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Assessment of the Activity of Selected Indian Medicinal Plants against *Mycobacterium tuberculosis*: A Preliminary Screening Using the Microplate Alamar Blue Assay

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Authors' contributions

This work was carried out in collaboration between all authors. TB was responsible for the study. DD was involved in co-ordination of the study, carrying out the assays, preparation and editing of the manuscript. MT was involved in carrying out the assays, undertaking the literature survey, preparation and editing of the manuscript. PD was involved in preparation of extracts, undertaking the literature survey, preparation and editing of the manuscript. VN was involved in carrying out the assays. PT collected and authenticated the plant material, and obtained the herbarium numbers. JCT was responsible for carrying out the external quality control of the MABA, editing the manuscript. SH was In charge of WHO Supranational Reference Laboratory for TB at Swedish Institute for Communicable Disease Control, editing the manuscript. All authors read and approved the final version of the manuscript.

Research Article

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ABSTRACT

Aim: Identification of anti-*Mycobacterium tuberculosis* agents of plant origin, against sensitive and multidrug resistant (MDR) strains.

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Study Design: Assessing anti-*M. tuberculosis* activity of five Indian medicinal plants, which have been reported in traditional literature for various uses including respiratory ailments.
Place and Duration of Study: Mumbai, India; May 2009 – December 2011.
Methodology of Study: The reference strain (H37Rv), three susceptible and three MDR clinical isolates of *M. tuberculosis* were used. Acetone, ethanol and aqueous extracts (prepared sequentially) of *Acorus calamus* L. (rhizome), *Andrographis paniculata* Nees. (leaf), *Ocimum sanctum* L. (leaf), *Piper nigrum* L. (seed) and *Pueraria tuberosa* DC. (tuber) were tested at 1, 10 and 100 µg/ml using the Microplate Alamar Blue Assay. The active extracts were assessed for cytotoxicity on the human lung epithelial cell line (A549) using the neutral red assay and a phytochemical analysis was made using High Performance Thin Layer Chromatography (HPTLC).
Results: Among the plants tested, the acetone extract of *P. nigrum* appears promising. It was effective against H37Rv, all susceptible isolates and one MDR isolate at 100 µg/ml. The ethanol extract caused some inhibition of growth, though less than the cut-off of 99%. A combination of acetone and ethanol extracts at 50 µg/ml each was effective against all isolates tested. The known active phytoconstituent of *P. nigrum*, piperine (also an efflux pump inhibitor), was effective against H37Rv in the presence of suboptimal concentration of Rifampicin, but not against the clinical isolates tested. Presence of piperine in the acetone and ethanol extracts was confirmed by HPTLC. Extracts of *P. nigrum* and piperine were not cytotoxic to the A549 cell line.
Conclusion: Amongst the five plants tested, *P. nigrum* was active. The acetone extract may have active components in addition to piperine. It is possible that the class and expression of efflux pumps in H37Rv is different from that in the clinical isolates, and hence piperine did not inhibit these isolates. Thus, it is necessary to screen clinical isolates in addition to reference strains. The observation of the increased efficacy of the combination of acetone and ethanol extracts is interesting.

Keywords: Traditional medicine; anti-Mycobacterium tuberculosis; microplate alamar blue assay; cytotoxicity; HPTLC; Piper nigrum.

1. INTRODUCTION

Tuberculosis (TB) caused by *M. tuberculosis* is a highly infectious disease, and the morbidity and mortality due to TB continue to be a cause of concern. Due to the global emergence of multidrug resistant (MDR) and extensively drug resistant (XDR) strains of *M. tuberculosis* (Singh, 2007) and more recently the reports of totally drug resistant TB (Udwadia et al., 2012; Velayati et al., 2009), there is an urgent need to develop new drugs and strategies to fight TB (Souza, 2006). The last few decades have witnessed a substantial increase in the investigation of medicinal plants for their biological efficacy in treatment of various disorders. In the field of anti-TB agents, a number of studies on potential medicinal plants have been reported from various parts of the world (Gautam et al., 2007; Green et al., 2010; Lakshmanan et al., 2011; Webster et al., 2010).

Over the years, a number of improved and high throughput techniques towards screening of anti-mycobacterial agents have been developed (Nayyar and Jain, 2005). Several methods exist for testing the anti-*M. tuberculosis* potential of plant extracts, such as fluorescence based testing on the Bactec MGIT960 system, use of redox indicator dyes such as Alamar Blue or Resazurin and MTT, using colony forming units (CFU) on solid agar plates. The CFU assay is too time consuming and tedious for use as a screening protocol, while assays using

indicator dyes are rapid and efficient. Techniques such as the agar diffusion and broth dilution method have been used, but they too have limitations (Gautam et al., 2007). The Microplate Alamar Blue Assay (MABA) is a colorimetric oxidation-reduction based assay. It is a non-radiometric, rapid, high-throughput and comparatively low cost assay producing results with a high degree of confidence (Collins and Franzblau, 1997; Kumar et al., 2005). Moreover, this technique has been used by a number of researchers for testing anti-mycobacterial activity of several plants (Camacho-Corona et al., 2008; Webster et al., 2010). Hence, in the present study five Indian medicinal plants viz., *Acorus calamus* L. (rhizomes), *Andrographis paniculata* Nees. (leaves), *Ocimum sanctum* L. (leaves), *Piper nigrum* L. (seeds) (along with piperine) and *Pueraria tuberosa* DC. (tubers) were studied for their anti-*M. tuberculosis* potential using the MABA.

The primary reasons for choosing these medicinal plants, besides their known antibacterial properties, were their use in respiratory ailments as cited in traditional literature. Use of these plants for respiratory disorders in reported ethnobotanical surveys were also documented (Table 1). All plants selected are native plants for which cultivation practices have been developed. Sustainable harvesting is possible for all plants except *A. calamus* and *P. tuberosa*. *In vitro* screening of organic (acetone and ethanol) and aqueous extracts of the selected plants against *M. tuberculosis* reference strain H37Rv and drug susceptible and MDR clinical isolates using the MABA was undertaken. Thus, in all, 15 extracts were studied. This preliminary study was intentionally restricted to testing crude extracts since it has often been noticed that crude extracts are more efficacious than the isolated compounds (Ginsburg and Deharo, 2011; Houghton, 2000; Sood et al., 2012). Anti-*M. tuberculosis* activity in the absence of cytotoxicity would confirm their anti-*M. tuberculosis* potential. Hence, cytotoxicity of these plant extracts was also carried out using A549, a human lung epithelial cell line, since *M. tuberculosis* is an intracellular pathogen.

2. MATERIALS AND METHODS

2.1 Plants Used and Preparation of Extracts

The plant material used for the present study was collected and authenticated by Dr. P. Tetali, Naoroji Godrej Centre for Plant Research (NGCPR, Shirwal, Maharashtra). Voucher specimens of the plants were deposited at the Botanical Survey of India (BSI), Western Center, Pune, India (*A. calamus*, *A. paniculata*, *O. sanctum*, *P. nigrum*); or at NGCPR (*P. tuberosa*). The area of collection and the herbarium numbers of the plants have been presented in Table 2.

Extracts were prepared in a sequential manner using acetone, ethanol and distilled water as solvents from 25g of shade dried and coarsely powdered plant material using the Soxhlet apparatus. For 25g of plant material 300ml of respective solvent was used and refluxed for a period of 24-30 hours. Continuous refluxing ensured that efficient extraction of the phyto-constituents in their respective solvents was achieved. The aqueous extract was prepared by boiling the plant material post ethanol extraction and evaporation of the solvent until the volume of water was reduced to 25% (Thakkur, 1976).

Table 1. Basis of selection of plants for the study

Botanical name (Family)	Common Name	Selection criteria		
		Traditional use[a]	Ethnobotanical survey[a]	Antibacterial[b]
Acorus calamus Linn. (Araceae)	Sweet Flag (Vekhand)	Rhizome: Cough and bronchitis (Sharma et al., 2000-2002)	Dried rhizome powder: Tuberculosis, expectorant, chest congestion (Motley, 1994)	Aqueous root extract: anti-TB activity (Chopra et al., 1957)
Andrographis paniculata Nees. (Acanthaceae)	Kalmegh	Whole plant: Bronchitis (Sharma et al., 2000-2002)	No reference found for use in India	Ethanol extract of aerial part: Antibacterial activity[c] (Mishra et al., 2009)
Ocimum sanctum Linn. (Lamiaceae)	Holy Basil (Tulsi)	Part used unspecified: Bronchitis and asthma (Sharma et al., 2000-2002)	Leaves crushed with onion bulbs: Cold and cough (Muthu et al., 2006)	Leaf extract: anti-TB activity (Farivar et al., 2006)
Piper nigrum Linn. (Piperaceae)	Black pepper (Kali Mirch)	Fruit: Cough (Sharma et al., 2000-2002)	Seeds: Throat infection (Ignacimuthu et al., 2006)	Aqueous and various organic extracts of the fruit: Antibacterial activity[c] (Khan and Siddiqui, 2007).
Pueraria tuberosa DC. (Fabaceae)	Bidaarikand	Tuberous root: Tuberculosis (Billore et al., 2004);	No reference found for use in India	Ethyl acetate extract of root tubers: Antibacterial activity[c] (Venkata Ratnam and Venkata Raju, 2009)

[a] *Selected references only for respiratory ailments*
[b] *Selected references only*
[c] *Activity reported against bacteria other than M. tuberculosis*

The aqueous extract was then lyophilized (Thermo Fisher Scientific, USA) and the acetone and ethanol extracts were allowed to air dry. The yield of the extracts thus obtained is presented in Table 2. For the assays, the extracts were reconstituted at 20 mg/ml concentration in dimethyl sulfoxide (DMSO), filtered through 0.2 µm, 25 mm DMSO resistant Acrodisc syringe filters (Pall Corporation, USA) and stored at -20ºC for up to 2 weeks.

Table 2. Details of the plants used for the study

Botanical name (Herbarium no.)	Area of collection	Part used	Percentage Yield (w/w)[a]		
			Acetone	Ethanol	Aqueous
A. calamus Linn. (BSI-131716)	Shindewadi, Satara district, Maharashtra	Rhizomes	10.00	7.04	10.80
A. paniculata Nees. (BSI-131744)	Shindewadi, Satara district, Maharashtra	Leaves	6.28	6.00	7.21
O. sanctum Linn. (BSI-131712)	Shindewadi, Satara district, Maharashtra	Leaves	6.00	3.62	24.00
P. nigrum Linn. (BSI-131714)	Kottayam, Kerala	Seeds	3.16	6.64	22.64
P. tuberosa DC. (NGCPR-670)	Mulshi, Pune district, Maharashtra	Tubers	6.72	5.92	22.83

[a] *Percentage yield after sequential extraction using Acetone, Ethanol and Water*

2.2 *M. tuberculosis* Strains and Culture Conditions

The *M. tuberculosis* reference strain H37Rv; three susceptible (S1, S2 and S3) and three MDR (MDR1, MDR2 and MDR3) clinical isolates were used to test the anti-*M. tuberculosis* activity of the extracts using the Microplate Alamar Blue Assay (MABA). The strains were selected from among those collected for a community based study on MDRTB transmission in Mumbai, during 2004-2007. The patients sampled were new pulmonary TB patients who accessed the Revised National Tuberculosis Programme for diagnosis and treatment of TB. The drug susceptibility profile was determined during the earlier study (D'souza et al., 2009). The MDR isolates were included to investigate possible cross resistance with anti-TB drugs.

All strains were inoculated into Middlebrook 7H9 medium (7H9) [Becton and Dickinson, USA] with added ADC (Albumin Dextrose Catalase) supplement (BD, USA) and incubated for 6 days at 37ºC in a shaker incubator. Twenty-four hours prior to the experiment the culture was centrifuged and 10 ml of fresh medium was added. At the Foundation for Medical Research (FMR) the number of Acid Fast Bacilli (AFB) was estimated microscopically and the density of the culture was adjusted to 0.5×10^6/ml. At the Swedish Institute for Communicable Disease Control (SMI), the inocula were prepared with the standard method used for drug susceptibility testing in the Bactec MGIT960 system (Becton and Dickinson, USA) according to the recommended procedures from the manufacturer.

2.3 Microplate Alamar Blue Assay

The MABA was carried out as reported before (Collins and Franzblau, 1997; Webster et al., 2010). Briefly, 100 µl of 0.5 x 10^6/ml of the *M. tuberculosis* (MTB) were cultured in 7H9 medium in the presence of the plant extracts (1, 10 and 100 µg/ml) in a Nunc™ flat bottom 96 well plate (Nunclon, Denmark). Standard piperine (Sigma-Aldrich, USA) was tested at 0.25, 2.5 and 25 µg/ml. The controls maintained for all the tested strains included: medium, DMSO (at a volume that would reflect that used for the highest concentration of plant extract tested i.e. 5 µl DMSO per ml of medium), 1:100 MTB and 2 µg/ml Rifampicin (RIF) (Sigma-Aldrich, USA). In case of the susceptible strains an additional suboptimal RIF (a concentration that allowed a percent reduction of Alamar Blue similar to that of the MTB) control was also maintained. The plant extracts were tested in both absence and presence of RIF (suboptimal concentration for susceptible isolates and 2 µg/ml for MDR isolates). Additionally, wells with plant extracts and medium but no bacteria were maintained to check the interaction of the extract with Alamar Blue. The plates were incubated at 37ºC for 7 days, after which 10 µl of Alamar Blue dye (Invitrogen, USA) [5% (v/v)] diluted 1:1 in 7H9 medium was added and incubated for 30 hours. The Optical Density (O.D.) of the wells was measured at 600 nm and 570 nm on an ELISA reader (Thermo Fisher Scientific, USA), and the percent reduction of Alamar Blue dye was calculated as per the manufacturer's instructions. Use of percent reduction to identify active plant extracts allowed identification of those extracts with marginal activity (not resulting in 99% kill). This permitted identification of extracts which could be tested in combination in cases where the extract demonstrated some activity when used individually at 100 µg/ml. Extracts thus identified were further tested at 25 µg/ml of each extract or 50 µg/ml of each extract. Overall, for *P. nigrum* (acetone and ethanol extracts) the concentrations tested were 1, 10, 25, 50 and 100 µg/ml. Triplicate wells were maintained for each variable in every assay and all the assays were performed thrice. In cases where the growth was insufficient in the controls, the assay was repeated.

2.3.1 Interpretation of the MABA results

Interpretations were based on the percent reduction of the dye which is directly proportional to the bacterial growth. The extracts were considered active if the percent reduction value of Alamar Blue dye was less than that observed for the 1:100 MTB control (Clinical and Laboratory Standards Institute, 2011).

2.4 Internal Quality Control for the MABA

The results of the MABA were compared with the Bactec MGIT960 system (BD, USA). The extracts of *P. nigrum* and piperine could not be tested as they auto fluoresced. Of the remaining four plants, extracts of two plants were randomly chosen to be tested against H37Rv and all the clinical isolates.

2.5 External Quality Control for the MABA

Extracts which showed activity at the FMR along with two other randomly chosen extracts were sent to the WHO Supranational Reference Laboratory for TB at SMI for testing against H37Rv using an identical protocol of MABA.

2.6 Cytotoxicity Testing of Efficacious Plant Extracts

The cytotoxicity of the active plant extracts was carried out using A549 cell line (National Center for Cell Sciences, Pune, India) by the neutral red uptake assay (Parish and Mullbacher, 1983). Briefly, appropriate dilutions of the plant extract stock (1, 10 and 100 µg/ml) prepared in RPMI medium (GIBCO BRL, UK) supplemented with 10% FCS (Biowest, South America) were incubated overnight onto a 24 hr culture of A549 cells and then subjected to the Neutral red assay. Wells with DMSO (at a volume that would reflect that used for the highest concentration of plant extract tested i.e. 5 µl DMSO per ml of medium) and without the plant extract were used as controls. The O.D. was measured at 540 nm (reference 630 nm) on an ELISA reader (Labsystems, Finland). The percent viability was calculated with respect to the DMSO control using the following formula: percent viability = [test/control] x 100. Triplicate wells were maintained for each variable in every assay and all the assays were performed thrice.

2.7 Phytochemical Profile of Efficacious Plant Extracts

High performance thin layer chromatography (HPTLC) fingerprinting of plant extracts showing activity in the MABA was carried out on pre-coated Silica gel G60 F254 TLC plates (Merck, Germany). The extracts were spotted along with appropriate reference standards using Linomat V Automatic Sample Spotter (CAMAG, Switzerland), run in a 'twin trough TLC chamber', dried and visualized in 'CAMAG TLC visualizer'.

3. RESULTS

3.1 Anti-*M. tuberculosis* Activity of the Plant Extracts Using MABA

Crude plant extracts did not show enhanced reduction of Alamar Blue as compared to the medium control. The range for the medium control was 7.5-10.9 percent reduction and for the plant extracts 7.4-12.2 percent reduction. In four of the five plants tested viz. *A. calamus*, *A. paniculata*, *O. sanctum* and *P. tuberosa*, no anti-*M. tuberculosis* activity was noted with any extract against the strains included in the present study (data not shown). We observed that the acetone extract of *P. nigrum* at 100 µg/ml showed activity against H37Rv, all three susceptible isolates and one of the three MDR isolates (MDR3) (Table 3). However, no activity was seen at 1 and 10 µg/ml. Though the ethanol extract (100 µg/ml) of *P. nigrum* showed a drop in reduction of Alamar Blue compared to the MTB control, it was not less than that of the 1:100 MTB control indicating that this extract had some degree of anti-*M. tuberculosis* activity. The aqueous extract did not show any anti-*M. tuberculosis* action. Thus, of the 15 extracts tested, the acetone extract of *P. nigrum* was found to have anti-*M. tuberculosis* activity.

Table 3. Percent reduction of Alamar Blue dye obtained for *P. nigrum* extracts at 100 µg/ml. Values are mean ± standard deviation of 3 independent experiments. Controls (DMSO, Rifampicin and suboptimal Rifampicin, where applicable) were within permissible range (data not shown)

Strain	Test parameter	1:100 MTB control	MTB control	MTB + *P. nigrum* extracts[a]		
				Aqueous	Acetone	Ethanol
H37Rv	Extract	26.22 ± 1.47	70.68 ± 3.44	70.09 ± 10.09	25.73 ± 4.74	65.47 ± 13.85
	Extract + sub optimal RIF[b]			72.87 ± 12.30	24.33 ± 4.35	66.04 ± 4.53
S1	Extract	20.91 ± 3.14	54.60 ± 1.10	62.35 ± 3.85	18.14 ± 1.95	49.81 ± 6.05
	Extract + sub optimal RIF[b]			48.82 ± 2.97	20.10 ± 5.76	40.04 ± 2.81
S2	Extract	16.83 ± 4.57	57.61 ± 5.55	58.48 ± 13.81	16.37 ± 7.17	37.19 ± 15.14
	Extract + sub optimal RIF[b]			38.45 ± 5.83	15.91 ± 5.84	34.13 ± 11.27
S3	Extract	22.89 ± 3.68	65.15 ± 1.70	50.08 ± 12.13	19.63 ± 1.43	49.21 ± 8.02
	Extract + sub optimal RIF[b]			43.56 ± 16.55	17.02 ± 1.43	45.51 ± 9.49
MDR1	Extract	20.78 ± 1.98	68.90 ± 10.72	58.91 ± 11.81	32.13 ± 4.96	46.31 ± 13.26
	Extract + RIF			42.17 ± 11.29	33.96 ± 7.46	30.03 ± 7.42
MDR2	Extract	18.93 ± 5.97	60.92 ± 7.40	49.31 ± 8.77	38.87 ± 9.52	40.66 ± 2.18
	Extract + RIF			48.23 ± 1.57	36.87 ± 7.78	46.10 ± 3.85
MDR3	Extract	16.20 ± 1.57	53.36 ± 2.93	43.87 ± 5.88	18.75 ± 4.78	33.73 ± 12.72
	Extract + RIF			38.17 ± 6.49	15.56 ± 2.94	31.67 ± 13.18

[a] *Cells shaded in grey indicate those parameters for which the percent reduction in test containing wells was less than that for the 1:100 MTB control.*
[b] *Suboptimal concentration of RIF used for H37Rv and sensitive strains 1, 2 and 3 was 0.0125, 0.08, 0.02 and 0.125 µg/ml respectively*

3.2 Anti-*M. tuberculosis* Activity of a Mixture of Acetone and Ethanol Extracts of *P. nigrum*

As the ethanol extract of *P. nigrum* showed marginal activity at 100 µg/ml, its combination with the acetone extract was tested for anti-*M. tuberculosis* activity. The mixture of these extracts was tested at 25 µg/ml each and 50 µg/ml each. It was seen that these mixtures showed inhibitory activity against H37Rv, all the susceptible isolates and two of the MDR isolates (MDR1 and MDR3), despite the lack of activity when used individually at 50 µg/ml (Table 4). In the case of MDR2, although the percent reduction of Alamar Blue was marginally higher than that of the 1:100 MTB, it could be considered as effective against the isolate. Studying the mechanism by which this mixture was active across the sensitive and MDR strains would be useful in developing anti-*M. tuberculosis* agents that have the potential to treat MDRTB.

Table 4. Percent reduction of Alamar Blue dye obtained for a mixture of *P. nigrum* extracts. Values are presented as mean ± standard deviation of 3 independent experiments. The remaining controls (DMSO, Rifampicin) were within permissible range (data not shown)

Strain	1:100 MTB control	MTB control	MTB + *P. nigrum* extracts[a]		
			Acetone 50 µg/ml	Ethanol 50 µg/ml	Acetone 50 µg/ml + Ethanol 50 µg/ml
H37Rv	19.89 ± 0.04	67.23 ± 3.77	21.53 ± 4.83	51.45 ± 1.74	17.39 ± 4.15
S1	18.95 ± 7.23	53.11 ± 3.17	22.33 ± 2.18	67.75 ± 18.94	15.82 ± 9.44
S2	15.17 ± 7.71	61.91 ± 6.72	36.62 ± 6.18	59.34 ± 5.34	15.87 ± 6.88
S3	20.34 ± 1.36	51.81 ± 6.79	28.95 ± 1.12	36.26 ± 5.24	18.96 ± 0.64
MDR1	22.98 ± 3.21	55.51 ± 4.42	35.60 ± 3.91	36.36 ± 7.21	14.66 ± 7.16
MDR2	16.72 ± 0.40	56.13 ± 1.84	32.51 ± 4.38	52.21 ± 2.36	20.55 ± 1.54
MDR3	22.10 ± 1.15	54.88 ± 9.43	25.99 ± 4.24	36.91 ± 1.96	22.18 ± 2.42

[a] *Cells shaded in grey indicate those parameters for which the percent reduction in test containing wells was less than that for the 1:100 MTB control.*

3.3 Anti-*M. tuberculosis* Activity of Piperine

As piperine is one of the active constituents of *P. nigrum* (Madhavi et al., 2009), this compound was also tested for its activity. Due to the limited solubility of piperine in DMSO, the highest concentration tested was 25 µg/ml. It was observed that piperine was active against H37Rv at 25 µg/ml but only in the presence of a suboptimal concentration of RIF. It was ineffective against all other strains tested (Table 5)

3.4 Internal Quality Control of the MABA

The acetone extracts of *A. paniculata* and *A. calamus* were tested against H37Rv and the clinical isolates in the MGIT960 system. They did not show any activity and were thus concordant with the MABA results.

Table 5. Percent reduction of Alamar Blue dye obtained for piperine at 25 µg/ml. Values are presented as mean ± standard deviation of 3 independent experiments. The remaining controls (DMSO, Rifampicin and suboptimal Rifampicin, where applicable) were within permissible range (data not shown)

Strain	1:100 MTB control	MTB control	MTB + piperine [a]	MTB + piperine + RIF (or sub optimal RIF[b]) [a]
H37Rv	21.71 ± 2.84	66.05 ± 5.03	59.93 ± 8.45	22.12 ± 2.14
S1	21.73 ± 2.33	54.68 ± 8.29	61.46 ± 6.53	45.52 ± 12.07
S2	17.92 ± 6.05	55.48 ± 5.86	45.48 ± 2.86	32.05 ± 7.28
S3	25.08 ± 1.43	66.55 ± 2.19	50.78 ± 14.37	38.97 ± 5.67
MDR1	19.95 ± 3.10	55.41 ± 13.72	31.37 ± 5.42	28.58 ± 5.84
MDR2	18.93 ± 5.97	60.92 ± 7.40	42.75 ± 7.43	44.16 ± 7.85
MDR3	16.20 ± 1.57	53.36 ± 2.93	29.72 ± 9.75	27.75 ± 5.76

[a] *Cells shaded in grey indicate those parameters for which the percent reduction in test containing wells was less than that for the 1:100 MTB control.*

[b] *Suboptimal concentration of RIF used for H37Rv and sensitive strains 1, 2 and 3 was 0.0125, 0.08, 0.02 and 0.125 µg/ml respectively.*

3.5 External Quality Control of the MABA

The results with acetone extract of *A. calamus*, *O. sanctum* and *P. nigrum* and the ethanol extract of *P. nigrum* tested against H37Rv at SMI were in concordance with the results obtained by FMR. The results for *P. nigrum* are depicted in Fig. 1.

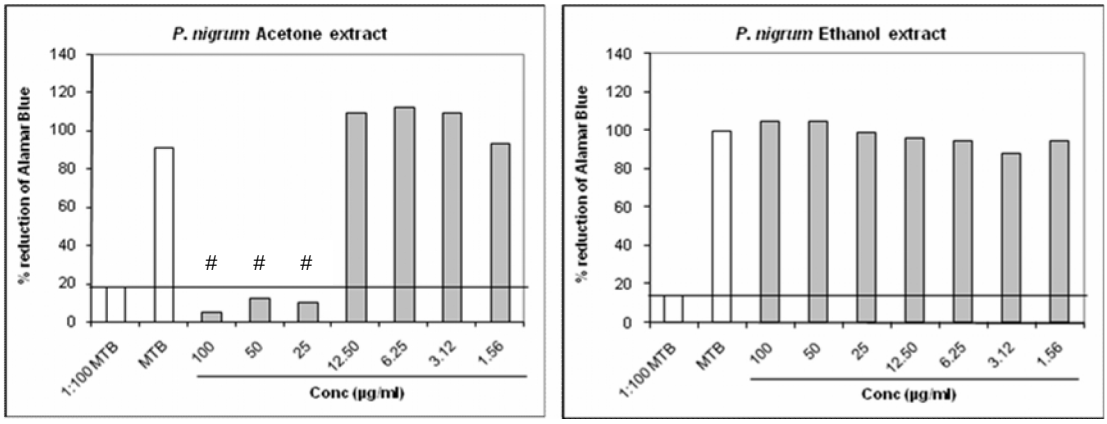

Fig. 1. External quality control by the MABA performed at the Swedish Institute for Communicable Disease Control, Karolinska. The extracts were tested against H37Rv (n=1)

MTB = M. tuberculosis; # Percent reduction of Alamar Blue of the tests is less than that of the 1:100 MTB control.

☐ *M. tuberculosis growth controls;* ▨ *M. tuberculosis + Plant extract*

3.6 Cytotoxicity of *P. nigrum* Extracts

The percent viability of A549 cells in presence of the highest concentrations of acetone (100 µg/ml), ethanol (100 µg/ml) extracts and mixture of acetone and ethanol extracts of *P. nigrum* (50 µg/ml each) was 90.49 ± 6.75, 97.58 ± 18.57 and 116.67 ± 13.82 respectively. The percent viability of A549 cells in presence of piperine (25 µg/ml) was 111.51 ± 23.85.

3.7 Phytochemical Profile of Acetone and Ethanol Extract of *P. nigrum*

The HPTLC profile and the chromatogram of the acetone and ethanol extract of *P. nigrum* extracts scanned at 254nm and 366nm is depicted in Fig. 2. The chromatogram was obtained using n-Hexane: Ethyl acetate: Acetic acid (5:5:0.05) as the mobile phase. Piperine (Sigma-Aldrich) was used as the reference standard. The Rf of piperine was noted to be 0.21.

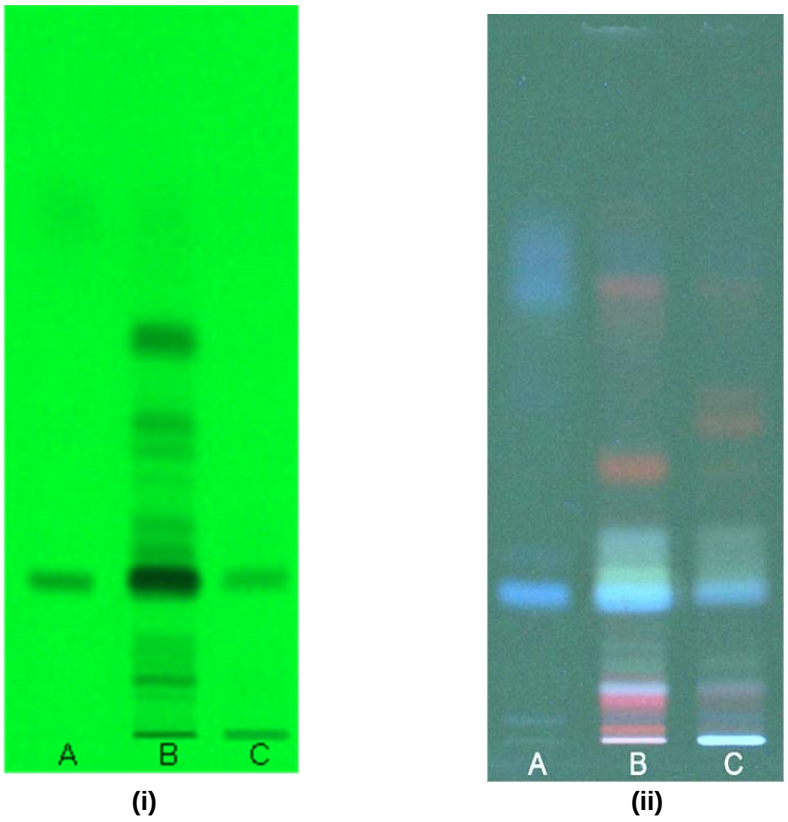

Fig. 2. HPTLC fingerprinting of *P. nigrum* scanned at (i) 254nm and (ii) 366nm
Lane A corresponds to the reference compound piperine, lane B and lane C correspond to the acetone and ethanol extracts respectively.

4. DISCUSSION

In the era of emerging drug resistance in infectious diseases, use of medicinal plants as an alternative therapy has been proposed (Oluyege et al., 2010; Sibanda and Okoh, 2007;

Singh et al., 2010). There are limited reports on the anti-TB activity of Indian medicinal plants (Gautam et al., 2007). We used the MABA to screen for potential anti-*M. tuberculosis* activity of five Indian medicinal plants which have been in use for various respiratory ailments viz., *A. calamus, A. paniculata*, *O. sanctum*, *P. nigrum* and *P. tuberosa*. Amongst these, only *P. nigrum* showed anti-*M. tuberculosis* activity. The external and internal validation of the results strengthens our observations.

Amongst the *Piper* spp, *P. imperiale* (Diaz et al., 2012), *P. longum* (Singh et al., 2011), *P. sarmentosum* (Hussain et al., 2008), *P. betle* (Gupta and Viswanathan, 1956) and *P. cubeba* (Grange and Davey, 1990) have been reported to have anti-mycobacterial activity. *P. nigrum* has also been shown to have anti-mycobacterial activity, though the plant part used was unspecified (Grange and Davey, 1990).

In our study, the acetone extract of *P. nigrum* at 100 µg/ml demonstrated inhibitory activity against H37Rv, all three susceptible isolates and one of three MDR isolates. The combination of the acetone extract of *P. nigrum* and suboptimal rifampicin was not more effective than the plant extract alone. Although the ethanol extract of *P. nigrum* showed only marginal activity, a mixture of the acetone and ethanol extracts (each at 25 and 50 µg/ml) were tested to check for possible anti-*M. tuberculosis* activity. These mixtures showed inhibitory activity against H37Rv and all the clinical isolates tested, despite the lack of activity when used individually at 50 µg/ml. This finding has two-fold value since it enabled the use of lower concentrations of the active extract and demonstrated that non active extracts may have the potential to show activity if used in combination. The efficacy of the extracts could be due to the interplay between the different active constituents present, leading to better activity. It has been demonstrated that different constituents of crude extracts act through different mechanisms (Mavar-Manga et al., 2008) or act synergistically (Birdi et al., 2010; Ncube et al., 2012).

The activity of the extracts of *P. nigrum* could be due to piperine, a principle active component of the plant (Ahmad et al., 2012; Madhavi et al., 2009). It has also been shown to inhibit the *M. tuberculosis* efflux pump Rv1258c (Sharma et al., 2010). The presence of piperine in the acetone and ethanol extracts was confirmed by HPTLC. In our study, piperine (sourced from Sigma-Aldrich) was active only against H37Rv at 25 µg/ml in the presence of suboptimal concentration of rifampicin. It is possible that the level of expression of Rv1258c in clinical isolates is altered as compared to H37Rv, resulting in the lack of bactericidal activity when using a combination of piperine and rifampicin. Though Phongpaichit et al. (2006) reported that piperine isolated from the fruit of *P. chaba* exhibits anti-mycobacterial activity, the lack of activity in our study could be due to the difference in the test strains screened. The active component and target of *P. nigrum* thus need to be identified. Based on the results obtained in this study, it is possible that the target molecules of the active component(s) of *P. nigrum* may be different from those of standard anti-TB drugs. A similar conclusion with respect to possible mechanism of action was reached by Lakshmanan et al., (2011) for the active molecule of *Kaempferia galanga*.

The *P. nigrum* results reiterate the necessity of screening plant extracts against multiple clinical strains with different clinical profiles and not only against H37Rv. A Mexican study on plants against drug resistant TB also stated that testing only a laboratory reference strain like H37Rv would be insufficient as an indicator of activity against clinical isolates, since the activity might differ against susceptible and resistant strains (Camacho-Corona et al., 2008).

Chopra et al. (1957) reported anti-mycobacterial activity of the essential oil of *A. calamus* rhizome and Webster et al. (2010) showed that the aqueous extract of the root of this plant was active against *M. tuberculosis* H37Ra. The lack of activity in our study can be attributed to the different plant part (rhizome), extract used and the test strains screened. Anti-mycobacterial activity of *O. sanctum* was reported by Reddi et al. (1986) and Farivar et al. (2006). However, the above factors, as well as the difference in the concentration of the extract (50-100 mg/ml) could explain the absence of activity of *O. sanctum* in this study. Additionally, this variation could be due to the sequential extraction procedure used by us. The aqueous extract prepared directly could possibly have contained two or more compounds that could have had anti-*M. tuberculosis* activity.

M. tuberculosis is an intracellular pathogen capable of infecting and replicating in epithelial cells (Castro-Garza et al., 2002). A549, a human lung epithelial cell line, was thus selected to test the cytotoxicity of the five plants. Hence in the absence of cytotoxicity, *P. nigrum* extracts seem promising for intracellular testing of newer anti-*M. tuberculosis* agents of plant origin.

5. CONCLUSION

In conclusion, the present study identifies *P. nigrum* as a promising anti-*M. tuberculosis* plant active against both drug sensitive and resistant strains. The study also highlights the combined activity of the ethanol and acetone extracts. Further studies to identify constituent(s) of *P. nigrum* (in addition to piperine) responsible for its anti-*M. tuberculosis* activity are being undertaken, along with the exploration of mechanisms of action of the plant and the possible interactions between *P. nigrum* and presently available anti-TB drugs.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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