Evaluation of Anti-Tubercular Activity of a Herbal Extract (Lawsonia.inermis) and its Comparison with Ethambutol

Medical Science

KEYWORDS : Anti-Tubercular activity, Herbal Extract (Lawsonia.Inermis) , Ethambutol

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ABSTRACT

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 evaluate the antitubercular activity of a herbal extract(Lawsonia inermis), and if any, to compare it with ethambutol by resistance ratio method.

Materials and Methods: Acid-fast bacilli were cultured and identified from sputum samples of patients of pulmonary tuberculosis, who had not received any antitubercular drug earlier. 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 192 mcg/ml were prepared. One set was inoculated with test strain and the other with standard H37Rv strain. Each set had an 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: The MIC for ethambutol for both test as well as standard H37Rv strain was 4 mcg/ml by Resistance ratio method. There were more than 100 colonies on all L-J slants containing L.inermis aqueous leaves extract.

Conclusion: Thus the aqueous extract of L.inermis leaves used, did not show any antitubercular activity in the concentrations of 3 to 192 mcg/ml in the Lowenstein – Jenson media in the present laboratory set up. It is possible that L.inermis may have some other activities; if not primarily as directly antituberculous; which may be beneficial in the treatment of tuberculosis.

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 is evidence that tuberculosis problem in developed countries has been decreasing for past 40 years after introduction of chemotherapy. However success in therapy gets limited due to emergence of drug resistance strains of M.tuberculosis and HIV pandemic. During last few years MDR has been on rise especially in HIV positive patients [1]. This 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 [2].

There is therefore a pressing need for new antitubercular agents. Nowadays, there is a definite and significant increase in demand for the herbal drugs because they are of natural origin, 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[3,4]. So far, few plants have been tested against mycobacteria and a few plants which showed anti-TB activity were Adhatoda vasica[5,6], Allium cepa[7,8] and Aloe vera [9-11]. Extract of leaves of Lawsonia inermis showed marked antibacterial, antifungal and antitubercular activity[12]. Well planned studies to screen the herbal medicines for their antitubercular activity are scarce, hence we have made an attempt in this study to assess the antitubercular activity of Lawsonia. inermis Linn .(henna).

MATERIALS AND METHOD

I. Establishment Of Culture Technique For M.tuberculosis

(A) Primary Culture

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 lifetime. Each sputum sample was collected in a sterile, wide mouth glass container with an airtight lid, then transferred to our laboratory. From purulent portion of sputum a loopful (diameter 3 mm) was taken and smears were made and 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[13,14]. 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 [13,14].

The inoculated L-J slants were incubated at 37 °C for 4 weeks. The slants were observed once every week for appearance of colonies. 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 [15].

(B) 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 were inoculated [16]. 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 were more than 100 on each L-J slant.

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II. Determination Of Minimum Inhibitory Concentration (MIC) of Ethambutol

Pure ethambutol

powder was used for finding the MIC values for ethambutol. Serial dilution of ethambutol was done. 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 [13]. H37Rv strain was procured from Cardio Thoracic Centre (Pune) who in turn had obtained from National Institute of Tuberculosis (Bangalore). 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 [13]. 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 [16] . At the end of 8 weeks 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.

III. Evaluation of Antitubercular Activity Of Lawsonia.inermis Extract

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". Culture 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. 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. 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, thus suggesting that the test as well as standard H37Rv strain were sensitive to ethambutol not sensitive to L.inermis. Hence additional 6 sputum samples were studied further by the same method as mentioned above on L-J slopes containing L.inermis aqueous extract in serially doubling concentration now ranging from 12 mcg/ml to 192 mcg/ml. Even at these concentrations L.inermis leaves aqueous extract in L-J media showed no antitubercular activity. The number of colonies on all the slants (test and standard H37Rv) were more than 100. No perceptible change in colony size or number was noted as compared to control. The morphology noted was same as noted earlier.

DISCUSSION & CONCLUSION

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. The "Resistance ratio" method is widely used by studies sponsored by World Health Organization (WHO), International Union Against Tuberculosis (IUAT), Tuberculosis Institute of Madras, National Institute of Tuberculosis (NTI) Bangalore. Resistance is determined by the ratio of the minimum inhibitory concentration (MIC) of the test strain to that of the MIC of standard H37Rv strain. WHO has recommended that the "resistance ratio" technique be commonly used in the various countries so that the results are comparable 16. 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 [16]. 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 (pale yellow) 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 [13,15].

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 antituberculous drug commonly used in the first line treatment of tuberculosis. It is a bacteriostatic and effective against mycobacteria resistant to INH, PAS, 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 [17]. In the present studies ethambutol was chosen for comparison with tuberculostatic activity of L.inermis aqueous leaves extract. The minimum inhibitory concentration (MIC) for ethambutol was found to be 4 mcg/ml in the L-J media for all the 6 samples tested. This was within the range reported earlier i.e. from 0.5 mcg/ml to 8 mcg/ml [18].

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. Various plants are being studied for their antitubercular action. 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 [19] . Bhatnagar, S.S. et al reported the antitubercular activity of few plants . L.inermis was one of them [12]. Satyavati, et al also supports the tuberculostatic activity of L. inermis [20]. Sharma, V.K. reported the tuberculostatic activity of L.inermis aqueous leaves extract at concentration of **6** mcg/ml "in vitro" and 5 mg/kg in guinea pigs and mice [26].

Hence in the present study the concentration of 3-192 mcg/ml of L. inermis extract was chosen 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 192 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. There was no marked difference in

the colony size or other morphological aspects as compared to control and with various concentrations. 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 cannot be completely ruled out. This should be taken into consideration especially because L.inermis (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.

L.inermis has been described in old literature as useful in the treatment of bronchitis and as an expectorant. Apart from this it has been claimed to be useful in conditions associated with inflammation.. Further it has been claimed to "enrich blood". Hence it is possible that L.inermis may have some other activities; if not primarily as directly antituberculous; which may be beneficial in the treatment of tuberculosis.

Thus to conclude from present study - A laboratory set up was established for testing agents claimed to have antituberculous activity. 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 192 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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