ISSN: 0974-2115
www.jchps.com Journal of Chemical and Pharmaceutical Sciences

Anti-Mycobacterial and Phytochemical Investigation of Methanol Extracts of few Medicinal Plants

D. Gnanslin Sheeba*[1], Dr. K. Suseela Gomathi[2] and DrT.Citarasu[3]
1. Biotechnology, Udaya College of Arts and Science, Vellamodi, KK District, Tamil Nadu, India,
2. Santhigiri IGNOU Community College, Trivandrum, Kerala, India,
3.Centre for Marine Science and Technology, Rajackamangalam, KK District, Tamil Nadu, India.
***Corresponding author: E.Mail:gnansitech@gmail.com**

ABSTRACT

Tuberculosis is a leading cause of death in the world. Treatment of tuberculosis has become a challenge due to the rapid increase of multidrug and extensive drug resistant Mycobacterium tuberculosis.Medicinal plants might represent a possible source for new potent antibacterial to which pathogen strains are not resistant. In this study 6 plant species *Quercus infectoria, Citrus aurantium, Caesalphinia pulcherima, Mimosa pudica, Mentha spicata* and *Chysanthemum parthenium* were screened for anti-mycobacterial activity against Mycobacterium tuberculosis and antibacterial activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Vibrio harveyi* by disc diffusion and agar well diffusion methods. The extracts were compared favourably with the standard antibiotics. The extract of Quercus infectoria showed the maximum inhibition zone (30mm) against Mycobacterium tuberculosis in Lownsteen Jensen medium. Phytochemical screening for secondary metabolites revealed the presence of various biochemical compounds such as flavonoids, tannins, phenols, alkaloids, proteins, glycosides, terpenoids, carbohydrates, amino acids and saponins. Results showed that methonal crude extract of Quercus infectoria screened exhibited potential antituberculosis activity against Mycobacterium tuberculosis and further work is required to identify the active molecule of these plants to get a novel anti tubercular drug.

Keywords: Mycobacterium tuberculosis, Medicinal plants, Secondary Metabolites, Anti-bacterial activity.

INTRODUCTION

Tuberculosis (TB) is a highly infectious disease currently infecting around 1/3 of the world's population. As per the WHO estimate, there are 9 million cases of active TB with 1.3 million reported deaths every year at present. Asian and African countries shares major TB burden with 55% and 30% of the total reported cases respectively. Currently used anti-mycobacterial drugs, namely, rifampicin, isoniazid, streptomycin and ethambutol, were introduced in TB control programs almost three decades ago. Indiscriminate use of these drugs has led to the development of multi-drug resistant (MDR) and extensively drug-resistant (XDR) strains of Mycobacterium tuberculosis (*M. tuberculosis*), making the treatment difficult and increasing TB burden. The emergence of MDR and XDR strains and increase in TB burden necessitated the urgent need for new anti-mycobacterial drugs.

Medicinal plants have been used worldwide by traditional medicinal practitioner for the treatment of various diseases. Approximately 60% of world's population still relies on medicinal plants for their primary healthcare. The advantage of plant based drug discovery is that the phytochemicals provide novel drug leads with novel mechanism of action. Anti-mycobacterial phytochemicals may be helpful in identification of new molecular targets in MDR and XDR strains of mycobacterium opening new vistas for anti-tubercular drug discovery. A number of medicinal plants have been screened for anti-mycobacterial activity in past few years. Some plants have shown promising results, but very few active molecules have been purified. In the Indian subcontinent which is rich in traditional knowledge of "Ayurveda", studies have been carried out on anti-mycobacterial activity of medicinal plants.

Present study was carried out to evaluate in-vitro anti-myco bacterial and anti-bacterial activity of *Quercus infectoria, Citrus aurantium, Caesalphinia pulcherima, Mimosa pudica, Mentha spicata and Chysanthemum parthenium* plants.*Quercus infectoria*is used for treatment of dysentery, bleeding, cough, haemorrhoids. Its extracts are used stomatitis, pharyngitis, laryngitis and tonsillitis. *Citrus aurantium*used in the treatment of stomach problems,stuffy sensation in the chest. The seed and the pericarp are used in the treatment of anorexia, chest pains, colds, coughs etc. *Caesalphinia pulcherima* used in the treatment of ulcers, fever, tumors, asthma and skin diseases.*Mimosa pudica*used in the treatment of leprosy, dysentery, vaginal and urinary tract infections.Its extract can cure skin diseases. It is also used to treat whooping cough and fevers in children. *Mentha spicata*used in the treatment of fevers, headaches, digestive disorders toothache, and muscle pain. Its essential oil was found to have some antifungal activity. *Chysanthemum parthenium*used in antimicrobial, antiulcer and analgesic activities. Screening of medicinal plant for anti-mycobacterial activity involves large number of solvent extracts. Screeninglarge number of extracts on slow growing, pathogenic *M. tuberculosis strain* is a major problem. In this study, Methanol extracts of six selected medicinal plants were screened against M. *tuberculosis* and *Pseudomonas*

aeruginosa, Staphylococcus aureus, Vibrio harveyi bacterial strains. This study revealed that Methanol extract of *Quercus infectoria* exhibited anti-mycobacterial activity.

MATERIALS AND METHODS

Strains and culture media: Pure cultures of *Mycobacterium tuberculosis*strain (MTTC -300) was obtained from MTTC Laboratory Chandigarh and activated in Lownsteen Jensen and Nutrient broth.Pure bacterial strains of *Pseudomonas aeruginosa, Staphylococcus aureus ,Vibrio harveyi*wereobtained from Vivek Laboratories, Nagercoil, Tamil Nadu. And activated in nutrient broth and maintained as pure culture in nutrient agar slants.Antibacterial activity in Muller Hinton agar plates.

Collection of plant materials: The Leaves of *Mimosa pudica, Mentha spicata*, Peel of *Citrus aurantium* and flowers of *Chrysanthemum parthenium, Caesalpinia pulcherima* have been used in the present syudy and they were collected locally from kanyakumari district and the taxonomical characters were studied for plant identification Quercus infectoria seed sample used in the study was obtained from Ayurvedic medical shop at Nagercoil, Tamil Nadu.And identified based on its physical characteristics.

Processing of plant materials: The whole plant or parts of plants were used to prepare extracts for the study. The healthy and disease free plants were collected and washed with water to remove the soil and dust particles.

Methanol Extraction: The flowers and leaves were dried under shade and ground into fine powder using an electric blender. 50gm, of the flowers, seed and leaves of the dried plants were soaked in 100ml of methanol and left for seven days for extraction. After extraction it was filtered through a layer of whatmann No:1 filter paper. The extracts were sterilized by UV and then stored at 4^{o}c for further use.

Preparation of sterile disc: Whatman's No.1 filter paper was punched into 5 mm disc form and they sterilized, each sterile disc was incorporated individually with 20-50 µl of extracts using micropipette. Precautions were taken to prevent the flow of the solvent extract from the discs to the outer surface. The condensed extracts were applied in small quantities on discs and they were allowed to dry in air. After sometimes another doses of extracts were applied on discs. Then they were stored at 4^{o}C.

Antimycobacterial activity against Mycobacterium tuberculosis: To determine the activity of the plant extracts as antimycobacterials, initial screening was carried out using the disc diffusion and agar well diffusion methods. The petriplates with Lownsteen Jensen media was seeded with 100 µl of *Mycobacterium tuberculosis culture.* The prepared discs were aseptically applied to the surface of the Lownstein Jensen media plate at well- spaced intervals. In agar well diffusion method the wells were cut at regular space in surface of the medium. The standard antibiotic substance-streptomycin was used as a positive control. The plates were incubated at 37^{o}c of 1-4 weeks and the results were recorded by measuring diameter of growth inhibition zone. All experiments were carried out in triplate.

Assay of antimicrobial activity using Disc diffusion method: The 20 ml of sterilized Muller Hinton Agar was poured into sterile petriplates, after solidification, 100 µl of fresh Cultures were swabbed on the respective plates. The discs were kept over the agar plates using sterile forceps. The standard antibiotic substance-Ampicillin disc was used in order to compare the result. The plates were incubated for 24 hrs at 37^{o}c and the zone of bacterial growth inhibition around disk was measured. The assay was repeated twice, and mean of the three experiments was recorded.

Assay of Antimicrobial activity using Agar well diffusion method: A sensitive radial diffusion technique was used for the assessment of antibacterial activity of the test samples. The 20 ml of sterilized Muller Hinton Agar was poured into sterile petriplate, after solidification, 100 µl of fresh cultures were swabbed on the respective plates. The Wells were made using a stainless steel sterilized cork borer under aseptic conditions. Subsequently, 50 mg/100 of crude extracts were loaded into corresponding wells. The standard antibiotic substance-Ampicillin was used (50 g\100 of sterile water) in order to compare the result. The plates were incubated for 24 hrs at 37^{o}c and the zone of bacterial growth inhibition around disk was measured. The assay was repeated twice, and mean of the three experiments was recorded.

Phytochemical analysis: The plant extracts were screened for phytochemicals using following techniques for the detection of the flavonoids, tannins, phenols, alkaloids, proteins, glycosides, terpenoids, carbohydrates,amino acids and saponins.

Test for Flavonoids:
Ferric chloride Test: 2ml of sample was treated with few drops of ferric chloride solution and observed for the formation of blackish red colour.

Alkaline Reagent Test: 2ml of sample was treated with sodium hydroxide solution,it showed increase in the intensity of yellow colour, which become colourless on addition of few drops of dilute HCL.

Test for Tannin: 2ml of sample and alcoholic ferric chloride solution was added, observed for the formation of bluish black colour which was then disappeared on addition of little dilute H2So4 yellowish brown precipitate was observed.

Test for Phenols: To 2ml of the sample was dissolved in few drops of dilute H2O, dark green colour was observed on addition of few drops of neutral 5% Ferric chloride.

Test for Alkaloids:

Hager's Test: Test sample was treated with few drops of Hager's reagent (saturated picric acid solution) yellow precipitate was formed.

Mayer's Test: To 2ml of sample, 1% HCL was added and to this 6 drops of Mayer's reagent. Test solution was observed for the formation of yellowish or ceramish or brown or red or orange precipitation.

Test for Proteins:

Biuret Test: 2ml of the sample was treated with 10% sodium hydroxide solution and two drops of 0.1% copper sulphate solution and observed for the formation of violet or pink colour.

Test for Glycosides:

Killer-killiani Test: Test solution was treated with few drops of glacial acetic acid was added and observed for the formation of two layers. Lower reddish brown colour layer and upper acetic acid layer which turns bluish green.

Test for Terpenoids:

Salkowaski Test: To 2ml of the extract, 2ml of chloroform was added concentrated H2SO4 was carefully added to form a layer. It was observed for reddish brown colouration at the interface.

Test for carbohydrate:

Benedict's test: 2ml of test solution was mixed with few drops of Benedict's reagent (alkaline solution containing cupric citrate complex) and boiled in water bath. Observed for the formation of reddish brown precipitate.

Test for free Amino acid:

Ninhydrin Test: 2ml of the test solution was boiled with 0.2% solution of ninhydrin, observed for the formation of purple colour.

Test for Saponin:

Frothing Test: 2ml of sample was added to 5ml distilled water and shaken vigorously for stable persistent froth.

RESULTS AND DISCUSSION

Antimycobacterial activity against Mycobacterium tuberculosis: The Antimycobacterial activities of plant extracts were tested against *Mycobacterium tuberculosis*. The extracts compared favorably with the standard antibiotic Streptomycin. In the disc diffusion assay, antibiotic control showed mean zones of inhibition ranging from 20 to 22mm.The plant extracts showed good anti-mycobacterial activity in the disc diffusion assay method. The extract of *Quercus infectoria* also showed maximum inhibition zone (30mm) against *Mycobacterium tuberculosis* in Lownstein Jensen medium. The results were showed in Table 1 and Graph 1.

Table.1.Inhibition zone diameters (mm) by various plant extracts against *Mycobacterium tuberculosis*

Name of the plant	Diameter of inhibition zone (mm) ± S.D.[a]		
	Disc Diffusion	Well Diffusion	Streptomycin
Quercus infectoria	27.66±2.51	27.66±2.08	40
Chrysanthemum parthenium	5.33±0.57	3.00±1.00	20
Mentha spicata	8.00±1.73	6.00±1.00	20
Citrus aurantium	18.00±1.73	14.66±1.52	30
Caesalpinia pulcherima	13.66±1.154	9.66±1.52	30
Mimosa pudica	7.00±1.00	4.33±0.577	25

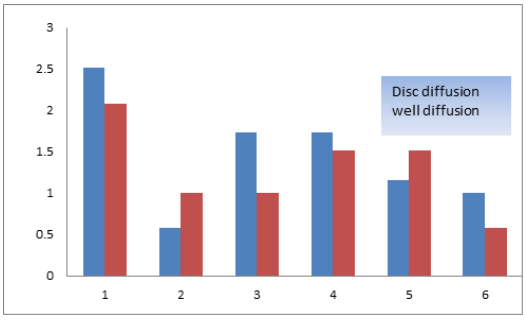

Figure.1. Inhibition zone diameters (mm) by various plant extracts against *Mycobacterium tuberculosis*
1.*Quercus infectoria*. 2.*Chrysanthemum parthenium*.3.*Mentha spicata* 4.*Citrus aurantium*.5.*Caesalpinia pulcherima*.6.*Mimosa pudica*

Assay of antimicrobial activity using Disc diffusion method: The antibacterial activity of methnol extracts of six medicinal plants were investigated using disc diffusion method (Tables2) and agar well diffusion method (Table3) against *Pseudomonas aeruginosa*, *Staphylococcus aureus and Vibriyo harveyi*.The extracts compared favorably with the standard antibiotic Ampicillin. In the agar diffusion assay,antibiotic control showed mean zones of inhibition ranging from 18 to 21mm. When compared to the well diffusion method the disc diffusion method have showed maximum zone of inhibition. The extract of Quercus infectoria showed highest inhibition activity against *Vibriyo harveyi* (30mm) followed by *Staphylococcus aureus*(29mm) and *Pseudomonas aeruginosa* (27mm). The extract of Chrysanthemum parthenium showed the lowest inhibition zone against *P.aeruginosa* (5mm).The results were showed in table 2 and graph 2.

Table.2.Inhibitionzone diameters by various plant extracts against pathogenic bacteria using Disc diffusion method

Name of the plant	Diameter of inhibition zone (mm) ± S.D(Disc diffusion)		
	Pseudomonas aeruginosa	***Staphylococcus aureus***	***Vibriyo harveyi***
Quercus infectoria	24.66±2.51	26.00 ±2.64	27.66±2.51
Chrysanthemum parthenium	4.33±1.15	8.66±1.52	8.00±1.00
Mentha spicata	11.76±1.52	13.33±1.15	10.00±2.00
Citrus aurantium	4.66 ±1.52	4.00±1.73	4.66±2.08
Caesalpinia pulcherima	12.00±1.73	12.66±1.52	4.00±1.00
Mimosa pudica	8.66±1.15	10.66±1.15	8.00±1.73

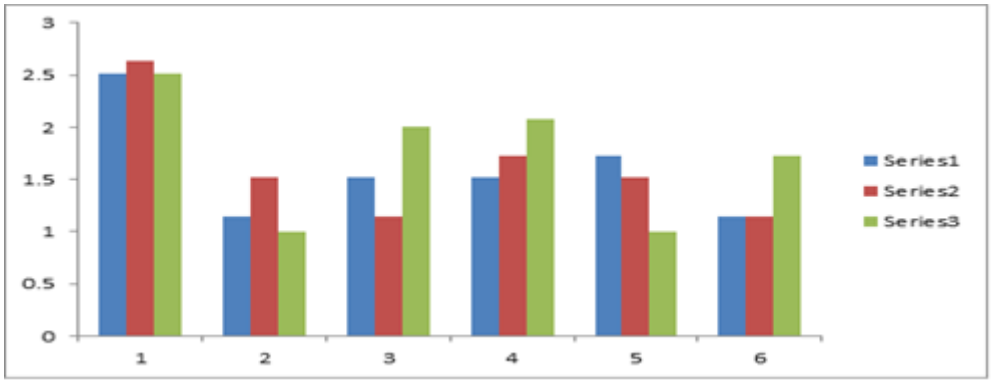

Figure.2. Inhibitionzone diameters by various plant extracts against pathogenic bacteria using Disc diffusion method
1.*Quercus infectoria*. 2.*Chrysanthemum parthenium*.3.*Mentha spicata* 4.*Citrus aurantium*.5.*Caesalpinia pulcherima*.6.*Mimosa pudica*

Phytochemical screening of plant extracts: The methanol solvent extracts of plants were tested for phytochemical presence such as flavonoids, phenolic compounds, tannins, alkaloids, Glycosides, carbohydrate, amino acids, terpenoids. Screening of phytochemical constituents of methanol extracts of plant showed positive results for the presence various biochemical compounds such as flavonoids, phenols, tannins, alkaloids, Glycosides, terpenoides. The results of phytochemical screening indicated that flavonoid content was found to be more in methanol extract followed by tannins. Results were showed in table 3.

Table.3.Phytochemical Analysis of Plant Extracts

Test	*Chrysanthemum parthenium*	*Mentha spicata*	*Citrus aurantium*	*Caesalpinia pulcherima*	*Quercus infectoria*	*Mimosa pudica*
Flavonoids 1.Alkaline	+	-	+	-	-	+
2.Fecl	+	+	+	+	+	+
Tannins	+	-	+	+	+	+
Phenol	+	-	+	+	+	+
Alkaloids 1.Mayer's	+	+	+	-	-	-
2.Hayer's	+	-	-	-	-	-
Glycosides	+	-	-	+	+	-
Terpenoids	-	-	+	-	+	-
Carbohydrate	-	-	-	+	+	-
Aminoacid	-	+	-	-	-	-

(+) = indicates presence; (-) = indicates absence

DISCUSSION

WHO declared TB as global health emergency because of the increase in HIV co-infection and the appearance of MDR and XDR strains. No new anti-mycobacterial drug has been introduced in past 30 years, hence, there is an urgent need to develop novel, safe, effective and affordable drug for treating resistant forms of TB. There has been a renewed interest in phytochemicals as source of novel therapeutics in the past decade, hence, plants have been investigated for various pharmacological effects including anti-mycobacterial activity. A number of plants have been reported to possess anti-mycobacterial activity. Phytochemicals may become the base for new drug development by providing a pharmacophore which could be used for the development of new drug with novel mechanism of action. Recently anti-mycobacterial activity of *Adhatoda vasica, Acalypha indica, Aloe vera, Allium cepa* and *Allium sativum* have been reported against MDR strains of *M. tuberculosis*. In current study, in vitro anti-mycobacterial activity of *Quercus infectoria, Chrysanthemum parthenium, Mentha spicata, Citrus aurantium, Caesalpinia pulcherima, Mimosa pudica*have been evaluated. There are reports of screening of large number of plant extracts against bacterial strains. The active extracts have also exhibited activity against *M. tuberculosis* out of 6 plants selected in this study, *Quercus infectoria* and *Citrus aurantium*were identified as potentially active in disc diffusion assay against *M. tuberculosis Quercus infectoria* produced 30mm inhibition zone and *Citrus aurantium* produced 20mm inhibition zone. The fresh extract of *Quercus infectoria* showed highest inhibition activity against *Vibriyo harveyi* (30mm) followed by *Staphylococcus aureus* (29mm) and *Pseudomonas aeruginosa* (27mm).The fresh extract of *Chrysanthemum parthenium* showed the lowest inhibition zone against *P.aeruginosa* (5mm).

The phytochemical screening carried out on these medicinal plants showed that it contained major classes of natural products. The activity shown by the crude extracts may be a synergistic action of complex phytochemicals. Investigation of wild medicinal plants is an efficient way of searching for new candidate chemotherapeutic drugs. Plant extracts are attractive and effective sources of new drugs. The use of herbs for the treatment of TB is increasing due to increased incidence of resistance to the available antibiotics. Natural products play a significant role in drug discovery and development of highly active anti-mycobacterial metabolites. Our investigation indicates the presence of flavonoids, phenoliccompounds, tannins, alkaloids, Glycosides, carbohydrate, amino acids, terpenoids and these phytochemicals are responsible for the anti-tubercular activity. Similar observations have been made in plants employed for traditional medicines, which were known to contain the said mentioned bioactive components. Literature survey indicated that these compounds have bioactivity. Anti-mycobacterial herbal medicine where some metabolites such as terpenes, steroids and alkaloids are found to be abundant in the plant extracts and possess potential structural skeletons that could provide useful scaffolds or templates for the development of new anti-mycobacterial drugs has been revived The bioactivity demonstrated by the extracts is attributable to the presence of secondary metabolites. There are reports about the potent activity of tannins isolated from *Combretum molle*against *M. tuberculosis*. Alkaloids isolated from medicinal plant *Alstonia scholaris* showed the anti-mycobacterial activity. The antibacterial, antifungal and anti-TB activity exhibited by the extracts of the plant *Cissampelos owariensis* (P. Beauv.) The preliminary results presented in this study showed the potential of extracts from these 2 medicinal plants to be used against *M.tuberculosis*. However, further *in-vitro* studies are needed to understand the mechanism of action of crude drugs.

CONCLUSION

The antimycobacterial activity appears to be more pronounced in certain fraction of plants as it has also possess compounds with antimicrobial properties.the most active extracts can be subjected to therapeutic antimicrobials

REFERENCES

Adaikan PG, Gauthaman K, Prasad RNV, History of herbalmedicines with insight on the pharmacological properties of Tribulus terrestris, The Aging Male, 4(3), 2001, 163-169.

Agarwal SP, Inter-sectoral cooperation for success of the RNTCP, Indian J Tuberc, 51(2), 2004, 59-62.

Akande R, Okwute SK, Iliya I, Efi om O. Chemical constituentsand anti-tuberculosis activity of the root extracts of *Cissampelosowariensis* (P. Beauv.) Menispermaceae, Afr J Pure Appl Chem, 7, 2013, 21-30.

Asres K, Bucar F, Edelsbrunner S, Kartnig T, Höger G, Thiel W, Investigations on antimycobacterial activity of some Ethiopian medicinalplants. Phytother Res, 15, 2001, 323-6.

Baby Joseph, Jency Joseph, Jeevitha Mohan, Pharmacology and Traditional uses of Mimosa pudica, *International Journal of Pharmaceutical Sciences and Drug Research,* 5 (2), 2013, 41- 44

Cantrell CL, Franzblau SG, Fischer N. Antimycobacterial plantterpenoids, Planta Medica, 67(8), 2001, 685-694

Celio TH, Fernando R, Clarice QF, Miriam S, Wagner V, Sergio RA, *et al*.Triterpenes and antitubercular activity of *Byrsonima crassa.* Quím Nova, 7, 2008, 1719-21.

Copp BR, Antimycobacterial natural products. Nat Pro Rep, 20(6), 2003, 535-557.

Copp BR. Antimycobacterial natural products, Nat Prod Rep, 20, 2003, 535-57.

Dolin PJ, Raviglione MC, Kochi A, Global tuberculosis incidence andmortality during 1990-2000. Bull World Health Organ, 72, 1994, 213-20.

Gupta PP, Srimal RC, Tandon JS. Antiallergic activity of sometraditional Indian medicinal plants. Int J Pharmacogn, 31(1), 1993, 15-18.

Gupta VK, Shukla C, Bisht GR, Saikia D, Kumar S, Thakur RL.Detection of anti-tuberculosis activity in some folklore plants byradiometric BACTEC assay, Lett Appl Microbiol, 52(1), 2011, 33-40.

Hannan A, Ullah MI, Usman M, Hussain S, Absar M, Javed K.Anti-mycobacterial activity of garlic (Allium sativum) againstmulti-drug resistant and non-multi-drug resistant *Mycobacterium tuberculosis*, Pak J Pharm Sci, 24(1), 2011, 81-85.

Iseman MD, Evolution of drug-resistant tuberculosis, A tale of twospecies. Proc Natl Acad Sci USA, 1994, 91(7), 1994, 2428-2429

Luo X, Pires D, Aínsa JA, Gracia B, Mulhovo S, Duarte A, Antimycobacterial evaluation and preliminary phytochemicalinvestigation of selected medicinal plants traditionally used in Mozambique, J Ethnopharmacol, 137(1), 2011, 114-120.

Macabeo AP, Krohn K, Gehle D, Read RW, Brophy JJ, Cordell GA, Indole alkaloids from the leaves of Philippine *Alstonia scholaris*, Phytochemistry, 66, 2005, 1158-62.

Mukesh Chandra Sharma and Smita Sharma; Phytochemical screening and in-vitro Antimicrobial activity of combined Citrus Paradisi and Ficus Carica Linn Aqueous Extract; *IDOSI Publication*, 2010, 162-165.

Newman DJ, Cragg GM, Natural products as sources of new drugs over the last 25 years, J Nat Prod, 70, 2007, 461-77.

Newton SM, Lau C, Gurcha SS, Besra GS, Wright CW.The evaluation of forty-three plant species for in vitroantimycobacterial activities: isolation of active constituentsfrom Psoralea corylifolia and Sanguinaria Canadensis, JEthnopharmacol, 79(1), 2002, 57-67.

Okunade AL, Elvin-Lewis MPF, Lewis WH, Natural antimyco bacterial metabolites: current status. Phytochemstry, 65(8), 2004, 1017-1032.

Otshudi AL, Foriers A, Vercruysse A, Van Zeebroeck A, Lauwers S. *In vitro* antimicrobial activity of six medicinal plants traditionally used forthe treatment of dysentery and diarrhoea in Democratic Republic ofCongo (DRC). Phytomedicine,7, 2000, 167-72.

Rahele Radmanesh, Bita Poukaveh,The antimycobacterium activity of Mentha piperita and Mentha spicata ethanolic extract against mycobacterium Bovis in comparison with isoniazid , Iran J clin Infect, 6(2), 2011.

Shah NS, Wright A, Bai GH, Barrera L, Boulahbal F, Martín Casabona N, et al. Worldwide emergence of extensively drug resistant tuberculosis, Emerg Infect Dis, 13(3), 2007, 380-387.

Singh MM. XDR-TB, Danger ahead, Indian J Tuberc, 54(2), 2007, 1-2.

Soejarto DD, Gyllenhaal C, Kadushin MR, Southavong B, SydaraK, Bouamanivong S, et al. An ethnobotanical survey of medicinalplants of Laos toward the discovery of bioactive compounds aspotential candidates for pharmaceutical development. Pharm Biol, 50(1), 2012, 42-60

World Health Organization. Anti-Tuberculosis Drug Resistance in the World. Fourth Global Report.WHO/HTM/TB.2008;394.[Online].Availablefrom:http://www.who.int/tb/publications/2008/en/index.html. [Accessed on December 27, 2012].

World Health Organization. Global Tuberculosis Control: A Short Update to the 2009 Report,March2009.[Online].Availablefrom:http://www.who.int/tb/features_archive/globalreport09_update_8dec09 /en/index.html. [Accessed on December 27, 2012].

World Health Organization. Tuberculosis fact sheet no. 104. March 2010. [Online]. Available from: http://www.who.int/mediacentre/factsheets/fs104/en/index.html. [Accessed on December 27, 2012].