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ANTI-MYCOBACTERIAL POTENTIAL OF *TECOMA STANS* L. SYN. *BIGNONIA STANS* (BIGNONIACEAE)

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In the present study antibacterial activity of aqueous and alcoholic extracts of stem bark and leaves of *Tecoma stans* L. syn. *Bignonia stans* (Bignoniaceae) was tested against MDR isolates DKU-156 and JAL-1236 of *M. tuberculosis*, reference susceptible strain *M. tuberculosis* H37Rv as well as fast growing mycobacterial pathogen *M. fortuitum* (TMC-1529). The leaves and bark collected between spring and summer season were dried and extracts was prepared using three portions of the dried powdered bark and leaves. It was soaked separately in 500 ml of distilled water and ethanol (98%) for 72 h and refluxed and filtrates were concentrated under vacuum at 40°C to obtain the dry extracts. Reference drug susceptible strain *M. tuberculosis* H37Rv as control, multi-drug resistant isolates DKU-156, JAL-1236 and fast growing mycobacterial pathogen *M. fortuitum* (TMC-1529) were used during the present investigation. Antimicrobial assays were performed in Lowenstein Jensen (L-J) medium and Middlebrook 7H9 broth in BacT/ALERT 3D system (Sigma-Aldrich, St. Louis, USA). The aqueous and alcoholic extracts of stem bark and leaves were incorporated in the media. Susceptibility testing of MDR isolates was also performed against streptomycin in the same batch of media for comparison of cfu on drug free controls. The results of the present investigation clearly showed that the aqueous extracts of stem bark were more effective as compared to aqueous and leaf extracts and alcoholic stem bark and leaf extracts.

Keywords : *M. tuberculosis*, Lowenstein Jensen medium and Middlebrook 7H9 broth in BacT/ALERT 3D system, streptomycin

The World Health Organization has listed 20,000 medicinal plants globally and about 2000 drugs used are of plant origin (WHO 2009). India's contribution is 15-20%. More than 7,500 species of medicinal plants grow in India which is considered as the botanical garden of the world. More than 70% of India's populations still use herbal drugs (Ayurveda, Yoga, Unani, Sidha, Homeopathy and Naturopathy).

Tuberculosis, MTB or TB (short for *tubercle bacillus*) is a common, and in many cases lethal, infectious disease caused by various strains of mycobacteria, usually *Mycobacterium tuberculosis* (Kumar *et al.* 2007). Tuberculosis typically attacks the lungs, but can also affect other parts of the body. Tuberculosis is a highly infectious disease with about one third of the world's population including 40 per cent from India estimated to be infected by it (WHO 2011). India accounts for one third of the global tuberculosis burden of the world. 40% of the Indian population is infected with the TB bacillus. Every day more than 20,000 people get infected with tuberculosis, more than 5,000 people develop TB and more than 1,000 people die of TB in India. Tuberculosis thus continues to be the leading single infection cause of death (Katoch 2004).

Medicinal plants offer a great hope to fulfill these needs and have been used for curing diseases for many centuries. These have been used extensively as pure compounds or as a crude material. Only a few plant species have been thoroughly investigated for their medicinal properties (WHO, 2010). 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 (WHO 2011). So far, few plants have been tested against mycobacteria and a few plants which showed anti-TB activity were *Salvia hypargeia*, *Euclea natalensis* etc. (WHO 2009, 2010, 2011). Gupta *et al.* (2010) carried a study to check the antibacterial activity of aqueous extracts of five plants (*Adhatoda vasica*, *Allium cepa*, *Aloe vera*, *Acalypha indica* and *Allium sativum*) against MDR isolates of *M. tuberculosis*, reference susceptible strain *M. tuberculosis* H37Rv as well as fast growing mycobacterial pathogen *M. fortuitum* (TMC-1529).

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In recent years more attention is being directed towards herbal medicines because these are inexpensive, non-toxic and eco-friendly. There are larger numbers of phyto-pharmaceuticals isolated from plants which are being used in modern medicine. Plants are known to contain innumerable biological active compounds (Alade and Irobi 1993), which possess antibacterial properties (Brantner and Grein 1994, Samy and Ignacimuthu 1998). Medicinal components from plants play an important role in conventional as well as in western medicine. Plant derived medicines have been a part of the evolution of human health-care for thousands of years. Plant based medicines were commonly used in India and China. Although a large number of plants have been tested for antibacterial properties against gram positive and gram negative bacterial organisms, but only a few have been tested against mycobacteria.

Worldwide, the Bignoniaceae are mostly tropical trees or shrubs comprising of 120 genera and about 800 species (Lohmann 2004). In India the family is represented by species found chiefly in western and southern parts and a few are found in Himalayan region (Chauhan 2008). Several studies have shown that the vegetative parts of several members of the family Bignoniaceae contain a wide variety of chemical compounds (amino acids, phenolics and alkaloids) known to have antimicrobial properties (Binuto and Lajubutu 1994, Rasadah and Houghton 1998, Binuto *et al*. 2000, Costantino *et al*. 1994, 2003, Park *et al*. 2005, 2006a, 2006b, Rojas *et al*. 2006, Zaveri *et al*. 2007, Omonkhelin *et al*. 2007, Doughari *et al*. 2008, Dutta and Chaudhary 2010, Rinawati 2010). However, they have not been tested for their anti-tubercular properties. Chauhan and Chauhan (2012) have shown antimicrobial activity of some members of Bignoniaceae (*Adenochalyma alliaceum*, *Jacaranda mimosifolia, Millingtonia hortensis, Pyrostegia venusta* and *Tabebuia argentia*).

In light of the facts enumerated above, present study was carried out to check the antibacterial activity of aqueous and alcoholic extracts of stem bark and leaves of *Tecoma stans* L. syn. *Bignonia stans* (Bignoniaceae) against MDR isolates of *M. tuberculosis*, reference susceptible strain *M. tuberculosis* H37Rv as well as fast growing mycobacterial pathogen *M. fortuitum* (TMC-1529).

MATERIALS AND METHODS

Plant Material used : Present study was carried out on *Tecoma stans* L. syn. *Bignonia stans* (Bignoniaceae) plant growing in the Paliwal Park, Agra. Leaves and bark of above mentioned plants were collected between spring and summer season during March to May in the years 2011 and 2012.

Extract preparation : The plant extracts was prepared using the modified method of Alade and Irobi (1993). Three portions of the dried powdered samples (bark and leaves) were soaked separately in 500 ml of distilled water and ethanol (98%) for 72 h. Each mixture was refluxed followed by agitation at 200 rpm for 1 h. The filtrates obtained were concentrated under vacuum at 40°C to obtain the dry extracts.

Mycobacterial strains/isolates : Reference drug susceptible strain *M. tuberculosis* H37Rv as control, multi-drug resistant isolates DKU-156, JAL-1236 and fast growing mycobacterial pathogen *M. fortuitum* (TMC-1529) were obtained from Mycobacterial Repository Centre, Department of Microbiology and Molecular Biology at National JALMA Institute for Leprosy and other Mycobacterial Diseases (ICMR), Agra.

Assay protocol : Antimicrobial assays were performed in Lowenstein Jensen (L-J) medium and Middlebrook 7H9 broth in BacT/ALERT 3D system (Sigma-Aldrich, St. Louis, UAS).

Lowenstein-Jensen (L-J) medium : Determination of Colony forming units (cfu) on Lowenstein-Jensen (L-J) - The ten-fold dilution of standard 1 mg/ml *M. tuberculosis*

suspension19 were streaked on L-J medium for determining cfu in the presence and absence of plant extracts. An *M. tuberculosis* suspension of 1 mg/ml is equivalent to MacFarland standard-120. One loopful (6 µl) of this suspension was streaked on the L-J slants using 3 mm external diameter loop. Reagents of L-J media included potassium di hydrogen phosphate anhydrous (Qualigens), magnesium sulphate anhydrous (Qualigens), magesium citrate (Loba Chemie), L-asparagine (Hi-media, Mumbai), glycerol (Fisher Scientific, Mumbai) and malachite green (Hi-Media, Mumbai).

Middlebrook 7H9 broth in BacT/ALERT 3D system : Exposure of mycobacterial suspension (0.2 ml, 1mg/ml) to the millipore (0.22 µm) filtered plant extract (4% v/v) was done for 15 min at room temperature. The resultant mixture was inoculated into Mycobacterial Process (MP) bottles containing Middlebrook 7H9 broth supplemented with reconstitution fluid (Oleic acid, glycerol, & bovine serum albumin) in colorimetric BacT/ALERT 3D system (BioMerieux, France).

Minimum inhibitory concentration (MIC): Minimum inhibitory concentration (MIC) of the aqueous and alcoholic extracts of stem bark and leaves was determined by the method after Andrew (2001). In order to determine the MIC, 2% and 4% v/v concentration of each plant extract was added to LJ medium. The resistance was expressed in terms of the lowest concentration of the plant extract that inhibited all the growth i.e. minimum inhibitory concentration. A parallel set of medium containing different concentrations of the plant extracts was inoculated separately with standard inoculums (4 mg/ml).

Determination of the effect of direct exposure of bacterial suspension to the water extracts of plants was done by counting the CFUs on LJ medium after different intervals of exposure : 0.2 ml inoculums of 1 mg/ml suspension of *M. tuberculosis* was added to 0.5 ml plant extract and will be kept for 15 minutes, 2 h, 40 h and 80 h; 600 µl distilled water added after the exposure time of 15 minutes to dilute the extract so that the effective exposure can be controlled for desired duration (15 minutes) of time 30 µl of each was inoculated on LJ slants.

RESULTS AND DISCUSSION

Average growth and percentage inhibition of *M. tuberculosis* H37Rv, MDR isolates and rapid grower *M. fortuitum* (TMC-1529) by aqueous and ethanolic stem bark and leaf extracts of *Tecoma stans* added on Lowenstein Jensen (L-J) and BacT/ALERT media and extract free control L-J and BacT/ALERT 3 D system slants after 42 days of incubation at 37^{0}C is shown in Tables 1-8.

The Effect of aqueous extract of stem bark of Tecoma stans in L-J medium is shown in Table 1. It is evident from the results that addition of aqueous stem bark extract of *Tecoma stans* in L-J medium there was an average growth and 85% inhibition of MDR isolate DKU-156 and 62% inhibition for another MDR isolate JAL-1236, while for sensitive *M. tuberculosis* H37 Rv inhibition was 60% at 4% v/v concentration in L-J medium by water extract of stem bark. There was no inhibition against rapid grower *M. fortuitum* (TCM-1529).

The effect of addition of bark extract of *Tecoma stans* in water on Middlebrook 7H9 broth in BacT/ALERT 3D system is shown in Table 2. It is evident from the results that addition of water extract of stem bark of *Tecoma stans* in Middlebrook 7H9 broth in BacT/ALERT 3D medium, water extract of leaf showed poor inhibition against *M. tuberculosis*. There was an average growth and 80% inhibition of MDR isolate DKU-156 and 59% inhibition for another MDR isolate JAL-1236, while for sensitive *M. tuberculosis* H37 Rv inhibition was 58% at 4% v/v concentration in BacT/ALERT 3D medium. There was no inhibition against rapid grower *M. fortuitum* (TCM-1529).

Table 1 : Results of the effect of aqueous stem bark extract of *Tecoma stans* on *M. tuberculosis* and *M. fortuitum* in Lowenstein Jensen (L-J) medium (five replicates).

Isolate code	L-J medium				
	Mean cfu on media			% Inhibition	
	Control	Plant extract		Plant extract	
		2% v/v	4% v/v	2% v/v	4% v/v
M. tuberculosis H37Rv	42	20	12	53	60
DKU-156	18	6	2	68	85
JAL-1236	70	25	21	65	62
M. fortuitum TCM-1529	2	2	2	0	0

Table 2 : Results of the effect of aqueous stem bark extract of *Tecoma stans* on *M. tuberculosis* and *M. fortuitum* in Middlebrook 7H9 broth in BacT/ALERT 3D system (five replicates).

Isolate code	BacT/ALERT 3D system				
	Mean cfu on media			% Inhibition	
	Control	Plant extract		Plant extract	
		2% v/v	4% v/v	2% v/v	4% v/v
M. tuberculosis H37Rv	40	18	11	50	58
DKU-156	15	7	3	65	80
JAL-1236	69	23	20	61	59
M. fortuitum TCM-1529	1	3	2	1	1

Table 3 : Results of the effect of aqueous leaf extract of *Tecoma stans* on *M. tuberculosis* and *M. fortuitum* in Lowenstein Jensen (L-J) medium (five replicates).

Isolate code	Lowenstein Jensen (L-J) medium.				
	Mean cfu on media			% Inhibition	
	Control	Plant extract		Plant extract	
		2% v/v	4% v/v	2% v/v	4% v/v
M. tuberculosis H37Rv	41	22	11	50	52
DKU-156	19	8	3	66	77
JAL-1236	71	26	23	62	55
M. fortuitum TCM-1529	3	2	3	0	0

Table 4 : Results of the effect of aqueous leaf extract of *Tecoma stans* on *M. tuberculosis* and *M. fortuitum* in Middlebrook 7H9 broth in BacT/ALERT 3D system (Five replicates).

Isolate code	BacT/ALERT 3D system.				
	Mean cfu on media			% Inhibition	
	Control	Plant extract		Plant extract	
		2% v/v	4% v/v	2% v/v	4% v/v
M. tuberculosis H37Rv	41	22	11	50	53
DKU-156	19	8	3	66	73
JAL-1236	71	26	23	62	52
M. fortuitum TCM-1529	3	2	3	0	0

Table 5 : Results of the effect of ethanolic extract of stem bark of *Tecoma stans* on *M. tuberculosis* and *M. fortuitum* in Lowenstein Jensen (L-J) medium (five replicates).

Isolate code	L-J medium.				
	Mean cfu on media			% Inhibition	
	Control	Plant extract		Plant extract	
		2% v/v	4% v/v	2% v/v	4% v/v
M. tuberculosis H37Rv	41	22	11	50	51
DKU-156	19	8	3	66	71
JAL-1236	71	26	23	62	54
M. fortuitum TCM-1529	3	2	3	0	0

Table 6 : Results of the effect of ethanolic stem bark extract of *Tecoma stans* on *M. tuberculosis* and *M. fortuitum* in BacT/ALERT 3 D system (Five replictaes).

Isolate code	BacT/ALERT 3D system.				
	Mean cfu on media			% Inhibition	
	Control	Plant extract		Plant extract	
		2% v/v	4% v/v	2% v/v	4% v/v
M. tuberculosis H37Rv	41	22	11	50	53
DKU-156	19	8	3	66	70
JAL-1236	71	26	23	62	55
M. fortuitum TCM-1529	3	2	3	0	0

Table 7 : Results of the effect of ethanolic stem bark extract of *Tecoma stans* on *M. tuberculosis* and *M. fortuitum* in Lowenstein Jensen (L-J) medium (five replicates).

Isolate code	L-J medium.				
	Mean cfu on media			% Inhibition	
	Control	Plant extract		Plant extract	
		2% v/v	4% v/v	2% v/v	4% v/v
M. tuberculosis H37Rv	41	22	11	50	51
DKU-156	19	8	3	66	68
JAL-1236	71	26	23	62	50
M. fortuitum TCM-1529	3	2	3	0	0

Table 8 : Results of the effect of ethanol leaf extract of *Tecoma stans* on *M. tuberculosis* and *M. fortuitum* in BacT/ALERT 3D system (five replicates).

Isolate code	BacT/ALERT 3D system.				
	Mean cfu on media			% Inhibition	
	Control	Plant extract		Plant extract	
		2% v/v	4% v/v	2% v/v	4% v/v
M. tuberculosis H37Rv	41	22	11	50	53
DKU-156	19	8	3	66	71
JAL-1236	71	26	23	62	51
M. fortuitum TCM-1529	3	2	3	0	0

The effect of water extract of leaves of *Tecoma stans* in Lowenstein Jensen (L-J) medium is shown in Table 3. It is evident from the results that addition of aqueous leaf extract of *Tecoma stans* in L-J medium there was an average growth and 77% inhibition of MDR isolate DKU-156 and 55% inhibition for another MDR isolate JAL-1236, while for sensitive *M. tuberculosis* H37 Rv inhibition was 52% at 4% v/v concentration in L-J medium. There was no inhibition against rapid grower *M. fortuitum* (TCM-1529).

The Effect of water extract of leaves of *Tecoma stans* in Middlebrook 7H9 broth in BacT / ALERT 3D medium is shown in Table 4. It is evident from the results that addition of aqueous leaf extract of *Tecoma stans* in Middlebrook 7H9 broth in BacT/ALERT 3D medium, showed poor inhibition against *M. tuberculosis.* There was an average growth and 73% inhibition of isolate DKU-156 and 52% inhibition for another MDR isolate JAL-1236, while for sensitive *M. tuberculosis* H37 Rv inhibition was 53% at 4% v/v concentration in BacT/ALERT 3D medium. There was no inhibition against rapid grower *M. fortuitum* (TCM-1529).

The effect of ethanol extract of stem bark on different strains of Mycobacterium in Lowenstein Jensen (L-J) medium is shown in Table 5. It is evident from the results that addition of ethanolic stem bark extract of *Tecoma stans* in L-J medium, caused significant inhibition against *M. tuberculosis*. There was an average growth and percentage inhibition of 71% for MDR isolate DKU-156 and 54% inhibition for another MDR isolate JAL-1236, while for sensitive *M. tuberculosis* H37 Rv inhibition was 51% at 4% v/v concentration in L-J medium. There was no inhibition against rapid grower *M. fortuitum* (TCM-1529).

The effect of ethanolic extract of stem bark on Mycobacterium strains in BacT/ALERT 3D system is shown in Table 6. It is evident from the results that addition of ethanol stem bark extract of *Tecoma stans* in Middlebrook 7H9 broth in BacT/ALERT 3D medium showed lower degree of inhibition against *M. tuberculosis* as compared to that of aqueous extracts. There was an average growth and 70% inhibition of MDR isolate DKU-156 and 55% inhibition for another MDR isolate JAL-1236, while for sensitive *M. tuberculosis* H37 Rv inhibition was 53% at 4% v/v concentration in BacT/ALERT 3D medium. There was no inhibition against rapid grower *M. fortuitum* (TCM-1529).

The effect of ethanol extract of leaves of *Tecoma stans* in Lowenstein Jensen (L-J) medium is shown in Table 7. It is evident from the results that addition of ethanol leaf extract of *Tecoma stans* in Lowenstein Jensen (L-J) medium, ethanolic extract of leaf showed poor inhibition against *M. tuberculosis*. There was an average growth and percentage inhibition of 68% for MDR isolate DKU-156 and 50% inhibition for another MDR isolate JAL-1236, while for sensitive *M. tuberculosis* H37 Rv inhibition was 51% at 4% v/v concentration in L-J medium. There was no inhibition against rapid grower *M. fortuitum* (TCM-1529).

The effect of ethanol extract of leaves of *Tecoma stans* on anti-tubercular activity in different strains in BacT/ALERT medium is shown in Table 8. It is evident from the results that addition of ethnolic leaf extract of *Tecoma stans* in Middlebrook 7H9 broth in BacT/ALERT 3D medium, ethanolic leaf extract caused significantly poor inhibition against *M. tuberculosis*. There was an average growth and percentage inhibition of 71% for MDR isolate DKU-156 and 51% inhibition for another MDR isolate JAL-1236, while for sensitive *M. tuberculosis* H37 Rv inhibition was 53% at 4% v/v concentration in this medium. There was no inhibition against rapid grower *M. fortuitum* (TCM-1529).

The minimum inhibitory concentration (MIC) of aqueous and alcoholic extracts of stem bark and leaves of *Tecoma stans* is shown in Table 9. It is evident that the aqueous extracts

Table 9 : Minimum Inhibitory Concentration (MIC) of aqueous and alcoholic extracts of stem bark and leaves of *Tecoma stans* against the MDR isolates DKU-156 and JAL-1236 of *Mycobacterium tuberculosis*.

Samples	MIC (mg/ml)	
	MDR isolates of *M. tuberculosis.*	
	DKU-156	JAL-1236
a. Aqueous stem bark extract	1. 0	1.5
b. Aqueous leaf extract	0.75	2.0
c. Alcoholic stem bark extract	1.75	2.5
d. Alcoholic leaf extract	2.0	2.75
Streptomycin	5.5	10.5

of both stem bark and leaves were more effective as compared to those of alcoholic extracts.

The results of the foregoing experiments have clearly shown that aqueous and alcoholic extracts of stem bark and leaves of *Tecoma stans* have inhibitory effect on all the strains of *Mycobacterium tuberculosis* used in this study. The aqueous extracts of stem bark was more effective as compared to aqueous leaf extracts and alcoholic stem bark and leaf extracts. Antimicrobial activity of large number of plants including several members of the family Bignoniaceae has been determined by several workers (Otero *et al*. 2000, Lans *et al*. 2001, Fleischer *et al*. 2003, Kiokias and Gordon 2003, Pizzolatti *et al*. 2003, Martinez and Valencia 2003, Oyedeji *et al*. 2005, Rojas *et al*. 2006, Chauhan and Chauhan 2012). The anti-mycobacterial activity of *Tecoma* laves and bark of both stem and root have been studied by several investigators. Ramesh *et al*. (2009) have analyzed the methanolic extract of roots of *Tecoma stans* for antibacterial activity against four clinical isolates. Results against the selected microorganism *Psuedomonas aeruginosa*, showed that the zone of inhibition was moderately higher than the other three selected microorganisms.

Harris *et al*. (1988) have obtained two major alkaloidal compounds from the bark of *Tecoma arequipensis* (-)-delta-N-normethylskytanthine. The stereochemistry of 1 at all four centers was enantiomeric with that of skytanthine previously reported from *Tecoma stans* and *Skytanthus acutus*. Gc-ms of the crude base fraction indicated the presence of several related alkaloids as very minor constituents. Their structure was proved by X-ray diffraction analysis. *Tecoma sambucifoluim* showed accumulation of Phenylpropanoid glycoside, the main components were identified as verbascoside, orobanchoside, isoverbascoside (Pletsch et al. 1993).

Costantino *et al*. (1994, 2003) have isolated an alkaloid called Tecostanine (1) from *Tecoma* leaves. Its stereochemistry was elucidated as well as its antihyperglycemic activity and its affinity to opioid and nicotinic receptors. The oxalate salt of 1 did not significantly affect blood glucose levels in normoglycaemic and hyperglycaemic rats. Binuto and Lajubutu (1994) have observed antimicrobial potentials of some plant species of the Bignoniaceae family. They have obtained the methanol extracts of the leaves and stem bark of *Jacaranda mimosifolia*, *Tecoma stans*, *Tabebuia rosea* and *Crescentia cujute* other members of the family Bignoniaceae. Both stem bark and leaves of most of these plant species showed variable but remarkable broad spectrum antimicrobial activity. Their preliminary phytochemical screening of these plants revealed the presence of tannins, flavonoids, alkaloids, quinines and traces of saponins.

In the light of the results of the present study it is concluded that the aqueous extracts of stem bark of *Tecoma stans* of the family Bignoniaceae is having potent anti-mycobacterial activity and it should be further tested to further explore its vitality of developing a natural drug for treatment of tuberculosis after proper clinical trials.

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