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In-Vitro anti-tuberculosis activity of selected ethnomedicinal plants

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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, highlight the urgent need to search for newer anti-Tb drugs. So, the present aim to carry out the evaluation of the anti-Tb activity of selected ethnomedicinal plants. Three different extracts were prepared and evaluated for anti-Tb activity on *Mycobacterium smegmatis* using cup and bore method. Isoniazid and Rifampicin were used as standard drug. The zone of inhibition was taken to assess antitubercular activity. Among tested all plants Tulsi, Vasaka and Rasna shows potent antituberculosis activity. Thus, its result supports the uses of these plants in traditional medicine and can be further studied using more specific methods for anti-Tb activity.

Keywords: *M. smegmatis*, ethnomedicinal plants, Antituberculosis activity

1. Introduction
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-tubercular drugs, drug resistance being one of the reasons. The increasing incidence of MDR and XDR-TB worldwide highlight the urgent need to search for newer anti-tubercular drug [1]. Tuberculosis is caused by the infection with *Mycobacterium tuberculosis.*
Mycobacterium smegmatis is an acid-fast bacterial species in the phylum Actinobacteria and the genus *Mycobacterium*. It is 3.0 to 5.0 µm long with a bacillus shape and can be stained by Ziehl-Neelsen method [2]. The bacteria will be finely wrinkled and creamy white while it is growing on accessible nutrients. When *Mycobacterium smegmatis* has been growing for quite some time (generally after 48 hr. growth) and is abundant, the color will turn from white to a non-pigmented creamy yellow. It will also be waxy because of the high amount of unique Gram-positive cell wall coated with mycolic acids. The bacteria also range in textures, being seen as smooth, flat and glistening or coarsely folded or finely wrinkled [3].
Mycobacterium smegmatis is very useful for the research analysis of other species in the genus *Mycobacteria* in cell culture laboratories. There are several *Mycobacterial species* that are common, harmful like *Mycobacterium leprae, Mycobacterium tuberculosis and Mycobacterium bovis*. *Mycobacterium smegmatis* is so important because it is fast growing and non-pathogenic compared to these species. There are many similarities between *Mycobacterium smegmatis* and the much more virulent obligate pathogens that are *Mycobacteria*. The most significant is the complementary uses of mycothiol biosynthesis of *Mycobacterium* for making an essential thiol that is responsible for life. If it is knocked out, the species will be terminated and a treatment will be found [4]. There is also research involved in finding drug therapies that will inhibit the myolic acid biosynthesis which is essential for creating the unique bacterial cell wall. Currently, there are many laboratories that are culturing and isolating this species to determine the pathological course of deleterious *Mycobacteria* [5].
So, the present study was aim to carried out the evaluation of the anti-tubercular activity of selected ethnomedicinal plants. There are nine different plants were selected on basis of their ethnomedicinal uses in the tribal areas.

2. Materials and Methods
2.1 Collection and authentication of selected ethnomedicinal plant material
All the selected ethnomedicinal plants were collected at the different geographical sources when it fully grown with flowering. Plants raw material was authenticated by taxonomist. Plants having Herbarium specimen number PH/015/001 to PH/015/009 as deposited at

Pharmacognosy Department, K.B.I.P.E.R., Gandhinagar, Gujarat and India for future reference. The raw material were dried under shade and reduced mechanically to moderate coarse powder.

Plants were selected on their ethnomedicinal used in tribal areas. Following nine plants were selected for their *In-Vitro* Anti-tubercular activity.

Selected ethnomedicinal plants for cytotoxicity study on Vero cell line

Plant name	Botanical source	Family	Part used
Amla [6]	*Emblica officinalis*	Euphorbiaceae	Fruits
Baheda [7]	*Terminalia bellerica*	Combretaceae	Fruits
Harde [8]	*Terminalia chebula*	*Combretaceae*	Fruits
Ashwagandha [9]	*Withania somnifera*	Solanaceae	Roots
Nagarmoth [10]	*Cyperus rotundus*	Cyperaceae	Rhizomes
Rasna [11]	*Alpinia galanga*	Zingiberaceae	Rhizomes
Tulsi [12]	*Ocimum sanctum*	Liliaceae	Leaves
Vasaka [13]	*Adhatoda vasica*	Acanthaceae	Leaves
Long pepper [14]	*Piper longum*	Piperaceae	Fruits

2.2 Preparation of plant extracts [15]

20 gm. of powder of selected plants were taken to prepare its different extracts. Aqueous, Hydro-alcoholic and alcoholic extracts were prepared by maceration of powder material for 48 hours. Solvents were removed by Rota evaporator. Percentage yield were calculated.

2.3 *In-Vitro* Anti-Tuberculosis activity

2.3.1 Material required

Bacterial strain: *Mycobacterium smegmatis (MTCC 6)*

Medium used: Lowenstein Jensen Medium (LJ Medium) (Hi-media)

Medium base: Middle Brooke 7H10 Agar Base (Hi-media)

2.3.2 Glassware and plastic wares: glass petri plates, media bottles, tips, centrifuge tubes, volumetric flask.

2.3.3 Equipment: Biosafety cabinet class II, BOD incubator, Deep freezer, Micropipettes, RO water system.

2.4 Preparation of medium

2.4.1 Lowenstein-Jensen medium (LJ medium)

Suspend 37.24 grams in 600 ml distilled water containing 12 ml glycerol. Heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 15 lb pressure (121 °C) for 15 minutes. Meanwhile prepare 1000 ml of whole egg emulsion. Aseptically add and mix egg emulsion base in prepared LJ medium. Autoclave the prepared medium at 85°C for 45 minutes. Then add freezed dried culture of *Mycobacterium smegmatis* (MTCC 6) in LJ medium. Incubate it at 37°C for seven days for the growth of bacteria [16].

Fig 1: Lowenstein-Jensen medium (LJ medium)

2.4.2 Preparation of Inoculum

Add one loopful of fresh cultured *Mycobacterium smegmatis* in freshly prepared LJ medium. Incubate prepared medium with bacteria for 24 hrs at 37 ºC. Check the growth of bacteria similar as 0.5 McFarland standards dilution for the In-Vitro assay. If the growth is more than 0.5 McFarland standards dilution then again serial dilute the bacterial culture. Purity of prepared culture was confirmed by Ziehl-Neelsen Staining specific for confirmation of acid fast bacilli.

2.4.3 Acid fast stain of *Mycobacterium smegmatis*

Mycobacterium smegmatis culture was heat fixed on clean slide, allow smear to air dry.

It was stained with Ziehl-neelsen-carbolfusin dye and allows it to stand for 5min, wash it with tap water. It was decolorized with 20% sulphuric acid and again washes with water. Counter stained with Methylene blue for 2 min. Wash the smear with tap water [17].

The slide was observed under microscope.

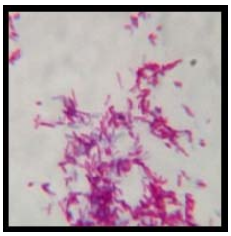

Fig 2: *Mycobacterium smegmatis*

2.5 The Agar Diffusion Cup Method [18]

This method is used to screen the anti-tuberculosis activity of selected ethnomedicinal plants. Agar plates were seeded with 0.5 McFarland standards bacterial culture of *M. smegmatis*. Agar plates were then bore holed using 6 mm diameter cork bore.100, 500, 1000 µg/ml concentration of extracts were prepared to perform this assay. 0.3 ml each of the extract concentration was introduced into the hole and allowed to diffuse for 5-10 minutes before incubation. The Petri dishes used for antitubercular screening were incubated at 37 ^{0}C for 48 hours. All the concentration was done in triplicate to minimize the error. The inhibition zone diameters (IZD) were determined and recorded for further analysis. Isoniazid and Rifampicin were used as a standard.

3. Result

3.1 Preparation of extracts

Alcoholic, aqueous and 70% hydro-alcoholic extracts were prepared to screen its anti-tubercular activity % yield of prepared extracts are shown in Table no. 1

Table 1: % Yield of prepared extracts of selected plants

Sr. No	Name of plant	%Yield of extracts		
		Aqueous	Hydro-alcoholic	Alcoholic
1	Amla	52.92%	63.52%	44.338%
2	Baheda	66.68%	55.16%	42.46%
3	Harde	46.56%	50.58%	45.08%
4	Ashwagandha	24.16%	15.32%	8.91%
5	Nagarmoth	15.46%	10.47%	8.28%
6	Rasna	6.63%	5.92%	8.26%
7	Tulsi	28.21%	21.10%	17.87%
8	Vasaka	13.30%	28.56%	36.26%
9	Long pepper	45.76%	40.78%	21.52%

3.2 In Vitro anti-tuberculosis activity of selected plant extracts

Plants shows significant zone of inhibition on *Mycobacterium smegmatis* at different concentration is shown in Table 2. From these plants vasaka shows maximum anti-TB activity comparing with standard drug.

Table 2: Zone of inhibition of different plant extracts

Sr. no.	Name of plant extract	Tulsi			Vasaka			Rasna		
		Alcoholic extract	Hydro-alcoholic extract	Aqueous extract	Alcoholic extract	Hydro-alcoholic extract	Aqueous extract	Alcoholic extract	Hydro-alcoholic extract	Aqueous extract
1	100 µg/ml	5mm	-	4mm	3mm	6mm	10mm	3mm	4mm	-
2	500 µg/ml	11mm	-	5mm	6mm	11mm	15mm	8mm	7mm	-
3	1000 µg/ml	13mm	1mm	14mm	10mm	16mm	19mm	10mm	11mm	2mm
4	Isoniazid+Rifampicin (100µg/ml)		21mm	23mm	22mm					

4. Discussion and Conclusion

All the ethnomedicinally selected plants were screened for their anti-tuberculosis activity. From these nine plants Vasaka, Tulsi and Rasna plant extracts shows maximum anti-tuberculosis activity on *M. smegmetis*. Amongst these three plants, aqueous extracts of vasaka shows maximum anti-TB activity as compare to all other plant extracts. Further studies using more specific methods are required to explore the constituents responsible for the activity and the mechanism of this activity which might prove important and improved therapies for the treatment and prevention of tuberculosis.

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