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Anti-Mycobacterial Activity of *Acalypha indica* and *Andrographis paniculata,* the Indian Medicinal Plants against *Mycobacterium tuberculosis* (MTB)

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ABSTRACT

Tuberculosis (TB) remains a serious health problem and has mortality globally. *Mycobacterium tuberculosis* (MTB) infections have been accounted for a couple of million deaths annually and this higher mortality has been associated with poor diagnostic methods and rapid emergence of multi-drug-resistant TB (MDR-TB). Medicinal plants are an important source of new antimicrobial agents and remain an attractive alternative strategy. The present study was performed to evaluate anti-MTB activity of three medicinal plants viz., *Acalypha indica (A. indica), Andrographis paniculata (A. paniculata)* and *Aloe barbadensis* Miller which is commonly called as *Aloe vera (A. vera)*. Different concentrations of aqueous leaf extracts of these plants were tested for their anti-MTB activity against *MTBH37Rv* strain and MDR-TB and the inhibitory activity was expressed as CFU inhibition, % inhibition and IC_{50}. The study showed that *A. indica* extracts out-performed other two plants by having 78% inhibition (IC_{50} value was 5.4). This was followed by *A. paniculata* which had 71% inhibition (IC_{50} value of 5.3). *Aloe vera* performed the poorest. Our study has identified novel plants which may have a therapeutic potential against MTB infections.

INTRODUCTION

Tuberculosis is a highly infectious and contagious disease with about one-third of the world's population infected which includes 40% of the Asian Indians[1]. According to the 2014 report, TB killed over 1.5 million people worldwide and over 9.6 million people are estimated to have fallen ill within the same year which included 5.4 million men, 3.2 million women and 1.0 million children. Globally, 12% of the 9.6 million TB cases were found to be HIV positive[2]. Significantly higher morbidity and mortality have been associated with availability of poor diagnostic methods and, of a greater importance that emergence of multi-drug resistant TB (MDR-TB).The resurgence of tuberculosis (TB) as a major disease in many parts of the world is prompting the search for novel compounds, active against the causative organism, *MTB*[3]. The World Health Organization (WHO) estimated that about 80 percent of world's population still relied on traditional medicinal plants for their primary health care. The uses of herbs and herbal products have been broadly being accepted in our modern way of life[4]. Moreover, the majority of new drugs introduced in the United States and other countries are derived primarily from the plants. As discussed, most of the chemical drugs cause adverse effects and are costlier, therefore, nowadays there is an increasing inclination towards the use of an alternative source of medicine, especially based on medicinal plants[5]. A number of medicinal plants have been reported for antimycobacterial activity across the globe[6,7]. The medical knowledge which was developed in India thousands of years ago and describes numerous plants to treat several diseases[8]. Of which over 250 plants were shown to have anti-MTB activity. The comprehensive safety, toxicity and clinical studies are needed for these plants before using them effectively as curative and/or preventive medications against TB[9]. 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. India is also one of the leading countries in herbal medicines and researchers are continuously engaged in searching novel drug molecules to combat MDR/XDR-TB. Since last few years, several plants have been reported for their Anti-Mycobacterial activity from India[10]. Medicinal plants offer a great hope to fulfill these needs and have been used for curing diseases for many centuries[11]. 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[12]. There are several reports on the presence of antimicrobial compounds in various plants but there are only few but promising reports on antimicrobial property on *A. indica, A. paniculata* and *A.vera*[13, 14].Therefore, the aim of the present study was to evaluate

the antimycobacterial activity of aqueous extracts of three medicinal plants (*A. indica, A. paniculata,* and *A. vera)* against MTB.

MATERIALS AND METHODS

Collection of plants:

Leaves of *A. indica L., A. paniculata* and *Aloe vera* were collected from Chennai, India and were authenticated at the Department of Botany, University of Madras-Guindy campus, Chennai-600113, India. These leaves were dried under shade and then powdered with an electric mixer and stored in air-tight container and stored at 4^{o}C until usage.

Preparation of plant aqueous extracts

Thoroughly washed, dried leaves of *A. indica*, *A. paniculata* and *A. vera* were weighed (25 g each) and pulverized using mixer separately. Approximately 100 ml water was added to each powdered samples and homogenized for 1 hour and filtered using Whatman filter paper No. 1. Filtered solutions were lyophilized. The powder of concentrated *A. indica*, *A. paniculata* or *A. vera* were diluted accordingly with sterile water.

MTB strains and culture:

MTB cultures were handled in Biosafety level laboratory and all assays were performed by trained personals. A standard strain of MTB (*H37Rv)* (drug-sensitive) and MDR-TB (drug resistant) isolate were used for the anti-mycobacterial assays. For the culture, few colonies from fresh cultures were collected from a Lowenstein-Jensen (LJ) solid medium with a sterile loop and then transferred into a sterile tube containing 4 ml of Middlebrook 7H9 broth medium with 5-6 sterile glass beads (culture suspension tube). This tube was then vortexed for 2 min to break up the clumps. The turbidity of the suspension was greater than the McFarland #1.0 standard. The tubes were allowed to settle for 20 min. The supernatant suspension was transferred into another sterile tube, which allowed to settle for an additional 15 minutes undisturbed. The supernatant was taken out with a pipette, without disturbing the sediment, and transferred into another sterile tube. The turbidity of this suspension was adjusted to 0.5 McFarland standard with sterile saline, and the adjusting was done by visual comparison. A 1:5 dilution of the above suspension in sterile saline was used as inoculums for plant extracts susceptibility testing.

Anti- Mycobacterial Assay

Antimicrobial assays were performed in LJ medium. The plant extract was incorporated in the medium at concentration of 2, 4 and 6 µg/ml prior to inspissation. For comparison, extract free control slants were used. The ten-fold dilution of standard 1 mg/ml H37Rv strain of *M. tuberculosis* and MDR-TB isolate suspensions were made and 10 µl of this suspension was streaked on LJ medium with the help of 4 mm (external diameter) loop in the presence or absence of plant extracts. The medium was incubated at 37°C for 60 days. Reading was taken at weekly basis and each test was done in triplicate. Percentage inhibition was calculated by mean reduction in number of colonies on extract containing as compared to extract free controls. The number of colonies grown on the LJ media was counted on extract containing and extract free control LJ slants after 42 days of incubation at 37°C were recorded. The results are represented as percentage of (%) inhibition and CFU/ml. The inhibitory concentration 50 (IC_{50}) values were calculated from data obtained graphically, using a mathematical method based on the principle of the right-angled triangle. [Eq-1] $IC_{50}=D - [(A - 50\%\ max\ response)\ X]/Y$, in which *A* represents immediately higher response of 50% max response; *B* represents immediately lower response of 50% max response; *D*= log concentration corresponding to *A* response; *C* = log concentration corresponding to *B* response; $X = D - C$; and $Y = A - B$ (Alexander *et al.*, 1999).

Statistical analysis

Data were expressed as mean + SD and significance of the results were compared using One-way analysis of variance (ANOVA) using statistical software.

RESULTS

TB is one of the long nagging human health concern and above that it is a nightmare situation among patients who are co-infected with HIV. To fight against TB there are several anti-TB drug regime in place. Such drugs are classified under first line, second line and third line of drugs and these drugs are chosen based on the clinical situation appropriately. Albeit there is a growing concern over TB now because of the emergence of MDR-TB and XDR-TB which makes the clinicians running out of drug options. With this background, the current study was conducted on the clinical utility of 3 Indian medicinal plants namely *A. indica. A. paniculata* and *Aloe vera* as drug against MTB. Aqueous extracts were prepared from each of those plants. Based on the previous literature, we prepared LJ medium containing 6 or 4 or 2 mg/ml

of each drug preparation. Then 10 µl of 1 mg/ml concentration was streaked on to LJ medium and incubated at 37^{o}C for 60 days. These cultures were observed every week for colony development. The results were expressed as reduction of colony forming units (CFU), % inhibition, and IC_{50} concentrations.

At first, we studied the plant extracts for their ability to inhibit colony development and found that *A. indica* extract outperformed the other two extracts in inhibiting both MDR-TB and H37RV MTB strains predominantly (Table-1). As shown in the table, 6 □ g/ml of*A. indica* showed comparable numbers of colonies (p=1.0) which were 21.5±2.7 colonies by MDR-TB and 17.5±□ □ □ colonies by H37Rv (control bacteria)One interesting aspect is that overall the inhibitory activity was marginally more with control strain than that the MDR-TB and these values statistically not significant (p≥0.1). Next to A. *indica, A. paniculata* exhibited anti-TB activity and these values were significantly higher than *A. vera* (p values shown in the table). Throughout the study, *A. vera* showed the minimum anti-TB activity on both strains. Another important observation with all the extracts was that all of them performed so well with 6 □g/ml and the anti-TB activity was directly proportional to the concentration of the extract. Not shown are the results that we obtained with drug positive control (rifampicin) where there was a complete absence of TB colonies and drug negative control (where there is no drug) developed large number of colonies (74.6±3.9).

Next, we evaluated the percentage inhibition of MTB by these extracts. As shown in the previous table *A. indica* showed the maximum but comparable levels (statistically insignificant) of inhibition as it had 71.5±2.0 and 75.6±3.5 percentage inhibition against control strain and MDR strain, respectively (Table-2). *A. paniculata* showed a percentage inhibition of 65.5±2.5 with H37Rv and 71.0±1.1 with MDR-TB which was marginally below *A. indica* (statistically insignificant). As shown above, there was a positive correlation with the percentage inhibition and concentration of the extract used. An inhibition percentage of 54±1.3 and 46.5±3.5 was disclosed by *A. vera* and remained the poorest in possessing anti-TB activity. These results confirmed the previous data shown above.

Finally, we wanted to further confirm our data on these extracts by evaluating the inhibitory concentration 50 (IC_{50}) of each extract against H37Rv strain and MDR-TB strain. This is illustrated in the Fig.2 and as shown in the figure maximum anti-TB activity against both strains was shown by *A. indica* as revealed by their IC_{50} values of 5.4 for control strain and 2.6 (Fig.5) for MDR strain. It is important to note that *A. indica* may be the first choice to

study further for its efficacy in controlling other MDR and XDR strains. Almost on the same note, it is important to mention that there was an almost similar level of inhibition was found with *A. paniculata*. Thus, it may be worth pursuing both extracts for the further evaluation of the anti-MTB activity against MDR-TB and XDR-TB.

Table-1: Colony Forming Units (CFU) after drug treatment:

BOTANICAL NAME	LOCAL/ TAMIL NAME	PART USED	SOLVENT	CFU					
				H37Rv MEAN ± SD			MDR-TB MEAN ± SD		
				2 µg	4 µg	6 µg	2 µg	4 µg	6 µg
A. indica	Kuppaimeni	Leaf	Aqueous	30.4±1.8	24.2±2.5	17.5±1.9[a]	38.5±2.5	35.5±1.5	21.5±2.7[d]
A. paniculata	Nilavembu	Leaf	Aqueous	34.4±2.1	28.9±1.5	22.7±2.6[b]	41.3±2.3	32.5±1.5	23.1±3.6[e]
A.vera	Katrazhai	Leaf	Aqueous	48.6±3.1	41.8±3.5	35.1±2.3[c]	50.5±3.9	43.3±3.5	40.6±2.9[f]

Plant extracts were added to LJ medium before inspissation. Cultures were incubated at 37^{o}C for 60 days and the cultures were observed every week and at the end of 60 days the number of colonies were counted and shown in table 1. Table values represent the mean± SD of three experiments.

Table 2. Anti-Mycobacterial activity of aqueous extracts of various plants were tested against *M. tuberculosis H37Rv* and MDR-TB isolate

BOTANICAL NAME	% inhibition of H37Rv MEAN ± SD			% inhibition of MDR-TB MEAN ± SD		
	6µg/ml	4 µg/ml	2 µg/ml	6 µg/ml	4 µg/ml	2 µg/ml
A.indica	71.5±2.0[a]	49.5±1.5	23.2±6.2	75.6±3.5[d]	59.5±2.5	27.5±13.5
A.paniculata	65.5±2.5[b]	55.8±1.5	21.5±2.5	71±1.1[e]	62.5±7.5	23.5±4.5
A.vera	46.5±3.5[c]	23.5±1.5	12.5±0.5	54±1.3[f]	29±7.2	15±1.0

Analysis of variance (Anova) Methods: a vs b=0.05, a vs c = 0.001, b vs c=0.003, a vs d=0.1, b vs e=0.9, c vs f=0.1, d vs e =0.6, e vs f=0.002, d vs f=0.001

Results are expressed as % inhibition of colonies on the LJ medium. Three different concentrations of each plant which were 2,4,6 µg/ml. Culture bottles were incubated at 37^{o}C for 60 days and % inhibition was calculated as mentioned in the methods. Table values represent the mean± SD of three experiments.

Analysis of variance (Anova) Methods: a vs b=0.03, b vs c= 0.002 ,a vs c=<0.001, d vs e= 0.1,e vs f<0.001 ,d vs f=0.001, a vs d=0.15 ,b vs e= 0.02,c vs f=0.03

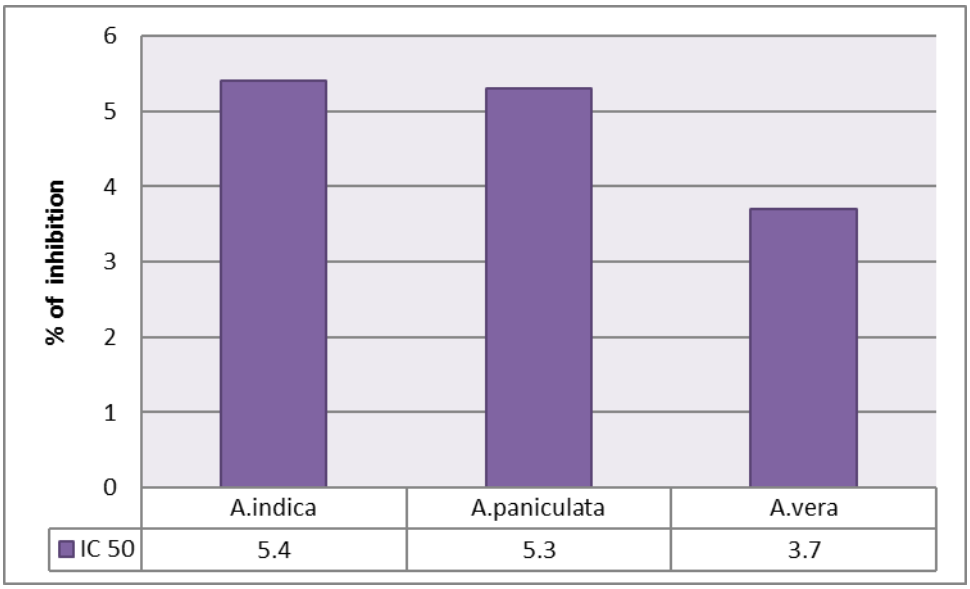

Fig-1. Percentage inhibition of H37Rv MTB by the extracts

From this the inhibitory concentration 50 (IC50) values were calculated as mentioned in the methods. This figures shows the IC 50 values for three different plants extracts viz. *A. indica, A. paniculata* and *A. vera.*

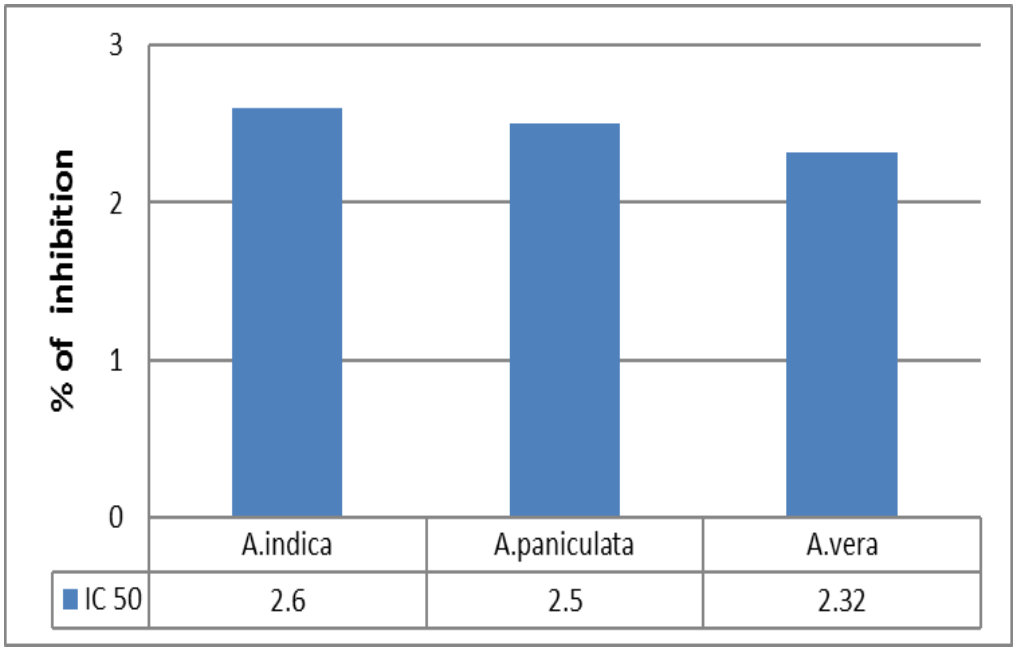

Fig-2: Percentage inhibition of MDR-TB by the extracts: From this the inhibitory concentration 50 (IC50) values were calculated as mentioned in the methods. This figures shows the IC 50 values for three different plants extracts viz. *A. indica, A. paniculata* and *A. vera.*

DISCUSSION

Tuberculosis has been a major health problem worldwide. Though MTB was discovered by Robert Koch in 1882 a good vaccine preparation was prepared by Albert Calmette and Camille Guerin in 1905 which they named as BCG vaccine[15]. This vaccine is a live attenuated strain of *M. bovis* strain produced in Pasteur Institute, Paris in 1921. Though its usage dates back about a century ago its efficacy in controlling TB is considered to be equivocal[16].One of the earliest anti-MTB drug was streptomycin (isolated from *Streptomyces griseous*) discovered in 1944[17] was the drug of choice until discovery of isoniazid in1952 and rifampicin in 1963[18]. Later on isoniazid, rifampicin and streptomycin were all considered first line of drugs to control MTB. Besides that second line and third line of drugs are being administered and during the course, drug resistance has emerged. Multi-drug resistant tuberculosis (MDR-TB) or Vank's disease show resistance isoniazid an rifampicin[19] and Extensively drug-resistant TB (XDR-TB) is a rare type of multidrug-resistant tuberculosis (MDR-TB) that is resistant to isoniazid and rifampin, plus any fluoroquinolones and at least one of three injectable second-line drugs (i.e. amikacin, kanamycin, or capreomycin)[20]. Due to increase in multi-drug-resistant (MDR) and extensive drug resistant (XDR) strains of *M.*

tuberculosis, there is an urgent need of finding newer antimycobacterial agents to combat this problem[21]. To overcome these hurdles alternative treatment strategies to be in place to manage such patients. It is in this backdrop the current study was conducted to evaluate the anti-MTB potential of aqueous extracts derived from 3 Indian medicinal plants viz. *A. indica*, *A. paniculata* and *A. vera* on H37Rv and MDR-TB. We found that *A. indica* yielded the maximum inhibitory activity (75.6%) with MDR-TB (Table-1). This was followed by *A. paniculata* with 71% inhibition. Poor inhibitory activity was noticed with *A. vera*. Almost similar levels of inhibitions were noticed by these plants with H37Rv strain suggesting that *A. indica* surpassed all the extracts (Table-1) and showed the maximum anti-TB activity. Based on the literature we tried 2 different concentrations namely 6, 4, 2 mg/ml and results showed a dose-response effect.

In a study conducted by Bernaitis L et al. 2013 they studied the anti-MTB activity of various plants they found that *A. indica, A. paniculata* and *A. vera* showed 75, 46 and 68% inhibition, respectively with an MDR-TB isolate and 69, 40 and 51% inhibition, respectively with H37Rv strain. This data suggested that *A. indica* showed the maximum inhibitory effect on both standard strain and MDR-TB strains. This result corroborates our results. Similarly, both studies showed a dose-response effect with varying concentrations. In their study, however, the second most powerful plant was Aloe vera and the poor performer was *A. paniculata* and the results are in contrast with ours. i.e. in our study the second powerful extract was *A. paniculata* and the worst performer was Aloe vera. The reason for this discrepancy may be due to we did this assay 3 times and we recorded only mean and standard deviation while they did the experiments only once suggesting that our results are much more meaningful.

In a study conducted by Batra, R et al. 2010 they compared the anti-MTB activities of aqueous leaf extract from 5 plants namely *Acalypha indica* L. (Euphorbiaceae), *Adhatoda vasica* Nees. (Acanthaceae), *Aloe vera* L. (Aloaceae) and bulbs of *Allium cepa* L. (Alliaceae) and *Allium sativum* L. (Alliaceae). In their study *A. indica* showed an inhibition of 68% and 95% against H37Rv and MDR strain DKU-156, respectively. The extract inhibited more of MDR isolate than susceptible isolate as we noticed in our study. The difference in percent inhibition was quite distinct in their study while in our study the difference is only trivial. When a similar study was done on *A. vera* they found 41% and 38% against susceptible and MDR isolates, respectively. In our study, the same was 46.5% and 54% suggesting the poor performance of *A. vera*. Overall their findings corroborated with ours.

Sathyanarayan et al. 2016 studied the anti-MTB activity of *A. indica* using 3 solvents namely n-hexane, dichloromethane and methanol and found that n-hexane and dichloromethane inhibited H37Rv MTB strain at a concentration of 25□ g/ml and methanol at 50 □ g/mlIn our study, we only used aqueous extracts and not any other solvents. With aqueous extracts, we found better anti-TB activity at 6 □ g/ml itself and that too against both H37Rv and MDR strains. Our study had shown the anti-MTB potential of *A. indica* and *A. paniculata* and both of them are well-known drugs of traditional medicine systems. However, a study using higher concentrations of the extract on its anti-TB activities and their toxicity would throw more light on their anti-TB potentials further. In addition, more studies are warranted to test our extracts against other forms of MDR and XDR-TB. Overall the study has yielded novel anti-MTB drugs namely *A. indica* and *A. paniculata* which are worth pursuing further.

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REFERENCES

1. Agarwal SP. Inter-sectoral cooperation for success of the 1. RNTCP. Indian J Tuberc 2004; 51: 59-62.
2. WHO; Global tuberculosis report. 2015; 20th edition.
3. Lall N, Meyer JJ. J Ethnopharmacol 1999; 66(3):347-54.
4. Cragg, G.M. and Newman, D.J. Natural Products: A Continuing Source of Novel Drug Leads. *Biochimica etBiophysica Acta* (*BBA*)—*General Subjects*,2013;**1830**, 3670-3695.
5. Santhosh, R.S. and Suriyanarayanan, B. Plants: A Source for New Antimycobacterial Drugs. *Planta Medica*,2014; 80,9-21.
6. Newton, S.M., Lau, C. and Wright, C.W. A Review of Antimycobacterial Natural Products. *Phytotherapy Research*,2000;**14**, 303-322.
7. Mohamad, S., Zin, N.M., Wahab, H.A., Ibrahim, P., Sulaiman, S.F., *et al*. Antituberculosis Potential of Some Ethnobotanically Selected Malaysian Plants. *Journal of Ethnopharmacology*,2011; 133, 1021-1026.
8. Arya, V. A Review on Anti-Tubercular Plants. International Journal of PharmaTech Research,2011; 3, 872-880.
9. Chattopadhyay, D., Arunachalam, G., Mandal, A.B., Sur, T.K., Mandal, S.C., et al. Antimicrobial and Anti-Inflammatory Activity of Folklore: *Mallotus peltatus* Leaf Extract. Journal of Ethnopharmacology.2002; 82, 229-237.
10. Sharma SK. 5. *Medicinal plants used in Ayurveda*. New Delhi, India: National Academy of Ayurveda, Ministry of Health and Family Welfare, Government of India; 1998.
11. Heinrich M, Gibbons S. Ethnopharmacology in drug 3. Discovery: an analysis of its role and potential contribution. *J Pharm Pharmacol* .2001;*53* : 425-32.
12. Gupta AK, Tandon N, editors.4. *Reviews of Indian medicinal plants*. New Delhi, India: Indian Council of Medical Research; 2004.
13. Ravindra, B., Malabadi, Gangadhar, S., Mulgund and Nataraja, k., Screening an antibacterial activity in the extracts of *Clitoria ternatea*, Jour medical and Aromatic Plant Sciences. 2005;27, 26-29.
14. Prusti, A., Misra, S.R., Sahoo, S. and Mishra, S.K., Antibacterial activity of some Indian medicinal plants, Ethnobotanical leaflets.2008; 12,227-230.

15. Gradmann, C (2001). "Robert Koch and the pressures of scientific research: Tuberculosis and tuberculin". Medical History. 45 (1): 1–32. doi:10.1017/s0025727300000028. PMC 1044696free to read. PMID 11235050.
16. Calmette A, Guerin C, Weill-Halle B. Bull Acad Med. Vol. 91. Paris: 1924. Essai d'immunisation contre l'infection tuberculeuse. pp. 787–96.
17. Schatz, A., Bugie, E. & Waksman, S. A. Streptomycin, a substance exhibiting antibiotic activity against Gram-positive and Gram-negative bacteria. Proc. Soc.Exp. Biol. Med. 55, 66–69 (1944)
18. Pym, A. S. & Cole, S. T. in Bacterial Resistance to Antimicrobials 2nd Edn (eds Wax, R. G., Lewis, K., Salyers, A. A. & Taber, H.) 313–342 (CRC, 2008)
19. Diagnosis and notification of multidrug-resistant TB" (PDF). WHO MDR TB Factsheet. Retrieved 9 May 2015.
20. Emergence of Mycobacterium tuberculosis with extensive resistance to second-line drugs – worldwide, 2000–2004. Morbidity and mortality weekly report. 2006;55(11):301–305.
21. Renu, G., T. Bandana, S. Pushpendra, H.B. Singh and rats. Int. J. Res. Nat. Prod., 3(1): 18-22. V.D. Sharma. Anti-tuberculosis activity of selected medicinal plants against multi-drug resistant. Ind. J. Med. sputum AFB smears in RNTCP, India: analysis of Res.2010; 131: 809-813.
22. Phillipson, J.D. Review -50 years of medicinal submitted to National Reference Laboratory for the plant research- every progress in methodology is a year, SAARC. J. Tuberc. Lung Dis., 4(1): 44-50. Progress in science, Planta Med., 2003;69(6): 491-495.
23. Cantrell CL, Franzblau SG, Fischer NH. Antimycobacterial plant terpenoids. Planta Med.2001; 67:1-10.
24. Pietro RCLR, Kashima S, Sato DN, Januário AH, França SC. *In vitro* antimycobacterial activities of *Physalis angulata* L. Phytomedicine .2000:7(4)335-8.
25. Vikrant A. A review on anti-tubercular plants. Int J Pharm Tech Res, 2011; 3(2): 872-80.
26. Torres-Romero, D., Jimenez, I.A., Rojas, R., Gilman, R.H., Lopez, M., et al. (2011) Dihydro-Beta-Agarofuran Sesquiterpenes Isolated from *Celastrus vulcanicola* as Potential Anti-Mycobacterium tuberculosis Multidrug-Resistant Agents. Bioorganic & Medicinal Chemistry, 19, 2182-2189.
27. Adegboye, M. and Babalola, O. (2013) Actinomycetes: A Yet Inexhaustive Source of Bioactive Secondary Metabolites. In: Mendez-Vilas, A., Ed., Microbial Pathogens and Strategies for Combating Them: Science, Technology and Education, Formatex, Badajoz, 786-795.
28. Alexander, B., Browse, D. J., Reading, S. J., Benjamin, I. S. A simple and accurate mathematical method for calculation of the EC50. *J. Pharmacol. Toxicol.* 1999, *41*, 55–58.