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Original Article

ANTIMYCOBACTERIAL ACTIVITY OF CRUDE EXTRACTS PRODUCED BY *BACILLUS* SP. ASSOCIATED WITH ENTOMOPATHOGENIC NEMATODE

SREERAG RS[1], DILEEP C[2], NISHANTH KUMAR SASIDHARAN[1*],

[1]Division of Crop Protection and Division of Crop Utilization, Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram 695017, Kerala, India, [2]Department of Botany, SD College, Kalarcode, Thookkukulam, Alappuzha, Kerala, India.
Email: micronishanth@rediffmail.com

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ABSTRACT

Objective: The World Health Organization estimates that about 8 to 10 million new Tuberculosis (TB) cases occur annually worldwide and its incidence is currently increasing. There are two million deaths from TB each year. The global threat of tuberculosis demands the search for alternative antimycobacterial drugs. The aim of the present study was to determine the antimycobacterial activity of nine crude extracts from a *Bacillus* sp. N strain associated with entomopathogenic nematode *Rhabditis* (*Oscheius*) sp.

Methods: The liquid media for fermentation was prepared in TSB alone, LB alone and TSB + LB (1:1) supplemented with six different carbon sources (fructose, maltose, dextrose, mannitol, sucrose and lactose) and after fermentation crude extract was extracted using ethyl acetate. The minimum inhibitory concentration (MIC) of extracts was determined using the broth dilution method on middle brook 7H11 against *M. tuberculosis* $H_{37}Rv$. The cytotoxicity of the extracts was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay against VERO cell line.

Results: Out of nine extract tested only two recorded activity and significant activity was recorded by TSB+LB+lactose, followed by TSB+LB+fructose. These two extracts were nontoxic to the normal cell line.

Conclusion: Purification of these extract will get pure compounds with antimycobacterial activity in future.

Keywords: Antimycobacterial, *Bacillus* sp, Entomopathogenic nematode, Fermentation.

INTRODUCTION

Tuberculosis (TB) is a chronic infectious disease caused mainly by *Mycobacterium tuberculosis* [1, 2]. The World Health Organization (WHO) estimated that almost 9 million new cases and 1.4 million TB deaths (990,000 among HIV-negative people and 430 000 HIV-associated TB deaths), occurred in 2011 [3]. Ninety-five percent of TB cases are produced in underdeveloped countries, 80% of them corresponding to the 15 to 29-year-old group, generating strong socioeconomic problems [3]. Furthermore, the lack of treatment adherence has given rise to antibiotic-resistant *M. tuberculosis* strains in many parts of the world.

Tuberculosis is mostly asymptomatic and is aggravated when impairment of immunity arises due to conditions like malnutrition, diabetes, malignancy, and AIDS. Weakening of the immune system by HIV increases vulnerability to other infections and diseases caused by *M. avium* complex has also increased [4]. This phenomenal increase is due to their weak immune system compromised by stress, immunosuppressive drugs, substance abuse, or HIV/AID [5]. HIV infection is the greatest single medical risk factor because cell-mediated immunity, which is impaired by HIV, is essential for defense against mycobacterial infection; other immunosuppressive illnesses (e. g., diabetes) or therapies (e. g., corticosteroids) are risks but less so than HIV. The problem of multi-drug resistant strains of mycobacteria has made the search for more efficacious, safer, cheaper, and more accessible drugs a priority. The search for new and better anti-mycobacterial drugs has informed our present study of medicinal plants with folk reputations as effective alternative therapies for mycobacteria infections.

A major problem for the control of TB is the requirement of drug regimens for six to nine months. These lengthy regimens lead to non-compliance with therapy, relapse and development of drug resistance. In order to shorten the duration of therapy, novel drugs that are active against *Mycobacterium*, which act through mechanisms different from those employed by the existing frontline and secondary anti-TB drugs are urgently needed. The emergence of antibiotic resistant mycobacterial strains underscores the need for novel effective drugs against resistant mycobacteria as first-line antituberculosis medications. According to World Health Organization (WHO), between 1980 and 2005, 90 million cases of TB worldwide were reported [6].

Bacteria of the genera *Xenorhabdus* and *Photorhabdus* are known to be symbiotically associated with the soil dwelling entomopathogenic nematodes (EPN) of the family Steinernematidae and Heterorhabditidae, respectively [7]. The antimicrobial nature of metabolites produced by *Xenorhabdus* spp. and *Photorhabdus* spp. is known, and several compounds with biological activity have been isolated and identified [8]. In the course of studies on EPN, a new EPN belonging to the genus *Rhabditis*and subgenus *Oscheius* was isolated from sweet potato weevil grubs collected from Central Tuber Crops Research Institute (CTCRI) farm, Thiruvananthapuram, Kerala, India. In the present study, we investigated the antimycobacterial activity of the crude extract from the above bacterium against *M. tuberculosis*$H_{37}Rv$.

MATERIALS AND METHODS

All chemicals used for extraction and purification were of AR grade (Merck, Mumbai, India). Microbiological media were purchased from the Hi-media Laboratories Limited, Mumbai, India. The carbon source used is fructose was purchased from the SRL Laboratories Limited, Mumbai.

Fermentation media preparation

Bacterial isolate was inoculated into the liquid medium. The liquid media was prepared in TSB alone, LB alone and TSB + LB (1:1) supplemented with 1% of six different carbon sources (fructose, maltose, dextrose, mannitol, sucrose and lactose). Thus a total of 9 different combinations where used in the study. The media pH was adjusted to 7.0 before autoclaving using NaOH or HCl solution.

Hundred mL aliquots of each media containing one each of different carbon and nitrogen sources were dispensed separately in 250 mL

Erlenmeyer flasks and this was inoculated with a loop full of the bacterial culture. The flasks were incubated in a controlled environment, gyrorotatory shaker (150 rpm at 30 °C in darkness for 24 – 48 hours. When the optical density of the culture at 600 nm was approx. 1.7 (AU), the bacterial cultures were transferred into 400 mL sterile medium and incubated in a gyrorotatory shaker (150 rpm) at 30 °C in darkness for 96 hours. The culture media were then centrifuged (10,000 rpm, 20 min, 4 °C) followed by filtration through a 0.45µM filter, to obtain cell free culture filtrate.

Preparation of crude organic extract

The cell free culture filtrate (500 mL) was neutralized with 1 N HCl and extracted with an equal volume (500 mL) of ethyl acetate thrice. The ethyl acetate extracts were combined, dried over anhydrous sodium sulphate, and concentrated using a rotary flash evaporator at 30 °C to obtain the crude extract.

Antimycobacterial activity of crude extracts

***Mycobacterium Tuberculosis* strains**

M. tuberculosis H37Rv was used as the sensitive strain used for the study. H_{37}Rv are internationally used as standard *M. tuberculosis* strain for sensitivity testing.

Growth media

Middle brook 7H10 agar supplemented with oleic acid-albumin-catalase (OADC) was used for reviving and culturing the mycobacterium for sensitivity testing. The medium was from Becton Dickinson Microbiology Systems of Becton Dickinson Company (Difco TM), 7 Loveton Circle, Sparks, Maryland, USA; Lot No. 8175150. The OADC, Lot 8136781, also from Becton Dickinson Company. No adjustments for pH were made.

Preparation of inoculum for drug sensitivity testing

Preserved strains were revived on middle brook 7H10 agar, prior to anti tubercular susceptibility testing. Colonies were scraped from freshly growing colonies (three weeks old) on middle brook 7H10 plates and introduced into 10 ml of saline. Bacterial suspensions with 0.5 McFarland standard turbidity equivalents to 10^5 CFU/ml were prepared by dilution with saline. The tubes were vigorously vortexed for 30 seconds in a glass bottle containing glass beads and the particles were allowed to settle.

Preparation of test compounds for susceptibility testing

A stock solution, 2 mg/ml for crude extract, was prepared by suspending each test extract in absolute ethanol. Rifampicin was prepared as a 1 mg/ml stock suspension in absolute ethanol. The stock solutions were stored in aluminum foil-wrapped bottles at 4°C. As a high percentage of ethanol could be bactericidal, the amount of ethanol added to the growth medium was kept as low as possible in order to minimize the potential effect on growth of *M. tuberculosis*. A preliminary experiment was carried out to determine the maximum percentage of ethanol which could be included in the growth medium without growth inhibition of *M. tuberculosis* and it was found to be 0.8% (v/v) (data not shown). The final concentration of ethanol present in the growth medium was standardized at 0.4% (vol/vol) in this study. Before the test, each stock solution was serially diluted (threefold) in middle brook 7H9 broth to yield final concentrations of 2 to 2000 µg/ml for crude extracts. For rifampicin, the final concentrations were 0.02 to 1 µg/ml. Ethanol at 0.4% (vol/vol) was added to the growth medium to serve as a negative control.

Determination of the Minimum Inhibitory Concentration (MIC)

The micro plate method was performed to determine the MICs of test extract [9]. Briefly, a 100 µl volume of Middlebrook 7H9 broth was dispensed in each well of a 96-well cell culture plate. Serial dilutions of the test extracts (2000-2 µg/ml) were used to determine the minimum inhibitory concentration, using middle brook 7H9 as the medium. Perimeter wells of the plate were filled with sterile water to avoid dehydration of the medium during incubation. A standard bacterial suspension equivalent in turbidity to that of a no. 1 McFarland standard was prepared and diluted 1:20 in 7H9 broth; a 100 ml inoculum was used to inoculate each well of the plate. A growth control containing no test extract and a sterile control without inoculum were also included. Plates were sealed and incubated at 37°C for 4 week. The lowest concentration of the test extract in the test tubes with no visible or detectable bacterial growth was considered to represent the MIC.

Disc diffusion susceptibility test

Bacterial suspension was prepared from 14-days *M. tuberculosis* cultures grown on Middlebrook 7H11 agar slant, supplemented with 10% oleic acid, bovine serum albumin (fraction V), dextrose, and catalase (OADC; Remel, Lenexa, KS, USA) and 0.05% Tween 80. The turbidity of the suspension was adjusted to a McFarland no. 3 (9×10^8 CFU/ml) in sterile normal saline. The bacterium was spread on Middlebrook 7H11 agar plates, then discs with MIC concentration of test compounds were placed on the plates. All plates were incubated at 37°C for 4-8 weeks before measuring the diameter of the zone of inhibition of extract[10].

Determination of cytotoxicity of crude in VERO cells

The MTT assay was used to determine the cytotoxicity (IC_{50}) in VERO cells at concentrations of 50 to 500 µg/ml of crude extract. After 72 h of exposure, viability was assessed on the basis of cellular conversion of MTT into a formazan product using the Promega Cell Titer 96 Non-radioactive Cell Proliferation Assay [11]. Briefly, 20 µl of MTT solution (5 mg/mL PBS) were added to each well. Samples were incubated for further 4 h at 37°C in 5% CO2 and humidified air atmosphere. Then, 100 µl of 10% SDS were added to extract the insoluble product formazan, resulting from the conversion of the MTT dye by viable cells. The number of viable cells in each well was proportional to the intensity of the absorbance of light, which was then read in an ELISA plate reader at 570 nm. Absorbance (A) at 570 nm was measured 24 h later. To get cell survival (%), A of a sample with cells grown in the presence of various concentrations of the investigated extracts was divided with control optical density (the A of control cells grown only in nutrient medium), and multiplied by 100. It was implied that A of the blank was always subtracted from A of the corresponding sample with target cells. IC_{50} concentration was defined as the concentration of an agent inhibiting cell survival by 50%, compared with a vehicle-treated control.

Results

MIC

The data of antimycobacterial activity of crude extract are shown in Table 1. Two out of the nine crude extract tested were found to inhibit the growth of *M. tuberculosis* strains during incubation at 37°C for up to 42 days. The most effective compound was TSB+LB+ lactose, with a MIC of 125 µg/ml, followed by TSB+LB+ fructose(250 µg/ml) (Table 1). It appeared that effective MIC also represents the effective bactericidal concentration of the bacteria tested. The activity of the crude extract was compared with that of rifampicin and the data is shown in Table 1.

Disc diffusion

The data of disc diffusion assay of test extract was also shown in Table 1. Best activity was recorded by TSB+LB+ lactose (35 mm).

Table 1: Antimycobacterial activity of crude extract

Media	MIC	Diameter of zone of inhibition (mm)
TSB	-	-
LB	-	-
TSB+LB	-	-
TSB+LB+ fructose	250	23±0.77
TSB+LB+ maltose	-	-
TSB+LB+ glucose	-	-
TSB+LB+mannitol	-	-
TSB+LB+ sucrose	-	-
TSB+LB+ lactose	125	35±0.52
Rifampicin	28	0.5

- Not active

Cytotoxicity test

TSB+LB+ fructose and lactose alone was subjected to cytotoxicity activity. The two extracts were nontoxic to VERO cell up to 500 µg/ml. This clearly indicated that these extracts were safe for therapeutical purposes and its action is selectively targeted against the bacteria.

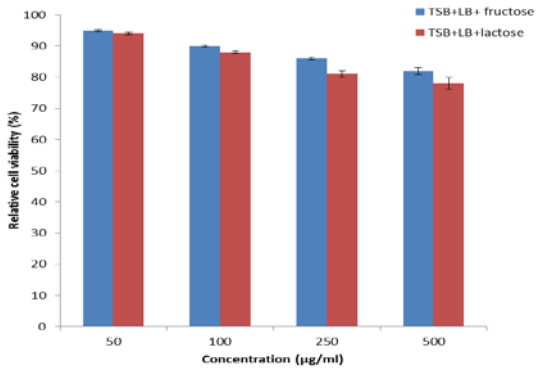

Fig. 1: Cytotoxic activity of crude extract against normal cell line

DISCUSSION

Natural products are a proven template for the development of new scaffolds of drugs [12] and they have received considerable attention as potential anti-TB agents20. The emergence of pathogenic microbes with increased resistance to established antibiotics provides a major incentive for the discovery of new antimicrobial agents [13]. Due to emergence of resistance to antibiotics amongst microorganisms especially *M. tuberculosis*, investigations for novel anti TB agents have always been one of the major preoccupations of medical society. Additional chemotherapeutic agents were recognized in the early 20th century. The discovery of antibiotics and other antimicrobial chemicals, and studies on their mode of action, have allowed us to control a great variety of TB. In the present study the crude extract from TSB+LB+lactose recorded significant antimycobacterial activity and purification of this extract in future may leads to pure compound which elevated antimycobacterial activity.

Rifampicin is typically used to treat *Mycobacterium* infections, including tuberculosis and Hansen's disease and the most serious adverse effect is related to rifampicin hepatotoxicity. In our present study significant activity and low cytotoxicity makes the crude extract as an ideal antimycobacterial drug after purification in the near future. This study is the first report of anti-mycobacterial activity of the crude extracts isolated from an entomopathogenic nematode against *M. tuberculosis*.

CONCLUSION

The results of this study have revealed that crude extract has reasonable antimycobacterial activity, and is relatively safe for use in as far as lethality is concerned. This therefore validates its use in the treatment of tuberculosis by traditional practitioners. However more studies on the purification of crude extract are needed for getting pure antimycobacterial active compounds.

CONFLICT OF INTERESTS

Declared None

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