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ANTIMYCOBACTERIAL SCREENING OF SELECTED MEDICINAL PLANTS USING MTT AND THE MICROPLATE RESAZURIN ASSAY

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Keywords:

M. smegmatis, Medicinal plants, Antimycobacterial activity, MTT assay, Microplate Risazurin Assay

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ABSTRACT: Tuberculosis holds one of the top places on the list of the main cause of death in India. At times, the patients fail to respond to treatment with anti-Tb drugs, drug resistance being one of the reasons. The increasing incidence of MDR and XDR-TB highlights the urgent need to search for newer anti-Tb drugs. For last many years, plants have beneficial activity in a different types of diseases producing in human beings. As per WHO calculation about 80% of the world's inhabitants problem should treat by medicinal herbal drugs for their primary health care. So, the present aim to carry out the evaluation of the anti-mycobacterial activity of selected eleven medicinal plants. Three different extracts were prepared and evaluated for their antimycobacterial activity against *Mycobacterium smegmatis* using MTT and Microplate Resazurin assay. Isoniazid was used as a standard drug. The percentage for *anti-mycobacterial smegmatis* activity among tested eleven medicinal plants, an aqueous extract of *Cocculus hirsutus* and *Leptedinia reticulata* shows potent antimycobacterial activity. Thus, its result supports the uses of these plants in traditional medicine and can be further studied using more specific methods for antimycobacterial activity.

INTRODUCTION: Tuberculosis (TB) is an ancient disease and it is among the world's most deadly epidemics. Like any other infectious disease, TB can happen to anyone and spares no age, sex, and nationality [1, 2]. Several strains of *Mycobacterium tuberculosis* are the common cause of this deadly infectious disease [3]. This disease is endemic in every country in the world, and death due to TB is more common when compared to other bacterial disease [4]. It is unfortunate that more than 75% of TB cases are found in adults [5].

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An unprecedented decision was taken in 1993 by WHO to declare TB as a public health emergency [6], and it is the first disease that has ever been declared as a global emergency by WHO [7]. In order to combat TB, chemotherapy is used, which is the modern TB treatment.

Since no new antimycobacterial drugs have been introduced into the market since 1967. Moreover, up to 50 million people are infected with drug-resistant forms of TB, with about 500,000 cases of multidrug-resistant TB a year worldwide [8]. Even though there are currently new lead compounds being characterized for TB treatment [9], they are challenged by poor accessibility, high costs, long treatment regimen, and low adherence owing to the toxicity of second-line drugs. The newly commercialized drug is likely to be exhausted with the emerging resistance, emphasizing the

imperative continuous search, identification, and characterization of more compounds for anti-mycobacterial drugs [10]. Medicinal plants have been used for centuries as non-exhaustive sources of metabolites for drug development and as an alternative remedy for treating human diseases, as they contain numerous active constituents of therapeutic value [11-14].

Rational chemistry, while essential to the development of many modern pharmaceuticals, often serves better to refine the chemical blueprints isolated from natural pro-duct screens than to devise entirely new molecular backbones. The enormous diversity of plant-derived compounds therefore makes them one of the most promising reservoirs of potentially novel antimycobacterial molecules [15].

M. smegmatis is commonly used in work on the *Mycobacterium* genus due to its being a "fast grower" and non-pathogenic. *M. smegmatis* is commonly used in work on the *Mycobacterium* genus due to its being a "fast grower" and non-pathogenic. This species shares more than 2000 homologous genes with *M. tuberculosis* and shares the same peculiar cell wall structure of *M. tuberculosis* and other mycobacterial species. The *M. smegmatis* strain is hypertransformable, and is now the work-horse of *mycobacterial genetics*. Furthermore, it is readily cultivatable in most synthetic or complex laboratory media, where it can form visible colonies in 3-5 days. These properties make it a very attractive model organism for *M. tuberculosis* and other mycobacterial pathogens. *M. smegmatis* mc^{2}155 is also used for the cultivation of mycobacteriophage [16].

By taking into consideration of all the above points our aim of this research work is to evaluate anti-mycobacterial activity of selected eleven medicinal plants and their different extract using two quantitative *in-vitro* assays. The names of all selected plants are given in following **Table 1**. There are two *in-vitro* assays performed MTT assay and RESAZURIN assay (microplate resazurin assay).

TABLE 1: LIST OF SELECTED PLANTS FOR THE EVALUATION OF ANTIMYCOBACTERIAL ACTIVITY

S. no.	Commom name	Bioliogical source	Family	Part used
1	Amla [17]	*Emblica officinalis*	*Euphorbiaceae*	Fruits
2	Baheda [18]	*Terminalia bellerica*	*Combretaceae*	Fruits
3	Harde [19]	*Terminalia chebulla*	*Combretaceae*	Fruits
4	Ashwagandha [20]	*Withania somnifera*	*Solanaceae*	Roots
5	Nagarmoth [21]	*Cyperus rotundus*	*Cyperaceae*	Rhizomes
6	Rasna [22]	*Alpinia galanga*	*Zingiberaceae*	Rhizomes
7	Tulsi [23]	*Oscimum sanctum*	*Liliaceae*	Leaves
8	Vasaka [24]	*Adhatoda vasica*	*Acanthaceae*	Leaves
9	Long pepper [25]	*Piper longum*	*Piperaceae*	Fruits
10	Kharkhodi [26]	*Leptedinia reticulata*	*Asclepiadaceae*	Roots
11	Vevadi [27]	*Cocculus hirsutus*	*Menispermeaceae*	Whole herb

MATERIALS AND METHODS:

Plant Collection and Authentication: Dried plant materials of nine selected plants [Fruits of *Emblica officinalis,* fruits of *Terminalia bellerica,* fruits of *Terminalia chebulla,* roots of *Withania somnifera,* rhizomes of *Cyperus rotundus,* rhizomes of *Alpinia galanga,* leaves of *Oscimum sanctum,* leaves of *Adhatoda vasica* and fruits of *Piper longum*] out of eleven selected plants were procured from Ayurvedic store of Gandhinagar and fresh plant material of two selected plants [roots of *Leptedinia reticulata* and the whole herb of *Cocculus hirsutus*] out of eleven selected plants were collected from Dhandhiya village of Rajkot district, Gujarat, India.

Preparation of Plant Extract:

Preparation of Alcoholic, Hydroalcoholic and Aqueous Extracts of the Selected Plants: 100 gm of the powder of the eleven selected plants *i.e.,* fruits of *Emblica officinalis*, fruits of *Terminalia bellerica*, fruits of *Terminalia chebulla*, roots of *Withania somnifera*, rhizomes of *Cyperus rotundus*, rhizomes of *Alpinia galanga*, leaves of *Oscimum sanctum*, leaves of *Adhatoda vasica* and fruits of *Piper longum*, roots of *Leptedinia reticulata* and the whole herb of *Cocculus hirsutus* were 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 the raw material of selected 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, percentage yield was calculated. The prepared extracts were labeled and stored in an airtight container for further use.

Antimycobacterial Activity Tests:

Procurement and Culturing of *M. smegmatis* Freeze Dried Culture: The *M. smegmatis* (MTCC 6) freeze-dried culture was procured from Microbial Type Culture Collection and Gene bank (MTCC), Chandigarh, India.

***M. smegmatis* Culture Preparation:** The *M. smegmatis* (MTCC 6) strain was used. 10 ml of Middle brook 7H9 broth supplemented with 10% oleic acid-albumin-dextrose-catalase and 0.2% of glycerol culture was inoculated with 0.4-0.6 ml of *M. smegmatis* in a 50 ml conical tube. The culture was grown to mid-log phase on the wheel at 37 °C, until bacterial culture shows growth is equal to 0.5 McFarland standards dilution. This resulted in a culture with approximately 1.5×10^8 Cfu/ml. To check the purity of prepared culture, Ziehl-Neelsen staining was performed for the confirmation of acid-fast bacilli. Then, 100µl of this culture was used to set up the assay plates, with each well containing 10^4 Cfu.

The antimycobacterial activities of the different extracts of the eleven plants were tested using the different *in-vitro* antimycobacterial assays. Two different antimycobacterial assays were performed to evaluate the potential of different extracts of eleven selected medicinal plants.

MTT Assay:

Principle of MTT Assay: This assay is based on the assumption that dead bacteria or their products do not reduce tetrazolium. The principle involved is the cleavage of tetrazolium salt 3- (4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a blue-colored product (formazan) by mitochondrial enzyme succinate dehydrogenase. The numbers of bacteria were found to be proportional to the extent of formazan production by the bacteria used is insoluble in aqueous solutions.

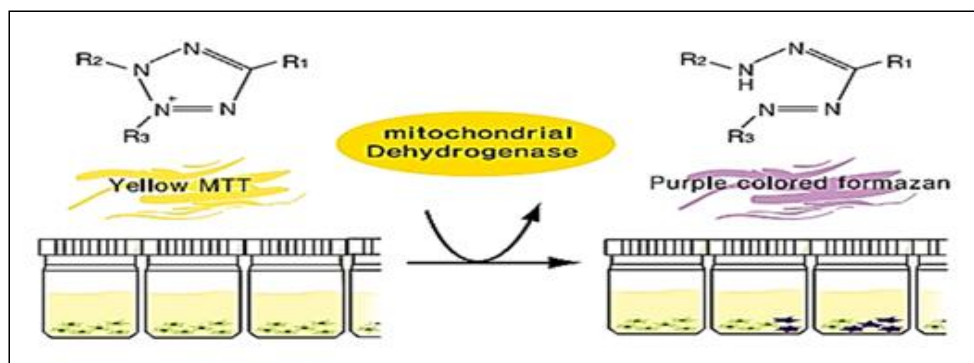

FIG. 1: PRINCIPLE OF MTT ASSAY

Chemicals and Instruments: Middlebrook 7H9 Broth (HiMedia), Middlebrook ADC Growth Supplements (HiMedia), Zeihl- carbol fusin dye, Methylene blue, MTT Reagent (3-[4,5-dimethyl-thiazole-2-yl]- 2, 5- diphenyltetrazolium bromide) (HiMedia), Trypsin–EDTA solution, 70% (v/v) Iso-Propyl alcohol, Dimethyl sulfoxide, Bio-safety cabinet II, Autoclave, Research Centrifuge (Eltrek), Incubator, Reagent Bottle Screw Cap- 500ml (Tarson), Reagent Bottle Srew Cap- 250 (Tarson), ELIZA reader, Digital Weigh Balance, 96 well plate flat-bottom, Inverted microscope, Micro-pipettes.

Prior to MTT Assay: Middlebrook 7H9 Broth Base with added enrichment is recommended for cultivation and sensitivity testing of *Mycobacterium* species. Suspend 2.35 gm in 450 ml distilled water. Add either 1 ml glycerol. Heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121 °C) for 10 min. Cool to 45°C or below and aseptically add contents of 1 vial of Middlebrook oleic acid–albumin-dextrose-catalase (OADC supplements) Growth Supplement. Mix well before dispensing. 7H9 broth supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC supplements) and 0.2%

of glycerol culture was inoculated with 0.4-0.6 ml of *M. smegmatis* in a 50 ml conical tube. The culture was grown to mid-log phase on the wheel at 37 °C, until bacterial culture shows growth is equal to 0.5 McFarland standards dilution. This resulted in a culture with approximately 1.5×10^8 Cfu/ml. To check the purity of the prepared culture, Ziehl-Neelsen Stain was performed for the confirmation of acid-fast bacilli. Then, 100µl of this culture was used to set up the assay plates, with each well containing 10^4Cfu.

MTT Assay: This assay is based on the assumption that dead bacteria or their products do not reduce tetrazolium. The assay depends both on the number of bacteria present and on the mitochondrial activity. The principle involved is the cleavage of tetrazolium salt 3- (4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a blue color product (formazan) by mitochondrial enzyme succinate dehydrogenase. The numbers of bacteria were found to be proportional to the extent of formazan production by the bacteria used. The bacterial suspension was trypsinized, and the bacterial count was adjusted to 1.0×10^5 bacterias/ ml Middlebrook 7H9 broth supplemented with oleic acid-albumin-dextrose-catalase. To each well of the 96 well microtitre plate, 0.1 ml of the diluted bacterial suspension (approximately 10,000 bacteria) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off; wash the monolayer once with medium and 100, 500, and 1000 µl of different concentration of plant extracts was added on to the partial monolayer in microtitre plates. The plates were then incubated at 37 ºC for 3 days in 5% CO_2atmosphere, and microscopic examination was carried out, and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded, and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37 ºC in 5% CO_2 atmosphere. The supernatant was removed, and 100 µl of DMSO was added, and the plates were gently shaken to solubilize the formed formazan.

The absorbance was measured using a microplate ELIZA reader at a wavelength of 540 nm. The experiments were performed thrice, and the results were obtained by calculating the mean absorbance of the triplicate wells. The percentage growth inhibition was calculated using the following formula, and the concentration of the test drug needed to inhibit cell growth by 50% (IC_{50}) values is generated from the dose-response curves for each cell line [28].

% Growth Inhibition = 100 - Mean OD of individual test group × 100 / Mean % Growth Inhibition

Trypan Blue Dye Exclusion Technique: The aliquot of 10 µl of the trypsinized bacterial suspension was taken in a micro-centrifuge tube, and 1:1 mixture of the bacterial suspension was prepared by adding 10 µl of the 0.4% trypan blue dye solution. It was gently mixed and kept at room temperature for about 5 min. Prior to use, the hemocytometer and the coverslip were washed with 70% (v/v) isopropyl alcohol and were allowed to air-dry. Fifteen microlitres of cell suspension and trypan blue dye mixture were applied to the edge of the chamber between the coverslip and the V-shaped groove in the chamber. It was allowed to sit for 1-2 min, and then the bacteria were counted for their cell viability and total cell count. This assay was based on the assumption that the dead bacteria will stain blue and viable bacteria are colorless [29]. The following formula calculated the percentage growth inhibition.

% Growth Inhibition = 100 – Total Cells – Dead Cells × 100 / Total Cells

As in the last step of the procedure of MTT assay, the supernatant was removed, and 100 µL of DMSO was added; the plates were gently shaken to solubilize the formed formazan. In this step, there may be chances of to flow of bacteria with the supernatant liquid as bacteria did not have an adherent ability. To overcome this problem in the antimycobacterial assay, another advanced new antimycobacterial assay was performed to evaluate antimycobacterial activity.

The Microplate Resazurin (Resazurin) Assay:
Principle of Microplate Resazurin Assay: Microplate Resazurin Assay work as a viability indicator through the conversion of RESAZURIN to RESORUFIN. Resazurin, a non-fluorescent indicator dye, is converted to highly red fluorescent resorufin *via* reduction reactions of metabolically viable organisms. The amount of fluorescence produced is proportional to the number of living organisms.

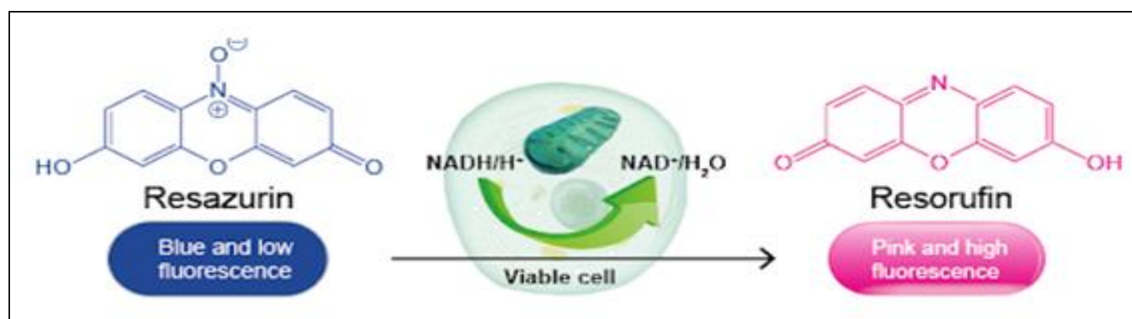

FIG. 2: GRAPHICAL REPRESENTATION OF PRINCIPLE OF MICROPLATERESAZURIN ASSAY

The quantitative *in-vitro* antimycobacterial activity of different extracts of selected plants was done in 96 microtiter plates using the Resazurin reagent as an indicator of bacterial viability. Working solutions of the tested extracts were diluted in Middlebrook 7H9 broth supplemented with oleic acid-albumin-dextrose-catalase to obtain the final sample concentrations that ranged from 1000µg/ml to 100µg/ml. Isoniazid was dissolved in dimethyl sulfoxide and used as a positive control drug at 50µg/ml as the starting concentration, and extracts/ drug-free medium with strain suspensions were used as the negative control. One hundred microliters of 7H9 broth was added into all wells of the 96 well plates, and 100µl of the different concentration of plant extracts was introduced to the wells in and mixed thoroughly. Then, 100µl of the inoculum was introduced into the corresponding wells. The final volume in each well was 200 µl. Each extract concentration was assayed in triplicate. Each microplate was then sealed with the optical sealing tape and incubated for 48 h at 37 °C. After the incubation period, 32.5µl of Alamar blue dye was added to each well. The plates were then reincubated for 4 h at 37 °C in the dark. The experimental results were computerized using the ELIZA microplate reader at 600nm for data analysis [30, 31].

The minimal inhibitory concentration (MIC) results were presented as the mean value. The lowest concentration that resulted to 90% inhibition was defined as the MIC. The MIC values determined by this method were cross-checked using the broth dilution methods. The blue color in the well was scored as ‘‘no mycobacterial growth,” and a pink color was scored as ‘‘growth occurrence”.

RESULTS:

Plant Collection and Authentication: The procured material of selected plants was authenticated by a taxonomist and further authenticated by comparing the microscopy with reported literature. Herbarium specimens of selected plant materials were deposited at Pharmacognosy department, K.B.I.P.E.R., Gandhinagar. Plant authentication numbers of selected medicinal plants are shown in **Table 2**.

TABLE 2: PLANT AUTHENTICATION NUMBERS OF SELECTED MEDICINAL PLANTS

Plant name	Plant part used	Herbarium sheet number
Emblica officinalis	Fruit	PH/15/001
Terminalia bellerica	Fruit	PH/15/002
Terminalia chebulla	Fruit	PH/15/003
Withania somnifera	Roots	PH/15/004
Cyperus rotundus	Rhizomes	PH/15/005
Alpinia galanga	Rizomes	PH/15/006
Oscimum sanctum	Leaf	PH/15/007
Adhatoda vasica	Leaf	PH/15/008
Piper longum	Fruit	PH/15/009
Leptedinia reticulata	Roots	PH/15/010
Cocculus hirsutus	Whole plant	PH/15/011

Percentage Yield of Selected Medicinal Plant Extract: Alcoholic, 70% hydroalcoholic, and aqueous extracts were prepared to screen its anti-tubercular activity using a different model. % yield of prepared extracts are shown in **Table 3**.

TABLE 3: PERCENTAGE YIELD OF PREPARED EXTRACTS OF SELECTED MEDICINAL PLANTS

S. no.	Name of the plant	% yield of extracts		
		Alcoholic extract	Hydro-alcoholic extract	Aqueous extract
1	*Emblica officinalis*	44.38%	52.92%	63.52%
2	*Terminalia bellerica*	42.46%	55.16%	66.68%
3	*Terminalia chebulla*	45.08%	50.58%	46.56%
4	*Withania somnifera*	08.91%	15.32%	24.16%

5	*Cyperus rotundus*	08.28%	10.47%	15.46%
6	*Alpinia galanga*	08.26%	05.92%	06.63%
7	*Oscimum sanctum*	17.87%	21.10%	28.21%
8	*Adhatoda vasica*	13.30%	28.56%	36.26%
9	*Piper longum*	21.52%	40.78%	45.76%
10	*Leptedinia reticulata*	08.80%	9.40%	10.39%
11	*Cocculus hirsutus*	16.00%	23.48%	30.12%

***In-vitro* Antimycobacterial Screening:** The present study was conducted to investigate the antimycobacterial activity of three different extracts of eleven selected plants against *Mycobacterium smegmatis*.

MTT Assay: The results of the MTT viability assay performed against *Mycobacterium smegmatis*. The mean absorbance values following 48 h of treatment with the selected plant extract were found to be significantly inhibiting the *Mycobacteria* as compared with antimycobacterial positive standard Isoniazid, as shown in Graph 2. On the basis of this assay, *Adhatoda vasica, Alpania galana and Oscimum sanctum* show good antimycobacterial activity. And the extract of *Cocculus hirsutus* and *Leptedinia reticulate* shows significant antimycobacterial activity against *Mycobacterium smegmatis*.

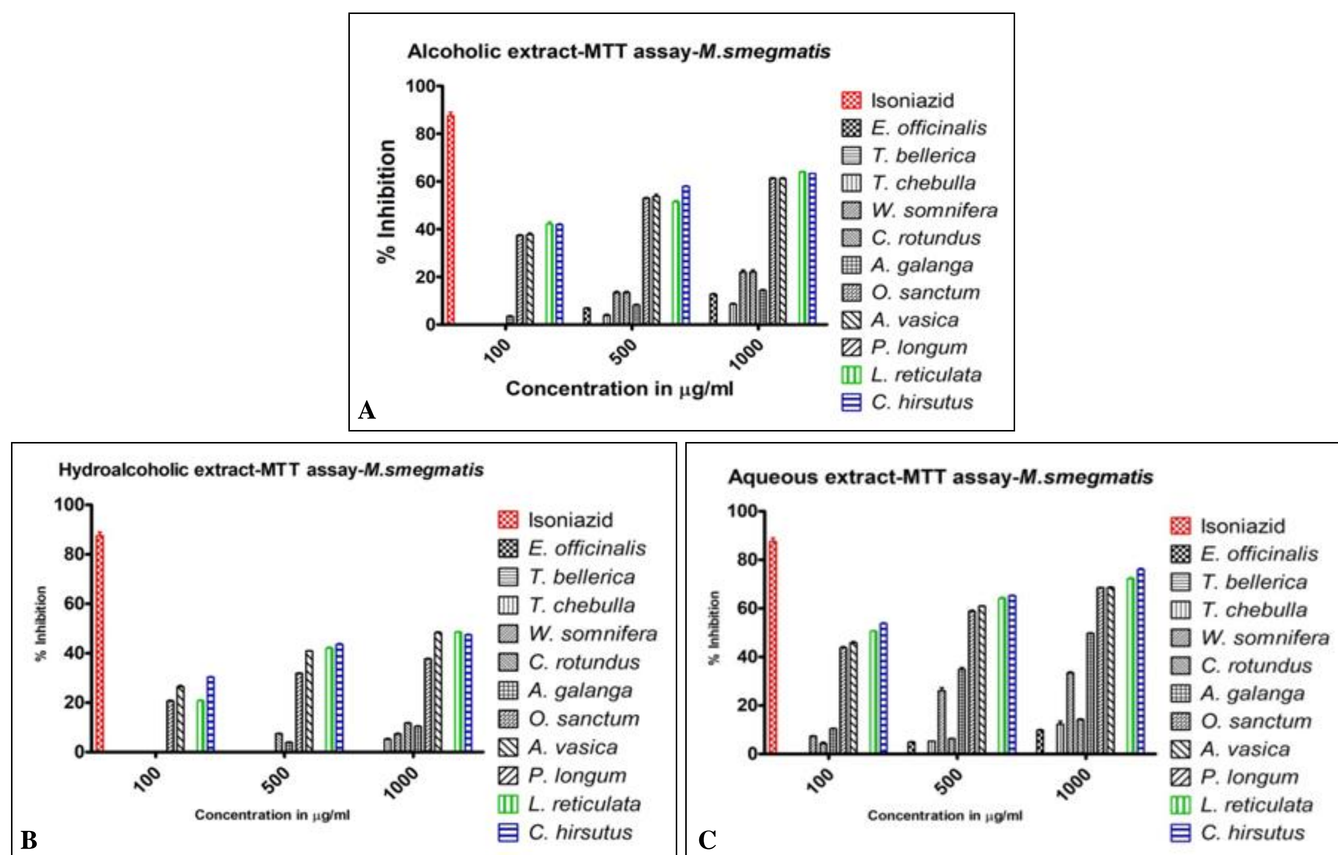

FIG. 3: PERCENTAGE INHIBITION OF THE ALCOHOLIC (A), HYDROALCOHOLIC (B) AND AQUEOUS (C) EXTRACTS OF THE SELECTED PLANTS AGAINST *M. SMEGMATIS VIA* MTT ASSAY. Results are presented as mean ± SD from at least three times (n=3). Statistical analysis of data was carried out by one-way ANOVA followed by Tukey *post hoc* test using GraphPad Prism for Windows (version 5). Values of $p<0.05$ were considered significant.

The Microplate Resazurin Assay: Crude plant extracts did not show an enhanced reduction of Resazurin as compared to the media control. Out of eleven selected plants, *Emblica officinalis, Terminalia bellerica, Terminalia chebulla, Withania somnifera, Cyperus rotundus, Piper longum* showed no anti-*M. smegmatis* activity, while aqueous extracts of *Cocculus hirsutus, Leptedinia reticulata Adhatoda vasica, Alpinia galanga, and Oscimum sanctum* showed significant activity against *M. smegmatis.*

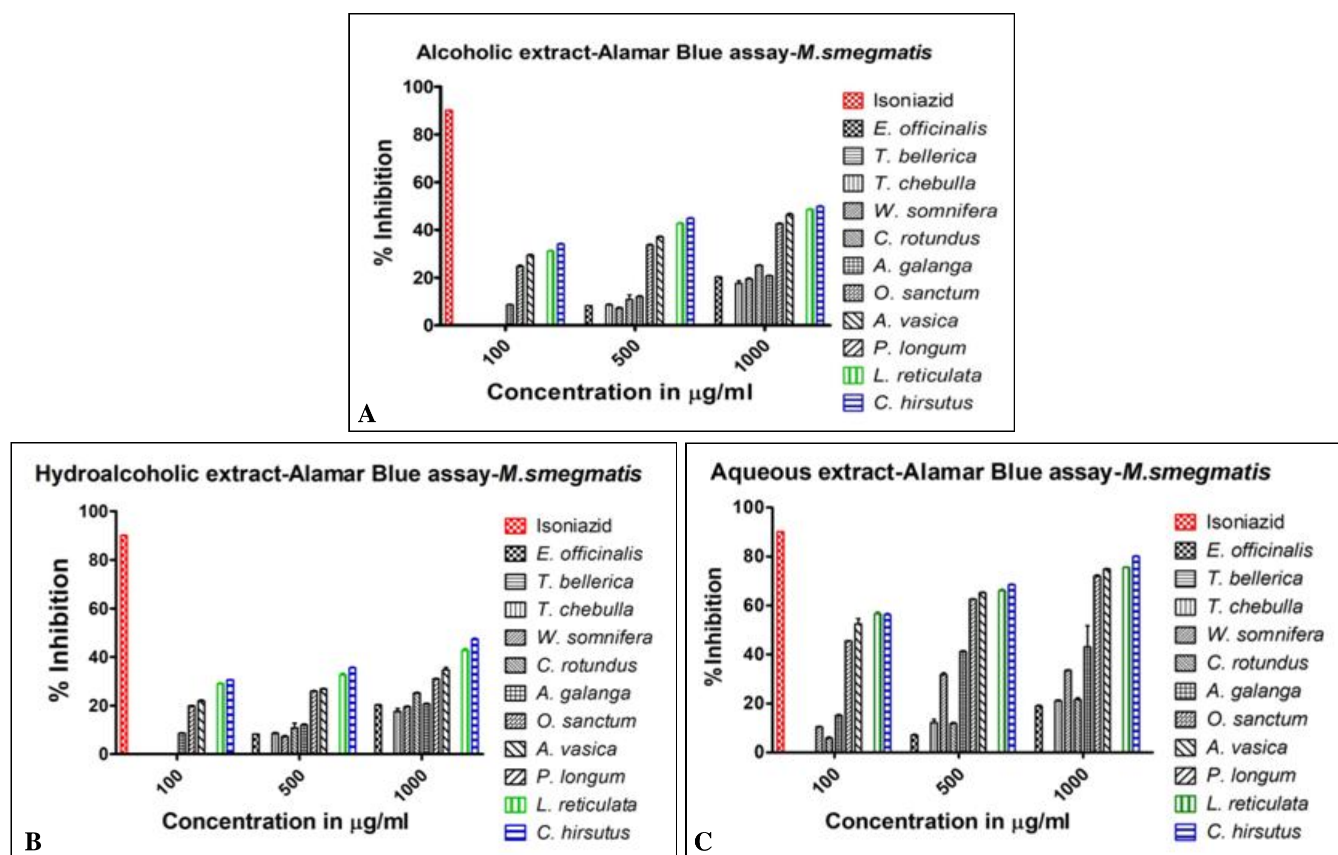

FIG. 4: PERCENTAGE INHIBITION OF ALCOHOLIC, HYDROALCOHOLIC AND AQUEOUS EXTRACTS OTHE SELECTED PLANT AGAINST *M. SMEGMATIS VIA* MICROPLATE RESAZURIN ASSAY. Results are presented as mean ± SD from at least three times (n=3). Statistical analysis of data was carried out by one-way ANOVA followed by Tukey *post hoc* test using GraphPad Prism for Windows (version 5). Values of $p<0.05$ were considered significant.

DISCUSSION: Tuberculosis has been a major health problem for developing countries, including India. The increasing resistance of the disease to first and second-line drugs has demanded the need for a new search for antimycobacterial agents that could be effective, efficient, non-toxic, and cost-effective [32].

There has been no antimycobacterial drug introduced in the past 30 years, and the rapid acquisition of drug resistance to the existing drugs necessitates the development of new, effective, and affordable antimycobacterial drugs [33].

Plant-derived antimycobacterial compounds belong to an exceptionally wide diversity of classes, including terpenoids, alkaloids, peptides, phenolics and coumarins. Hence medicinal plants remain an important resource to find new therapeutic agents [34].

In this study, there are eleven ethanomedicinal plants selected on the basis of a literature survey were evaluated for their antimycobacterial activity against *M. smegmatis.* Prior to testing, selected plants were each extracted using three different solvents *i.e.,* alcoholic, hydro-alcoholic, 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 [35]. There were two antimycobacterial assays performed to evaluate antimycobacterial activity of different extracts of selected eleven medicinal plants i.e., MTT assay and RESAZURIN assay.

The MTT assay is based on the assumption that dead bacteria or their products do not reduce tetrazolium. The numbers of bacteria were found to be proportional to the extent of formazan production by the bacteria used is insoluble in aqueous solutions [36]. The MTT assay employed in this study provides preliminary data as evidence that the aqueous extracts of *Oscimum sanctum* (68.54%), *Adhatoda vasica* (68.14%), *Leptedinia reticulata* (71.26%), *and Cocculus hirsutus* (76.96%) shows a satisfactory percentage of inhibition against *M. smegmatis.*

The RESAZURIN ASSAY is a non -radiometric, rapid, high-throughput assay that allows for the detection of bacterial activity with a high degree of confidence [37]. The results obtained showed thataqueous extracts of*, Oscimum sanctum, Adhatoda vasica, Leptedinia reticulata and Cocculus hirsutus* shows strong antimycobacterial activity as compared to MTT assay. On the basis of result from these four different extracts of plants, aqueous extract of *Cocculus hirsutus* shows 80.26% and *Leptedinia reticulata* shows 75.38% percentage of *Mycobacterial* inhibition against *M. smegmatis.* The results were comparable to those of the standard drug Isoniazid.

CONCLUSION: In conclusion, with increasing rates of tuberculosis worldwide and the rise of MDR-TB and XDR-TB, there is a need for novel antimycobacterial agents. Using the MRA as a screening tool, this study assessed the antimycobacterial properties of traditional medicinal plants. The aqueous extracts of *Leptedinia reticulate* and *Cocculus hirsutus* were found to exhibit very strong antimycobacterial activity. This study serves to validate traditional knowledge. The current growing literature on botanical sources identified as providing important novel antimycobacterial compounds. The antimycobacterial activity of the plant extracts investigated in this study was evaluated against the *M. smegmatis* strain of *Mycobacteria,* as the assays were performed in a biosafety containment level 2 setting. The results obtained indicate that it would be relevant to continue our investigations of *Leptedinia reticulate* and *Cocculus hirsutus*, and further studies using the virulent *M. tuberculosis* $H_{37}Rv$ strain are currently underway.

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CONFLICTS OF INTEREST: None

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