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SCREENING AND IDENTIFICATION OF ANTI-MYCOBACTERIAL PLANTS

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ABSTRACT

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (MTb) and is one of the majorcauses of death in the world. One third of the population suffers with TB and there is no potential drug introduced in the past 5 decades. The drugs currently used are ineffective due to the emergence of MDR (Multidrug Resistant Strain), XDR (Extreme Drug Resistant strain) and other co-infections. Hence there is a need for a potent molecule that can inhibit the growth of mycobacterium effectively. The plant kingdom provides a large variety of naturally occurring phytochemicals and other secondary metabolites which are known to be antibacterial. Therefore the present study focuses on screening and identification of potent anti-mycobacterial plants. Various parts of the plant including leaves are traditionally used for treating a wide spectrum of diseases. In the screening studies of dry leaf (methanolic extracts) of plants from the Western Ghats region gave rise to two potent anti-mycobacterial plants. These plants were identified as *TithoniadiversifoliaHemsl. A. Gray*(TD) and *CouroupitaguianensisAubl.*(CG). Both TD and CG plants showed significant anti-mycobacterial activity conducted by well diffusion assay having clearance zone of 15.6 mm and 17.3 mm respectively. These plant extracts were further purified with polar solvents and potential anti-mycobacterial extracts were tested for their minimum inhibitory concentrations (MICs). The MICs for TD ethyl acetate extract and CG chloroform extract were reported as less than 64µg/ml and 64µg/ml respectively. The entire investigation of anti-mycobacterial study was conducted against *Mycobacterium smegmatis* whichis a model organism for mycobacterial studies. These results show that both plants contain potent anti-mycobacterial compound(s) that can be further investigated for purification and isolation of bioactive compound which can be useful in the production of novel drugs.

KEYWORDS: ***Tuberculosis, Anti-mycobacterial, Phytochemical, Antibacterial, Tithoniadiversifolia, Couroupitaguianensis.***

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INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by single infectious agent *Mycobacterium tuberculosis* (MTb) that exists for more than thousand years. Nowadays, TB is a major concern worldwide as it has found a place among the top ten causes of death ranking above the human immunodeficiency virus (HIV) according to the WHO reports.[1] Though TB is curable, diagnosis and treatment becomes challenging in the latent stage of tuberculosis infection (LTBI) due to no clinical manifestation of the active disease.[1]MTb spreads through aerosols and causes both pulmonary and extrapulmonary infections and this results in multiorgan disease. The MTb bacteria has the ability to remain dormant for long period and gets reactivated in immunosuppressed conditions.[2] In general, TB treatment takes 6-9 months with multiple drug regimen which in turn causes health adversities in the patient.[3] Patients with non-compliance during the period of treatment lead to resistance to the drugs giving rise to multiple drug resistant (MDR), extreme drug resistant (XDR) and totally drug resistant (TDR) strains.[4] Such drug resistant strains are difficult to control and it leads to the increased risk of transmission.Rifampicin, a potent anti-tubercular drug was introduced as first line drug along with isoniazid, pyrazinamide and ethambutol.[5] Amongst the recent additions of drugs against MDR-TB, bedaquiline and delamanid are approved as successful drugs.[1] To eradicate TB infection in the coming years, development of more potent and new anti-mycobacterial compound/s is necessary. For many centuries plant based medicines are being used for the treatment of various human ailments including TB. Plants are an abundant source of biologically active compounds serving as prototype for drug discovery.[2,6] Eighty percent of the world's population rely on medicinal plants as their primary source of healthcare especially in developing countries.[7] Natural products derived from any part of the plant serves as a lead molecule in the field of pharmacology as they are known to be sterically complex and target specific.[8,9] Use of eco-friendly, natural plant based products for treating different diseases has increased due to adverse effects of synthetic drugs. India, a country with rich biodiversity, spanning with variety of ecosystem across the altitudes has many untapped natural resources.[10]A systematic screening of plant species may lead to the development of a potent anti-mycobacterial drug/s. According to the report by UNESCO, the Western Ghats or the Sahyadrimountains running parallel to the west coast of India is a "hot spot" of biological diversity in the world. Amongst which, many plants species are used in traditional medicine system for curing TB or TB symptoms. The antimycobacterial studies are normally conducted on the *Mycobacterium smegmatis* (MSM), a soil-dwelling mycobacterial model as it is non-pathogenic and fast-growing bacteria. The bacteria also shares similar cell wall composition and drug resistance properties of MTb with a biosafety level 2. The usage of MSM as a surrogate has been used successfully to identify some target drugs and in understanding the physiology of the mycobacterial infection.[11] Therefore, the present study is focused on the screening of plants having potential anti-mycobacterial activity against MSM, a model organism, and evaluates the minimum inhibitory concentration of the screened plants.

MATERIALS AND METHODS

Media and Chemicals

All the chemicals and solvents were procured from Hi-media and Fischer Scientific respectively. LB agar, peptone, yeast extract, sodium chloride, kanamycin A, dimethyl sulphoxide, methanol, hexane, ethyl acetate and tween 80.

Bacterial Strain

The *M. Smegmatis* culture was purchased from MTCC (wild type -mc^{2}155). The lyophilized culture was activated by sub-culturing on LB-Tween plate. On better growth of single colonies, the loop full of bacteria was further inoculated in 5 ml of LB-Tween broth and incubated at 37°C for 48hrs. The incubated broth was used for antimycobacterial activity of the plant extracts. The parent culture was subjected to prepare glycerol stock and stored at -80ºC deep refrigeration for further use.

Plant Material

The matured, uninfected leaves of plants (15 numbers) used as remedy by the farmers and locals were collected from Western Ghats region of Balehonnur village, Chikkamagaluru district, Karnataka, India. The leaves were washed and allowed to dry completely. They were then powdered and then used for the extraction.

Preparation of the Extracts

Crude extract was prepared using 1g of powdered

plant sample homogenised with 10 ml of methanol and filtered using Whatman filter paper no.1. The filtrate was collected in a pre-weighed crucible and then concentrated by evaporating the solvent at room temperature. The crude extract was weighed and resuspended in a known volume of dimethyl sulphoxide (DMSO) and stored at 4ºC.

Antimicrobial Assay

The screening of the various plant extracts was performed by Agar well diffusion method as described by Sivakumar*et al*.with minor modification.[12] 0.1 ml of 48hrs grown MSM culture (OD-0.6nm) was smeared uniformly on the LB-Tween agar plates. Wells were made on the LB-Tween agar plates with a standard cork borer of 8 mm diameter. 20 µl of the crude plant extract was added to each well and the plates were incubated at 37°C for 48hrs. The kanamycin A and DMSO was used as positive and negative control respectively. The anti-mycobacterial activity was assayed by measuring the diameter of zone of inhibition (ZI) formed around the well in millimetres (mm). The experiment was performed in triplicates. The plant which shows highest anti-mycobacterial activity was subjected to identification.

Fractionation of Crude Plant Extract

The screened plant powder was subjected to hot extraction by soxhlet using methanol as the solvent. The solvent extract was allowed to concentrate using rotary evaporator (Buchi rotavapor-R3) and poured in a pre-weighed crucible. The obtained concentrated methanolic extract was subjected to solubilization using methanol-water (2:3 ratio). Then the extract was fractionized in liquid-liquid solvent extraction with varying organic solvents (n-hexane, chloroform and ethyl acetate) using a separating funnel. Fractions collected were subjected to evaporation at room temperature and dissolved in a known volume of DMSO. Anti-mycobacterial activity of the different fractions was determined by well diffusion assay.

Minimum Inhibitory Concentration [MIC] of the Plant Extracts

Minimum inhibitory concentration of the most potent fractionation of plant extracts was determined by broth microdilution method using sterile LB-Tween broth (test media). 100µl of test media was added to 96 well microtiter-plates. The active fractions of plants extract along with control antibiotic (kanamycin A) was prepared in a series of two-fold dilution ranging from 0.25µg/ml to 128 µg/ml. 50 µl of these diluted extracts was added to the test media in microtiter plates. 100 µl of *M. smegmatis* inoculum (OD-0.6nm) was added to all the wells and the plates were covered with lid and sealed with parafilm to avoid contamination and evaporation. The plates were placed in shaker incubator at 37°C for 48hrs. The lowest concentration of the plant extract that did not give any visible growth was considered as the MIC.[13]

RESULT

Bacterial Strain

Figure 3.1 shows the growth pattern of *M. smegmatis* MTCC wild type mc^2155 on LB-Tween media. Quadrant streaking gave rise to single isolated colonies was used for the anti-mycobacterial assay.

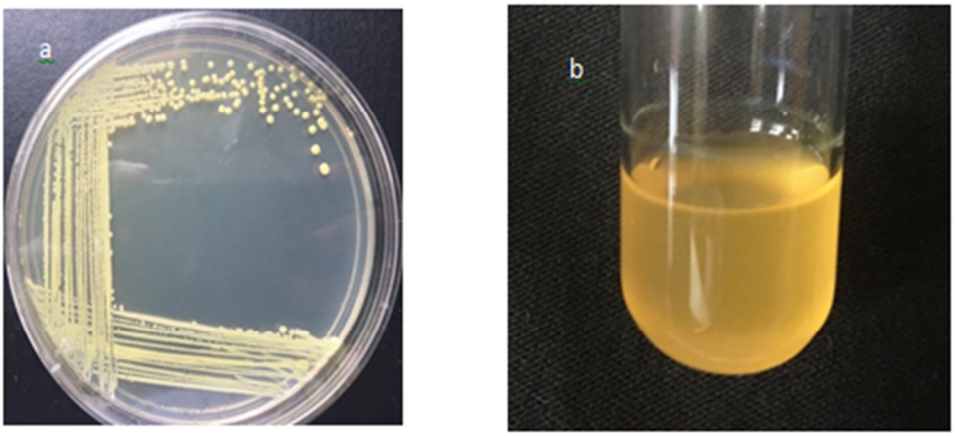

Figure 3.1.
Growth of M. smegmatis MTCC wild type mc^2155 on LB-Tween media a) Streak plate b) Broth.

Anti-mycobacterial screening of Plant extract against M. smegmatis

In the preliminary screening, five plants showed anti-mycobacterial activity against *M. Smegmatis*. Among these, only two plants (JU-5, JU-13) showed high potency of anti-mycobacterial activity with a significant zone of clearance. Figure 3.2 shows the ZI of plant extract on LB-Tween media. Similarly Table 3.1 shows diameter of ZI against MSM.

Table 3.1
Anti-mycobacterial activity against MSM

Sample	Average Zone of Clearance (mm)
PC- Positive control	6.6
NC – Negative control	-
JU-5	15.6
JU-7	6.0
JU-8	7.6
JU-13	17.3
JU-14	3.6

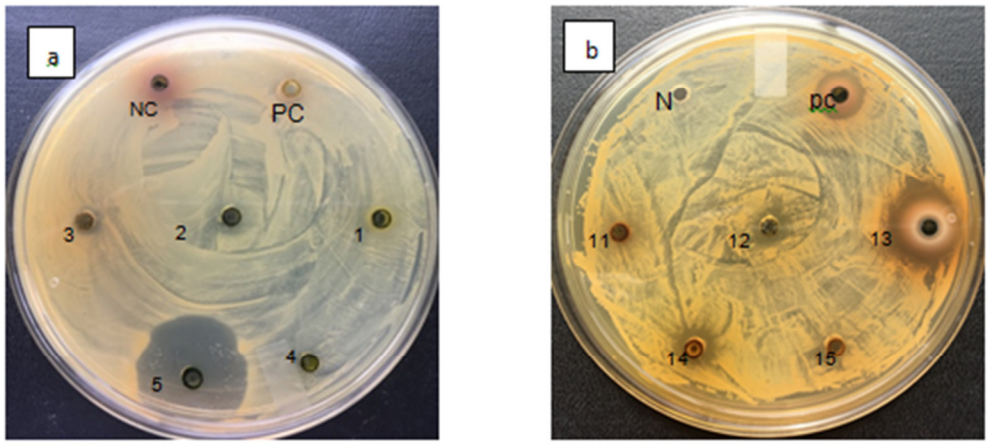

Figure 3.2
Well Diffusion Assay of plant extracts showing largest zone of clearance were sample (a) JU-5 and (b) JU-13

Identification of the Plant Samples

The active plant samples were identified at the Regional Ayurveda Research Institute for Metabolic Disorders, Bangalore. The plant samples were confirmed by giving reference numbers. (Table 3.2).

Table 3.2
Plant samples identified by Regional Ayurveda Research Institute for Metabolic Disorders, Bangalore.

S.no.	Sample	Plant name	Reference No.
1.	JU-5	Tithoniadiversifolia (Hemsl.)A.Gray	RRCBI-mus 193
2.	JU-13	CouroupitaguianenisisAubl.	RRCBI-mus 129

Fractionation of Plant Extract

Table 3.3 (Fig. 3.3 a, b) shows the average ZI of plants having potential anti-mycobacterial activity in different solvent fractions.

Table 3.3
Fractionated plant extract showing Zone of inhibition (ZI in mm)

S. No.	Solvent	Average zi	
		JU-5	JU-13
1.	Hexane	0	0
2.	Methanol water	0	0
3.	Chloroform	0	22
4.	Ethyl acetate	20	0
5.	Control (DMSO)	0	0

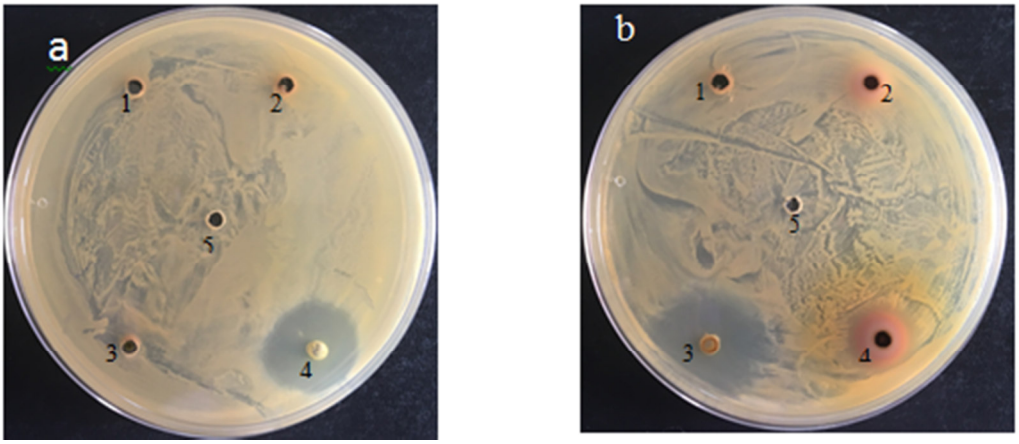

Figure 3.3a-
JU-5.1-hexane fraction, 2- methanol water fraction, 3-chloroform fraction,4-ethyl acetate fraction,5-control.
Figure 3.3b-
JU-13. 1- hexane fraction,2-methanol water fraction ,3-chloroform fraction,4-ethyl acetate fraction,5-control.

Minimum Inhibitory Concentration
Table 3.4 shows the MIC of the two active plant fractions with Kanamycin A and DMSO as the positive and negative control respectively.

Table 3.4
Minimum inhibitory concentration (MIC in µg/ml)

S. No.	Plant extract	Mic
1.	JU-5 (Ethyl acetate)	>64
2.	JU-13 (Chloroform)	64
3.	Kanamycin-A (positive control)	2
4.	DMSO broth (negative control)	0

DISCUSSION

To eradicate the MTb in the coming years, new and potent drug molecules are to be discovered. Due to complexity and toxicity of the animals, the effect of molecules might differ when tested in-vivo although most of the potent anti-tubercular molecules give excellent in-vitro results. The spread of TB and TB related co-infections have increased due the side effects of synthetic drugs, inadequate prognosis and non-compliance of the patients to the drug regime.[4] Developing MDR and LTBI strains has also become a major limitation in synthetic drug treatment.To overcome these challenges, a potential plant based molecules are considered in this study. Plants are known to be a repository of natural compounds that fight against **a** large number of diseases. Plant based medicines have been used traditionally to treat various

diseases in Ayurveda and Siddha.[14] Since India has rich plant diversity, screening a potential anti-tubercular plant could contribute significantly to the health sector. Results of the present study screened potential anti-mycobacterial plants from the Western Ghats of India. The identified plants - *Tithoniadiversifolia (Hemsl.) A. Gray* (TD) and Couroupita*guianensis*Aubl.(CG) are of Mexican and tropical South American origin that later got distributed all over the Europe and Asian subcontinents.TD leaves are traditionally used for treating a wide spectrum of diseases that include topical application for skin diseases, wound healing, stomach ache and oral administration for treating fever, diabetes, malaria, hepatitis and other infectious diseases.[15] CG tree also known as the cannon ball tree grown in India is a sacred tree in the Shiva temples. The tree possesses antibiotic, anti-inflammatory, antifungal, antiseptic, antipyretic and analgesic properties.[16]theamazonian natives are also known to use infusions obtained from the flower, bark and leaves of the cannonball tree to treat hypertension, tumours and pain.[17]the leaves of the CG are used specifically for the treatment of the skin diseases, toothache, wound healing and malaria.[18]The study performed by Obafemi and co-workers reported that ethyl acetate extract of TD leaves have most potent antibacterial activity against gram positive and negative bacteria.[19] The aqueous extract of TD leaves are inactive against gram positive (*M.foliorum , B.subtilis, S.aureus and R.equi*) and gram negative (*P.aeruginosa*) bacteria. A similar study reported that the dichloromethane extract of TD leaves gave a MIC of 25 mg/ml and ZI of 18mm against *S.aureus.*[20] Another research carried out by Liao *et al.*reported that one of the bioactive component tagitinin C induces cell death in cancer cells by down-regulating the survivinexpression.[21] The results of the present study also proved that TD plant has potential anti-mycobacterial activity (ZI of 20mm and MIC of 64µg/ml). The phytochemical analysis of CG plant leaves, fruits and flowers shows antibacterial activity in the ethyl acetate and chloroform fractions.[22] The experimental reports have shown that chloroform extract of the CG fruit shows less activity against MTb in comparison with rifampicin whereas the leaves of CGpossess a broad spectrum antibiotic activity against various human pathogens.[23] In another experiment, 50µl concentration of CG leaves extracted with acetone gave an antibacterial activity of 22 mm and 19 mm ZI for *Bacillus* sp. and *Staphylococcus* sp. respectively. The dichloromethane fraction shows 24 mm ZI for *Staphylococcus* sp. alone.[12]similarly the present study also shows a significant anti-mycobacterial activity in chloroform extract. Gautam and co-workers have reported around 25 plant species from Himalayas to tropics as potential antitubercular with MIC values ranging from 10-100µg/ml.[14] Similarly a study of Himalayan orchids also showed the active antibacterial components in the low polar solvent extracts.[10] According to Kuete*et al.* MIC less than 100µg/ml should be considered as potent and MIC between 100-625 µg/ml is moderate. This is a well-documented standard for antibacterial activity of any extract or compound/s.[24]In that case *Tithoniadiversifolia* (Hemsl.) *A. Gray* and *CouroupitaguianensisAubl.*Of this study can be considered as potent anti- mycobacterial plants.

CONCLUSION

The plants that were screened and identified in this study have significant anti-mycobacterial activity and they are eventually traditional medicinal plants used for the treatment of various other diseases as well as for curing symptoms of TB. Further investigation includes purification and characterisation of metabolites which may lead to the development of potent drug candidates against TB.

AUTHOR'S CONTRIBUTIONSTATEMENT

The study was framed and designed under the guidance of Dr.Aranganathan V. and all the experiments to support the study were performed by NiveditaPriyadarshini (Research scholar). The manuscript writing was also initiated under the guidance of Dr.Aranganathan V.

CONFLICT OF INTEREST

Conflict of interest declared none.

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