**In-vitro Antimycobacterial Activities of Endophytic Bacteria Associated with Medicinal Plant of Manipur**

**Abstract**

Endophytic bacteria isolated from indigenous medicinal plant *Solanus xanthocarpum* were screened for antimycobacterial activities against *Mycobacterium smegmatis* using MTT assays. Of 18 isolates obtained 3 showed antimycobacterial activity. The crude extract of 3 bioactive isolates were tested against attenuated *Mycobacterium bovis* strain and pathogenic strain of *Mycobacterium tuberculosis* and their IC₅₀ values were calculated. Crude metabolites of 3 strains showed IC₅₀ values less than 100µg/ml. The 2 bioactive strains were identified as *Streptomyces* sp. and other as *Balkholderia fungorum*.

**Keywords:** *Solanus xanthocarpum*; Endophytic bacteria; Antimycobacterial activities, MTT assay; Mycobacterium

**Abbreviations:** TB: Tuberculosis; *M. tuberculosis*; *Mycobacterium tuberculosis*; MDR: Multi-Drug Resistant; XDR: Extensively-Drug Resistant; SCN: Starch Casein Nitrate Agar; *M. smegmatis*: *Mycobacterium smegmatis*; *M. bovis*: *Mycobacterium bovis*; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

**Introduction**

Tuberculosis (TB) is a well-known infectious disease caused by *Mycobacterium tuberculosis*, which commonly affects the lungs. It has afflicted humans since ancient times. Death causes by TB crosses 2 million globally with another 9 million new cases each year [1]. TB is responsible for more years of healthy life lost than any other infectious disease, bar AIDS and malaria [2]. The major problem with treatment of TB however lies in lack of effective treatment methods. With the emergence of multi-drug resistant (MDR) and extensively-drug resistant (XDR) strains of *M. tuberculosis* the disease has become a serious concern [3,4]. The alarming increase of MDR-TB cases therefore, requires a urgent development of new, more effective and less toxic side effects with nearly as active as rifampicin under *in vitro* condition.

Endophytic bacteria from medicinal plants have recently generated significant interest in the search for anti-TB drugs due to their immense potential to contribute to the discovery of new bioactive compounds. Due to close biological association between endophytes and their host plant there is more potential for discovering greater number of bioactive molecules compared to epiphytes or soil related bacteria [10,11]. The anti-TB produce by endophytes are likely to possess reduced cell toxicity as the bioactive compounds may not affect the eukaryotic host cell due to symbiotic relationship between endophytes and host plants. This is of significance to the medical community as potential anti-TB drugs may not adversely affect human cells [11].

**Keywords:** *Solanus xanthocarpum*; Endophytic bacteria; Antimycobacterial activities, MTT assay; Mycobacterium

The current study is based on the antimycobacterial screening of endophytic bacteria associated with indigenous medicinal plant of Manipur. It also deals with the study of antimycobacterial activity by crude extracts of bioactive strains.
Materials and methods

Isolation of endophytic bacteria

Endophytic bacteria were isolated from roots, stem, leaves and fruits of medicinal plant Solanum xanthocarpum (local name: Leipung-khanga) following the protocols of Qin et al. [12]. Starch Casein Nitrate Agar (SCN), Tap Water peptone Agar, Tap Water Yeast Extract, 2.5% Water Agar and Yeast Malt Agar were used as isolation medium. The purified cultures were preserved as agar slants (4°C) and glycerol stocks (20% v/v, -20°C) for further use [13].

Primary screening for antimycobacterial activity

Endophytic isolates were subjected to a preliminary determination of mycobacterial growth arrest and toxicity by culture filtrates (described in the following section) and MTT assays [14] using Mycobacterium smegmatis (mc2 155) as the indicator. Isolates showing antimycobacterial activity were selected for further experiment. All the experiments were performed in the laboratory (F-60) approved by the Institutional Biosafety Committee (N0.UH/SLS/IBSC/Review/SB-R-11 and SB-R-14) for Mycobacterial cultures by University of Hyderabad Institutional Biosafety Committee under Department of Biotechnology, Govt. of India.

Antimycobacterial activity by culture filtrates

Strains were inoculated in Starch Casien Nitrate (SCN) broth and kept incubated in a shaker (150 rpm, 30°C, 7d). The cultures were centrifuged (10,000 rpm for 10mins) and the supernatant collected were filtered through a membrane filter (0.2µm pore size). The pathogenic strain M. smegmatis was grown till the log phase in 7H9 [15] media and about 1x10⁶ cfu/ml of mycobacterium were spread onto the 7H10 [15] plates. 100µl of the culture filtrates were then incorporated in a well (6mm) and the plates were incubated at 37°C for 36 h. Clearing zone surrounding the well indicated antimycobacterial activity.

Extraction of crude metabolites

Extraction was done according to Kaaria et al. [16] with some modifications. Bioactive isolates were allowed to grow in 6 L SCN broth (150 rpm, 30°C, 7d). Culture broth was then centrifuged (10,000 rpm, 10 mins) and the supernatant collected were extracted two times with ethyl acetate. The organic phase was allowed to pass through a pad of anhydrous sodium sulphate and evaporated to dryness using Rotary Evaporator (Stuart, Bibbly Scientific Limited).

The extracts were used for determining the cytotoxicity assay against attenuated M. bovis BCG and pathogenic strain of M. tuberculosis H37Rv. Mycobacterium strains were allowed to grow in 7H9 media supplemented with 10% Oleic Albumin Dextrose Catalase (OADC) till the log phase and about 1x10⁶ cfu/ml were seeded into microplate, incubated for 24-36 hours at 37°C. Inculum size was prepared using Macfarland standards (0.1 OD₅₅₀ corresponds to 1x10⁶ cfu/ml, 20µl of 5mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and incubated for 4h. The formazan crystals formed were dissolved in DMSO and the plates were read at 540 nm in Enzyme-linked immunosorbent assay (ELISA reader) and IC₅₀ values were calculated.

Characterization of bioactive strains

Genomic DNA isolation was done according to Li et al. [17]. The 16S rRNA gene sequence was amplified using the primers 8F (5’-CAGAGTTTGATCCTGCTGCT-3’) and 1522R (5’-AGGAGGTGATCCTGCA-3’) (IDT, USA). The primers were designed based on the 16S rRNA gene sequence of E. coli [18]. The almost complete 16S rRNA gene sequence of the strain was identified using the EzTaxon-e server database [19] and aligned with the 16S rRNA gene sequences of related species using CLUSTAL X version 2.1 [20].

Results and Discussion

Traditionally, soil-derived bacteria have been most frequently screened for bioactive compounds against M. tuberculosis. Unfortunately, the frequency of finding new bioactive compounds from normal soil-derived bacteria is declining because of the redundancy in the isolation of known bacteria and antibiotics. Alternatively, bacteria from previously unexplored or under explored environments such as medicinal plants, marine, desert and forest ecosystems are screened [21]. In recent years, pathogenic microorganisms are gaining resistance against antimicrobial agents; hence the search for new, safe and more effective antimicrobial agents is an urgent need for the emerging multidrug resistance [6]. Endophytes from medicinal plants have recently given significance due to their immense potential to contribute to the discovery of novel anti-TB compounds. Endophytic bacteria have been reported to possess wide spectrum activity against many pathogenic fungi and bacteria [22,23].

A total of 18 putative strains were isolated from medicinally important plant of Manipur. Based on the preliminary screening for antimycobacterial activity against M. smegmatis, 3 most promising isolates viz. SxF1 (isolated from Fruit), SxF2 (Fruit) and SxL6 (Leaves) were selected for study. The culture filtrates of the 3 strains also exhibited clearing zone of inhibition against M. smegmatis.

Crude extract strains SxF1 and SxL6 showed showed good antimycobacterial activity with IC₅₀ values of 25 and 12.5µg/ml. However, the IC₅₀ value of SxF2 was larger than 100 (Table 1 & Figure 1). Crude extract of strains SxF1, SxF2 and SxL6 showed IC₅₀ values of less than 100µg/ml against H37Rv. The extracts from SxF2 though effective against M. bovis BCG (IC₅₀=22.6µg/ml) was not as effective against M. tuberculosis H37Rv (IC₅₀=652µg/ml) (Table 1). The culture filtrates and crude metabolites extract of Streptomyces sp. has been reported to exhibit antimycobacterial activity against M. tuberculosis H37Rv [6,24]. Similarly, culture filtrate and crude extract of Brevibacillus laterosporus isolated from soil inhibit the growth of Mycobacterium sp. [25]. Penialidin C produced by endophytic fungi Penicillium sp. showed antimycobacterial activity against M. smegmatis [26]. Strain SxF1 was found to be closely related to Streptomyces harbinensis (99.51%), SxL6 was closely related to Streptomyces herbinensis (99.76%). SxF2 was identified as Bulkholderia fungorum (100%). To our knowledge, this is the first report of Bulkholderia sp. having antimycobacterial activity [27-32].
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Table 1: IC50 values of the crude extracts of bioactive strains.

| S. No. | Isolate | M. Bovis (BCG) | M. Tuberculosis (H37Rv) |
|--------|---------|----------------|------------------------|
| 1      | SxL6    | 83.76 µg/ml    | 62.48 µg/ml            |
| 2      | SxF1    | 24.902 µg/ml   | 74.70 µg/ml            |
| 3      | SxF2    | 22.6 µg/ml     | 652.5 µg/ml            |

Conclusion

Of 18 endophytic bacteria obtained from medicinal plant *Solanum xanthocarpum*, 3 strains showed antimycobacterial activity under primary screening. Crude metabolites of the 3 strains also exhibited good antimycobacterial activity against the tested *Mycobacterium* sp. However, crude extract of *Bulkholderia fungorum* was not effective against *M. tuberculosis H37Rv*. The antimycobacterial compound(s) release by the bioactive strains can be used for further study for development of new, more effective and safer anti-tuberculosis in order to control the alarming increase of MDR-TB cases in the developing country especially India.

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References

1. Tsara V, Serafi E, Christaki P (2009) Problems in diagnosis and treatment of tuberculosis infection. Hippokratia 13(1): 20-22.
2. Corbett EL, Watt CJ, Walker N, Maher D, Williams BG, et al. (2003) The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. Arch Intern Med 163(9): 1009-1021.
3. Venkataraman P, Paramasivan CN (2003) Drug resistance in tuberculosis and issues related to multi-drug resistance in planning for TB control in India. Health Administrator 15: 127-136.
4. Pandya K, Patel P, Patel G, Parikh V (2012) In Vitro Antimycobacterial study of essential oil of few selected plants - Part 2. International Journal of Universal Pharmacy and Bio Sciences 2(2): 150-155.
5. Harley RM, Atkins S, Budantsev A, Cantino PD, Conn BJ, et al. (2004) The families and genera of vascular plants. (7th edn), Springer Verlag, Berlin, Germany, pp. 167-275.
6. Radhakrishnan M, Suganya S, Balagurunathan R, Kumar V (2010) Preliminary screening for antibacterial and antimycobacterial activity of actinomycetes from less explored ecosystems. World J Microbiol Biotechnol 26(3): 561-566.
7. Radhakrishnan M, Balagurunathan R, Selvakumar N, Doble M, Kumar V (2011) Bioprospecting of marine derived actinomycetes with special reference to antimycobacterial activity. Ind J Geo-Marine Sc 40(3): 407-410.
8. Heinrich M, Gibbons S (2001) Ethnopharmacology in drug discovery: an analysis of its role and potential contribution. J Pharm Pharmacol 53(4): 425-32.
9. Halberstein R (2005) Medicinal plants: historical and cross-cultural usage patterns. Ann Epidemiol 15(9): 686-699.
10. Newton SM, Lau C, Wright CW (2000) A Review of antimycobacterial natural products. Phytother Res 14(5): 303-322.
11. Strobel G, Daisy B (2003) Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol Rev 67(4): 491-502.
12. Alvin A, Miller KJ, Neilan BA (2014) Exploring the potential of endophytes from medicinal plants as sources of antimycobacterial compounds. Microbiol Res 169(7-8): 483-495.
13. Qin S, Wang HB, Chen HH, Zhang YQ, Jiang CL, et al. (2008) *Glycomyces endophyticus* sp. nov., an endophytic actinomycete isolated from the root of Carex baccans Nees. Int J Syst Evol Microbiol 58(pt 11): 2525-2528.
14. Tamreihaok N, Nimaichand S, Chanu SB, Devi KA, Lynda R, et al. (2016) Acidosulfuric *Streptomyces sp*. MBR 10 from limestone quarry site showing antagonism against fungal pathogens and growth promotion in rice plants. Journal of King Saud University-Science.
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15. Montoro E, Lemus D, Echemendia M, Martin A, Portaels F, et al. (2005) Comparative evaluation of the nitrate reduction assay, the MTT test, and the resazurin microtitre assay for drug susceptibility testing of clinical isolates of mycobacterium tuberculosis. J Antimicrob Chemother 55(4): 500-505.

16. Kaaria P, Maturu V, Ndungu M (2012) Antimicrobial activities of secondary metabolites produced by endophytic bacteria from selected indigenous Kenyan plants. Afr J Microbio Res 6(45): 7253-7258.

17. Li WJ, Xu P, Schumann P, Zhang YQ, Peckall R, et al. (2007) Georgenia ruanii sp. nov., a novel actinobacterium isolated from forest soil in yunnan (China), and emended description of the genus georgenia. Int J Syst Evol Microbiol 57(pt 7): 1424-1428.

18. Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, et al. (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl Environ Microbiol 64(2): 795-799.

19. Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, et al. (2012) Introducing EzTaxon-e: a prokaryotic 16S rRNA Gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Microbiol 62(pt 3): 716-721.

20. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, et al. (2007) Clustal w and clustal X version 2.0. Bioinformatics 23(21): 2947-2948.

21. Berdy J (2005) Bioactive microbial metabolites. J Antibiot (Tokyo) 58(1): 1-26.

22. Castillo U, Strobel GA, Ford EJ, Hess WM, Porter H, et al. (2002) Munumbicins, wide spectrum antibiotics produced by Streptomyces munumbi, endophytic on Kennedia nigriscans. Microbiology 148(pt 9): 2675-2685.

23. See WT, Lim WJ, Kim EJ, Yun HD, Lee YH, et al. (2010) Endophytic bacterial diversity in the young radish and their antimicrobial activity against pathogens. J Korean Soc Appl Bio Chem 53(4): 493-503.

24. Radhakrishnan M, Balagurunathan R, Selvakumar N, Doble M, Kumar V (2011) Bioprospecting of marine derived actinomycetes with special reference to antimycobacterial activity. Ind J Geo Marine Sci 40(3): 407-410.

25. Hassi M, Guendouzi SE, Haggoud A, David S, Ihsnouda S, et al. (2012) Antimycobacterial activity of a Brevibacillus laterosporus strain isolated from a Moroccan soil. Braz J Microbiol 43(4): 1516-1522.

26. Jouda JB, Mawabo IK, Notedji A, Mbazoa CD, Nkenfou J, et al. (2016) Anti-mycobacterial activity of polyketides from Penicillium sp. endophyte isolated from Garcinia nobilis against Mycobacterium smegmatis. Int J Mycobacteriol 5(2): 192-196.

27. Bauer AW, Kirby WM, Sherris JC, Turck M (1966) Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol 45(4): 493-496.

28. Brewer TF, Heymann SJ (2004) To control and beyond: moving towards eliminating the global tuberculosis threat. J Epidemiol Community Health 58(10): 822-825.

29. Hui Sun, Yan He, Qing Xiao, Renyuan Ye, Yongqiang Tian (2013) Isolation, characterization, and antimicrobial activity of endophytic bacteria from Polygonum cuspidatum. Afr J Microbiol Res 7(16) 1496-1504.

30. Madigan MT, Martinko JM, Parker J (1997) Brock biology of microorganisms. (8th edn), Prentice Hall International Inc, New Jersey, USA, pp. 440-442.

31. Razzagh Mahmoudi (2014) An overview of using some essential oils in functional dairy products from Iran. Malaysian J Sci 33(1): 3-8.

32. Yuan WM, Crawford DL (1995) Characterization of Streptomyces lydicus WYEC 108 as a potential biocontrol agent against fungal root and seed rots. Appl Environ Microbiol61(8): 3119-3128.