Anti-tubercular Activity of Aqueous Extract of L.inermis Leaves and Its Comparison with Ethambutol

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Abstracts: Background: 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 drugs. Objectives: The present study was carried out to assess the antitubercular activity of Lawsonia inermis, and if any, to compare it with ethambutol by "in vitro" method. Materials and Methods: From sputum samples of patients of pulmonary tuberculosis, who had not received any antitubercular drug earlier, the acid-fast bacilli were cultured and identified. Resistance ratio method was followed. For each isolate, (originally from the sputum samples) two sets of L-J slants containing ethambutol, in serially doubling concentration ranging from 1mcg/ml to 16 mcg/ml and L-J slants containing L.inermis leaves aqueous extract (2%) in serial doubling concentration ranging from 3 mcg/ml to 48 mcg/ml were prepared. One set for inoculating with test strain and the other with standard H37Rv strain. Each set had a drug/extract free L-J slant (control). All the L-J slants were labeled with appropriate drug concentrations and incubated at 37° C for 8 weeks for growth. Result & Conclusion: The MIC for ethambutol for both test as well as standard H37Rv strain was 4 mcg/ml by Resistance ratio method. The number of colonies (test and standard H37Rv) on all the L-J slants containing L.inermis extract were more than 100. Thus the aqueous extract of L.inermis leaves used, did not show antitubercular activity in the concentrations of 3 to 48 mcg/ml in the Lowenstein – Jenson media in the present laboratory set up. It is suggested that further studies may be undertaken to confirm the results of the present study.[Joseph R et al NJIRM 2012; 3(5) : 36-42]

Key Words: Anti-tubercular Activity , L.inermis Leaves , Ethambutol

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Introduction: Tuberculosis has always had and still has distinction of being a leading contender for one of the top places on the list of the main cause of death in India. There still existed 20 million cases worldwide, with 10 million new cases added annually and only 7 million treated every year [1]. There is reliable evidence that tuberculosis problem in developed countries has been decreasing at least for past 40 years after introduction of chemotherapy. However success in therapy gets limited due to increase in incidence of emergence of drug resistance strains of M.tuberculosis and HIV pandemic.

Multiple drug resistance (MDR) and XDR has become a new buzz word. The term covers the mycobacterial strains resistant to more than one commonly used highly potent antitubercular drugs. During last few years MDR has been on rise especially in HIV positive patients[2]. Emergence of drug resistance has reduced the efficacy of treatment to almost the level of pre-chemotherapeutic era and is a limiting factor in our attempts to cure individual patients and thereby to eradicate the disease from community[3]. There is therefore a pressing need for new antitubercular agents preferably those that can readily and cheaply be made available from some local source.

Nowadays, there is a definite and significant increase in demand for the herbal drugs because they are of natural origin, easily available, cheap and apparently have low toxicity as compared to the allopathic medicines.

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 [4-5].

In the Indian medical traditions of Ayurveda and Unani many plants such as *Piper longum, Withania somnifera, Sida cardifola, Ocimum, Embilica*

officinalis are useful in treatment of tuberculosis[6]. Extract of leaves of *Lawsonia inermis* showed marked antibacterial, antifungal and antitubercular activity[7]. So far, few plants have been tested against mycobacteria and a few plants which showed anti-TB activity were *Salvia hypargeia, Euclea natalensis, etc.*[8-12] . *Adhatoda vasica* [13,14], *Allium cepa*[15,16] and *Aloe vera* [17-19].

The increasing incidence of MDR- and XDR-TB worldwide highlight the urgent need to search for newer anti-tuberculosis compounds/drugs. Therefore, the present study was carried out to assess the antitubercular activity of aqueous extracts of Lawsonia inermis Linn (henna) leaves, and if any, to compare it with standard antitubercular drug ethambutol by "in vitro" method.

Materials and Methods: This study is conducted at B.J .Medical college, Pune. After ethical approval and written consent of human volunteers, 6 sputum samples were collected early in the morning from proved cases of pulmonary tuberculosis who had not received any antitubercular drug earlier in their life time. Each sputum sample was collected in a sterile, wide mouth glass container with an airtight lid, then transferred to our laboratory for primary culture. From purulent portion of sputum a loopful (diameter 3 mm) was taken and smears were made. These were stained by Ziehl-Neelson technique. M. tuberculosis appeared as small red colored rods against faint blue background. Each sputum sample was homogenised and concentrated by Petroffs method [20,21] . A loop full of this was inoculated on 2 L-J slants and on one L-J slant containing para-nitro benzoic acid (PNBA). M. tuberculosis is sensitive to PNBA while atypical mycobacteria are insensitive to PNBA [20,21].

The inoculated L-J slants were incubated at 37 °C for 4 weeks. The slants were observed once every week for appearance of colonies. To prevent dryness of, the medium, sterile distilled water was added whenever necessary. When growth appeared; pigmentation, consistancy shape etc. of colonies were looked for. M. tuberculosis colonies appeared to be buffy (pale yellow), irregular and rough. To confirm the presence of acid-fast bacilli, smears were prepared and stained by Ziehl-Neelson technique. All the acid-fast bacilli were speciated by colony morphology, time taken to grow and growth on PNBA.

Only strains which did not grow on PNBA medium and were niacin and nitrate reduction test positive were identified as M. tuberculosis [22]. For standardization of inoculum size, with a 22 SWG (wire diameter 0.7mm), a representative sweep from the growth of primary culture was taken on the loop of $2mm^3$ (approximately 2 mg moist weight). The growth taken was then discharged into 0.4 ml of sterile distilled water in sterile screw capped bijou bottles, together with 6 glass beads 3 mm in diameter. A suspension was prepared by shaking the bijou bottles for 1 minute on cyclomixer and then with a 27 SWG (wire diameter 0.4mm) nichrome loop 3 plain L-J slopes was inoculated [23]. It was done by first touching the centre of the slope and then spreading the suspension evenly over the entire medium. All the slopes were arranged serially and incubated at 37° C and read at the end of 4 weeks. The number of colonies was more than 100 on each L-J slant.

Pure ethambutol powder was used for finding the MIC values for ethambutol. Serial dilution of ethambutol was done as well as resistance ratio method was followed for which we had to include the standard H37Rv strain of M.tuberculosis. "Resistance ratio" is expressed as the ratio of the MIC of the test strain to the MIC of the standard strain. If resistance ratio is less than 2 the mycobacteria is considered sensitive, if it is 4 then doubtful (test has to be repeated) and if 8 and above they are considered resistant[20]. H37Rv strain was procured from Cardio Thoracic Centre (Pune) who in turn had obtained from National Institute of Tuberculosis (Bangalore). The H37Rv strain is used as a standard strain because its known high degree of virulence and well established metabolic, pathogenic and immunologic characteristics and because of its wide spread employment for investigations on the bacteriological aspects of tuberculous, including

evaluation of drugs effective against M. tuberculosis.
For each isolate,(originally from the sputum samples). L-J slants containing ethambutol, in serially doubling concentration ranging from 1mcg/ml to 16 mcg/ml were prepared [20.] . Two such sets were prepared: one for inoculating with test strain (obtained from sputum samples) and the other for inoculating with standard H37Rv strain. Each set had a drug free L-J slope (control). Inoculum was prepared in the same way as done for standardizing the inoculum size, for both test and standard strain. One set of L-J slants was inoculated with test strain and the other set with standard H37Rv strain. All the L-J slants were labeled with appropriate drug concentrations and incubated at 37° C for 8 weeks. Every week the bottles were examined for "growth." "Growth" was defined as presence of 20 colonies or more [23].
At the end of 8 weeks, readings were noted down. The control of the test and standard H37Rv strain showed more than 100 colonies, while growth in 1mcg/ml showed approximately 45-50 colonies, with 2 mcg/ml more than 20 colonies. No colonies were seen on slopes containing 4 mcg/ml, 8 mcg/ml & 16 mcg/ml. The observations were same for all the 6 test isolates as well as standard strain H37Rv. Therefore MIC for ethambutol was found out to be 4 mcg/ml. Resistance ratio was 1 indicating the test strains being sensitive to ethambutol.

For each isolate L-J slants containing L.inermis leaves aqueous extract (2%) in serial doubling concentration ranging from 3 mcg/ml to 48 mcg/ml were prepared. One L-J slant devoid of any drug and one L-J slant containing 4 mcg/ml of ethambutol was also prepared. Similar set was prepared for the standard H37Rv strain as the resistance ratio method was followed .

6 sputum samples were studied i.e. the same primary cultures were used which were used for determining MIC of ethambutol. Inoculum of the test strain as well as standard H37Rv strain was prepared as mentioned earlier. One set of L-J slants was inoculated with test strain and the other set with standard H37Rv strain. All slants were labeled with appropriate drug concentrations and incubated at 37° C for 8 weeks for appearance of "growth". Cultures were observed once a week for 8 weeks for appearance of "growth". However, there were more than 100 colonies on each of L-J slants having aqueous extract of L.inermis in 3 mcg/ml to 48 mcg/ml concentration. Further, the observations were similar to control L-J slant while the result of ethambutol was positive i.e. there was absolutely no "growth" on L-J slant having ethambutol concentration of 4 mcg/ml.

Results: The control L-J slants during the study of L. inermis aqueous extract showed growth of more than 100 colonies of test strains. The colonies were buff coloured, irregular, dry and heaped up. Similar was the observation for standard H37Rv strain. The H37Rv colonies appeared to be buff coloured, irregular, dry and rough. Presence of M.tuberculosis was confirmed by acid fast staining by Ziehl-Neelson technique i.e. M. tuberculosis appeared as red coloured rods and biochemical tests i.e. the nitrate reduction test and niacin test which were positive i.e. there was development of pink colour.

The MIC for ethambutol for both test strain as well as standard H37Rv strain was 4 mcg/ml. Resistance ratio method i.e. ratio of the MIC of test strain to that of std H37RVs strain was also calculated which was found to be 1 indicating the strains being sensitive to ethambutol

In our studies, growth were observed on all the L-J slants containing the L-inermis leaves extract with the test as well as the standard H37Rv strain. Colonies started appearing after 2 weeks of inoculation. In the initial weeks they were few in number and gradually there was increase in the number of colonies and its size on further incubation.

At the end of 8 weeks of incubation, the number of colonies on all the L-J slants (test and standard H37Rv) were more than 100. The colonies appeared to be buffy (pale coloured), irregular, dry, heaped up and rough. There was no marked difference observed in the colony size or number

with different concentration of L.inermis extract. L.inermis leaves extract showed no antitubercular activity at concentrations of 3 mcg/ml to 48 mcg/ml in the L-J media.

Discussion: One of the most important problems encountered in the treatment of tuberculosis is drug resistance. The estimation of drug resistance termed 'sensitivity testing' is therefore of vital importance. Sensitivity testing is also done for various drugs/plants extracts which are still under investigation for their antitubercular action.

The "Resistance ratio" method is widely used by studies sponsored by World Health Organization (WHO), International Union against Tuberculosis (IUAT), Tuberculosis Institute of Madras and National Institute of Tuberculosis (NTI) Bangalore. Resistance is determined by the ratio of the MIC of the test strain to that of the MIC of standard H37Rv strain[24]. Therefore the "resistance ratio" method was followed in the present study.

In the present study sputum samples were collected from proved cases of pulmonary tuberculosis who had not received any antitubercular drugs earlier in their life time. All samples were subjected for smear examination by Z-N technique. All samples were smear positive for acid-fast bacilli which appeared to be small red coloured rods on a faint blue background. The culture was necessary for species identification and for obtaining the primary culture for investigation of antimycobacterial activity.

The WHO expert committee on tuberculosis has recommended Lowenstein Jenson medium without potato starch for culture [23]. Hence this medium was employed in the present study. All the sputum samples were decontaminated by Petroffs method and subjected for culture on L-J slants. After incubation of 4 weeks results were noted. M.tuberculosis was speciated by colony morphology i.e. they appeared to be buff coloured, rough, irregular and heaped up; by their inability to grow on PNBA medium, and the biochemical test i.e. the nitrate reduction and niacin test which were positive. Smear positivity by Z-N technique was also looked for, which show acid fast bacilli, for confirmation.

For any sensitivity testing it is important to standardize the inoculum size. It was done so by taking a representative sweep from primary culture of colony of 2 mm^3 and discharging in 0.4 ml sterile distilled water to form a suspension. When a loopful of this suspension was inoculated onto drug free L-J slants and incubated for 4 weeks, growth appeared was more than 100 colonies which was an ideal for a control.

Ethambutol is one of the antitubercular drug commonly used in the first line treatment of tuberculosis. It is a bacteriostatic drug and is effective against mycobacteria resistant to INH, PAS and Ethionamide, Streptomycin as well as many atypical mycobacteria. Primary resistance to this drug has not been reported. When used along with other drugs resistance to ethambutol develops slowly [25]. In the present studies ethambutol a bacteriostatic drug was chosen for comparison with tuberculostatic activity of L.inermis aqueous leaves extract.

In the present study the MIC for ethambutol was found to be 4 mcg/ml in the L-J media for all the 6 samples tested as well as for H37Rv strain. This was within the range reported earlier i.e. from 0.5 mcg/ml to 8 mcg/ml [26].

Nowadays, there is an increasing demand for herbal drugs as they are natural, cheap, easily available and less toxic as compared to other allopathic medicines. Surveys carried out by different workers point to the wide distribution of antibiotic principle in plants active against tuberculosis. Various plants such as Ocimum sanctum, Withania somnifera, Sida cardifolia, Piper longum are used in treatment of tuberculosis in Ayurveda. Japanese workers have isolated alkaloids from Stephonia apharantha and S. sasakii which are used in prophylactic treatment of tuberculosis [27]. Bhatnagar, S.S et al reported the antitubercular activity of few plants. Lawsonia.inermis was one of them[28]. Sharma, V.K. reported the tuberculostatic activity of L.inermis aqueous leaves extract at concentration of **6** mcg/ml "in vitro". He also studied its effect in

guinea pigs and mice which he had infected with the standard H37Rv strain. The dose showing antitubercular effect was 5 mg/kg in guinea pigs and mice[29].

Hence in the present study the concentration of 3-48 mcg/ml of L. inermis extract was chosen for study. However, the present study do not support the above findings, L.inermis aqueous leaves extract showed no antitubercular activity "in vitro" at concentrations 3 mcg/ml to 48 mcg/ml in the L-J media for all the test as well as standard H37Rv strain i.e.more than 100 colonies were observed on all these slants.. Hence resistance ratio method was not applicable as MIC could not be found out for L.inermis. The test strain could not be considered resistant as no antitubercular activity was seen for standard H37Rv strain either. However, the possibility of test strains as well as standard H37Rv strain employed being sensitive to Ethambutol but resistant to aqueous extract of leaves of L-inermis (in concentration of 3 to 48 mcg/ml on L-J media) cannot be completely ruled out. This should be taken into consideration especially because L.inermis i.e. Mehndi / Henna is commonly employed by Indian people as local applications; the organisms being already exposed to the active principle present; if any. The difference in the observations may be because of biological variation in the properties of the plants or the bacilli both being biologicals. It is probable that it may have some activity at higher concentration or may only have "in-vivo" action. The antitubercular activity "in-vivo" cannot be completely ruled out.

Drug susceptibility testing is carried out on sub-cultured bacteria after the initial positive culture is obtained for diagnosis. It usually takes 3–6 weeks to obtain the initial positive culture with an additional 3-4 weeks for susceptibility testing. Thus, susceptibility testing is time consuming and costly, and there are numerous problems associated with the standardization of tests and the stability of the drugs in different culture media. The slow diagnosis of drug resistance may be a major contributor to the transmission of MDR-TB .

PCR amplification followed by DNA sequencing is the most widely used technique to identify mutations associated with drug resistance in TB. This technique is costly and require expertise, which make it unpractical for use in routine laboratories, especially in developing countries, where simple, cost effective drug susceptibility testing is needed. The next generation of molecular methods for the prediction of drug resistance in *M. tuberculosis* will possibly consists of matrix hybridization formats such as DNA oligonucleotide arrays on slides or silicon micron chips[30]. It is essential that developments for new techniques must consider the fact that the majority of drug resistant cases occur in resource poor countries and therefore the methodologies must not only be cheap but also robust.

Limitations ; The conventional microbiological procedures, though quite robust, take several months and as a result the search for alternatives has been accelerated in recent years. After comprehensive review, WHO has endorsed certain new DST methodologies, including molecular line probe assays and liquid culture systems. *M. tuberculosis* is a very slow growing organism and the use of molecular methods for the identification of mutations in resistance-causing genes may offer a means to rapidly screen *M. tuberculosis* isolates for antibiotic resistance. Mutation screening methods are fast and include methods such as DNA sequencing, probe based hybridization methods, PCR-RFLP, single–strand conformation polymorphism (SSCP), eteroduplex analysis (HA), molecular beacons and ARMS-PCR .

Conclusion : The minimal inhibitory concentration (MIC) of Ethambutol was found to be 4 mcg/ml with "Resistance ratio" of 1.The aqueous extract of L.inermis leaves used, did not show antitubercular activity in the concentrations of 3 to 48 mcg/ml in the Lowenstein – Jenson media against the test and the standard H37Rv strain used in the present laboratory set up. It is suggested that further studies may be undertaken, taking into account the variables discussed earlier to confirm the results of the present study.

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