



Research Article

Effect of *Piper nigrum* (Linn.) seeds extract and second line anti-tuberculosis drugs on a few *Mycobacterium tuberculosis* strains

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Abstract

Piper nigrum (Linn.) belonging to the family Piperaceae have been reported for its multitudinous medicinal values. The present study was undertaken to examine the direct effect of Ethionamide (ETH), Para amino salicylic acid (PAS), ethanolic extracts of *P. nigrum* on *Mycobacterium tuberculosis* (MTB) strain H37Rv and Multi drug-resistant (MDR)-strains-12, 19 and 21. The proportion method was used to detect the anti-mycobacterial drug susceptibility testing for mycobacteria using Lowenstein Jensen (LJ) medium. It was found that *P. nigrum* does not interfere with single or in the combination of both ETH and PAS showing the bioenhancer activity. *In vitro* study of ethanolic extract of *P. nigrum* observed that the extract inhibited the growth of H37Rv strains and MDR strains-12, MDR strains 19, and MDR strains 21. The present results will pave new avenues to find a new medicine that possesses *P. nigrum* alone or in combination with drugs to combat MDR-strains controlling tuberculosis.

Keywords: Ethionamide, *Mycobacterium tuberculosis*, Multidrug-resistant, Para aminosalicylic acid

INTRODUCTION

Tuberculosis (TB) is a transmittable disease caused by *Mycobacterium tuberculosis*. Despite medical advancement, tuberculosis is very lethal and is the second most frequent cause of death in many countries (WHO, 2014). The world's quarter population is infected with TB. In the report of WHO (2020), it is estimated that about ten million of the people developed TB and 1.4 million died of it. Most new cases were found in the populated nations such as India and China (WHO 2002; and Raviglione et al., 1995). Due to the global emergence of multi-drug resistant (MDR) and extensively drug-resistant (XDR) strains of *Mycobacterium tuberculosis* and totally drug resistant TB, there is an urgent need to develop new drugs and strategies to

fight TB (Singh, 2015). The standard treatment for tuberculosis susceptible (non-resistant) is given with first-line tuberculosis drugs Isoniazid, Rifampicin, Pyrazinamide for over two months followed by four or more than four months with the combination of Isoniazid and Rifampicin (NTCP. 2008). Resistance to first-line drugs is due to the continuous unlinked chromosomal mutation. It is studied that the development of MDR-TB is due to misuse of proper antibiotic treatment by patients and unfocused physician observations to these patients. Due to a very modern issue associated with MDR-TB, the Second line Tuberculosis drugs are used (Al-Humadi, 2017). Due to the resistance of *Mycobacterium tuberculosis* to first-line drugs, it is mandatory to study the potential of second-line drugs and the controlling agent to fight against TB. Therefore, the

objective of the present study was 1) To find the effect of second-line anti-TB drugs on *M. tuberculosis* strains, 2) To find the combined effect of second-line anti-TB drugs and *Piper nigrum* against Multi-drug resistant strain no. 12, 19 and 21.

MATERIALS AND METHODS

Collection of samples

Fresh seeds of *P. nigrum* were procured from the botanical garden of Kokan Krushi Vidyapeeth, Dapoli, Ratnagiri Maharashtra, India. The initial identification was made by referring related literature. The authentication and identification of seeds were made by Dr. R.G. Khandekar at the Department of Horticulture, Kokan Krushi Vidyapeeth, Dapoli, Ratnagiri. Maharashtra, India. The final confirmation of *P. nigrum* was made at the "Government of India, Ministry of Environment, Forest and Climate change, Botanical Survey of India, Western Regional Centre, 7, Koregaon, Road, Pune, India and the Voucher specimen No. BSI/WRC/100-1/TECH./2019/53 were deposited in the said repository.

Extraction

The ethanolic extract of the *Piper nigrum* seeds were carried out by using Soxhlet extraction method at the Department of Zoology S. S & L.S. Patkar College Goregaon (west), Mumbai, India. The seeds of *P. nigrum* were crushed in pestle and mortar. The crushed powder was weighed and loaded in a porous cellulose thimble. The process was further followed by adding 250 ml ethanol to the round bottom flask. The apparatus was assembled and extraction was carried by heating at 55°C. The three cycles of extraction were carried out to get the compound. Solvents were filtered through Whatman filter paper No.1. The remaining solvent was evaporated at low pressure using a Rotary Vacuum Evaporator at 45°C. The resultant compound was subjected to the Millipore filter system and finally dried in a vacuum desiccator and stored at -20°C in a refrigerator till further use.

Purchase of drugs

The drugs ETH (Ethionamide) (Macleods Pharmaceuticals Ltd) and PAS (Para amino salicylic acid) (Lupin Ltd) were purchased following the prescription of Physician from B.J. Govt Medical College and Sassoon General Hospital, Pune, Maharashtra.

Procurement of *M. tuberculosis* strains

The Mycobacterium tuberculosis strain H37Rv and MDR-strains-12, 19 and 21 were provided by the Department of Microbiology, B.J. Govt Medical College and Sassoon General Hospital Pune, Maharashtra. The experiments were carried out under the supervision of Dr. Sujata Dharmshale at the BSL3 TB Laboratory facil-

ity at B.J. Govt Medical College, Pune, Maharashtra.

One set of media bottles for testing one culture consisted of five Lowenstein-Jensen (LJ) slope- one for neat, two for 10⁻² and two for 10⁻⁴; twelve drug-containing LJ slopes- two each for drugs ETH, PAS, Extract, ETH+ Extract, PAS+ Extract and ETH + PAS + Extract (one each for 10⁻² and 10⁻⁴ suspensions) and one for PNB slope and thus total 18 LJ slopes were used.

Drug containing media preparation

The LJ medium was prepared as proposed by Gruft (1965). All strains of tuberculosis contained some sub-population of bacilli that were resistant to anti-TB drugs. However, in resistant strains, the proportion of such bacilli is considerably higher than the sensitive strains. The proportion method was used to calculate the proportion of resistant bacilli present in a strain. Two appropriate dilutions of the bacilli, 10⁻² and 10⁻⁴ dilutions (undiluted = 10⁶ to 10⁸ CFU/ml) were inoculated on drug-containing and drug-free media in order to obtain countable colonies on both media. The ratio of the number of colonies observed on the drug-containing media to drug-free medium indicated the proportion of resistant bacilli present in the strain. The strain was classified as sensitive below a certain proportion (critical proportion = 1%); above as resistant. The concentration of drugs were 0.2 µg /ml and 40 µg /ml added to LJ Media with a critical proportion 1% to determine the effect of ETH and PAS against mycobacterial strains for interpretation of the economical variant.

RESULTS AND DISCUSSION

The present study observed that there was no growth in standard strain H37Rv and S2 inoculums in drug medium as well as in drug-free medium. In Strain H37Rv S4 inoculums, around 25 large colonies in drug-free LJ media were observed. At the same time, no growth was reported in drugs containing media and in ethanol extract. In MDR strain-12 in S2 inoculums 2+ colonies were observed in ETH and 1+ in PAS in drug-free LJ media. On adding the *P. nigrum* extract in PAS and in ETH, it was observed that the numbers of colonies were reduced in PNS + PAS extract and PNS +ETH extract with and without LJ media, indicating the sensitivity of *P. nigrum* against MDR strain, thus confirming its anti-tuberculosis potential. The MDR strains-19 and 21, the S2 and S4 inoculums incorporated in LJ media or without media with ethanolic extract of *P. nigrum* in combination with PAS and ETH also showed sensitivity, indicating a positive effect against MDR strains 19 and 21 (Table 1).

Various traditional plants have shown anti-TB activity. It is reported that *Emblica officinalis* was effective in preventing *Klebsiella pneumoniae* ATCC43816 bacterial colonization with the decrease in the bacterial load

(Tasduq et al., 2005). The aqueous extracts of *Acalypha indica*, *Adhatoda vasica*, *Allium cepa*, *Allium sativum*, *Aloe vera* have been reported to have anti-tuberculosis activity against MDR isolates of *M. tuberculosis* (Gupta et al., 2010). Aqueous extract of *Withania somnifera* (0.01-1.0 mg/mL) had significant effect against *M. tuberculosis* H37Rv whereas, *P. tuberosa* in higher concentration showed no inhibition (Periyakaruppan et al., 2012). The plants like *Citrullus colocynthis* (L.) (Leon-Diaz et al., 2010), *Plumeria bicolor* (Changtam et al., 2010), *Curcuma longa* L. (Mehta et al., 2013) have also been reported to have anti-TB activity. *Alpinia galanga* (L.) exhibited anti M.TB activity with multiple modes of action (Gupta et al., 2014). *Curcuma longa* and *Nigella sativa*, modulated the hepatotoxic effect of anti-tuberculosis drugs and acetaminophen (Eman et al., 2018). *Syngizium aromaticum* Linn. also showed activity against *M. tuberculosis* (Fadipe et

al., 2020). Zodape and Bhise, (2017), in their study on effect of *Aloe vera* extract and isoniazid - rifampicin drug on *M. tuberculosis* bacterial (Mtb) Strain -H37rv, reported that the *Aloe vera* had anti-TB potential against the H37Rv strain.

In case of *Piper nigrum*, Singh et al. (2011) studied its properties and constituent piperine and tested against multi-drug resistant strains of *Mycobacterium tuberculosis*. They found that piperine has the potential to inhibit the growth of *M. tuberculosis* species. Barua et al. (2014) studied the antioxidant and anti-mycobacterial activity against *P. longum* seeds extracts and reported that the chloroform extracts have effective activity against *Micobacterium smegmatis*. They found that the chloroform extract had the best antioxidant activities when compared to hexane, ethyl acetate, ethanol and aqueous extract. In our previous study it was found that, the second line anti-tuberculosis drugs ETH and

Table 1. Showing the effect of *P. nigrum* seed extract, and ETH and PAS drugs on different strains of *M. tuberculosis*.

Strain Number	Drug Free LJ	ETH Drug	PAS Drug	PNS Extract (Ethanolic)	ETH+ PNS Extract (100µg/ml)	PAS+PNS Extract (100µg/ml)	ETH+ PAS+PNS Extract (100µg/ml)
H37Rv							
S1	3+	XX	XX	XX	XX	XX	XX
S2	2+	NG	NG	NG	NG	NG	NG
S2	2+	XX	XX	XX	XX	XX	XX
S4	25 large CFU (1+)	NG	NG	NG	NG	NG	NG
S4	26 CFU (1+)	XX	XX	XX	XX	XX	XX
MDR 12							
S1	3+	XX	XX	XX	XX	XX	XX
S2	2+	2+	>50CFU (1+)	NG	1+	Very Tiny 1+ colonies	Very Tiny 1+ colonies
S2	2+	XX	XX	XX	XX	XX	XX
S4	1+	<50 CFU with contamination	20mall CFU with contamination	NG	20 tiny CFU	18 tiny CFU	20 tiny CFU
S4	1+	XX	XX	XX	XX	XX	XX
MDR 19							
S1	3+	XX	XX	XX	XX	XX	XX
S2	2+	NG	NG	NG	NG	NG	NG
S2	2+	XX	XX	XX	XX	XX	XX
S4	1+	NG	NG	NG	NG	NG	NG
S4	1+	XX	XX	XX	XX	XX	XX
MDR 21							
S1	3+	XX	XX	XX	XX	XX	XX
S2	2+	NG	NG	NG	NG	NG	NG
S2	2+	XX	XX	XX	XX	XX	XX
S4	1+	NG	NG	NG	NG	NG	NG
S4	1+	XX	XX	XX	XX	XX	XX

Abbreviations: PNS- Piper nigrum (linn.); ETH- Ethionamide; PAS- Para amino salicylic acid; XX=Not done, 1+= 20-100 colonies, 2+=>100 colonies, 3+=confluent growth, NG=No Growth (Indicate Sensitivity) S1-Neat inoculums ($1 \times 10^{-1} \times 10^8$ CFU/ml) inoculated single LJ with standard strain; S2- 10^{-2} & S4 10^{-4} inoculated with standard strains in duplicates.

PAS caused hepatic damage in *Sprague-dawley* rats. The seed ethanolic extract of *P. nigrum* (L.) showed hepatoprotective effect on *Sprague-dawley* rats; when administered to the test groups with ETH and PAS with or without combination, it ameliorated the toxic effect of the drugs (Zodape and Gaikwad, 2019). In the present study, it was observed that the ethanolic extract of *P. nigrum* did not affect the activity of ETH and PAS with or without combination *in vitro*. This ethanolic extract was found to inhibit the growth of H37Rv strains and MDR strains-12, MDR strains 19, and MDR strains 21. Thus, *P. nigrum* can play an important role in liver protection and can have anti-tuberculosis activity against *M. tuberculosis*. Therefore, this study would serve as a baseline model system as new templates in the bioprospection and development of new and more effective plant-based antibiotics to prepare multi-drug therapy against *M. tuberculosis*.

Conclusion

The present study found that *P. nigrum* seed ethanolic extract did not affect the activity of the antitubercular drug ETH and PAS either independently or in combination. This seed ethanolic extract alone seemed to have the anti-tuberculosis activity against the H37Rv strains. It was also observed that *P. nigrum* had clear cut sensitivity against MDR strains-12, MDR strains 19, and MDR strains 21, thereby showing anti-tuberculosis activity. Therefore, it is suggested that *P. nigrum* seed ethanolic extract supplementation with ETH and PAS drugs may be used in the pharmaceutical industry for the manufacture of multi-drugs therapy for combating MDR and XDR-TB.

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Conflict of interest

The authors declare that they have no conflict of interest.

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