Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 4, Suppl 3, 2012

Research Article

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

MRS. PADMA. L. LADDA*[1], DR. CHANDRAKANT. S. MAGDUM[2]

[*1] Appasaheb Birnale College of Pharmacy, South Shivaji nagar, Sangli. 416416. (M.S.) India, [2]Rajarambapu college of Pharmacy, Kasegaon (M.S)
Email: *P_ladda@rediffmail.com, magdum_cs@yahoo.co.in

Received: 28 Jan 2012, Revised and Accepted: 05 Apr 2012

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 out-breaks; 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 TB deaths in HIV-positive people, and 13.7 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 research efforts to address the urgent need for new anti-tb drugs. Moreover 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 *erandah* describes the property of the drug to dispel diseases. It is considered as a reputed remedy for all kinds of rheumatic affections. 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 lumbago[9, 10]. This plant as a folk medicine reported for treatment for asthma, as bactericide, expectorant, hepatoprotective & in scrofula [11]. A poultice of castor seeds is also applied to scrofulous sores and boils due to tuberculosis of lymph nodes[12]. *Ricinus communis* Linn. whole plant juice used in scrofula/tuberculosis was reported traditionally[8].

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 regents 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 tubercle

Pure culture of standard strain *M. tuberculosis* H37RV procured was inoculated in freshly prepared Loweinstein-Jensen (LJ) solid media in McCartney bottles and incubated at 37^{0}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 *Ricinus 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 placed in a spherical bottle 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[15].

Conventional proportion method

The tubercle bacilli were tested for its sensitivity by use of all the Different concentrations (50, 100, 150 and 200 µg /ml) of extracts of *Ricinus 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 diluted inoculums inoculated on drug containing media and control media that becomes 10^{-4}.

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 42ndday. 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 *Ricinus communis* Linn. The LJ media prepared and potassium nitrate (KNO_3- 30 mg/ml) was added to the media. The Golyshevskaia et al and Angeby et al reported the concentration of (KNO_3) 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 (KNO_3- 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 inoculam 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^{0}C for 14 days.

0.5 ml of a mixture of three reagents (25 µl of concentrated HCl, 50 µl of 2% sulphanilamide and 50 µl of 1% n-1-napthyl-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, Boxtels) 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, Boxtels, 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× 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.

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 [22, 23]. All positive bottles were smeared and stained by the Ziehl-Neelsen method to confirm the presence of acid-fast bacilli[15].

Statastical 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 [19].

It was observed by proportion method that no. of colonies decreases as concentration of extract increases. Results of anti-tubercular activity by proportion method revealed that complete inhibition of growth of *M. tuberculosis* i.e. no growth of colonies was observed at 150 & 200 µg/ml of acetone extract and at 200 µg/ml of ethanol and successive ethanol extract.

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 possesses most significant while 150 µg / ml of 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.

S. No.	Name of the test drug/ extracts of *Ricinus communis* Linn.	Mean number of colonies of *Mycobacterium tuberculosis* (Mean ± SEM) in different concentration of test drug / extract (µg/ml)		
		100	150	200
1	Control	77.66 ±1.453	77.66 ±1.453	77.66 ± 1.453
2	Aqueous extract	68 ± 1.155	53.33 ± 1.20	21.33 ± 1.764
3	Ethanol extract	20.33 ± 0.888	4.33 ± 0.888*	0**
4	Successive ethanol extract	28 ± 1.1	8.3 ± 1.4	0**
5	Acetone extract	17.33 ± 0.8819	0**	0**
6	Chloroform extract	55.66 ± 0.8819	36 ± 0.5774	18.33 ± 0.8819

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 coloration 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 possesses 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:

S. no.	Name of the test drug/ extracts of *Ricinus communis* Linn.	Growth of *Mycobacterium tuberculosis* indicated by reddish/violet colouration					
		150 µg / ml			200 µg / ml		
		I	II	III	I	II	III
1	Control	+	+	+	+	+	+
2	Aqueous extract	+	+	+	+	+	+
3	Ethanol extract	+	+	+	--	--	--
4	Successive ethanol extract	+	+	+	--	--	--
5	Acetone extract	+	+	+	+	+	+
6	Chloroform extract	+	+	+	--	--	--
7	Benzene extract	+	+	+	+	+	+
8	Petroleum ether extract	+	+	+	+	+	+
9	Methanol extract	+	+	+	+	+	+
10	Rifampicin	--	--	--	--	--	--
11	Isoniazide	--	--	--	--	--	--

-- 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 3 D system.

S. No.	Name of test drug	Concentration of extract µg/ml	No. of days required for growth of *Mycobacterium tuberculosis* (Mean + SEM)
1	Proportional control	--	10.4233± 0.01333
2	Direct control	--	6.2333 ± 0.01667
3	Ethanolic extract	150	6.2766 ±0.01333
		200	13.3566 ± 0.01333**
4	Petroleum ether extract	150	7.2766 ± 0.01333
		200	9.3833±0.01333
5	Acetone extract	150	5.2166 ± 0.01667
		200	7.2166 ± 0. 01667
6	Successive ethanolic extract	150	6. 2633 ±0. 01333
		200	11.4233 ± 0. 01333**
7	Benzene extract	200	7.3033 ±0. 01333
8	Chloroform extract	200	11.3833 ± 0. 01333**
9	Methanol extract	200	5.2333 ±0.01667
10	Aqueous extract	200	6.4833± 0.01667
11	Rifampicin	1	24.1466±0.003333**
12	Isoniazide	0.1	19.5666 ± 0.03333**

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.

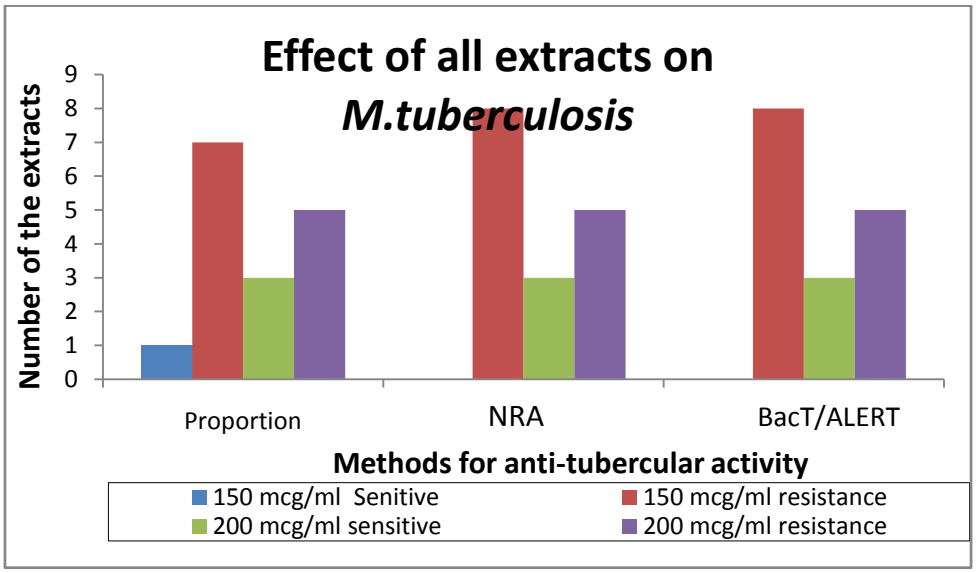

Fig. 1: Effect of all extracts on *M. tuberculosis* by different methods used for screening of anti-tubercular activity

BacT/Alert 3 D system is a novel, completely automated system and we obtain results in 8-12 days which is useful for susceptibility testing of *M. tuberculosis*. But it is expensive and the chances of contamination are more. Drug susceptibility testing (DST) of *M. tuberculosis* by NRA is simple, cost effective and reproducible. The shorter turnaround time (7 to10 days) is an advantage over the proportion method, which requires≥ 28 days for culture and 42 days for DST. Therefore it is concluded in the present study that the NRA is rapid and superior to LJ proportion method.

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 Mc Nemar 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 3 D 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.

ACKNOWLEDGEMENT

The authors are thankful to the Principal Prof. D. D. Chougule and Management of Appasaheb Birnale College of Pharmacy, Sangli for providing facility to carry out the work and special thanks to All India Council of Technical Education, New Delhi for funding this project and provided BACT/ALERT 3D system for evaluation of anti-tubercular activity .

REFERENCES

1. Rang HP, Dale MM, Ritter JM, Moore PK. Basic principles of chemotherapy. Pharmacology 5th edition. Churchill Livingstone; 2005. p. 620-653.
2. Govt. of India, Ministry of health and family welfare, Times of India. 24th March, 1999.
3. http://www.who.int/tb/publication/global-report2009/en /index.html accessed on August 2010.
4. Dye C, Scheele S, Dolin P, Pathania V, Raviglione, MC. Global Burden of Tuberculosis: Estimated incidence prevalence and mortality by country. JAMA. 1999; 282: 677-686.
5. Higuchi CT, Sannomiya M, Pavan FR, Leite SRA, Sato DN, Franzblau SG, et al. Byrsonima fagifolia Niedenzu Apolar Compounds with Anti- tubercular Activity. eCAM Advance Access published December 17, 2008.
6. Shrikumar S., T.K Ravi, Short Review Approaches towards Development and Promotion of Herbal Drugs Pharmacognosy Reviews 2007; 1: 23-26.
7. Kumar Vivek, Shinde Prann and Sobhia ME. Trends in anti-tubercular drug discovery: Current targets and future prospects. CRIPS. 12, (1): 2011.
8. http://waynesword.palomar.edu/plmar 99.htm accessed on Aug 2009.
9. Duke JA and Wain KK. Medicinal Plants of the World. 1981.
10. Kirtikar KR, Basu BD. Indian Medicinal plants. 2nd edition. Allahabad: Bishan Singh Mahendra Pal Singh 1991, (III): p.2274-2277.
11. http://www.home-remedies- guide.com/herbs/castor-seeds.htm accessed on Aug 2008.
12. Rangari V.D., Pharmacognosy and Phytochemistry. Ist edition. Part-1 Nashik, Career Publications; 2002. p. 130.
13. Kokate CK, Purohit AP & Gokhale SB. Pharmacognosy., 27th edition. Nirali Prakashan Pune; 2004. p. 106.
14. Godkar PB, Godkar DP, Staining methods and aseptic techniques. Textbook of medical Laboratory technology. 2nd edition. Bhalani Publishing house, Mumbai; 2003. P. 517-526.
15. Laszlo Adalbert, Raviglione Mario, Rieder Hans, Espinal Marcos. Aziz Mohamed Abdel, Guidelines for surveillance of drug resistance in tuberculosis. (WHO/CDS/CSR/RMD/2003.3). World Health Organization. 2nd ed. 2003; 1-32.
16. R. Cruickshank, Duguid JP, Marmion S, Medical Microbiology. 12th ed. 1975; 9 p. 204-205.
17. Sachdava R, Gadre DV, Talwar V, Characterisation and drug susceptibility patterns of extrapulmonary mycobacterial isolates. Indian Journal of Medical Research. 2000; 1-5.
18. Canetti G, Froman S, Grosset J, Hauduroy P. Mycobacteria: Laboratory methods for testing drug sensitivity and resistance. Bulletin of World health organization. 1963; 29: 565-578
19. Sethi, Sunil, Sharma, Sachin, Sharma, S K, Meharwal, S K, Et al, Drug susceptibility of Mycobacterium tuberculosis to primary anti-tubercular drugs by nitrate reductase assay. Indian Journal of Medical Research, Nov 2004.
20. Golysheyskaia VI, et al. New microbiological techniques in diagnosis of tuberculosis. Probl Tuberk. 1996; 6: 22-5.
21. Ängeby KA, Klintz L, Hoffner SE, Rapid and inexpensive drug susceptibility testing of *Mycobacterium tuberculosis* with a nitrate reductase assay, Journal of Clinical Microbiology. 2004, 553-555.
22. Monterola JM, et al. Comparison of a non-radiometric system with BACTEC 12B and culture on egg based medium for recovery of mycobacteria from clinical specimens. Eur J Clin Microbiol Infect Dis 1998; 17: 773-777.
23. Tenover F C, Crawford J T, Huebner R E, Geiter L J, Horsburgh C R, Jr, Good R C. The resurgence of tuberculosis: is your laboratory ready? J Clin Microbiol. 1993; 31: 767–770.