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In-vivo Screening for Mucuna imbricata Plant and its Synergism with Rifampicin against Mycobacterium tuberculosis

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The global threat of tuberculosis (TB) demands for the search for alternative antimycobacterial drugs. Present study dealt with the antimycobacterial potential of methanolic extracts of seeds of *Mucuna imbricata* and its synergistic effect with rifampicin against *M. tuberculosis* by molecular identification of mycobacterium in the lung tissue of mice by PCR. Mice were intravenously infected with *M. tuberculosis* H37Rv and plant extract/drugs were administered orally for first 10 days of challenge. The mice were sacrificed on the 30th day of challenge and macrophages from peritoneal fluid of mice were isolated and tested for various antioxidant enzymes. Mice were sent for biopsy for recording necropsy score on lungs, smear examination, & histopathological studies. DNA isolation from mice lung tissues and PCR was done targeting *MPB-64* gene to detect the TB infection in mice. The result revealed that, mycobacteria induced cellular damage in mice peritoneal macrophages is associated with enhanced Lipid peroxidation (LPO), Reactive Nitrogen Intermediates (RNI) generation and decrease enzymatic antioxidant (SOD, CAT, GPx, and VIT.C) activity, which were protected by co-administration of extracts as well as standard drugs in group III, IV, V and VI. Giant cell formation and tuberculous granuloma was observed in lung tissue. PCR of lung samples showed band at 240 bp when targeted for *MPB-64* gene which confirms the tuberculosis infection in mice and therefore confirms enzymatic changes were due to TB infection only. Preliminary phytochemical screening showed positive for the presence of principle antimycobacterial agents in the active extracts. Seeds of *Mucuna imbricata* were found to be practically nontoxic on acute oral toxicity study in the female mice. Present study provide scientific basis for the use of *Mucuna imbricata* alone and in combination of standard drug (Rifampicin) against *M. tuberculosis*.

Keyword: Tuberculosis, Synergistic, Antimycobacterial, Antioxidant, Polymerize chain reaction (PCR).

1. Introduction

Tuberculosis (TB), a zoonotic disease is the leading cause of death worldwide with the resurgence of HIV infection and emergence of multidrug resistance strains of mycobacterium and has become a major public health problem in both developed and developing countries [1]. It is known fact that herbal plants have medicinal

values and play an important role in the treatment of various infectious diseases [2]. Novel approach to study the antimycobacterial activity of volatile components of some selected Indian herbs has been made by a few researchers. *Mucuna imbricata* belongs to family *Fabaceae* is found in both temperate and tropical countries including India. The seeds of the various species of *Mucuna imbricata* plants are used traditionally in dysentery, diarrhea, sexual debility, cough, tuberculosis, muscular pain, gout, diabetes, uterine stimulant, nerve tonic, diuretic, anti-Parkinson's drug, anticancer and as a blood purifier [3]. The present paper describes the antimycobacterial action of *Mucuna imbricata* through *in vivo* test and potency of its activity with combination of known antitubercular drug.

2. Material and methods

2.1 Plant extract

The seeds of *Mucuna imbricata* were collected from college of Veterinary science, Khanapara campus, Guwahati and hilly areas of Diphu, Assam, during the month of March-April, 2012. The samples were identified by the Botanical Survey of India, Shillong. Methanolic extract of *Mucuna imbricata* (MEMI) was prepared by standard cold extraction method [4]. The freshly prepared MEMI was subjected to phytochemical screening tests for the presence of various constituents as per standard methods [5].

2.2 Standard Drug

Rifampicin was purchased from Sun Pharma, Sikkim. Those were preserved at 4 ^{0}C.

2.3 *M. tuberculosis* strains

M. tuberculosis strain H37Rv were originally obtained from IVRI, Izatnagar, UP, and maintained on Lowenstein-Jensen (LJ) slants.

2.4 Animal Experiments

In vivo animal experiments were performed as per Institutional Animal Ethics Committee, College of Veterinary Science, AAU, (IAEC No: 770/03/ac/CPCSEA/FVSc., AAU / IAEC/06) guidelines for the care and use of laboratory animals. Specific pathogen free Swiss albino mice of 5-6 weeks old (weight 16-20 g) were used. Animals were maintained in animal house at standard conditions at 1±1 ^{0}C. Water and diet were given *ad libitum.*

2.5 Experimental Model

Mice were divided into 6 groups of 6 animals each. Group I served as normal Group (uninfected and untreated), Group II: Negative Group (Mice infected with *M. tuberculosis* H37Rv), Group III: Standard Group (Mice infected and treated with standard drug rifampicin 10 mg/kg of body wt.), Group IV: Plant extract Group (Mice infected and orally administered with MEMI 100 mg/kg of body weight), Group V: Plant extract Group (Mice infected and orally administered with MEMI 200 mg/kg of body weight), Group VI: Synergistic Group (Mice infected and orally administered with both 100 mg/kg of plant extract + Rifampicin). In mice, the *M. tuberculosis* H37Rv suspension (0.2 ml containing approximately 1×10^9 CFU) was injected in lateral tail vein. Drug administration was done according to groups. The test material was fed orally and the treatment duration for general screening was 10 consecutive days. The last dose of treatment was given on day 28th and on the day 30th all mice were sacrificed humanely and peritoneal exudates cells (PEC) from the mice was isolated by standard methods [6, 7] and subjected to various antioxidant assay viz., Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Reductase (GSH), Ascorbic acid (VIT.C), Lipid peroxidase (LPO) and Reactive nitrogen intermediate (RNI) by standard method [8, 9, 10, 11, 12]. At the time of termination of experiment all surviving mice were sent for biopsy for recording necropsy score on lungs, smear examination, & histopathological studies. DNA isolation from mice lung tissues and PCR was done targeting *MPB-64* gene to conform the TB infection in mice.

2.6 Bacterial enumeration in mice organs

Lungs and spleen of sacrificed mice were aseptically removed subjected to homogenization in sterilized PBS. Three serial 10-fold dilutions of the sterile sample were plated out on LJ media

with 5 tubes per dilution and examined for growth of the infecting *M. tuberculosis* at 37 ^{0}C for 4 weeks.

2.7 DNA isolation and PCR

Small piece of tissue was taken, measured and homogenate with equal volume of Tris buffer (pH-8). 30 µl lysozyme (50 mg/ml) was added to 1.5 ml micro centrifuge tube (MCT) containing 200 µl homogenate, vortex and incubate for 1 hour at 37 °C. Further, 70 µl of 10% SDS, 10 µl proteinase K (20 mg/ml) was added, vortex and incubate for 15 min at 65 °C. Then 100 µl 5 M NaCl and 100 µl CTAB/NaCl (pre warmed at 65 °C) was added and vortex. Then incubated for 10 min at 65 °C. Samples were cooled and equal volume of Phenol/Chloroform was added. MCT were turned upside down few times (in order to avoid breaking the DNA chain), and centrifuged at room temp. for 15 min at 11,000 g. Aqueous supernatant was transferred to a new MCT carefully. Same volume of Phenol/Chloroform was added, mixed by turning the MCT upside down and centrifuged at room temp for 10 min at 13,000 g. Aqueous supernatant was added to a new MCT carefully. Chilled isopropanol (0.6 volume of supernatant) was added to precipitate the nucleic acids and placed at least 30 min at -20 °C (or longer), spine for 10 min at room temp. at 13,000 g. The supernatant was discarded and 1 ml of cold 75% ethanol, spine for 5 min at room temp. in a MCT and supernatant was discarded. Pellet was dried at room temp (about 10 min) and dissolved in 100 µl TE buffer. The DNA was subjected to PCR targeted against *MPB-64* gene. DNA amplification were an initial denaturation of 94 ^{o}C for 6 min, followed by 40 cycles of denaturation at 94 ^{o}C for 1 min, annealing at 55 ^{o}C for 1.5 min, elongation at 72 ^{o}C for 1.5 min and final extension at 72 ^{o}C for min and holding at 4 ^{o}C. 10 µl of amplified samples were examined in 1.5% w/v of agarose gel in Tris-acetate – EDTA (TAE) buffer containing ethidium bromide 0.5 µg/ml by electrophoresis for 45 min at 80V. The amplicons of 240 bp visualized by gel doc system (Sigma) were considered to be positive for the presence of *M. tuberculosis.*

2.8 Statistical analysis

Data were expressed as MEAN ± SD. One way ANOVA and Dunnett's test was done to analyze the level of significance [13].

3. Results and discussion

The result of in vivo antimycobacterial study of the extract of *Mucuna imbricata* (seed) shows a remarkable activity against virulent strain of *Mycobacterium tuberculosis* (H37Rv). In the present study, the phytochemical analysis of MEMI showed the presence of alkaloids, terpenoids, steroids, tannins and saponins. Percentage yield of MEMI was found to be 6.24% w/w.

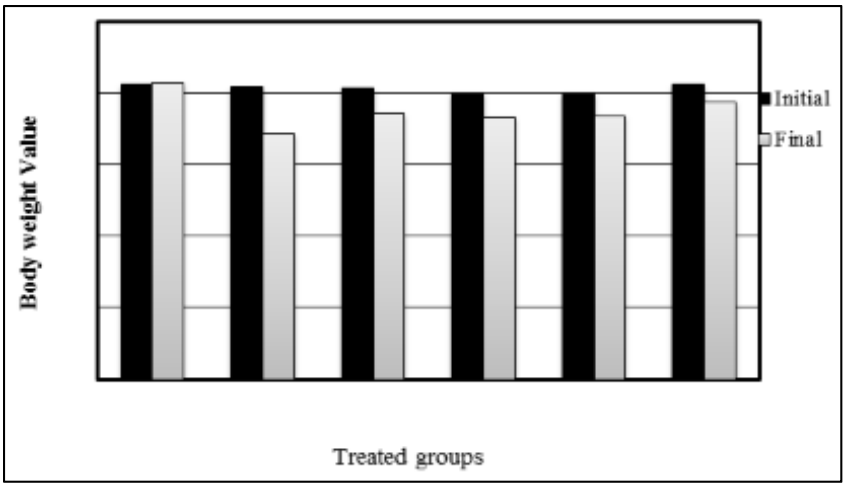

Fig 1: Physical changes observed in different experimental groups of mice.

Mice of the experimental negative group II showed maximum reduction in weights after a period of 10 days (fig.1). There was also hair loss and mice appeared weak. On oral administration of the extracts and treatment with rifampicin to those mice of group III and group IV, the weight of mice decreased from the initial weight of mice but slightly increased as compared to group II. The decrease in weight might be because of infection or stress due to frequent handling or drug administration.

3.1 *In-vivo* antioxidant assay

The present study showed comparisons of all groups with standard groups. Negative group shows elevated levels of lipid peroxidation products, however, extract treatment cause concentration dependent significant protection of MDA production, indicating a reduction in lipid peroxidation mediated cell injury in macrophages, thus plays protective level against immune cell damage preserving cell integrity. The changes in antioxidant levels of SOD, CAT and GPx as free radical scavenging enzymes and are the first line of cellular defense against oxidative stress. They showed a considerable decrease in group II (negative). The antioxidant enzymes level was found to be high in plant extract and standard groups. It has been shown that the deficiency of ascorbic acid (VIT.C) result in accumulation of lipid peroxide, which is intermediate product in lipid peroxidation [14]. Result shows that level of ascorbic acid decreased in infections and increased with treatment more than even standard group, which may be due to the sparing effect of antioxidant defense system as the drug has quenched the generation of free radical by the presence of flavonoids. Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons, etc and involved in regulation of various physiological processes. Oxygen reacts with excess nitric acid to generate nitrite (NO_2^-) and nitrate (NO_3^-) are termed RNI, which play important role in host defense against *M. tuberculosis* [15]. NO level was significantly increased in treated mice macrophage as compared to negative mice macrophage (Table 1).

Table 1: Activities of antioxidant enzymes in PEC- GP_x and VIT.C in experimental mice

Groups	LPO	SOD	CAT	GP_X	VIT.C	RNI (NO)
Negative	36.8±1.20***	0.015±0.002***	1.110±0.06***	12.29±0.76***	2.56±0.07***	1.40±0.01NS
Standard	13.23±0.64	0.165±0.002	3.69±0.09***	21.29±0.31	4.30±0.12	2.06±0.03
MEMI 100	22.50±0.04***	0.035±0.007***	1.70±0.2	19.91±0.58**	3.80±0.12**	1.51±0.5NS
MEMI 200	19.14±0.63***	0.078±0.008***	2.25±0.15***	19.83±0.73**	5.30±0.12**	1.67±0.08NS
Synergistic	16.17±0.73*	0.103±0.006***	2.95±0.38NS	23.02±0.34***	4.77±0.07***	2.02±0.02NS

***P<0.001, **P<0.01, *P<0.05, NS- non significant. The values are expressed as Mean ± SD.

Giant cell formation and tuberculous granuloma was observed in lung tissue (fig. 3&4). Heat fixed impression smear from infected lung and liver showed presence of acid fast bacilli on staining with acid fast stain (fig. 2).

Present study supports a link between oxidative stress and mycobacterial infection. Plant extracts protects murine peritoneal macrophage from mycobacteria by decreasing free radical generation, lipid damage and also increasing antioxidant status. Synergistic effect could be useful in fighting emerging drug resistance problem especially mycobacterial infections as

from present investigation synergistic group found to be similar like standard one.

PCR of lung samples showed band at 240 bp (fig.5) when targeted for *MPB-64* gene which confirms the tuberculosis infection in mice and therefore confirms the enzymatic changes were due to TB infection only.

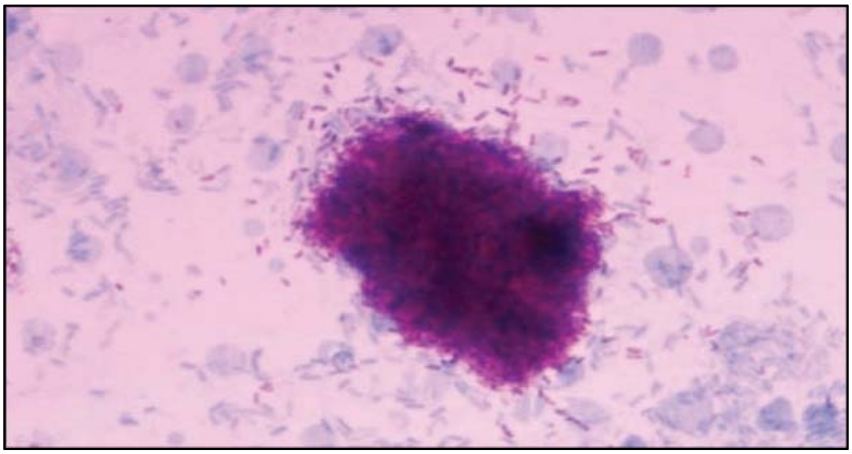

Fig 2: *M. tuberculosis* ZN stain (100X)

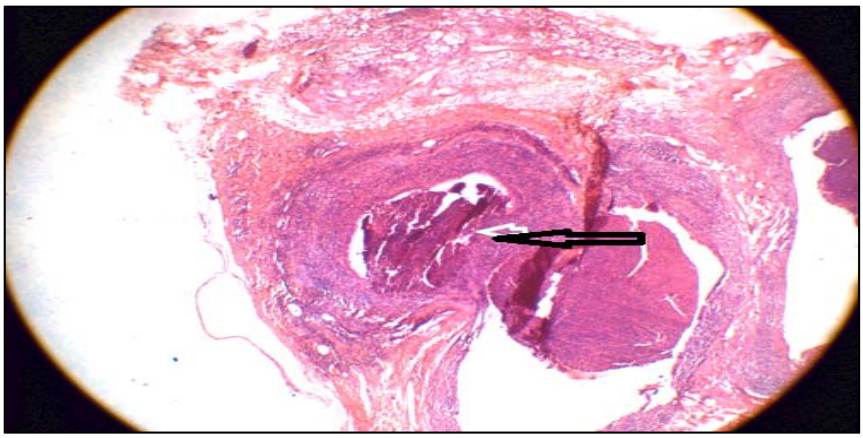

Fig 3: Giant cell formation (400X, H& E)

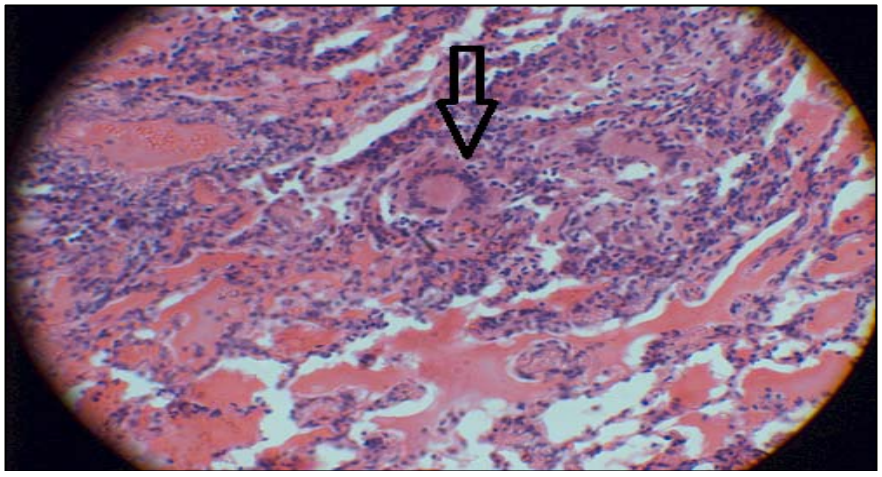

Fig 4: Tuberculous granuloma of lung tissue (100X, H&E).

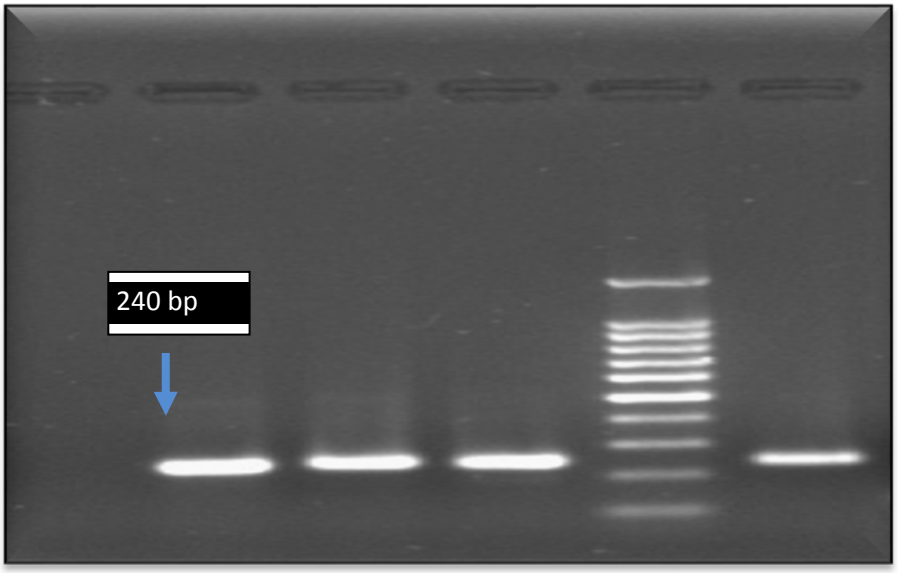

Fig 5: *M. tuberculosis* specific PCR pattern tissue targeted for *MPB*-64 gene shows band at 240 bp.

4. Conclusion

The present study showed that *Mucuna imbricata* played a significant role in antimycobacterial activity and provides scientific basis for its use as synergistic with rifampicin, and that may be because of presence of various phytochemical constituents. Further study can be done for detection of bacterial load through real-time PCR.

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