric assay. The isolates were screened based on the maximum day of inhibition. Maximum inhibition was exhibited by 5mg/ml of all the fungal crude extract followed by 2mg/m, 1mg/ml and finally 0.5 mg/ml. The crude extract of *Trichoderma harzianum* showed negative growth up to maximum inhibition days in 5mg/ml and 2mg/ml, whereas *Trichoderma viridae, Trichoderma inhamatum and Trichoderma harzianum (2)* showing maximum inhibition only at 5mg/ml. The research study revealed that the four fungal crude extracts showed good anti-mycobacterial activity against virulent Mycobacterium *tuberculosis* H37Rv. Therefore, these crude extracts can be used as a potential source of antituberculosis agents to develop new drug molecules.

KEYWORDS: Marine microbes, *Trichoderma sps*, anti-tuberculosis, inhibition.

INTRODUCTION

Tuberculosis is a pandemic deadly disease, TB along with HIV as a leading cause of death worldwide and there are 9.6 million people are suffered with TB in 2014. It includes 5.4 million men, 3.2 million women and 1.0 million children. In 2014, 6 million new cases of TB were reported to WHO. To minimize this burden, diagnosis and treatment method should be improved. Tuberculosis death has declined sharply in the western world but due to high poverty rate, poor nutrition, overcrowded and HIV it is gradually increase major health hazard problem in Asia, Africa and the Western Pacific region. Currently using first line anti-TB drugs increases the opportunity to develop drug resistant tuberculosis all over the world and it is ineffective to completely eradicate the infection.[1]

Multiple drug resistant (MDR) strains has been reported in recent days and this a major emergency situation in human society due to an inappropriate TB management and drug-resistant bacteria. The emergence of multiple drug resistant (MDR) strains of *Mycobacterium tuberculosis* (defined resistance against isoniazid and rifampin) and extensively drug-resistant (XDR) tuberculosis resistant to (isoniazid, rifampin, a fluoroquinolone) usually found in patient infected with tuberculosis are the critical issue to public health because of the high rate of death in people co infected with human immunodeficiency virus (HIV).[2]

There are one million tons of antibiotics produced every year most of which are produced from soil.[3] The persistence of resistance mechanisms and side effects of present anti-mycobacterial drugs makes an urgent need to discover the alternative drug molecules that is effective against tuberculosis. Marine soil organisms are important sources of bio-active molecules that have been used to treat various diseases and are associated with chemical diversity, leading to a resource of novel active substances for the development of bio-active products. Recently numerous compounds have been found in marine organisms with wide range of potent activities against the deadly bug, *Mycobacterium tuberculosis*. Therefore, marine organisms are believed to be a potential source of important and novel biologically active substances for the development of therapeutics and create the clinical and pharmaceutical attention due to their potential effects in promoting health and reducing disease.[4]

Marine microorganisms have a diverse range of enzymatic activity because of its nature of living in extreme condition like high pressure, salinity, low temperature, absence of light etc.[5] Sea water is saline in nature and chemically more close to the human blood plasma that could be safer and side effects are most probably nil if it is used in humans for therapeutic purposes.[6]

The drug resistant infections caused by *M. tuberculosis* is considerable global concern. Since no new anti-tuberculosis drugs have become available during the last forty years hence the need for new and effective anti-tubercular drugs discovery is needed for the society. Since traditionally, terrestrial microorganisms were explored as a source of biologically active products.[7] Hence research is now focused on marine fungi for bioactive compounds. Marine secondary metabolites can easily impede other microorganisms.[8] Among marine microorganisms, particularly fungi have gained an important role as a source of biologically active secondary metabolites. Marine-derived fungal strains majorly produce polyketide derived alkaloids, terpenes, peptides and mixed biosynthesis compounds and are yielding major group of secondary metabolites.[9] Recent research only targeted the antimicrobial and cytotoxic effect of mangrove fungi.[10] but the anti-mycobacterial activity of marine associated study was limited, hence this study mainly focused on anti-mycobacterial activity of marine related fungi.

MATERIALS AND METHODS

Fungal cultures used

The fungal cultures used for the study were isolated from mangrove rhizosphere soil. The cultures were identified as *Trichoderma harzianum(*S1), *Trichoderma viridae*(S2), *Trichoderma inhamatum*(S3) *and Trichderma harzianum(2)* (S4).

Bacterial cultures used

The bacterial strain used for the investigation was *Mycobacterium tuberculosis H37Rv*.

Preparation of crude fungal extracts

Preparation of crude extracts of Mangrove rhizosphere fungi *Trichoderma spps.* and extraction of were done by using method.[11] Mass scale fermentation of required fungi were transferred into sterile Erlenmeyer flask (1L) containing 100 ml of distilled water and 100 g of rice. Then the culture was incubated at room temperature for 30 days. After 30 days of incubation, 250 ml of Ethanol were added to the culture and left overnight. The contents were

filtered under vacuum using Buchner funnel, for optimal extraction of fungal biomass, the extraction was repeated up to three times using ethanol until the colour fades. All the filtrates were combined and washed with distilled water. The aqueous and Ethyl acetate phases had left in a separation funnel until complete separation of the two immiscible liquid phase is achieved. The dry residue was obtained from Ethyl Acetate extracts were partitioned between *n*-hexane and 90% Methanol in the ratio of 1:1 (vol/vol) (~150 ml each). Separation of two immiscible liquid phase is achieved and Methanol phase were dried under vacuum (~200 mbar) using rotary evaporator at 40°C. Finally solid residue were obtained and used for further purification.

Anti-mycobacterial testing by using conventional method-Lowenstein Jensen slants

The fungal extracts (*Trichoderma spps*) were dissolved in 0.01% dimethyl sulfoxide (DMSO) (so as to get the desired final concentrations of 5, 2, 1 and 0.5 mg/ml) and added to the LJ medium before heating at 85°C for 50 minutes in a slanting position. The LJ slants were then stored at room temperature for 48 hours to exclude contamination. The tubes were inoculated with strains of mycobacteria. A stock solution of 2.0 mg/ml of isoniazid was used as positive control and 0.01% DMSO as the negative control. Isolates of the mycobacteria H37Rv were prepared for drug susceptibility testing. Using a 3 mm internal diameter (24 standard wire gauge) wire loop, about 4 mg fresh culture was scraped from LJ medium into 500µl sterile distilled water in a bijou bottle with five glass beads and vortexed for about 30 seconds to homogenize. The suspension was made up to 4 ml volume by adding 3.5 ml sterile distilled water. The suspension was allowed to settle for about 30 minutes before gently aspirating the upper portion into a fresh bijou bottle. The suspension was further diluted to obtain the turbidity of 0.5 McFarland standard. The prepared culture was inoculated into extract-free and extract-containing LJ slopes and incubated at 35°C. Growth was recorded weekly (for 6 weeks) as: +++ for confluent growth, ++ moderate growth, + less growth and – for no growth. Cultured tubes were examined visually and sample tubes showing less growth than negative control tubes were considered inhibitory.

Anti-mycobacterial testing by using Semi-automated method by BACTEC-MGIT

The extracts and the drug were prepared just like in the LJ slants. Into the 7 ml BBL Mycobacteria Growth Indicator Tube (MGIT) tubes, 0.8 ml of a mixture of growth OADC supplement (added to provide essential substances for rapid growth of mycobacteria) and BBL MGIT PANTA (a mixture of antimicrobial agents) was added. Then 1 ml of the extract

was added into the BBL MGIT tubes to attain appropriate concentrations of 2, 1 and 0.5 mg/ml. Then 0.5 ml of the mycobacterium suspension was introduced into the BBL MGIT tubes. The BACTEC MGIT system was loaded using manufacturer's instructions at 37°C. Culture vials which remained negative for a minimum of 42 days (maximum 56 days) were removed and recorded as negative, while growth units (GUs) for the positive ones were recorded appropriately.[12]

RESULT AND DISCUSSION

This study focused the bio-active component from marine related fungi *Trichoderma Spp*. from the mangrove rhizosphere soil was analyzed for the anti-mycobacterial activity. The recent research for anti-dormant mycobacterial substances from marine organisms, isolated three new aminolipopeptides from the culture of the marine sponge-derived fungus of Trichoderma *Spp*. exhibited anti-mycobacterial activity.[13] In both conventional and radiometric assay the crude metabolites of all the *Trichoderma Spp.* exhibited inhibitory activity against the test pathogens.[14] MGIT is an rapid effective method for confirming multidrug resistant tuberculosis and it was found an effective method when compared to the 'gold standard' conventional method.[15] Addition of antibiotics will minimize the contamination rate and improve the result than the conventional method.[16] The studies of volatile metabolites are necessary to determine the secondary metabolites and are highly involved in the multifaceted interactions between filamentous fungi and their living environment. Thus, analytical methods for the identification of volatiles are yielding more than 278 different components in *Trichoderma Spp* which involved in antibacterial activity.[17] Some soil filamentous fungi such as *Penicillium Spp* produce many bioactive small molecules, or secondary metabolites that range from beneficial bioactive compounds and the bioactive potential of brown seaweed from ocean to exploit its bioactive potential for the production of valuable therapeutics drugs.[18] Hence this study confirmed that the fungus from the ocean sources yielded act as a bio-active compound against pathogen.

COMPARATIVE SENSITIVITY OF CONVENTIONAL AND RADIOMETRY ASSAY

Table 1: Inhibition of the *Mycobacterium tuberculosis H37Rv* at concentration of 5mg/ml

Fungus	Week-1	Week-2	Week-3	Week-4	Week-5	Week-6	Week-7	Week-8
S-1 R	-	-	-	-	-	-	-	-
S-1 C	-	-	-	-	-	-	-	+

S-2 R S-2 C	- -	- -	- -	- -	- -	- +	- +	- +
S-3 R S-3 C	- -	- -	- -	- -	- -	- +	- +	- +
S-4 R S-4 C	- -	- -	- -	- -	- -	- +	- +	- +

Table 2: Inhibition of the *Mycobacterium tuberculosis H37Rv* at concentration of 2mg/ml

Fungus	Week-1	Week-2	Week-3	Week-4	Week-5	Week-6	Week-7	Week-8
S-1 R S-1 C	- -	- -	- -	- -	- -	- -	- -	- +
S-2 R S-2 C	- -	- -	- -	- -	- -	- -	- +	+ ++
S-3 R S-3 C	- -	- -	- -	- -	- -	- +	- +	+ +
S-4 R S-4 C	- -	- -	- -	- -	- -	- +	+ +	+ ++

Table 3: Inhibition of the *Mycobacterium tuberculosis H37Rv* at concentration of 1mg/ml

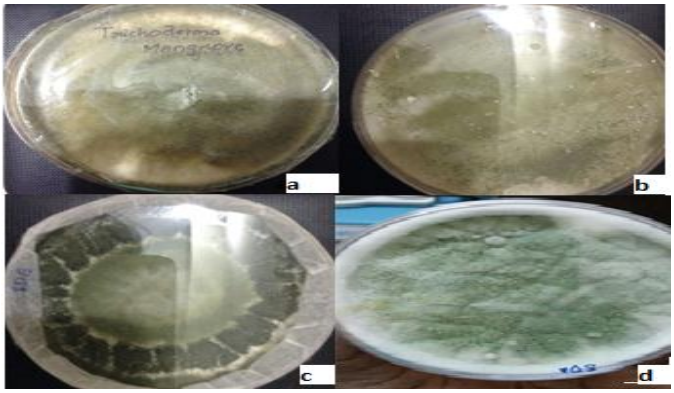
Fungus	Week-1	Week-2	Week-3	Week-4	Week-5	Week-6	Week-7	Week-8
S-1 R S-1 C	- -	- -	- -	- -	- -	- ++	+ ++	++ ++
S-2 R S-2 C	- -	- -	- -	- -	- -	+ ++	+ ++	+++ +++
S-3 R S-3 C	- -	- -	- -	- -	+ +	++ ++	+++ +++	+++ +++
S-4 R S-4 C	- -	- -	- -	+ ++	++ +++	+++ +++	+++ +++	+++ +++

Table 4: Inhibition of the *Mycobacterium tuberculosis H37Rv* at concentration of 0.5mg/ml

Fungus	Week-1	Week-2	Week-3	Week-4	Week-5	Week-6	Week-7	Week-8
S-1 R S-2 C	- -	- -	- -	+ +	++ +++	+++ +++	+++ +++	+++ +++
S-2 R	-	-	-	+	++	+++	+++	+++

S-2 C	-	-	-	++	+++	+++	+++	+++
S-3 R	-	-	-	+	++	+++	+++	+++
S-3 C	-	-	+	++	++	+++	+++	+++
S-4 R	-	-	+	+	++	+++	+++	+++
S-4 C	-	-	++	++	+++	+++	+++	+++

+++: Indicate confluent growth, ++: Indicate moderate growth, +: Indicate less growth and –: Indicate no growth



"Fig. 1": Isolation of rhizosphere soil fungus *Trichoderma spps.* S1, S2, S3 & S4 in plate a, b, c, d, respectively

Sensitivity of test organisms against various concentration of crude extracts

"Fig. 2": LJ tube: 1, 2, 3, 4 and 5 showing inhibition of *Mycobacterium tuberculosis* at various concentration of crude 0.5,1,2 and 5mg/ml showing respectively

CONCLUSION

The need of a novel and safe bioactive compound in human's life is ever increasing because of the emergence of new deadly diseases, development of drug resistant bugs. The focus of

marine environment is unique in terms of its specific composition in both organic and inorganic substances and it produces specific natural products from marine fungi and may give us a solution to this alarming drug resistant problem.

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