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Effect of ethanomedicinal plants on *Mycobacterium smegmatis* Tubercular bacilli using mycobacteria growth indicator tube assay

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Abstract
Despite all of the control approaches, tuberculosis (TB) is a major cause of death worldwide and one-third of the world's population is infected with TB. Plant-derived medicines have been used in traditional medicinal system for the treatment of many ailments worldwide. From last number of years, plants have advantageous in different type of diseases producing in human beings. The present aim to carry out the evaluation of the antimycobacterial activity of selected eleven medicinal plants. Three different extracts were prepared and evaluated for its antimycobacterial activity against *Mycobacterium smegmatis* using Mycobacterial Growth Indicator Tube (MGIT) assay. The MGIT assay consists of liquid broth medium that is known to yield better recovery and faster growth of *mycobacteria.* Isoniazid was used as standard antituberculosis drug. The percentage for *anti-mycobacterial smegmatis* activity among tested eleven medicinal plants, aqueous extract of *Oscimum sanctum, Adhatoda vasica, Leptadenia reticulata and Cocculus hirsutus* shows good antimycobacterial activity. Among these four *Leptadenia reticulata* and *Cocculus hirsutus* shows potent inhibition as compared to isoniazid. Thus, its result supports the uses of these plants in traditional medicine and also helps to cure and prevent tuberculosis. It can further have studied using more specific methods for antimycobacterial activity.

Keywords: *Mycobacterium smegmatis*, medicinal plants, antimycobacterial activity, mycobacterial growth indicator tube assay

1. Introduction
From the ancient times, Herbal medicinal plants used for treatment and alleviate the many diseases [1]. The World Health Organization (WHO) has estimated that up to 70% of the world's population is purely depends on the traditional system of medicine for their preliminary health care [2]. A number of factors can be attributed by dependence of large portion of the population on traditional medicine like: comparatively good approachability to the plants and wide-ranging of local information and knowledge amongst the societies [3]. Even though the availability of modern treatment and medicines, maximum population is still using an extensive pharmacopoeia of inborn plants [4-5].

Tuberculosis like infections are a significant throughout the world with medical condition cluttered by raising steps of anti-infection obstacle with an predictable 8-9 million new cases happening yearly [6]. Tuberculosis is mainly caused by *Mycobacterium tuberculosis*, an infection responsible for 26% of all possibly mortality rate throughout the world [7]. The main drawback of the existing TB treatment comprise the risk of multi drug-resistant (MDR) and extensively drug resistant (XDR) strains. These led to introduce again interest in the finding of new agents for tuberculosis treatment.

Recently, there has been increasing interest in the molecules that is isolated or derived from the plants. The medicinal plants are generally harmless and more reliable, as compare to synthetic drugs which are very expensive with many adverse effects [8]. In the past years, number of reports were appeared about natural products having anti-mycobacterium activity [9-11]. A number have confirmed *in vitro* anti-mycobacterial activity of compounds belonging to various classes that have been isolated [12].

As tuberculosis is the most communicable disease mostly connected with hepatotoxicity and patient's immunity. On the basis of this consideration, mainly there are four criteria selected to select the plants for antituberculosis activity. Criteria like antituberculosis effect, hepatoprotective effect, immunomodulatory action and to enhance bioavailability. In the present study eleven different plants were selected on the basis of ethanomedicinal based review and literature based review as shown in table 1.

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

Sr. No.	Common name	Biological source	Family	Part used
1.	Amla [13]	*Emblica officinalis*	*Euphorbiaceae*	Fruits
2.	Baheda [14]	*Terminalia bellerica*	*Comtn.etaceae*	Fruits
3.	Harde [15]	*Terminalia chebulla*	*Combretaceae*	Fruits
4.	Ashwagandha [16]	*Withania 30mM/era*	*Solanaceae*	Roots
5.	Nagarmoth [17]	*Cyperus rotundas*	*Cvperaceae*	Rhizomes
6.	Rasna [18]	*Alpinia galanga*	*Zingiberaceae*	Rhizomes
7.	Tulsi [19]	*Oscimum sanctum*	*Liliaceae*	Leaves
8.	Vasaka [20]	*Adhatoda vasica*	*Acanthaceae*	Leaves
9.	Long pepper [21]	*Piper lonzum*	*Piperaceae*	Fruits
10.	Kharkhodi [22]	*Leptadenia reticulata*	*Asclepiadaceae*	Roots
11.	Vevadi [23]	*Coccullts hirsutus*	*Menispermeaceae*	Whole herb

Different culture or broth media are used for the isolation and sub-culturing of *Mycobacteria species*. The most common is Lowenstein Jensen Medium (LJ medium) (Himedia), which is an egg-based medium. LJ medium includes very high concentrations of malachite green which helps to prevent contamination with other species of bacteria. Mycobacterium Growth Indicator Tube was better media as compared to LJ medium in retrieval rate of *Mycobacterial* growth [24]. MGIT should be used mostly for assessing antituberculosis effect of different agents. By taking consideration of all above points our aim of this research work is to evaluate antimycobacterial activity of selected eleven medicinal plants and their different extract using MGIT assays.

2. Methods and Materials

2.1 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 *Leptadenia reticulata* and Whole herb of *Cocculus*) of eleven selected plants were collected from Dhandhiya village of Rajkot district, Gujarat, India.

The procured material of eleven selected medicinal plants were authenticated by taxonomist and further authenticated by comparing the microscopy with reported literature. Herbarium specimens of selected plant materials (PH/015/001-PH/015/011) were deposited at Pharmacognosy department, K. B. Institute of Pharmaceutical Education and Research, Gandhinagar.

2.2 Preparation of plant extract

2.2.1 Preparation of alcoholic, hydro-alcoholic and aqueous extracts of the selected plants:

100 gm of the powder of each eleven selected medicinal 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 *Leptadenia reticulata* and Whole herb of *Cocculus hirsutus* were taken to prepare its different extracts. Alcoholic, hydroalcoholic (30:70 water: alcohol) and aqueous extracts were prepared by maceration of raw material of selected plants for 48 hours in respective solvents. It was refluxed for about 1 hour with shaking. This process will repeat consecutively three times and filtered. The filtrates were pooled and evaporated to make it concentrated up to dryness. Percentage yield was calculated. The prepared extracts were labelled for further storage in an air tight container for future use.

2.3 Antimycobacterial activity

2.3.1 Procurement and culturing of *Mycobacterium smegmatis* (*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.

2.3.2 *M. smegmatis* culture preparation

The *M. smegmatis* (MTCC 6) strain was used for the culture preparation. 10 ml of Middlebrook 7H9 broth was taken with supplemented of 10% oleic acid–albumin–dextrose–catalase (OADC) 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-up to mid log phase on the wheel at 37 °C temperature, until the growth of bacterial culture shows equal to 0.5 McFarland standards dilution. This resulted in a culture with approximately $1.5*10^8$ Cfu/ml of *M. smegmatis*. Purity of prepared culture was checked by Ziehl-Neelsen staining. It was performed for the confirmation of acid fast bacilli. 100 µl of culture was used to perform the antituberculosis assay in the assay plates, in which each well containing 10^4 Cfu.

The antimycobacterial activity of the three different extracts of the eleven selected medicinal plants were screen using the MGIT antituberculosis assay.

2.3.3 Mycobacterial growth indicator tube assay

2.3.3.1 Principle of MGIT Assay

A fluorescent compound is implanted in silicone on the bottommost of 16 x 100 mm round-bottom tubes. The fluorescent compound is very sensitive to oxygen which is dissolved in the broth. Initially, the large amount of dissolved oxygen quenches emissions from the compound and very little fluorescence can be detected. Later on, when the bacteria starts growing, it will consume the oxygen. It allows the fluorescence to be observed using a 365 nm UV transilluminator or long wave UV light. Microbial growth can also be identified by the presence of turbidity in the culture medium due to unwanted growth.

The broth is mainly consists of Oleic acid, Albumin, Dextrose and Catalase (OADC) are the substances necessary for the rapid or fast growth of *Mycobacteria*. Oleic acid is used by *Mycobacteria* and plays an important role in the metabolism. Albumin acts as a protective agent and helps to binds with the free fatty acids, which may be toxic to *Mycobacterium species*. Dextrose is rich in energy source. Catalase destroys lethal peroxides present in the broth medium.

2.3.3.2 Reagents

The MGIT tube is mainly contains 7 ml of broth medium with 110 µl of fluorescent indicator. The fluorescent indicator contains Tris 4, 7 - diphenyl-1, 10-phenanthroline ruthenium chloride pentahydrate in a silicone rubber base.

Approximate Formula per litre Purified Water,
Modified Middlebrook 7H9 Broth base …..... 05.90 gm
Casein peptone ..... 01.25 gm

MGIT OADC contains 15 ml Middlebrook OADC enrichment.

Approximate Formula per litre Purified Water,
Bovine albumin..... 50.00 gm
Catalase ..... 00.03 gm
Dextrose ..... 20.00 gm
Oleic acid .....00.60 gm

The MGIT PANTA vial contains a lyophilized mixture of antimicrobial agents. Approximate Formula per Vial Lyophilized PANTA,
Polymyxin B .....6,000 units
Trimethoprim..... 600 µg
Amphotericin B .....600 µg
Azlocillin .....……..600 µg
Nalidixic acid ..... 2,400 µg

2.3.3.3 Chemicals and instruments

MGIT tube (BBL™ MGIT™ Bactec and Dickinson,USA), Middlebrook oleic acid–albumin–dextrose–catalase (OADC supplements) (HiMedia), Zeihl- carbol fusin dye, Methylene blue, Iso-Propyl alcohol, Bio-safety cabinet II(Photon cleantech inc), Autoclave, Research Centrifuge (Eltrek), Incubator, MGIT reader(BBL™ MGIT™ Bactec and Dickinson,USA), Digital Weigh Balance, Micropipettes

2.3.3.4 Procedure

In this study, *M. smegmatis* species was used to evaluate the antimycobacterial activity. *M. smegmatis* bacteria was cultured on the Mycobacteria growth indicator tube (MGIT) with growth supplement. MGIT 960 instrument system was used in this study to screen the antimycobacterial activity of selected medicinal plants. Before inoculating with the bacteria, MGIT tubes contains 7 ml of broth media primarily supplemented with Mycobacteria growth supplement (Oleic acid, Albumin, Dextrose, Catalase; OADC) as specified in the manufacturer's protocol (800 µl/tube). After that add 100 µl aliquots of different concentration (100,500 and 1000µg/ml) of extracts of selected eleven medicinal plants aseptically into the MGIT tubes. For inoculation, the bacterial stock was thawed and prepared in 7H9 broth media so that the inoculation volume (500 µl) contained sufficient numbers of bacterial cell (1×10^5 Cfu/ml in a tube). The contents were gently mixed by upsetting the tube 3-4 times. Tubes were incubated for 24-72 hour at 37 ºC. All inoculated tubes were then injected into the MGIT 960 automated culture system after the barcode scanning and incubated again at the temperature of 37 °C. For positive control, isoniazid or no drugs were used. The growth control (GC) tube was prepared by adding 800 µl growth supplement and 500 µl bacterial suspensions into MGIT tube. In the case of positive growth, the system automatically detects growth and signals positive. After a maximum of 72 hour the instrument flags a tube as negative if no growth occurs [25].

3. Results

3.1 Plant collection and authentication

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

3.2 Percentage yield of selected plant extract

Alcoholic, 70% hydro-alcoholic and aqueous extracts were prepared to screen its antimycobacterial activity using different model. % yield of prepared extracts are shown in Table.2.

Table 2: Percentage yield of selected plant extracts

Sr. No	Name of the plants	% yield of extracts		
		Alcoholic extract	Hydro-alcoholic extract	Aqueous extract
1	*Emblicgofficinalis*	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	*Operus rotundas*	08.28%	10.47%	15.46%
6	*Alpinia galanga*	08.26%	05.92%	06.63%
7	*Oscimum sanctum*	17.87%	1.10%	28.21%
8	*Adhatoda vasica*	13.30%	28.56%	36.26%
9	*Piper longue:*	21.52%	40.78%	45.76%
10	*Lep:ago/a reticulata*	08.80%	09.40%	10.39%
11	*Cocculus hirsutus*	16.00%	23.48%	30.12%

3.3 Mycobacterial growth indicator tube assay (MGIT ASSAY)

The present study was conducted to investigate the percentage inhibition against *Mycobacterium smegmatis* of three different extracts of eleven selected medicinal plants.

The *anti-mycobacterial smegmatis* effects of the extracts were evaluated using the MGIT 960 system assay. The bacteria were incubated with various concentrations (100, 500 and 1000 µg/ml) of the all three extracts of selected medicinal plants and *anti-mycobacterial smegmatis* inhibition compared with first-line drugs Isoniazid (50 µg/ml) in an MGIT growth media tube of the BACTEC™ MGIT 960 system device testing for 72 hour. The growth units were markedly inhibited in a concentration-dependent manner. The Percentage inhibition of the alcoholic extract, hydroalcoholic extract and aqueous extract is shown in Figure 1, 2 and 3.

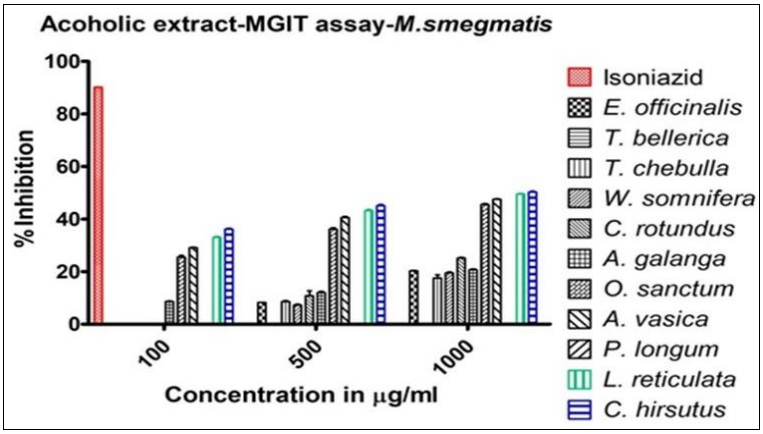

Fig 1: Percentage inhibition of the Alcoholic extracts of the selected plants again M. *smegmatis* via MGIT Assay

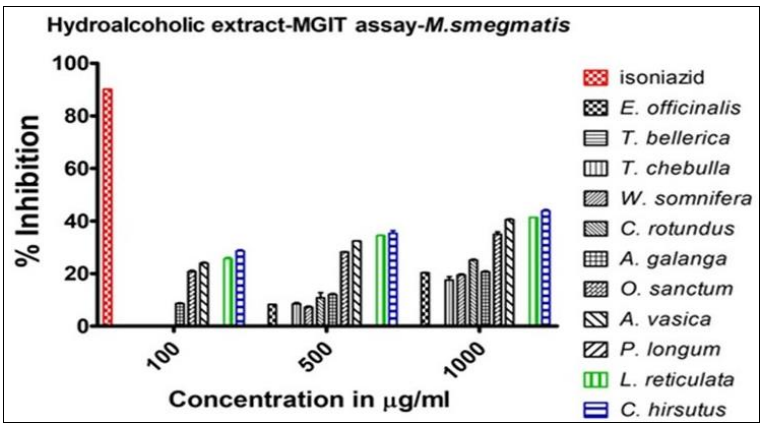

Fig 2: Percentage inhibition of the Hydroalcoholic extracts of the selected plants against *M. smegmatis* via MGIT Assay

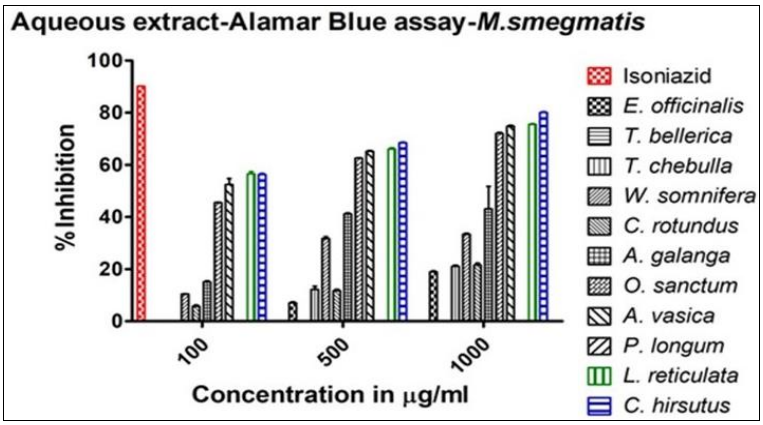

Fig 3: Percentage inhibition of the aqueous extracts of the selected plants against *M. smegmatis* via MGIT Assay

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.

4. Discussion

The worldwide increase in the incidence of tuberculosis [26] and the increasing number of *Mycobacteria* in immune compromised patients [27] which needs fast and effective cultivation approaches that can easily be useful in *Mycobacteriology* laboratory.

The present anti-mycobacterial drugs induce various side effects including hepatotoxicity and nephrotoxicity [28-30]. However, anti-tuberculosis agents of active next-generation that can be used as possible first line anti-tuberculosis drugs, mainly, the extracts, natural products, and/or semisynthetic compounds, which are not yet reported in the global pharmaceutical market.

One of the greatest developments in the direction of MGIT: it is very easy to handle, non-radiometric, and it does not require any sophisticated instrumentation. Our study compared MGIT with known cultivation methods for Acid Fast Bacteria and which defines the most significant parameters 1) the rate of recovery and mean time to detection, and 2) speed.

Rapid diagnosis of *Mycobacterial* infections is very hazardous or unsafe. The BACTEC MGIT 960 system is a totally automated culture system, due to nonstop monitoring of O_2 consumption, it allows detection, without delay, of the *mycobacteria* growing within a liquid medium.

The MGIT assay consists of liquid broth medium for better recovery and faster growth of *Mycobacteria*. The MGIT Tube contains modified Middlebrook 7H9 broth base. It also

contains an oxygen-quenched fluorochrome, fixed in silicone at the bottom of the tube. When bacterial growth appears within the tube, the free oxygen is used and is replaced by carbon dioxide. The decrease of free oxygen made the fluorochrome was no longer inhibited and produces fluorescence within the MGIT tube when visualized under UV light [31]. The percentage of inhibition of bacterial culture between the sample tube and culture tube with free of sample was evaluated by the software, until the growing culture tube became positive. If the comparative growth of the plant extracts containing tube was equal to or exceeded that of the growth culture tube, the bacterial was considered as a resistant, if the comparative growth was fewer than in the growth culture tube, the bacteria was considered susceptible. The selected plants were already proven for its anti-tuberculosis activity [32, 33]. The results obtained from this MGIT assay showed the significant anti-mycobacterial activity of aqueous extracts of *Oscimum sanctum* (71.26), *Adhatoda vasica* (74.63), *Leptadenia reticulata (*77.91%) *and Cocculus hirsutus (*82.77%) out of eleven selected plants at three different concentrations. It showed aqueous extracts of *Leptadenia reticulata* and *Cocculus hirsutus* shows potent anti-mycobacterial activity at 500 µg/ml concentration. *Leptadenia reticulata* and *Cocculus hirsutus* are reported for their anti-tuberculosis use in the literature along with their other reported uses like immunomodulators, hepatoprotective, etc. which not only helps in preventing *tuberculosis* but also acts as chemoprotective, ultimately resulting in the improvement of the overall health of the patient.

5. Conclusion

In conclusion, with growing rates of tuberculosis throughout the world and the rise of MDR-TB and XDR-TB, there is a need for novel anti-mycobacterial drugs. Using the MRA as an evaluating tool, this study measured the anti-mycobacterial properties of medicinal plants. The aqueous extracts of *Leptadenia reticulata* and *Cocculus hirsutus* were found to exhibit very strong anti-mycobacterial activity. The anti-mycobacterial activity of the plant extracts investigated in this study was screened 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 *Leptadenia reticulata* and *Cocculus hirsutus* and further studies using the virulent *M. tuberculosis* H37Rv strain are currently underway.

6. Abbreviations

TB: Tuberculosis, MGIT: Mycobacterial Growth Indicator Tube, WHO: World Health Organization, MDR: Multi Drug Resistant, XDR: Extensively Drug Resistant, LJ Medium: Lowenstein Jensen Medium, MTCC: Microbial Type Culture Collection and Gene bank, *M. smegmatis: Mycobacterium smegmatis*, OADC: Oleic acid, Albumin, Dextrose and Catalase, GC: Growth Control.

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