

ANTI MYCOBACTERIAL ACTIVITY OF THE PLANT EXTRACTS OF *ALSTONIA SCHOLARIS*MOLLY ANTONY¹, JOEL JAMES², CHANDRA SHEKHAR MISRA², LIPIN DEV MUNDUR SAGADEVAN², ARUN KUMAR THALIYIL VEETIL² & THANKAMANI V^{2*}¹Sree Chitra Tirunal Institute for Medical Sciences and Technology Trivandrum 695011, ²School of Bio Sciences and Technology, VIT University, Vellore-632014, Tamil Nadu, India. Email: dr.thankamani@gmail.com

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ABSTRACT

The present study was carried out to find out the susceptibility of *Mycobacterium tuberculosis* to butanolic extracts of bark of *Alstonia scholaris*. Luciferase reporter phage (LRP) assay and an in vitro bioassay based on inactivation of viability by a procedure similar to neutralization test were used to study the inhibition of *Mycobacterium tuberculosis*. The LRP method showed that butanol extracts of flower and bark at concentrations of 100 and 500 µg/ml respectively showed moderate bactericidal activity against clinical strains of sensitive and drug resistant *Mycobacterium tuberculosis* whereas the results of in vitro bioassay showed complete inhibition of the fast grower *Mycobacterium* sps even after 6 days of incubation compared to control when treated with the butanolic extract. The present results are possibly one of the first few investigations showing a clear indication of the potent anti tubercle effect of *Alstonia scholaris* and with more in vivo tests using purified fractions would throw light on the specific phytochemicals in *Alstonia scholaris* bark and the elucidation of the structure would help understand the mechanism of anti *M.tuberculosis* potential of the plant.

Keywords: *Alstonia scholaris*, Anti *M. tuberculosis* activity, Luciferase Reporter Phage Assay.

INTRODUCTION

Mycobacteria are major causes of morbidity and mortality in the world. About 2 million people are infected with *Mycobacterium tuberculosis*. Globally, there are an estimated 13.7 million chronic active cases, 9.3 million new cases and 1.8 million deaths mostly in developing countries in 2007¹. It is estimated that there are currently 2 million deaths from tuberculosis annually². Moreover, up to 50 million people are infected with drug-resistant forms of TB with about 500,000 cases of MDR tuberculosis a year worldwide³. Tuberculosis is mostly asymptomatic and is aggravated when impairment of immunity arises due to conditions like malnutrition, diabetes, malignancy, and AIDS. Weakening of the immune system by HIV increases vulnerability to other infections and diseases caused by *M.avium* complex has also increased⁴. A major problem for the control of TB is the requirement of drug regimens for six to nine months. These lengthy regimens lead to non-compliance with therapy, relapse and development of drug resistance. In order to shorten the duration of therapy, novel drugs that are active against *Mtb*, which act through mechanisms different from those employed by the existing frontline and secondary anti-TB drugs are urgently needed. The use of herbs and other alternative therapies for the treatment of tuberculosis is on the increase.

Phytochemical aspects of most medicinal plants have been known and used since time memorial^{5,6}. Ethanobotanical advantages conferred by these plant based products have surpassed the chemical counter parts owing to their lesser side effect and more potent therapeutic effect. Natural products continue to play the most significant role in the drug discovery and development process⁷. Hence it is a demanding need of the hour to study the various pharmacologically valuable aspects of these medicinal plants and one of which is *Alstonia scholaris* in treating human diseases. The anti Mycobacterial activity can be detected by the conventional method such as incorporating the various dilutions of the test antibiotic compound into Lowenstein Jensen Medium and inoculating a known amount of the test organism and by modern techniques like Luciferase Reporter Phage assay.

Alstonia scholaris (Apocynaceae) is a large evergreen tree commonly found in the subtropical regions of South Asia and Africa. Almost all parts of the plants are used in medicine. The bark is used as an anthelmintic, astringent, antiperiodic and also used to treat chronic diarrhoea, dysentery, and bowel movements⁸.

Leaves are used in the treatment of beri-beri, congestion of liver, dropsy and ulcers. The latex obtained from the exudates of the tree

has been in application for ulcers, sores, tumours, and in rheumatoid pain⁹. Methanolic extracts of roots and flower have exhibited potent antimicrobial activity^{10, 11}. Therefore the present study is carried out to study the anti mycobacterial effect of the butanolic bark extract of the *Alstonia scholaris*.

MATERIALS AND METHODS

Collection and Processing of Plant Materials

The fresh plants of *Alstonia scholaris* were collected from VIT University campus, Vellore and authenticated by Plant Biotechnology division, VIT University, Vellore. The dried bark, leaves, flowers and fruits were pulverized and 100g of the powered samples were extracted with ethyl acetate, butanol and water in a Soxhlet apparatus. The extracts obtained were dried and used for anti-mycobacterial studies.

Microbial strain for anti-*Mycobacterium tuberculosis* Assays

Standard strain H37RV, one clinical sensitive strain and the other a clinical resistant strain were used for the anti mycobacterial assays.

Susceptibility Testing of *Mycobacterium tuberculosis*

Luciferase reporter phage (LRP) assay

Standard strain H37RV, a clinical sensitive strain and a clinical resistant strain were grown in Middlebrook 7H9 complete medium¹² with and without extracts of *Alstonia scholaris* for 3 days at 37°C.

Luciferase Reporter Phage Assay¹³ was done using concentrations of 100 and 500 µg/ml of *Alstonia scholaris* extracts. Rifampicin was included as an assay control and DMSO as the solvent control. LRP phage AETRC21 was added and the samples were incubated for four hours. Equal volume of the cell phage mixture was mixed with 0.3Mm D-Luciferin in 0.05M sodium citrate buffer of pH 4.5 and light output was immediately measured as RLU (Relative light units) in the luminometer at 10seconds integration. Compounds exhibiting a reduction of 50% or more in RLU in the test vials compared to that of the control were considered to have anti mycobacterial activity. These LRP assays^{13,14,15} offer an elegant means of detecting viable mycobacteria and provide a rapid tool for drug susceptibility screening.

In vitro bioassay to demonstrate anti mycobacterial activity

We had improvised a test based on the principle of bacterial viability neutralization test to be used as a screening test for detecting anti mycobacterial activity using a rapid grower of atypical mycobacteria grown on Lowenstein Jensen (LJ) medium¹⁶.

Growth was emulsified in 3 ml of sterile 0.9% saline and turbidity was adjusted to 0.5 on a densimeter. 0.5 ml each of this suspension was distributed aseptically in a Bio-safety hood into 2 sterile tubes marked as one "test" and the other "control". Into the "test" tube 0.5 ml of *Alstonia scholaris*-leaf-Butanol fraction (150 mgms) of Methanol extract dissolved in DMSO was added and into the control tubes 0.5 ml of DMSO was added in place of *Alstonia* extract. The tubes were vortexed on a cyclomixer and incubated at 37°C. After 24 hours 0.5 ml from the control tube and "test" tube were inoculated

on to LJ slopes each and incubated for 6 to 20 days until sufficient growth was observed on the culture control LJ slope.

RESULTS AND DISCUSSION

The result of the anti mycobacterial activity by Luciferase reporter phage assay is presented (Tables 1 & 2) against H37RV a standard strain of *Mycobacterium tuberculosis*, a clinical sensitive and a clinical resistant strain of *Mycobacterium tuberculosis*.

Table 1: Percentage reductions in relative light units (RLU) by 100µg/ml against clinical isolates of H37RV Standard, Clinical Sensitive and Clinical Resistant cultures of *Mycobacterium tuberculosis*

Extract Code (Source)	H37RV Standard Strain	Clinical Sensitive Strain	Clinical Resistant Strain
MFR (Fruit)	0	0	11.8
MFL (Flower)	0	0	50.69
CR2 (Bark)	37.01	50.40	61.76
CR3 (Leaf)	0	35.6	15.80

Consistent reduction in RLU against all 3 strains were observed for both the concentrations 100 and 500µgms /ml of *Alstonia scholaris* bark extracts ranging from 37.01 to 73.09%. Extracts of *Alstonia scholaris* flower also showed a moderate reduction in RLU of 21.59 to 55.28% at 500µgm/ml concentration.

Table 2 depicts a comparison of anti mycobacterial activity of fruit, flower, bark and leaf extracts of *Alstonia scholaris* at 500µgm concentration when tested against the standard strain and two clinical strains.

Table 2: Percentage reductions in relative light units (RLU) by 500µg/ml against clinical isolates of H37RV Standard, Clinical Sensitive and Clinical Resistant cultures of *Mycobacterium tuberculosis*

Extract Code (source)	H37RV Standard Strain	Clinical Sensitive Strain	Clinical Resistant Strain
MFR (Fruit)	44.10	21.65	0
MFL (Flower)	33.56	21.59	55.28
CR2 (Bark)	47.40	51.46	73.09
CR3 (Leaf)	11.93	26.36	47.59

The highest inhibitory activity was seen in the bark extract which is 73.09% inhibition and it was even more significant as this was noted against a clinical strain which was resistant to Streptomycin, Isoniazid, Rifampicin and Pyrazinamide.

The second set of experiment was done with a rapid grower of a typical mycobacterium. Rapid grower was chosen as it would

hasten the results of antimycobacterial testing of plant extracts that would eventually give leads for probable drug development. The invitro bioassay to test for anti mycobacterial activity was based on the principle of neutralization of viability of the organism by incubation with test material check if the test extracts have any potential to kill *Mycobacterium*.



Fig. 1: Inhibitory action of Butanol fraction of Methanol extract of *Alstonia scholaris* on *Mycobacterium* (fast grower strain) on LJ medium

Bottle 1, Culture control, growth +++; 2- Reagent control, growth +++; 3- Test (20 days) No Growth

Growth of rapid grower strain of *Mycobacterium* was detected on the normal LJ medium control bottle after 4 days and by the 6th day there was good growth. DMSO control also showed similar growth by 6 days while growth was completely inhibited in the rest tube inoculated with *Mycobacterium* pre incubated with *Alstonia* extracts for 24 hours at 37°C. All bottles were further incubated up to 20

days to rule out possibility of delayed suppressed growth indicating static or incomplete killing by the phyto chemicals in the plant extract. But even after three weeks no growth could be observed in the "test".

The results of the preliminary investigations were very encouraging that all the *Mycobacteria* exposed to Butanol fraction of *Alstonia scholaris* leaf – methanol extract at a concentration of 150 mg had mycobactericidal action. Medium and reagent control bottles showed

good and luxuriant growth of Mycobacterium while test bottle which received the same load of inoculum showed complete inhibition of Mycobacterium thus proving the bactericidal action even after prolonged incubation of 20 days.

Natural products are a proven template for the development of new scaffolds of drugs^{17, 18, 19} and they have received considerable attention as potential anti-TB agents²⁰. The emergence of pathogenic microbes with increased resistance to established antibiotics provides a major incentive for the discovery of new antimicrobial agents²¹. Antimicrobial screening for the phytochemicals from plant extracts then represents a starting point for antimicrobial drug discovery²², especially, antimycobacterial drugs. There are reports from folklore which claim that extracts of *Alstonia scholaris* are used by tribals and natives in cases of Tb infection, but to our knowledge such anti M tuberculosis activity is reported for the first time using pure culture in vitro and that *Alstonia scholaris* extracts do possess phytochemicals with remarkable anti Mycobacterial activity. Similar anti mycobacterial activity from herbal extracts – hexane extract of *Adhatoda vasica* has been reported by Ignacimuthu S and Shangumam N²³. Gupta K.C and Chopra I.C.²⁴ have reported similar findings about *Adhatoda vasica* having anti mycobacterial activity. There are also reports that anti mycobacterial activity is noted in *Aloe vera* and garlic when tested against H37Rv²⁵⁻²⁸. Gupta R et al have reported anti tuberculosis activity from *Acalypha indica* and *Allium cepa* extracts against *Mycobacterium tuberculosis*²⁹.

CONCLUSION

The anti mycobacterial activity showed that *Alstonia scholaris* bark extract has the potential to cure tuberculosis and is a promise for future therapeutic interventions. To our knowledge it is for the first time that it has been possible to demonstrate experimentally in the laboratory that *Alstonia scholaris* plant extracts possess remarkable anti mycobacterial activity. The results assume significance and throws some light on the basis of the ancient use of the tree in our traditional systems of medicine and in folklore.

Further detailed phytochemical screening and bio activity studies need be carried out using crude solvent extracts as well as further purified constituents to comprehend their role in anti-tuberculosis activity and develop suitable drugs so that the most deadly disease in the world can be combated. The present study also could pave the way towards possibility to obtain anti mycobacterial moieties against other Mycobacterial species.

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