Original Article

Antimycobacterial Screening of Selected Medicinal Plants against *Mycobacterium tuberculosis* H_{37}Rv using Agar Dilution Method and the Microplate Resazurin Assay

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Abstract

Background: Tuberculosis (TB), has been serious disease to the global human population causing millions of deaths worldwide. The recent increase in the number of multi-drug resistant clinical isolates of *Mycobacterium tuberculosis* has created an urgent need for the discovery and development of new anti-TB drugs. Medicinal plants have had a great influence on the daily lives of people living in developing countries, particularly in India. Medicinal plants were selected, and they were evaluated for its anti-TB activity against the pathogenic strain of *M. tuberculosis* H_{37}Rv. **Methods:** Eleven medicinal plants were selected on the basis of its literature survey, and three different extracts were prepared. Antimycobacterial activities were screened using two *in vitro* assays, namely agar dilution assay and microplate resazurin assay against *M. tuberculosis* H37Rv at different concentrations of prepared extracts. We analyzed minimal inhibition concentrations and percentage of inhibition of the used strain of *Mycobacteria*. Isoniazid was used as a standard anti-TB drug. **Results:** The results of this study showed that aqueous extracts four selected medicinal plants *Ocimum sanctum*, *Adhatoda vasica*, *Leptadenia reticulata*, and *Cocculus hirsutus* having minimum inhibitory concentration at 500 µg/ml, 500 µg/ml, 250 µg/ml, and 250 µg/ml, respectively, and *O. sanctum* (60.24%), *A. vasica* (62.89%), *L. reticulata* (74.26%), and *C. hirsutus* (81.67%) showed significant anti-TB activity against *M. tuberculosis*. **Conclusion:** This study helps society to found new anti-TB agents having better anti-TB activity with lesser or no side effects.

Keywords: Agar dilution method, 11 selected medicinal plants, isoniazid, microplate resazurin assay, *Mycobacterium tuberculosis*

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Introduction

Human tuberculosis (TB) is a transmittable infectious disease chiefly caused by *M. tuberculosis*, which is an aerobic pathogenic bacterium that creates its septicity usually in the lungs.[1] The World Health Organization estimates that active cases of TB afflict 7–8 million people annually and lead up to three million deaths per year.[2,3]

Because natural products are a proven template for the development of new scaffolds of drugs, they have received considerable attention as potential anti-TB agents. Antimycobacterial active compounds have been found not only from terrestrial plants but also from other organisms such as fungi and marine plants and animals.[4] Due to the important role medicinal plants play in the process of drug discovery and development, they are widely recognized as sources of active antimicrobial metabolites.[5] Thus, there is a great potential in finding medicinal plants with activity against *Mycobacterium* species. Therefore, 11 medicinal plants were selected to screen anti-TB activity against the pathogenic strain of *Mycobacteria,* i.e., *M. tuberculosis* H_{37}Rv using two *in vitro* assays, namely agar dilution assay and the microplate resazurin assay.

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Methods

Selection of medicinal plants

The selection of plants was mainly based on ancient literature review and research article based review. The plants were selected having anti-TB effect, hepatoprotective effect, immunomodulatory action, and having ability to enhance bioavailability.

Plant collection and authentication

Dried plant materials of 9 selected plants (fruits of *Emblica officinalis,* fruits of *Terminalia bellerica*, fruits of *Terminalia chebula*, roots of *Withania somnifera*, rhizomes of *Cyperus rotundus*, rhizomes of *Alpinia galanga*, leaves of *Ocimum sanctum*, leaves of *Adhatoda vasica,* and fruits of *Piper longum*) out of 11 selected plants were procured from Ayurvedic Store of Gandhinagar, and fresh plant materials of 2 selected plants (roots of *Leptadenia reticulata* and whole herb of *Cocculus hirsutus*) out of 11 selected plants were collected from Dhandhiya village of Rajkot district, Gujarat, India. The procured materials of the 11 selected medicinal plants were authenticated.

Preparation of plant extracts

Preparation of alcoholic, hydroalcoholic, and aqueous extracts of the 11 selected medicinal plants

One hundred grams of the powder of the 11 selected plants, i.e., fruits of *E. officinalis*, fruits of *T. bellerica*, fruits of *T. chebula*, roots of *W. somnifera*, rhizomes of *C. rotundus*, rhizomes of *A. galanga*, leaves of *O. sanctum*, leaves of *A. vasica,* fruits of *P. longum*, roots of *L. reticulata,* and whole herb of *C. hirsutus,* was taken to prepare its different extracts. Three different extracts, i.e., alcoholic, hydroalcoholic (30:70 water: alcohol), and aqueous extracts, were prepared by maceration of raw material of the 11 selected medicinal plants for 48 h in respective solvents. It was then refluxed for about 1 h with occasional shaking, consecutively three times, and filtered. The filtrates were pooled and concentrated to dryness, and percentage yield was calculated. The prepared extracts were labeled and stored in an airtight container for further use.

Antimycobacterial activity

Procurement and culturing of Mycobacterium tuberculosis freeze-dried culture

Mycobacterium tuberculosis $H_{37}Rv$ strain is the most pathogenic strain among all the different species of *Mycobacterium*. It is procured from the American Type Cell Culture (ATCC), USA, *M. tuberculosis* (strain ATCC 27294/$H_{37}Rv$). It is obtained as a freeze-dried (frozen) culture.[6]

Maintenance of the *Mycobacterium tuberculosis*

Chemicals and instruments

Lowenstein Jensen Medium M-162 (LJ Medium) (HiMedia), Middlebrook 7H10 Agar Base (HiMedia), Middlebrook 7H9 Broth (HiMedia), Middlebrook ADC Growth Supplements (HiMedia), glycerol, Ziehl–Neelsen carbol fuchsin dye, methylene blue, Tween 80 (Sigma), biosafety cabinet II, autoclave, Research Centrifuge (Eltek), incubator, Reagent Bottle Screw Cap – 250 ml and 500 ml (Tarson), digital weight balance, Disposable Petri Plates (HiMedia), colony counter machine, and micropipettes were used.

Mycobacterial strains and growth conditions

The mycobacterial strains were maintained on Lowenstein–Jensen slopes and cultured on enriched media comprising Middlebrook 7H9 broth supplemented with 0.05% (v/v) Tween 80, 10% (v/v) oleic acid–albumin–dextrose–catalase (OADC), 1.0 g of casitone per liter, and 0.2% (v/v) glycerol. Cultures were incubated at an aerobic atmosphere at 37°C.

Agar dilution method

Principle of agar dilution method[7]

Agar dilution is the most commonly used technique to evaluate the minimal inhibitory concentration of test substances that helps to kill or inhibit the growth of microorganisms.[8] The main aim of this method is to determine the lowest concentration of plant extract under defined test conditions and inhibit the visible growth of the bacteria being investigated. Minimum inhibitory concentration (MIC) values are used to determine the susceptibilities of bacteria to drugs and also to evaluate the activity of new antimicrobial agents. This method is used when a quantitative method is required for *Mycobacteria* with a variable growth rate and aerobic microorganisms.[9]

Method of agar dilution assay[10]

Prepare Middlebrook 7H10 agar media and autoclave this medium at 15 lbs pressure (121°C) for 10 min. Aseptically add OADC supplements and glycerol in prepared 7H10 agar media. Different concentrations of the 11 selected medicinal plant extracts were prepared and sterile all the extracts by passing through syringe filter of pore size 22 µm. Different concentrations of the 11 selected medicinal plant extracts (500, 250, 125, 62.5, 31.25, and 15.62 µg/ml) were incorporated into the prepared autoclaved agar medium before it solidifies. By the application of a standardized number (0.5 McFarland standard dilutions) on the surface of the agar plate, the Petri plates used for antitubercular screening were incubated at 37°C for 7 days. All the concentrations were done in triplicate to minimize the error. The MIC was determined by visual growth of *Mycobacteria* on agar plates. Record the MIC of all tested extracts for further analysis. Isoniazid was used as a standard anti-TB drug. There are several advantages of the agar dilution method including capacity to test different strains at once, easy detection of contamination, and capacity to test opaque materials.

The microplate resazurin assay

Principle of microplate resazurin assay

Alamar Blue or microplate resazurin assay works as a viability indicator through the conversion of resazurin to resorufin. Resazurin is the low or nonfluorescent indicator dye is converted to highly red fluorescent resorufin dye. This is due to the reduction reactions of metabolically viable bacteria. The amount of fluorescence produced is directly proportional

to the number of living bacteria. The principle of microplate resazurin assay is shown in Figure 1.

Chemicals and instruments

Middlebrook 7H9 Broth (HiMedia), Middlebrook OADC supplements (HiMedia), Ziehl–Neelsen carbol fuchsin dye, methylene blue, Alamar Blue dye (HiMedia), trypsin–EDTA solution, isoniazid (Sigma-Aldrich, USA), 70% (v/v) isopropyl alcohol, biosafety cabinet II, autoclave, Research Centrifuge (Eltek), incubator, Reagent Bottle Screw Cap – 500 ml (Tarson), Reagent Bottle Screw Cap – 250 ml (Tarson), ELISA reader (Thermo Fisher Scientific, USA), digital weight balance, 96-well plate flat bottom, inverted microscope, and micropipettes were used.

Method of Alamar Blue assay[11,12]

The quantitative *in vitro* anti-TB activities of different extracts of the 11 selected medicinal plants were evaluated in 96 well microtiter plate using the Alamar Blue reagent as an indicator of bacterial viability. One hundred microliters of 0.5 McFarland standards (0.5×10^6/ml) of the viable *M. tuberculosis* H37Rv was cultured in 7H9 Middlebrook medium (supplemented with ADC and 0.5% glycerol) in flat bottom 96 microtiter plate. One hundred microliters of 7H9 broth was added into all wells of the 96 microtiter plate, and 100 µl of the different concentrations (100, 500, and 1000 µg/ml) of plant extracts was added to the wells and mixed thoroughly. Each extract concentration was assayed in triplicate. The controls maintained for all the tested strains included: medium, DMSO (at a volume that is used for the highest concentration of plant extract), 1:100 viable *M. tuberculosis,* and 50 µg/ml isoniazid. To check the interaction of the plant extracts with Alamar Blue, additionally, wells with plant extracts and media were also maintained. The microliter plates were sealed with the optical sealing tape and incubated at 37°C for 14 days. Post incubation, 20 µl of Alamar Blue dye (5% [v/v]) diluted 1:1 in 7H9 medium (supplemented with ADC and 0.5% glycerol) was added, and the plates were reincubated for 30 h. The optical density of the wells was measured at 600 nm and 570 nm in an ELISA reader, and the percentage reduction of Alamar Blue dye was calculated as per the manufacturer's instructions. The use of percentage reduction to screen the plant extracts allowed the identification of those extracts with marginal activity (not resulting in 99% kill). Triplicate wells were maintained for each variable in every assay, and all the assays were performed thrice. The data were analyzed as mean ± standard deviation (SD). A color changed from blue to pink indicated mycobacterial growth. The results were interpreted based on the percentage reduction of the dye which is directly proportional to the bacterial growth. The extracts were considered to be active if the percentage reduction value of Alamar Blue dye was less than that observed for the 1:100 Viable *M. tuberculosis* controls.[13]

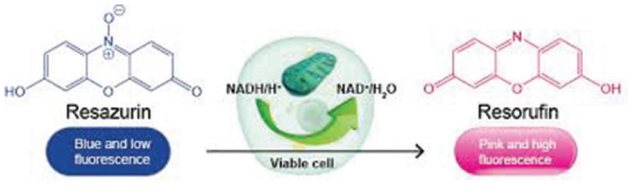

Figure 1: Graphical representation of principle of microplate resazurin assay

Results

Selection of plants

Eleven medicinal plants were selected on ethnomedicinal-based review. All the selected medicinal plants were reviewed from the ancient literature for all mentioned activities. Table 1 shows the selected medicinal plants and its ancient literature review.

Plant collection and authentication

The procured material of the 11 selected medicinal plants was authenticated by a taxonomist and further authenticated by comparing the microscopy with reported literature. Herbarium specimens of the 11 selected medicinal plant materials (PH/015/001-PH/015/011) were deposited at Pharmacognosy Department, K.B.I.P.E.R., Gandhinagar.

Percentage yield of the selected plant extracts

Alcoholic, hydroalcoholic (30:70), and aqueous extracts of the 11 selected medicinal plants were prepared to screen its antitubercular activity using different models. The percentage yield of prepared extracts is shown in Table 2.

In vitro antimycobacterial screening

The present study was conducted to investigate the antimycobacterial activity of three different extracts of the 11 selected medicinal plants against *M. tuberculosis* H_{37}Rv.

Agar dilution method

The antitubercular potency of the active plant extracts was determined by agar dilution method. Inhibition of *M. tuberculosis* H_{37}Rv was observed for 4 of the 11 medicinal plants. Inhibition of *M. tuberculosis* H_{37}Rv by agar dilution method was recorded on the four Petri plates containing aqueous extracts of *A. vasica, O. sanctum, L. reticulata,* and *C. hirsutus* after 14 days of incubation at 37°C. All the aqueous extracts of four plants were found to be active in agar dilution assays with MIC of 500 µg/ml, 500 µg/ml, 250 µg/ml, and 250 µg/ml, respectively.

The microplate resazurin assay

Crude plant extracts did not show an enhanced reduction of resazurin as compared to the media control. Out of the 11 selected medicinal plants, *E. officinalis, T. bellerica, T. chebula, W. somnifera, C. rotundus,* and *P. longum* showed no anti-*M. tuberculosis* activity, whereas aqueous extracts of *O. sanctum, A. vasica, L. reticulata,* and *C. hirsutus* showed significant inhibition activity against *M. tuberculosis* H_{37}Rv, as shown in Figure 2.

Results are presented as mean ± SD from at least three times (n = 3). Statistical analysis of data was carried out by one-way ANOVA, followed by Tukey *post hoc* test using

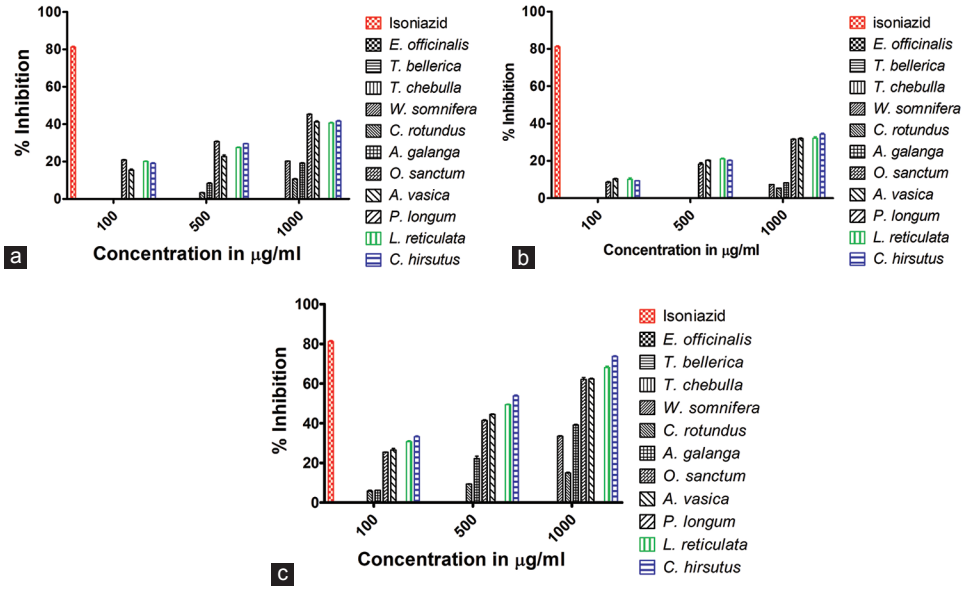

Figure 2: Percentage inhibition of alcoholic (a), hydroalcoholic (b) and aqueous (c) extracts of the 11 selected medicinal plants against *Mycobacterium tuberculosis* $H_{37}Rv$ via microplate resazurin assay

Table 1: List of selected plants for the evaluation of antimycobacterial activity

Common name	Botanical source	Family	Part used	Activity mentioned	Reference
Amla	*Emblica officinalis*	*Euphorbiaceae*	Fruits	Asthma, bronchitis	The Wealth of India, 2002[14]
Baheda	*Terminalia bellerica*	*Combretaceae*	Fruits	Asthma, cough	ICMR, 2003[15]
Harde	*Terminalia chebula*	*Combretaceae*	Fruits	Asthma, bronchitis	The Wealth of India, 1982[16]
Ashwagandha	*Withania somnifera*	*Solanaceae*	Roots	Tuberculosis	Nadkarni, KM,1982[17]
Nagarmotha	*Cyperus rotundus*	*Cyperaceae*	Rhizomes	Tuberculosis	Kirtikar and Basu 2007[18]
Rasna	*Alpinia galanga*	*Zingiberaceae*	Rhizomes	Tuberculosis	ICMR, 2003[19]
Tulsi	*Ocimum sanctum*	*Liliaceae*	Leaves	Tuberculosis	Medicinal plants of Gujarat[20]
Vasaka	*Adhatoda vasica*	*Acanthaceae*	Leaves	Tuberculosis	ICMR, 2005[21]
Long pepper	*Piper longum*	*Piperaceae*	Fruits	Bioavailability enhancer	Kirtikar and Basu 1933[22]
Kharkhodi	*Leptadenia reticulata*	*Asclepiadaceae*	Roots	Tuberculosis	Kirtikar and Basu 1933[23]
Vevadi	*Cocculus hirsutus*	*Menispermaceae*	Whole herb	Tuberculosis	Kirtikar and Basu 1933[24]

ICMR: Indian Council of Medical Research

Table 2: Percentage yield of prepared extracts

Name of the plant	Percentage yield of extracts		
	Alcoholic extract	Hydroalcoholic extract	Aqueous extract
Emblica officinalis	44.38	52.92	63.52
Terminalia bellerica	42.46	55.16	66.68
Terminalia chebula	45.08	50.58	46.56
Withania somnifera	08.91	15.32	24.16
Cyperus rotundus	08.28	10.47	15.46
Alpinia galanga	08.26	05.92	06.63
Ocimum sanctum	17.87	21.10	28.21
Adhatoda vasica	13.30	28.56	36.26
Piper longum	21.52	40.78	45.76
Leptadenia reticulata	08.80	09.40	10.39
Cocculus hirsutus	16.00	23.48	30.12

GraphPad Prism for Windows (version 5). Values of $P < 0.05$ were considered statistically significant.

Discussion

There has been no anti-TB drug announced in the past 30 years, and the rapid acquirement of drug resistance to the already available drugs demands the development of new and affordable anti-TB drugs.[25] The increasing rate of multidrug resistance and extensively drug-resistant tuberculosis made anti-TB remedy more challenging. Although approaches have been projected in an attempt to control the spread of the infection, the search for new methods to treat drug resistance stimulates the investigation of new natural compounds, which is an alternative treatment of TB infections.[26]

In this study, there are 11 medicinal plants selected on the basis of literature survey, which were evaluated for their antimycobacterial activity against *M. tuberculosis*. Before testing, the selected medicinal plants were each extracted using three different solvents, i.e., alcoholic, hydroalcoholic, and aqueous. The highest percentage yield of extract was in

polar solvents. It is, therefore, not surprising that traditional healers use mostly water for extraction processes.[27] There were two antimycobacterial assays performed to evaluate the antimycobacterial activity of different extracts of the 11 selected medicinal plants, i.e., agar dilution assay and microplate resazurin assay.

Agar dilution is the most commonly used technique to determine the MIC of antimycobacterial agents.[28] The MIC is defined as the lowest concentration of the antimicrobial substance that inhibits the visible growth of the tested *Mycobacteria*. The growth of a single colony caused by the inoculum should be disregarded. When the growth of the tested *Mycobacteria* occurs on all agar plates with extracts of the selected medicinal plants, the MIC is recorded as greater than the highest concentration tested. The MIC is recorded as less than or equal to the lowest concentration when no growth occurs on any of the agar plates but on the growth control.[29] Antimycobacterial TB activity of aqueous extracts of *O. sanctum, A. vasica, L. reticulata,* and *C. hirsutus* showed the MIC at 500 µg/ml, 500 µg/ml, 250 µg/ml, and 250 µg/ml, respectively, against the tested strains of *M. tuberculosis*.

The microplate resazurin assay is a very rapid and nonradioactive assay which allows for the exposure of bacterial activity with a high degree of confidence.[30,31] As with the tetrazolium salts, Alamar blue is a soluble redox dye that is stable in culture medium and nontoxic.[32] Alamar blue has been successfully used to assess the susceptibility of *M. tuberculosis* to various antimicrobials in several laboratories.[33,34] The results obtained showed that aqueous extracts of *O. sanctum, A. vasica, L. reticulata, and C. hirsutus* show significant antimycobacterial TB activity with the *Mycobacterial tuberculosis* inhibition percentage of 71.26%, 74.58%, 75.21%, and 80.26%, respectively. The results were comparable to those of the standard drug Isoniazid. This may be due to the bioactive constituents, such as flavonoids, alkaloids, steroids, terpenoids, saponins, and tannins in the aqueous extracts of plants.[35]

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Conflicts of interest

There are no conflicts of interest.

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