Evaluation of in vivo antimycobacterial activity of some folklore medicinal plants and enumeration of colony forming unit in murine model

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Abstract
Objectives: The present study was carried out to investigate the in vivo antimycobacterial activity of methanol extract of Alstonia scholaris and Mucuna imbricata in murine model.

Materials and Methods: Female BALB/c mice were infected with the Mycobacterium tuberculosis H₃₇Rv suspension. Extracts were administered orally for 2 weeks from 7th day postinfection at a dose of 200 mg/kg and rifampicin at 20 mg/kg as standard. The synergistic groups were 10 and 100 mg/kg for rifampicin and extract, respectively.

Results: The final body weight of mycobacteria-infected group was significantly reduced (15.41 ± 0.42, P < 0.01), but following treatment with the plant extract plus rifampicin could elevate the body weight. Colony forming unit (CFU) count of lung (8.71 ± 0.01) and spleen (8.59 ± 0.01) was significantly higher in infected and untreated group (P < 0.01). It was observed that activity of the synergistic group displayed powerful and maximum response against tuberculosis (TB) infection with lower CFU counts. Histopathology study showed cells such as lymphocytes, epithelioid, Langhans giant cell, and fibrous tissue proliferation in lungs; depletion of lymphocytes in the spleen.

Conclusions: The data indicate that methanol extract of A. scholaris has potential antimycobacterial activity, and the synergistic group consisting of rifampicin and A. scholaris could be a rational choice for the treatment of TB.

Key words: Alstonia scholaris, colony forming unit, histopathology, Mucuna imbricata, Mycobacterium tuberculosis
date, a very few information on antimycobacterial activity of Alstonia scholaris (MEAS) and M. imbricata (MEMI) is available.[8-11] Therefore, the present study was carried out to authenticate the folklore claim of A. scholaris and M. imbricata against mycobacteria. The objective of the study was designed to evaluate in vivo antimycobacterial activity of methanol extract of leaves and seeds of these plants in murine model along with synergistic effect with rifampicin.

Materials and Methods

Organism
M. tuberculosis H₃₇Rv was procured from Indian Veterinary Research Institute, Izatnagar, India. The Mycobacterium strains were grown in Middlebrook 7H9 medium (HiMedia, India) supplemented with 10% OADC (HiMedia, India). Log phase cultures were centrifuged, washed with sterile saline, and adjusted to McFarland standard corresponding to 1 × 10⁶ colony forming unit/ml (CFU). The size of inoculum was confirmed by plating serial dilutions on Middlebrook 7H11 media plates supplemented with 10% OADC. The plates were incubated for 4 weeks before CFU enumeration.

Plant Materials and Extraction
Leaves of A. scholaris were collected from Medicinal Garden of College of Veterinary Science, Guwahati, and seeds of M. imbricata were collected from Karbi Anglong District in the month of March-April of 2015. Plants were identified and authenticated by Botanical Survey of India, Shillong, Meghalaya, India. The voucher specimen number of A. scholaris and M. imbricata was 4732 and 4757, respectively. Leaf of MEAS and seed of MEMI were prepared as per the method of Mann et al.[8]

Animals
Female BALB/c mice of 19–21 g body weight were obtained from the Department of Pharmacology and Toxicology and maintained in 12 h light/dark cycle. The animals were provided food and water ad libitum. All studies were performed as per guidelines approved by the Institutional Animal Ethics Committee (CPCSEA/770/ac/IAEC/22).

Mice were divided into seven groups of 10 animals each, namely, Group I: Control (uninfected and untreated), Group II: Infected and placebo treated, Group III: Standard drug rifampicin (RIF) treated, Group IV: MEAS treated, Group V: MEMI treated, Group VI: Rifampicin + MEMI treated, and Group VII: Rifampicin + MEAS treated. Mice were weighed and data were recorded at the initiation of the experiment. BALB/c mice were infected via tail vein method with 0.2 ml of M. tuberculosis H₃₇Rv suspension in phosphate-buffered saline (PBS) supplemented with 0.05% Tween 80. Reproducibility of the challenging dose was ensured by standardizing its optical density to obtain the desired CFU in Middlebrook 7H9 broth. On day 7, following infection, six of the infected mice were sacrificed, spleens and lungs were recovered and homogenized. Five-fold serial dilutions of organ homogenates in PBS with 0.05% Tween 80 were placed on Middlebrook 7H11 agar to determine CFU counts in organs.[12] Drugs were administered for 2 weeks from 7th day postinfection at an oral dose of 20 mg/kg for rifampicin and 200 mg/kg for extracts in individual groups. The doses for synergistic groups were 10 and 100 mg/kg for rifampicin and extract, respectively.

Histopathology
Three mice per infected group were sacrificed under ether anesthesia on day 0, 7, and 20. The right lobe of lungs was fixed with ethanol then embedded in paraffin. The left lobe of lung and spleen was rapidly frozen and kept at −80°C for microbiological studies.[13,14]

Colony Forming Unit Enumeration
Organs were homogenized with a Polytron homogenizer in sterile tubes containing 1 ml of PBS with 0.05% Tween 80. Ten microliters of the original concentration and five-fold dilutions of each homogenate were spread onto Bacto Middlebrook 7H11 agar (HiMedia) medium supplemented with 10% OADC to enumerate the total CFU of M. tuberculosis per organ per mouse.

Statistical Analysis
CFU counts were converted to log₁₀ values and compared using Student’s t-test. Correlation was calculated between CFU and bodyweight.

Results
The TB challenged model was generally assessed by recording weight gain or loss as an indicator of time of onset of disease and drug activity. By 20 days, all the untreated mice lost about 35–40% of their body weight [Figure 1]. Mice treated with rifampicin or rifampicin + MEAS did not lose weight; they even continued to gain weight. After 20 days, the body weights of 2–3 mice of the infected but nontreated group had dropped to a weight of 14.5 g, whereas body weights of the drug-treated mice attained weight near to the pretreatment level. The difference between body weight of untreated mice and the other group was highly significant [P < 0.01, Table 1].

Histopathology showed edema with lymphocytic infiltration, fibrous tissue proliferation, giant cell formation, and TB granuloma in lung tissues of infected but nontreated group. This confirmed the TB infection in tissues [Figures 2 and 3].

Figure 1: Differences in mean body weights at day 1, 7, and 20 of different group of mice
Extensive edema was observed in lung tissues of mice of infected and untreated group on day 20. Figure 4 shows the effect of synergistic treatment of rifampicin + MEAS; the tissues were devoid of giant cell, tissue proliferation, and other symptoms of TB. The response of rifampicin + MEAS was almost equivalent to the single treatment of rifampicin. Development of secondary follicles of spleen in isolated areas commonly observed. Very less or no depletion of lymphocytes in spleen indicating curing stage of day 20 sample of Group VII (rifampicin + MEAS). In our study, most of the mice treated with effective doses of rifampicin, MEAS, and MEMI were alive and maintained body weights longer than 20 days postinfection, whereas 75% of infected, nontreated mice died during the experiment.

Mice were inoculated with $1 \times 10^6$ CFU of *M. tuberculosis* H37Rv to develop a rapid and progressive TB disease. Chemotherapy was initiated 7 days after inoculation, when bacteria in spleens and lungs achieved significant levels (6.11±0.01 and 6.26±0.01 mean log$_{10}$ CFU, respectively, at *P* < 0.01). Mean log$_{10}$ CFU in organs of infected and untreated mice continued to increase and reached 8.59±0.01 in spleen and 8.71±0.01 in lungs by 20 days (*P* < 0.01). Mean log$_{10}$ CFU in organs of untreated mice was lower than that of infected mice (*P* < 0.01). Untreated infected mice which were inoculated with $1 \times 10^6$ CFU of *M. tuberculosis* H37Rv per mouse, started dying from day 10, only 25% of the untreated control survived after 20 days. Ninety percent survival rate was observed in mice treated with standard drug rifampicin at 20 mg/kg; on the contrary, 85% survival rate was observed in the synergistic groups, i.e. the mice treated daily with rifampicin + MEAS or rifampicin + MEMI. The survival rates for mice treated daily only with MEAS or MEMI at 200 mg/kg were 62.5% and 50%, respectively, so the survival rate for the mice treated with MEAS was higher than group treated with MEMI, significantly lower than those for the above-mentioned synergic and rifampicin-treated groups (*P* < 0.01).

The drug efficacy and weight loss in mice at the end of the experiment were inversely correlated in this study. The drugs that reduced bacterial counts in lung and spleen (from $1 \times 10^6$ to $1 \times 10^3$ CFU) showed body weights near to level of uninfected mice. The mice lost body weight if their lung CFU was $>1 \times 10^6$. Without treatment, the infection became severe day by day with the gradual loss of body weight. The corresponding CFU in lung and spleen of the mouse was determined after sacrifice for all the mice separately, and the correlation of body weight with CFU was assessed. A high correlation (*r* = 0.972) was noticed between lung and spleen CFU calculated throughout the experiment. The correlation between CFU in lung and mouse body weight was −0.937 (*P* = 0.0058) and that of spleen and body weight was −0.975 (*P* = 0.0009, Figure 5), indicating higher CFU in lungs and spleen had a negative impact on body weight.

**Discussion**

In this murine model, we studied antimycobacterial activity of MEAS and MEMI along with synergistic effect with rifampicin. The comparison was done among MEAS, MEMI, rifampicin, and synergistic activity on the basis of CFU in lung, spleen, mortality rate as well as body weight changes including histopathological changes. Infected but untreated mice began to lose weight after few days of inoculation. This might be due to...
The present study provided the scientific basis for the therapeutic potential of MEAS and MEMI against TB. The data suggested that the body weight of mouse was good evidence of CFU levels in organs. Therefore, the CFU in organs such as lung or spleen equally reflected the specificity and sensitivity toward the drug treatment.

A drug which prevents body weight loss could be considered as a very protective agent against TB. The data suggested that the rapid screening assay could easily distinguish effective drugs from drugs with lower efficacy. In this study, the protective effect of synergistic treatment of MEAS or MEMI with rifampicin was superior to the single treatment of MEAS or MEMI. The rapid screening model of this study differs in intent from the acute and chronic TB models that are usually used for a detailed study of drug activity. To date, not much information about the *in vitro* and/or *in vivo* action of *A. scholaris* and *M. imbricata* against *M. tuberculosis* is available but need much more specific study. The phytochemical study of the extracts of *A. scholaris* and *M. imbricata* led to the isolation of compounds with different scaffolds, most of them alkaloids, flavonoids, and glycoside that might have potent antimycobacterial activity.

Treatment of established TB infection in animal models, as well as in patients, requires long-duration therapy. This study showed that the additive effect of MEAS and/or MEMI to the standard regimen of rifampicin resulted in increased killing of bacilli in the 1st month of treatment. For further studies, one can use a high-dose challenge or a chronic model of TB on these selected plants extracts for effective doses and mechanism(s) of action.

**Conclusions**

Long period of TB treatment makes the case more complicated, thus this protocol could be a very essential and useful tool to investigate and develop a new drug against TB. Although we have enumerated CFU in lung and spleen, bacteria may harbor in other organs such as lymph nodes and liver. The research has been performed with crude extracts only, but isolation of pure and active compound from the plants may show a better result. The present study provided the scientific basis for the
use of *A. scholaris* and *M. imbricata* against TB, which have been traditionally used against TB/respiratory diseases in Northeast India. Synergistic treatment with rifampicin and *A. scholaris* against TB could be a powerful and effective regimen.

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**Conflicts of Interest**

There are no conflicts of interest.

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