EVALUATION OF ANTI-TUBERCULAR ACTIVITY OF *RICINUS COMMUNIS* LINN. BY PROPORTION, NRA AND BACT/ALERT METHODS

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### ABSTRACT

Background: Tuberculosis infection is becoming an increasing problem worldwide due to MDR-TB, with approx 8 million of TB emerging every year and at the same time killing 2 million people. *Ricinus communis* Linn. can be used as anti-tubercular is reported traditionally. This led to investigate the anti-tubercular activity of *Ricinus communis* Linn.

Methods: The aqueous extract was prepared by maceration process. The leaves of the plant extracted with different solvents by successive solvent extraction method and with ethanol separately in soxhlet extractor. The anti-tubercular activities of all extracts were screened by Conventional proportion method, Nitrate reductase assay (NRA) and BacT/ALERT 3D system against *Mycobacterium tuberculosis* Std. strain H37RV. All positive tests extract samples confirmed by Ziehl Neelsen staining which was routinely performed for *M. tuberculosis*.

Results: Proportion method, NRA and BacT/ALERT 3D system were evaluated anti-tubercular activity. The ethanolic extract of *Ricinus communis* Linn. possess significant anti-tubercular activity. Results with the BacT/ALERT 3D system agreed 100% with those obtained by the NRA and conventional proportion methods. The susceptibility test results were obtained in average 10 days by NRA and BacT/ALERT 3D system and 42 days by conventional proportion method. BacT/ALERT 3D system is a novel, completely automated system which is useful for susceptibility testing of *M. tuberculosis*.

Conclusion: It also showed good concordance i.e. 100% agreement concluded between conventional proportion method, NRA and BacT/ALERT 3D system for ethanolic extract, which concluded anti-tubercular activity i.e. susceptible to *M. tuberculosis*.

**Keywords:** *Mycobacterium tuberculosis, Ricinus communis*, Proportion method, NRA, BacT/ALERT 3D system.

### INTRODUCTION

Infectious disease represents a critical problem to health and they are one of the main causes of morbidity and mortality. It has surpassed the AIDS. Tuberculosis is a major threat killing about 2 million people each year. HIV outbreak; India can have an additional impact on the increase of TB in India as India accounts for 1/4th of global TB burden 1,2. The latest estimates of the global burden of TB show that there were 9.27 million new cases of TB in 2007 (including 1.37 million cases among HIV-positive people). 1.32 million deaths from TB in HIV negative people with an additional 0.46 million deaths in HIV positive people, and 1.37 million prevalent cases (of which 687 000 were HIV-positive cases). There were 0.5 million cases of MDR – TB. Collectively, these statistics show that TB remains a major global health problems 3.

The current lengthy drug regimen, the emergence of drug resistant strains and HIV co-infection have resulted in a resurgence in the work were of analytical grades. The current chemotherapy relies on drugs which were developed 5 decades ago and the bacteria have started to show resistance to it. There are several reasons that justify the need to search for new drugs for TB, e.g., improvement of current treatment by shortening its duration, to get efficient treatment for MDR TB and to eradicate the latent infection. So, the development of new drugs for shortening the duration of the treatment and to fight against multidrug resistant tuberculosis strains is urgent 4,5,6. The challenge of discovering new, urgently needed anti-TB drugs from natural sources requires a truly interdisciplinary research 7. Innovative natural products chemistry tools have to be developed and employed in order to meet these demands. Recent acceptance of natural herbal medicines as an alternative form of health care and development of multiple drug resistance-TB has increasing 8.

*Ricinus communis* Linn. commonly called Castor is a perennial evergreen shrub. The Sanskrit name *ernadah* describes the property of the drug to dispel diseases. It is considered as a reputed remedy for all kinds of rheumatic afections. They are useful in gastropathy such as gulma, amadosa, constipation, inflammations, fever, ascitis, bronchitis, cough, leprosy, skin diseases and vitiated conditions of vata, colic and hambago.8,9 This plant as a folk medicine reported for treatment for asthma, as bactericide, expectorant, hepatoprotective & in scrofula 10. A poultice of castor seeds is also applied to scrofulous sores and boils due to tuberculosis of lymph nodes 11. *Ricinus communis* Linn. whole plant juice used in scrofula/tuberculosis was reported traditionally. Hence aim of our study consists of the evaluation of anti-tubercular activity of *Ricinus communis* Linn. leaves by Proportion method, NRA and BacT/ALERT 3D System for testing the susceptibility of *M. tuberculosis*.

### MATERIAL AND METHODS

*Rifampicin* and *isoniazide* were obtained as gift sample from Lupin, Mumbai. The standard strain of bacteria *M. tuberculosis* H37RV procured from Jalma institute of Leprosy and other mycobacterial diseases, Agra, BacT/ALERT 3D System (Biomeriux) is purchased from Biomeriux, Delhi. Loweinstein-Jensen media and McCartney bottles procured from Himedia. All other chemicals and reagents used in the work were of analytical grades.

**Collection and preparation of extracts**

Fresh leaves of *Ricinus communis* Linn. were collected from Sangli and Miraj areas and authenticated by Dr. Yadav, U. S., Botany department, of Willingdon College, Sangli. The leaves were washed and were shade dried to obtain coarse powder and powder subjected to following different extraction procedures.

- The aqueous extract was prepared by maceration process 13.
- The petroleum ether, benzene, chloroform, acetone, ethanol and methanol extracts of leaves were obtained by successive solvent extraction method by using soxhlet apparatus 14.
- The ethanolic extract was prepared in soxhlet apparatus.

### Evaluation of anti-tubercular activity

Anti-tubercular testing against standard strain of *M. tuberculosis* H37RV.
Inoculation and Cultivation of Mycobacterium tuberculosis

Pure culture of standard strain M. tuberculosis H37RV procured was inoculated in freshly prepared Lowenstein-Jensen (LJ) solid media in McCartney bottles and incubated at 37°C for 2-3 weeks for cultivation until growth of M. tuberculosis in the form of colonies was observed. It was identified and confirmed the colonies was only of Mycobacterium tuberculosis by performing Ziehl Neelsen staining.

Preparation of different concentration of drugs

Preparation of test extracts of Ricius communis Linn. - Prepared the stock solutions of each different extract as 1mg/ml and from stock solution, the different concentrations i.e. 50, 100, 150 and 200 μg/ml of each extract sample were prepared in solvents like water, dimethyl formamide and dimethyl sulphoxide according to their solubility.

Std. anti-TB drugs- Isoniazide (0.2 μg/ml) and rifampicin (40 μg/ml)

The control medium without drugs is prepared at the same time as the drug-containing media.

Preparation of Mycobacterium tuberculosis inoculum for anti-tubercular testing

A loopful of the colonies was taken from the primary culture and was shaken for 20-30 seconds; distilled water was added slowly under continuous shaking. The opacity of the bacterial suspension adjusted to a McFarland standard solution no.1.3

Conventional proportion method

The tuberculosis bacilli were tested for its sensitivity by use of all the Different concentrations (50, 100, 150 and 200 μg/ml) of extracts of Ricius communis Linn. in LJ media by proportion method against std. strain of M. tuberculosis H37RV.

0.2 μg/ml of isoniazide and 40 μg/ml of rifampicin were selected as standard control and added to the LJ media. Each media was distributed as 6-8 ml in to sterile McCartney bottles in triplicate and inspissated. The control media was prepared at the same time as the drug containing media. The two bacterial dilutions required for inoculation with the loop is 10^(-2) mg/ml. A loopful of 10(-2) mg/ml dilute inoculums inoculated on drug containing media and control media that becomes 10(-4) dilution of each extract sample were prepared in solvents like water, dimethyl formamide or dimethyl sulfide according to their solubility.

All the bottles were incubated at 37°C and results were obtained after 28 days followed by further incubation until growth was visible and confirmatory on 42nd day. The same procedure was repeated twice.

Reading of tests

The results were read for the first time on the 28th day. The inoculum is the same for the control and the drug-containing slopes. The average number of colonies obtained for the drug-containing slopes indicates the number of resistant bacilli contained in the inoculum. The results were calculated by using formula and the critical resistance for resistant strains is 1% for rifampicin and isoniazide.

R (%) = No. of colonies on drug media / No. of colonies on control medium ×100

If R ≥1 per cent, the bacilli was taken as resistant and if R ≤1 the bacilli was taken as sensitive.

If according to the criteria indicated above, the result of the reading made on the 28th day was resistant, the strain was classified as resistant. If the result at the 28th day is "sensitive", a second reading was made on the 42nd day, this provides the definitive result 15,16,17,18.

Nitrate Reductase Assay method

NRA was performed as described by Golyshevskaia et al and Angeby et al for anti-tubercular activity. The critical concentrations used were 0.2 μg/ml for INH, 40 μg/ml for rifampicin and different concentrations (100, 150, 200 μg/ml) of each test extract sample of Ricius communis Linn. The LJ media prepared and potassium nitrate (KNO3- 30 mg/ml) was added to the media. The Golyshevskaia et al and Angeby et al reported the concentration of (KNO3) as 1 mg/ml of the media. However growth was not observed of M. tuberculosis H37RV (in the form of pink colour) in control bottles when the reported concentration of (KNO3 - 1 mg/ml) was used in the media. Hence method was modified as above and growth was observed of M. tuberculosis H37RV (in the form of pink colour) in control bottles. For each test extract sample for one concentration three bottles were prepared and used for inoculation of M. tuberculosis H37RV and all the bottles were inspissated same as described in proportion method.

0.2 ml of inoculum suspension was inoculated into the bottles containing LJ medium with potassium nitrate and each different concentration of test extract. Similarly 0.2 ml of inoculum suspension was inoculated into the std. anti-tubercular drugs; 0.2 ml of the 10% inoculum was inoculated into drug free media which served as growth controls. McCartney bottles in triplicate utilized for each test sample extracts, anti-tubercular drugs and control bottles. After inoculation all the bottles were incubated at 37°C for 14 days.

0.5 ml of a mixture of three reagents (25 μl of concentrated HCl, 50 μl of 2% sulphanalidamide and 50 μl of 1% n-l-naphthyl-ethylenediamine dihydrochloride (ratio 1:2:2)) was added to one drug free control bottle after 7 days of incubation. When the colour of control bottle changes to pink then bottles with drugs were tested with this reagent.

Result interpretation

M. tuberculosis H37RV was considered as resistant if there were colour changes (pink or deep red to violet) in the test extract samples bottle greater than in the 10 % diluted growth control on the same day. The bottles that did not showed any colour change & remains the same were considered as M. tuberculosis H37RV sensitive to the test extract sample.

Those test extracts were further incubated for 10 days and 14 days as described by Angeby et al for confirmation of results. On the 10th day and 14th day again 0.5 ml of a mixture of three reagents was added in all these bottles and confirmed results 19, 20, 21.

BacT/ALERT 3D system: The colorimetric BacT/ALERT 3D system (BioMérieux) previously designated MB/BACT (Organon Teknika, Bosteels) has been reported to be useful for rapid and reliable susceptibility testing of M. tuberculosis.

Each MB/BacT broth bottle contains Middlebrooke 7H9 broth (0.47% w/v) plus pancreatic digest of casein (0.1% w/v), bovine serum albumin (0.5% w/v) and catalase. If microorganisms are present in the test broth, carbon dioxide is produced as the organisms metabolize substrates in the culture medium.

The rationale: When growth of the microorganisms produces carbon dioxide, the color of the gas permeable sensor at the bottom of each culture broth bottle changes from dark green to lighter green or yellow. The lighter color results in an increase of reflectance units monitored by the system. Bottle reflectance is monitored and recorded by the instrument every 10 minutes.

Method: The colorimetric BacT/ALERT 3D system (BioMérieux Inc., Durham, NC), previously designated MB/BACT (Organon Teknika, Bosteels, The Netherlands) has been reported to be useful for rapid and reliable susceptibility testing of M. tuberculosis. In this method, the Mycobacteria Process bottles with 7H9 medium were supplemented with reconstitution fluid (oleic acid, glycerol and bovine serum albumin) stock solution was added to the bottles so as to attain a final concentration of standard anti-tubercular drugs 0.1 μg/ml of isoniazide and 1μg/ml of rifampicin. Similarly the different test extracts drug stock solutions were prepared to get final concentration 150 and 200 μg /ml. Equal amounts of sterile distilled water were added to the drug-free control bottles. A 0.5 ml bacterial suspension adjusted to a 0.5 McFarland turbidity was used as an inoculum for the drug-containing bottles, and a 100 x diluted suspension was inoculated in the drug-free control bottles (1% proportional control). The bottles were loaded in the instrument's incubation module at 37°C.

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The Rationale for result interpretation: When the bottle containing test extract sample flagged positive at the same time as or before the bottle containing the drug-free proportional control, the organism was considered resistant [15, 16]. All positive bottles were smeared and stained by the Ziehl-Neelsen method to confirm the presence of acid-fast bacilli [15].

Statistical analysis

Statistical analysis was performed using Dunnet’s test. Data was expressed as Mean ± SEM.

RESULTS AND DISCUSSION

Proportion method

The proportion of resistant cells in the total viable population of the original inoculum was then calculated and expressed as a percentage. The proportion of growth above which the M. tuberculosis was labelled as resistant was taken as 1%, as per the recommendation [17].

Results from table no.1 reveals that M. tuberculosis is resistant to all extracts at 100 µg/ml. Statistical analysis was performed using Dunnet’s multiple comparison test for which probability values was found to be (p <0.05). Therefore it is concluded that 150 & 200 µg/ml of acetone extract and 200 µg/ml of ethanol and successive ethanol extract possesses mild anti-tubercular activity. Rifampicin and isoniazide at 40 µg/ml and 0.2 µg/ml respectively showed anti-tubercular activity. Petroleum ether, benzene, chloroform, methanol and aqueous extracts of Ricinus communis Linn. did not showed any anti-tubercular activity.

Table 1: Table shows mean no. of colonies Mycobacterium tuberculosis in extracts of Ricinus communis Linn.

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Name of the test drug/ extracts of Ricinus communis Linn.</th>
<th>Mean number of colonies of Mycobacterium tuberculosis (Mean ± SEM) in different concentration of test drug / extract (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>100</td>
<td>150</td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>77.66 ±1.453</td>
</tr>
<tr>
<td>2</td>
<td>Aqueous extract</td>
<td>68 ± 1.155</td>
</tr>
<tr>
<td>3</td>
<td>Ethanol extract</td>
<td>20.33 ± 0.888</td>
</tr>
<tr>
<td>4</td>
<td>Successive ethanol extract</td>
<td>28 ± 1.1</td>
</tr>
<tr>
<td>5</td>
<td>Acetone extract</td>
<td>17.33 ± 0.8819</td>
</tr>
<tr>
<td>6</td>
<td>Chloroform extract</td>
<td>55.66 ± 0.8819</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. *p<0.05 and **p<0.01 was taken as the criterion of significance when compared to control where n=3.

Nitrate reductase assay method

The NRA method utilizes the standard detection of nitrate reduction as an indication of growth and therefore results can be obtained much faster than visual detection of colonies.

From the observations of table no. 2, in all control bottles and in each extract containing bottles of Ricinus communis Linn. Reddish/violet colouration was observed on the surface of the slants indicative of a positive NRA and growth of standard strain of M. tuberculosis on addition of specific reagent. Therefore it is concluded that all the extracts did not showed anti-tubercular activity at 150 µg/ml by NRA method. No reddish/violet colouration was observed on the surface of the slants in std. anti-tubercular drugs rifampicin and INH and absence of colouration interpreted as negative NRA.

No reddish/violet coloration was observed on the surface of the slants in ethanol, successive ethanol and chloroform extract containing bottles of Ricinus communis Linn. and absence of colouration interpreted as negative NRA. Therefore it is concluded that aqueous, pet. ether, acetone, benzene and methanol extracts at 200 µg/ml did not showed any anti-tubercular activity while ethanol, successive ethanol and chloroform extracts possess anti-tubercular activity by NRA method. The results were obtained after seven days of inoculation in all the control and extract containing bottles.

Table 2: Table shows effect of extracts of Ricinus communis Linn. on growth of M. tuberculosis by nitrate reductase assay method:

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Name of the test drug/ extracts of Ricinus communis Linn.</th>
<th>Growth of Mycobacterium tuberculosis indicated by reddish/violet colouration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>150 µg / ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Aqueous extract</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Ethanol extract</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Successive ethanol extract</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Acetone extract</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Chloroform extract</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Benzene extract</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Petroleum ether extract</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>Methanol extract</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Rifampicin</td>
<td>--</td>
</tr>
<tr>
<td>11</td>
<td>Isoniazide</td>
<td>--</td>
</tr>
</tbody>
</table>

-- indicates no change in colour means no growth of M. tuberculosis.
+ indicates development of reddish violet colour means growth of M. tuberculosis.

BacT/ALERT method

In BacT/ALERT method from table no. 3, it was observed that the growth of M. tuberculosis was found after 10.42 days in the proportional control bottle and after 6.23 days in direct control bottle after inoculation. Aqueous, acetone, benzene, pet. ether, chloroform and methanol extracts 150 µg/ml used as test bottles, the growth of M. tuberculosis was found in all above extracts during period 5.23-9.28 days after inoculation, since the growth of M. tuberculosis compared to the proportional control bottle, which is earlier than proportional control bottle, hence it indicates these extracts did not possesses any anti-tubercular activity. The standard rifampicin and INH bottles showed M. tuberculosis growth at 24.14 and 19.5 days respectively after inoculation compared to the
proportional control bottle, since growth was observed later than the proportional control bottle, which indicates anti-tubercular activity.

From this result, it is concluded that all these extracts did not showed anti-tubercular activity at 150 µg/ml of *Ricinus communis* Linn. by BacT/Alert 3D system which was similar as shown by nitrate reductase assay method. Ethanol, chloroform and successive ethanol extracts of *Ricinus communis* Linn. at 200 µg/ml observed the growth of *M. tuberculosis* after 13.35, 11.38 and 11.42 days respectively which was later than proportional control bottle (after 10.42 days) after inoculation. Growth of *M. tuberculosis* identified by yellow colour at the bottom of bottle and indicated +ve signal. Therefore the results of BacT/Alert 3D system concluded that chloroform and successive ethanol extracts of *Ricinus communis* Linn. at 200 µg/ml showed significant and ethanolic extract showed highly significant anti-tubercular activity while other extracts of *Ricinus communis* Linn. did not showed anti-tubercular activity at 200 µg/ml.

Table 3: Table shows effect of 150 and 200 µg/ml extracts of *Ricinus communis* Linn. on *M. tuberculosis* by BacT/ALERT 3D system.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of test drug</th>
<th>Concentration of extract µg/ml</th>
<th>No. of days required for growth of <em>Mycobacterium tuberculosis</em> (Mean + SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Proportional control</td>
<td>--</td>
<td>10.4233± 0.01333</td>
</tr>
<tr>
<td>2</td>
<td>Direct control</td>
<td>--</td>
<td>6.2333 ± 0.01667</td>
</tr>
<tr>
<td>3</td>
<td>Ethanolic extract</td>
<td>150</td>
<td>6.2766 ± 0.01333</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>13.3566 ± 0.01333***</td>
</tr>
<tr>
<td>4</td>
<td>Petroleum ether extract</td>
<td>150</td>
<td>7.2766 ± 0.01333</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>9.3833±0.01333</td>
</tr>
<tr>
<td>5</td>
<td>Acetone extract</td>
<td>150</td>
<td>5.2166 ± 0.01667</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>7.2166 ± 0.01667</td>
</tr>
<tr>
<td>6</td>
<td>Successive ethanolic extract</td>
<td>150</td>
<td>6.2633 ± 0.01333</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>11.4233 ± 0.01333**</td>
</tr>
<tr>
<td>7</td>
<td>Benzene extract</td>
<td>200</td>
<td>7.3033±0.01333</td>
</tr>
<tr>
<td>8</td>
<td>Chloroform extract</td>
<td>200</td>
<td>11.3833 ± 0.01333**</td>
</tr>
<tr>
<td>9</td>
<td>Methanol extract</td>
<td>200</td>
<td>5.2333 ± 0.01667</td>
</tr>
<tr>
<td>10</td>
<td>Aqueous extract</td>
<td>200</td>
<td>6.4833±0.01667</td>
</tr>
<tr>
<td>11</td>
<td>Rifampicin</td>
<td>1</td>
<td>24.1466±0.003333**</td>
</tr>
<tr>
<td>12</td>
<td>Isoniazide</td>
<td>0.1</td>
<td>19.5666 ± 0.03333**</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. *p<0.05 and **p<0.01 was taken as the criterion of significance when compared to proportional control where n=3.

![Effect of all extracts on M. tuberculosis](image)

**CONCLUSION**

From the results obtained by all three methods and from fig no.1 it is observed that *M. tuberculosis* H37RV is sensitive to 150 µg/ml ethanol extract of *Ricinus communis* Linn. On correlation analysis using McNemar Chi-square test, no significant difference among all three methods is observed (p>0.05). Hence 100 % agreement is observed in between conventional proportion method, NRA and BacT/ALERT 3D system. However this is true for ethanol extract and not for methanol extract of *Ricinus communis* Linn.

Hence it concludes that ethanol extract of *Ricinus communis* Linn. leaves has potent anti-tubercular activity which may be probably be due to the phytoconstituents present in the plant and could be a function of either the individual or the additive effects of the phytoconstituents. All these findings justified the claim made in the indigenous system of medicine *Ricinus communis* Linn. for the treatment of tuberculosis.
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