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DETERMINATION OF ANTI-TUBERCULAR ACTIVITY OF FOUR INDIAN MEDICINAL PLANTS AGAINST *MYCOBACTERIUM TUBERCULOSIS* BY BROTH MICRO DILUTION METHOD

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ABSTRACT: Tuberculosis is one of the oldest killer diseases of mankind. It is an airborne communicable disease caused by transmission of aerosolized droplets of *Mycobacterium tuberculosis*. The increasing incidence of MDR- and XDR-TB worldwide highlighted the urgent need to search for newer anti-tuberculosis compounds/drugs. The World Health Organization (WHO) estimated that approximately 80% of world population relies mainly on traditional medicines, mostly plant drugs in their health care. The higher plant extracts are promising sources of novel anti-TB leads. Screening and estimation of lyophilised aqueous, aqueous ethanol and ethanolic extracts of four traditional Indian medicinal plants *Acalypha indica*, *Adhatoda vasica*, *Allium cepa* and *Allium sativum* for anti-tubercular activity to standard strain H37Rv, clinical isolate of Isoniazid mono resistant and poly resistant isolates by the sensitive and reliable Broth Microdilution Method (BMM). Inhibition of *M. tuberculosis* isolates was observed for two of the four medicinal plants. All the three extracts of *Allium sativum* were found to be active in broth dilution assays with MIC of 500µg/ml and higher against the tested strains. The ethanolic extract of *Allium cepa* was found to be active with MIC of 500µg/ml against the tested strains. While the extracts of *A. vasica* and *A. indica* did not inhibit the growth of *M. tuberculosis* even at 500 µg/ml. The extracts active against *M. tuberculosis* were evaluated for cytotoxicity. The maximal toxic free concentration on Vero cells were at 500µg/ml showing that the extracts were not toxic to Vero cells in the concentrations tested.

INTRODUCTION: Tuberculosis is one of the oldest killer diseases of mankind. It is an airborne communicable disease caused by transmission of aerosolized droplets of *Mycobacterium tuberculosis* affecting almost all the organs of the body, the lungs being most commonly involved.

Despite the availability of effective antituberculosis chemotherapy for over 60 years, TB remains a major global health problem. TB remains the leading single cause of death due to an infectious agent throughout the world. As the incidence of TB continued to increase, the World Health Organization (WHO) in 1993 declared TB a global emergency.

In 2010, there were an estimated 8.8 million incident cases of TB globally, equivalent to 128 cases per 100 000 population. Most of the estimated number of cases in 2010 occurred in Asia (59%) and Africa (26%).

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The five countries with the largest number of incident cases in 2010 were India (2.0 million–2.5 million), China (0.9 million–1.2 million), South Africa (0.40 million–0.59 million), Indonesia (0.37 million–0.54 million) and Pakistan (0.33 million–0.48 million). India alone accounted for an estimated one quarter (26%) of all TB cases worldwide, and China and India combined accounted for 38%.¹

The World Health Organization (WHO) estimated that approximately 80% of world population relies mainly on traditional medicines, mostly plant drugs in their health care². The higher plant extracts are promising sources of novel anti-TB leads. India is one of the few countries in the world which has unique wealth of medicinal plants and vast traditional knowledge of use of herbal medicine for cure of various diseases.

Broth Microdilution method is easy to perform and does not require special equipment or expensive supplies. In contrast to radiometric method it does not use radioactive material. The BMM reads faster (14-20 days) than the gold standard proportion method (20-28 days)³. It is reliable and reproducible and easily quality controlled⁴.

According to Ayurvedic and Greek systems of medicine garlic is one of the established remedies for tuberculosis⁵.

The water extract of garlic inhibited the *M. tuberculosis* H37Rv and an isoniazid resistant strain with a minimum inhibitory concentration slightly above 80 µg/ml and less than 160 µg/ml for susceptible strain and slightly above 100 µg/ml and less than 200 µg/ml for resistant strain⁶. The water and ethanolic extracts of selected medicinal plants (*A. sativum* -bulb, *A. cepa* -tissue, *S. aromaticum* – flowerbud, *C. verum* -bark) were reported to have anti-TB activity for *M. tuberculosis* H37Ra by Microtiter Alamar Blue assay⁷.

The Ethanolic extract of garlic was prepared by maceration method. Minimum inhibitory concentration (MIC) was performed by using 7H9 middle brook broth dilution technique. MIC of garlic extract was ranged from 1 to 3 mg/ml; showing inhibitory effects of garlic against both non-MDR and MDR *M. tuberculosis* isolates.⁵

The study on *in vitro* anti-tubercular activity of five medicinal plants viz., *Acalypha indica*, *Adhatoda vasica*, *Allium cepa*, *Allium sativum* and *Aloe vera* suggested the estimation of minimum inhibitory concentration (MIC) in suitable broth based media as MICs defined in broth are more accurate⁸.

The clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug-resistant pathogens. The increasing incidence of MDR- and XDR-TB worldwide highlighted the urgent need to search for newer anti-tuberculosis compounds/drugs.

Hence the present study was aimed for screening and estimation of four traditional Indian medicinal plants for anti-tubercular activity and determination of MIC by the sensitive and reliable Broth Microdilution Method (BMM).

MATERIALS AND METHODOLOGY:

Plants chosen for study:

1. *Acalypha indica* L. known as kuppaimani in tamil is an annual weed. It belongs to the family Euphorbiaceae. It is a common weed in many parts of Asia. It grows in the common farmlands, gardens, roadside waste lands. Parts used are leaves, root, stalk and flowers. This plant is used as diuretic, antihelmintic and for respiratory problems such as bronchitis, asthma and pneumonia.⁹
2. *Adhatoda vasica* nees (Acanthaceae) commonly known as vasaka is distributed throughout India up to an attitude of 1300m. The leaves, flowers, fruit, and roots are extensively used for treating cold cough, whooping cough, chronic bronchitis and asthma as sedative, expectorant and antispasmodic.

The plant is recommended for a variety of ailments such as bronchitis, asthma, fever, jaundice etc. The leaves & roots are efficacious in coughs, arthritis, diarrhoea and dysentery and have the best chemostatic quality. Leaves are anti-inflammatory, analgesic effective in skin disorders, cardiotoxic^{10,11}.

- Allium cepa* linn. belonging to the family Liliaceae, commonly known as 'Onion' having green stems and hollow leaves and can grow up to 3ft in height. The plants bear small flowers that are usually white or purple in color. Medicinally it is used in treatment of cold, cough, laryngitis, allergies So far; a few plants have been tested and showed to have anti-TB activity^{8,12}.
- Allium sativum* L. belongs to the family Liliaceae, commonly known as garlic. Garlic is a well-known indigenous herbal medicine since ancient times and held a place of honor in Indian traditional Ayurvedic medicine. Most of therapeutic effects are ascribed to specific

oil and water-soluble organosulphur compounds, which are responsible for the typical odor and flavor of garlic. They exhibit different antibacterial, antifungal, antiseptic antiviral, expectorant, antihistamine properties.¹³

Collection of plant parts: Leaves of *Acalypha indica* L., *Adhatoda vasica* Nees., bulbs of *Allium cepa* L. and cloves of *Allium sativum* L., were chosen. The potential & healthy plant parts were collected. The accuracy of the plant parts and family were ascertained with the Department of plant Biology & Biotechnology, Presidency College (Aut), Chennai. The names of plants and parts of plant used are shown in **Table 1**.

TABLE 1: PLANTS USED IN THE STUDY

Botanical name/ Family	Common name	Part used	Extracts taken	Ethano medicinal use
<i>Acalypha indica</i> L. (Euphorbiaceae)	Acalypha	Leaf	Aqueous/aqueous ethanolic/ethanolic	bronchitis, asthma
<i>Adhatoda vasica</i> Nees. (Acanthaceae)	Vasaka	Leaf	Aqueous/Aqueous Ethanolic/Ethanolic	cold cough, whooping cough, chronic bronchitis and asthma
<i>Allium cepa</i> L. (Alliaceae)	Onion	Bulb	Aqueous/Aqueous Ethanolic/Ethanolic	cold, cough, laryngitis, allergies
<i>Allium sativum</i> L. (Alliaceae)	Garlic	Clove	Aqueous/Aqueous Ethanolic/Ethanolic	cough

Preparation of Aqueous, Aqueous ethanolic and Ethanolic Extracts: Crude extracts of the medicinal plants were obtained according to traditional use for treating respiratory diseases in the indigenous system of medicine in India and also for the treatment of tuberculosis based on literature.

The healthy parts were surface sterilized with 70% ethanol. The parts were finely ground in a mixer. 20 gms of the ground plant part was soaked as follows

- Ethanolic (95%) Extract:** 20 gms of the specified finely ground plant part was soaked with 100 ml Ethanol and stored overnight at 4° C. Filtered and centrifuged to get clarified extract. This method repeated 3 times more, all extracts pooled together and concentrated, filtered through a 0.22µm pore sized millipore filter and lyophilized.
- Aqueous - Alcoholic Extract:** 20 gms of the specified finely ground plant part was soaked in 50 ml Ethanol – 50 ml water (1:1) and stored overnight at 4° C. Filtered and

centrifuged to get clarified extract. This method repeated 3 times more, all extracts pooled together and concentrated, filtered through a 0.22µm pore sized millipore filter and lyophilized.

- Aqueous Extract** - 20 gms of the specified finely ground plant part was soaked with 100 ml water and stored overnight at 4° C. Filtered and centrifuged to get clarified extract. This method repeated 3 times more, all extracts pooled together, concentrated, filtered through a 0.22µm pore sized millipore filter and lyophilized.

Lyophilization: The sterile extract was transferred to lyophilization flask & frozen at -80°C in deep freezer. The frozen extract was loaded to Lyophilizer. The lyophilised extract was stored in – 20° C till bioevaluation.

Preparation of plant extracts for Broth Microdilution Assay (BMM): Stock solution of the extract (1mg/ml) was prepared using DMSO. It was verified that DMSO did not suppress or delay

the growth of *M. tuberculosis* strains. Reference drug controls with Rifampicin and Isoniazid were also prepared by dissolving in water for INH and DMSO for Rifampicin mixed with Middlebrook 7H9 broth medium.

The stock solutions were sterilized through 0.2 µm millipore single use filter.

Determination of Minimal Inhibitory concentration (MIC) by Broth Microdilution Method:

1. **Test organism:** Clinical resistant isolates and reference strains for *M. tuberculosis* (H37Rv) was obtained from Bacteriology Department, Tuberculosis Research Centre (TRC), Chennai, India.
2. **Inoculum preparation:** Freshly grown colonies from LJ medium were transferred to a tube containing 3-4 ml phosphate buffered saline and 6 to 9 sterile glass beads. Tubes were vigorously agitated on a vortex mixer and clumps were allowed to settle for 30 min. The supernatant was transferred to sterile tubes. The supernatant was then adjusted with phosphate buffer saline to equal the density of 0.5 McFarland standards for use as the standard inoculum in the Broth Micro dilution Method (BMM).¹⁴
3. **The Broth Microdilution Method:** The BMM was performed in 96-well microtitre plates with U-shaped wells. Wells were filled with 0.1 ml amounts of Middlebrook 7H9 broth, supplemented with oleic acid, albumin, dextrose and catalase (ADC) enrichment. Serial two fold dilution in Middlebrook 7H9 broths were made of each of the extract with concentrations ranging from 500 µg/ml to 3.9µg/ml.

Each well was inoculated with 5 µl of 0.5 McFarland standard bacterial suspensions. A well without antimycobacterial agents was also inoculated with of 5 µl of 0.5 McFarland standard as a growth control. 0.1 ml amounts of Middlebrook 7H9 broth were added and the microtitre plates were sealed and incubated at 37°C for 14 days.¹⁴

Cytotoxicity assay: Assaying the toxicity of the isolated compounds on cell line was carried out in tissue Culture 96 well microtitre plates following the methods adopted and described by Julia Serkedjieva *et al* (1999).

Cytotoxicity is mainly for evaluating the safety of the separated plant components for determining the toxic free concentration of the test drugs on the cell line (Vero cells)¹⁵.

0.1 ml cellular suspension was transferred into each of the wells containing growth medium. The plate was incubated at 37°C in 5% CO₂ atmosphere in CO₂ incubator for 12 hours. The growth medium was removed after confluence was obtained by micropipette. 1mg of the extracts was dissolved (standard/test) in 1 ml 2% FBS MEM. The extracts mixed medium was serially diluted in two fold manner in 2% FBS MEM, from an initial concentration of 500µg / ml to a final concentration of 3.9 µg/ml. 0.1ml of the serially diluted drug and was added into the wells. For standard, adopting the same procedure, similar preparation of Isoniazid and Rifampicin (standard drug) were added to another row of cells for sensitive and INH resistant isolates respectively. The plate incubated at 37°C in 5% CO₂ atmosphere for 72 hours and was observed under inverted phase contrast microscope for determination of toxic free concentration

RESULTS: The anti-tubercular potency of the active plant extracts were determined by Micro Broth dilution Method. Growth and inhibition of *M. tuberculosis* H37Rv, mono resistant and poly resistant isolates by Broth Microdilution method on extract containing and extract free control wells after 14 days of incubation at 37°C were recorded (Table-2). Inhibition of *M. tuberculosis* isolates was observed for two of the four medicinal plants. All the three extracts of *Allium sativum* were found to be active in broth dilution assays with MIC of 500µg/ml and higher against the tested strains. However only the ethanolic extract of *Allium cepa* was found to be active with MIC of 500µg/ml against the tested strains.

The plate incubated at 37°C in 5% CO₂ atmosphere for 72 hours was observed under inverted phase contrast microscope for determination of cytotoxicity.

The cytotoxicity of the extracts active against *M. tuberculosis* were evaluated showing that the extracts were not toxic to VERO cells from 500µg/ml.

DISCUSSION: The increasing occurrence of MDR and XDR-TB made anti-tuberculosis therapy more difficult. Although strategies have been proposed in an attempt to control the spread, the search for new ways to treat drug-resistant infections stimulates the investigation of natural compounds as an alternative treatment of these infections¹⁶.

The present study was carried out to find the *in vitro* antimycobacterial potential of the four selected Indian medicinal plants based on literature on avirulent strain of H37Rv and isoniazid resistant clinical isolates. A few studies have also been proving antimycobacterial activity of garlic against different species of mycobacteria. Several plants investigated for their antituberculosis activity like *Adhatoda vasica*, *Allium cepa*, *Acalypha indica* and *Aloe vera* and have been suggested to have anti-tuberculosis activity, but determination of MIC has been attempted in this study by Broth Microdilution Method. Estimation of toxic free concentration by *in vitro* cytotoxicity assay was carried out. This study has proven the extract as toxic free in the concentrations tested.

All the three extracts from *Allium sativum* showed activity and the Minimum inhibitory concentration was at a concentration of 500µg/ml against the tested strains of *Mycobacterium tuberculosis*.

This is in agreement with the earlier by Gupta *et al* 2010. However Gupta *et al* have not estimated the MIC of the extracts and also have estimated the activity of water extract only. Whereas the present study have been carried out with aqueous, aqueous ethanolic and ethanolic extracts.

The ethanolic extract of *Allium cepa* alone exhibited activity and the Minimum inhibitory concentration was at a concentration of 500µg/ml against the tested organisms rather than aqueous extract and aqueous ethanolic extract of *Allium cepa*.

All the three extracts of *Adhatoda vasica* and *Acalypha indica* did not however show any significant anti-tubercular activity in the concentrations tested.

Our study demonstrated the MIC of both *Allium sativum* and *Allium cepa* and these plants could be evaluated further for developing a drug to control *M. tuberculosis*.

TABLE 2: MINIMUM INHIBITORY CONCENTRATION OF PLANT EXTRACTS IN MIDDLEBROKE 7H9 MEDIUM BY BROTH MICRO DILUTION METHOD

Strain	Lab code	<i>A. indica</i>	<i>A. vasica</i>	<i>A. cepa</i>	<i>A. sativum</i>	INH 0.2 (µg/ml)	Rif 2.0 (µg/ml)
		Aqueous/Aqueous ethanolic/ethanolic extracts (µg/ml)	Aqueous/Aqueous ethanolic/ethanolic extracts (µg/ml)	Ethanolic extract (µg/ml)	Aqueous & aqueous ethanolic/ethanolic extracts (µg/ml)		
<i>M. tuberculosis</i> H37Rv (Sen)	H37Rv	NA	NA	500	500	-	-
Resistant (H-Res)	H-485	NA	NA	500	500	+	-
Resistant (SH-Res)	SH-577	NA	NA	500	500	+	-
Resistant (SHOF-Res)	SHOF-567	NA	NA	500	500	+	-

Sen – sensitive strain, H res- INH resistant, SH res - Streptomycin, Isoniazid, SHOF res – Streptomycin, Isoniazid and Ofloxacin resistant (+) presence of growth (-) absence of growth, RIF- Rifampicin; INH- Isoniazid

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