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Anti-mycobacterial activity of *Piper longum* L. fruit extracts against multi drug resistant *Mycobacterium* Spp.

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Abstract

A long tradition of using pepper as to fight against several ailments by the local tribal people is still in the practice, in many parts of the rural India. So utilizing this tribal knowledge base for this highly medicinal plant, an attempt was made to isolate some novel natural bioactive compounds with potential activity against multidrug resistant (MDR) *Mycobacterium*. A bioassay guided fractionation of Pippali (*Piper longum* L.) was performed in five different organic solvents and their activities were monitored against different pathogenic bacteria including MDR *Mycobacterium*. Different fractions were screened for the bioactivity against *Mycobacterium,* and the structure of bioactive compound was characterized with H^1 and C^{13} NMR. An ethyl acetate fraction of Pippali extract was found active against *M. smegmatis* (3000µg ml^{-1}) and *M. tuberculosis* (39 µg ml^{-1}). It also shows very significant activity against other bacterial strains like *E.coli* (152 µg ml^{-1}), *Staphylococcus aureus* (14 µg ml^{-1}), *Salmonella typhi* (180 µg ml^{-1}), *Enterococcus faecalis* (15 µg ml^{-1}), and *Pseudomonas aeruginosa* (52 µg ml^{-1}). This fraction of ethyl acetate was then purified and characterized as piperine [5-(1, 3-benzodioxol-5-yl)-1-piperidin-1-ylpenta-2,4-dien-1-one], a well known alkaloid from this plant. Bioactivity guided fractionation concludes that Piperine is the only active ingredients in various fractions of fruit extract evaluated for antibacterial activity. Fraction having piperine has significant activity against multi drug resistant strains of *Mycobacterium* spp. than other purified fractions of fruit extract. The current finding encourages us to develop new alternative medicine that includes piperine alone and/or in combination with other drugs to fight against the drug resistance among Mycobacterial strains.

Keywords: *Piper longum*, piperine, bioassay guided fractionation, NMR, *Mycobacterium tuberculosis*.

Introduction

Natural bioactive compounds are the ultimate source for innovative therapeutic agents to cope with communicable and non communicable

diseases and other ailments [1]. Ethno pharmacologists, botanists, microbiologists and natural product chemists are searching novel bioactive metabolites which could be developed as new pro-drugs for treatment of infectious diseases and other biomedical challenges including drug resistance among various infectious microbes [2]. The genetic ability of pathogenic bacteria to develop resistance against commonly used antibiotics is a major medical problem and challenge worldwide, posing a big threat to human society [3-5]. Medicinal plants that have a well defined ethanobotanical history as well as tribal and folk medicinal uses are of great concern among the natural product researchers, for getting new bioactive leads [6-7]. Many efforts have been made to discover new antimicrobial compounds from various kinds of sources including microbes and plants. Systematic screening of them may result in the discovery of novel effective compounds [8] with potential of new drug design and development.

Antimicrobials of plant origin are efficient in the treatment of infectious diseases, while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials [9]. A wide variety of plant secondary metabolites have been identified as active principles for the treatment of various ailments [10-11]. A number of studies have been conducted for the selection of the crude plant extracts in a therapeutic treatment of bacterial infections [12-14]. Many plant extracts have shown the ability to overcome drug resistance in many pathogenic micro-organisms, which led the researchers to isolate active principles and investigate mechanisms. The discovery and development of structurally novel chemical entities to control the multi-drug resistant pathogenic bacteria is desperately desired by the pharmaceutical industries and drug developers that are looking towards the underexplored natural sources for developing the front line drugs. Many drug leads isolated from plants are active against a wide range of microbes including plant and human pathogens also rectify this approach [15]. The use of the plant extracts and the phytochemicals can be of great significance in therapeutic treatments and could be helpful to cure the problem of these multi-drug resistant microorganisms. World Health Organization has also advocated that the medicinal plants would be the best source for obtaining a variety of drugs for malaria, tuberculosis and immune-compromised patients [16]. Several secondary metabolites of plant origin that include a wide chemistry like alkaloids and flavonoids has already been in use as anti-viral, anti-bacterial, anti-amoebal and anti-cancer agents [17-20].

In this context, we have selected a well known spice plant *Piper longum*, to screen and isolate new bioactive metabolites that are active against multidrug resistance *Mycobacterium*. *Piper* sp. are distributed widely in the tropical and subtropical regions of the world, have multiple applications in different folk medicines, including Indian system of medicine 'Ayurveda' and have been reported to possess numerous biological activities [21-25]. *P. longum* used as bioavailability enhancer [26], digestive and in the treatment of bronchitis [27] and as effective hepatoprotective agent [28-29]. The main objective of this study was to screen extracts from fruits of *Piper longum* plant and isolate bioactive compounds with potential activity against multidrug resistance strains of *Mycobacterium*.

Materials and Methods

Plant materials and microbes

Healthy plants of *Piper longum* L. selected and fruits were randomly collected from herbal garden of faculty of Ayurveda, Banaras Hindu University Varanasi. Plant specimens were identified by Prof. A.K. Singh, Department of Dravya Guna, Banaras Hindu University BHU). A voucher specimen (CEMS/PL-054/SKS) was also deposited in the Centre of Experimental medicine and Surgery, BHU. Samples were brought to the laboratory and used to prepare extracts within 48h of collection. The following bacteria were used for anti-bacterial bioassay

besides *Mycobacterium tuberculosis* GN/mt-75, *M. smegmatis* GN/ms-43, *Escherichia coli* GN/ec-98, *Staphylococcus aureus* GN/sa-16, *Pseudomonas aeruginosa* GN/pa-40, *Salmonella typhi* GN/st-56, *Enterococcus faecalis* GN/ef-32. All cultures were obtained from culture collection of Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University India. All these strains were isolated and cultured on synthetic media, from the samples collected from the infected subjects in the OPD (outpatient department).

Instruments

Column chromatography was carried out by using 'silica gel G' of 60-120 mesh size (Qualigens, India). TLC analysis was done with Merck 0.25mm (silica gel G, 13% Calcium sulphate as binder) ready to use glass plates of 5×10 cm. Melting point was recorded with a Stuart-SMP 10 melting point apparatus in open capillaries. HPLC was performed on a Shimadzu LC 10. Infra red spectra were recorded in KBr (4000-350 cm^{-1}) with Shimazdu 8201 PC, while UV spectra was recorded with a dual beam spectrophotometer Jasco 7800, Japan. NMR spectra were recorded in $CDCl_3$ and DMSO- d6 solvent by using TMS as internal standard on a Buker DRX -300 Mz FTNMR.

Preparation of extract and fractions

The collected fruits were shade dried at room temperature and ground in a pulvelizer. 2-3 kg of dried fine powder was thus obtained, which was extracted with 2L of methanol for 2-3 times. The total of 6L of extracted methanol was then evaporated to dryness with a rotary evaporator, which yield 500-600g of dry extract. It was further partitioned in petroleum ether (37g), hexane (58g), chloroform (45g), ethyl acetate (100g), methanol (35g). The fractions were filtered through buchner funnel and the filtrates were evaporated to dryness under reduced pressure using rotary evaporator at 35°C. All the dried fractions were stored in airtight vials at 4°C for subsequent use.

Isolation and identification of bioactive compound

Since the ethyl acetate extract showed promising antibacterial activity in initial screening, it was subjected to column chromatography over silica gel (60-120 mesh) and eluted using 500ml of ethyl acetate:methanol:water (10:1:0.1 v/v). Total 22 fractions were collected and fraction PE-014 was showed maximum activity in preliminary bioassay. A HPLC study has been performed of this bioactive fraction with authentic reference standard of Piperine. Separation was achieved using a C18 column (250 × 4.6 mm, 5µ ID), (Merck, India). The solvent system consisted of 50mM potassium dihydrogen orthophosphate (pH adjusted to 3.5 using orthophosphoric acid): acetonitrile (40:60 v/v) was pumped isocratically at a flow rate of 0.6 ml/min. The detection was carried out using a diode array detector. The final structure determination of the bioactive fraction was performed by C^{13} and H^{1} NMR.

Anti-bacterial Assay

Anti-bacterial assay was performed by using the modified Kirby-Bauer disk diffusion susceptibility method [30]. The bacterial strains were suspended in 4ml of normal saline (0.85 %) and the density of suspension was adjusted to approximately 10^8 CFU ml^{-1} using 0.5 M barium sulphate buffer as the turbidity standard. The surface of the sterile 3.8% mueller hinton agar (Himedia, India) in petri dishes was dried and the test strain was inoculated with a sterile swab to obtain a homogenous bacterial lawn. The sterilized 6mm discs (Himedia) containing 10µl of fraction was placed onto the agar, and the inhibition zones was measured (in mm) after incubation for 18 hours at 37°C. DMSO was taken as positive control for organic extracts. Different organic solvent extracts (1% v/v) in DMSO did not affect the growth of microorganisms in accordance with our control experiments.

In vitro anti-mycobacterial activity against *M. smegmatis*

Minimum inhibitory concentration (MIC) was performed according to the standard reference method [31]. Different dilutions ranging from 5-10 mg ml^{-1} were prepared in DMSO and added into muller hinton broth medium. The bacterial suspension was prepared in normal saline and match with Mc farland turbidity standard to obtain concentration 10^6 CFU ml^{-1}. This suspension was added to fractions and incubated at 37^0C for 72h. 500µl of suspension from each of the broth which contains different concentration of compound was spread on plate and incubated at 37^0C to observe the bacterial growth. The lowest concentration of compound that showed no visible growth in media was considered as MIC.

In vitro anti-mycobacterial activity with *M. tuberculosis*

For bioactive compound, stock solution of 10 mg ml^{-1} in DMSO was prepared and stored at room temperature. Compound concentrations of 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039 and 0.01µg ml^{-1} were made accordingly by adding required volume of stock solution respectively to 15ml of lowenstein jensen (LJ) medium in Mc cartney bottles. The molten media at 75^0C poured and slanted till the media solidify at room temp. The whole media were incubated at 37°C for 24 hours as a check of sterility before inoculation. Cells from 3 week old culture were first scraped into a bottle containing saline and glass beads, to obtain a homogenous suspension of *M. tuberculosis* as inoculum. The bottle was vortexed and kept standing for 20 min. the supernatant was then transferred into another sterile tube and turbidity was matched with Mc farland standard. 150 µl of this inoculum was added to control as well as compound treated media. The bottles were incubated at 37^0C and bacterial growth was checked after four weeks. The lowest concentration of compound that showed no visible growth in media was considered as MIC.

Results and Discussion

Petroleum ether, hexane, chloroform, ethyl acetate and methanol extracts were screened against an array of pathogenic bacteria representing both gram positive and negative strains, including *Mycobacterium*. Among all the organic extracts the ethyl acetate extract showed notable activity against test microbes. The disk diffusion assay showed a wide range of growth inhibition of different organic solvent extracts against test bacterial strains. Table 1 showed the antibacterial activity of different fractions. The petroleum ether fraction was found ineffective against bacterial strains, while hexane extract was least inhibitory (6.33±1.52) among all the extracts against all test strains (Table 1).

Hexane fraction is showing highest activity against *E. faecalis* (12.66±2.08). Ethyl acetate fraction of *Piper longum* fruits was showing highest activity against all the tested bacterial strains together with *Mycobacterium*. *S. aureus* (24.33±1.52) and *P. aerugenosa* (22.66±1.52) were the highly sensitive strains towards ethyl acetate fraction with average inhibition zones 24.33 mm and 22.66 mm respectively. For methanolic fraction *E. fecalis* (21.00±1.00) was the highly sensitive strain while *E.coli* (9.33±1.15) strain was the least sensitive with inhibition zones 21 and 9.33mm respectively (Table 1). Hexane fraction was showing no inhibitory effect towards *M. smegmatis*. These findings revealed that each fraction was inhibitory to all tested gram positive and gram negative bacterial strain except petroleum ether. Ethyl acetate, methanol and chloroform fractions were having potent inhibitory property against bacteria, supported the view that most of the antimicrobial active fractions have been dissolved in these high polar solvents. After successful characterization of piperine from fruit extract of *Piper longum*, an anti-mycobacterial activity bioassay was performed against two multidrug resistant strains *Mycobacterium tuberculosis* and

Table 1 Antibacterial activity of fruit extracts of *Piper longum* extracted in different organic solvent.

Bacterial strains	Zone of inhibition (in mm), (mean±SD), n=3			
	Hexane fraction	Chloroform fraction	Ethyl acetate fraction	Methanol fraction
E .coli GN/ec-98	6.33±1.52	11.33±1.15	14.33±0.57	9.33±1.15
S. aureus GN/sa-16	10.66±1.52	20.33±2.08	24.33±1.52	16.66±1.52
P. aeruginosa GN/pa-40	12.00±1.00	20.66±1.52	22.66±1.52	15.66±0.57
S. typhi GN/st-56	10.33±0.57	11.66±1.52	19.33±1.15	10.66±0.57
E. faecalis GN/ef-32	12.66±2.08	19.00±2.64	21.66±1.52	21.00±1.00
M. smegmatis GN/ms-43	-	12.33±0.57	15.66±1.52	11.66± 0.57

Table 2 Minimum inhibitory concentration (MICs) of piperine from fruit extracts against different bacteria.

Tested Organisms	MICs (μg ml^{-1})
Escherichia coli GN/ec-98	152
Staphylococcus aureus GN/sa-16	14
Pseudomonas aeruginosa GN/pa-40	52
Salmonella typhi GN/st-56	180
E. faecalis GN/ef-32	15
Mycobacterium smegmatis GN/ms-43	3000
Mycobacterium tuberculosis GN/mt-75	39

M. smegmatis, together with some other pathogenic bacteria to assess the wide spectrum bioactivity. The minimum inhibitory concentration of piperine was observed against *Staphylococcus aureus* (14 μg ml^{-1}), while significant inhibitory activity was also found against *E. faecalis* (15 μg ml^{-1}), *Pseudomonas aeruginosa* (52 μg ml^{-1}), apart from *Mycobacterium* (Table 2).

Interesting differences were observed in the inhibition concentration against two strains of *Mycobacterium*, the piperine from extract was more efficient against *M. tuberculosis* (39 μg ml^{-1}), while against *M. smegmetis* it was around 3000 μg ml^{-1}, however this difference at this level is beyond any prediction. This seems that multi drug resistance *M. tuberculosis* strain is more susceptible to the piperine, rather than the *M. smegmetis*, which atypical strain of *Mycobacterium* (Table 2). Pure crystals of piperine obtained by purification of fraction PE-014, were also assay on the plate for inhibition measurements against *Mycobacterium tuberculosis,* and observed significant inhibition of the drug resistance *M. tuberculosis* strain. Thus after this assessment the ethyl acetate fraction was consider for further purification and characterization study. Further column purification of the active fraction was performed, and concentrated in vacuo. It was then resolved on TLC plate together with the reference standard of piperine. The bioactive fraction (PE-014) extracted in ethyl acetate was then further purified by adopting bioassay guided

fractionation, followed by HPLC and identified as piperine $C_{17}H_{19}NO_3$ after comparison with authentic reference standard (Figure 1and 2).

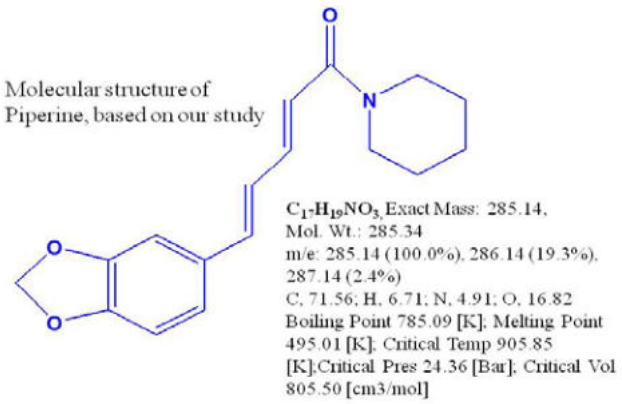

Figure 1 the molecular of pipreine crystals obtained from *Piper longum*, and its chemical properties.

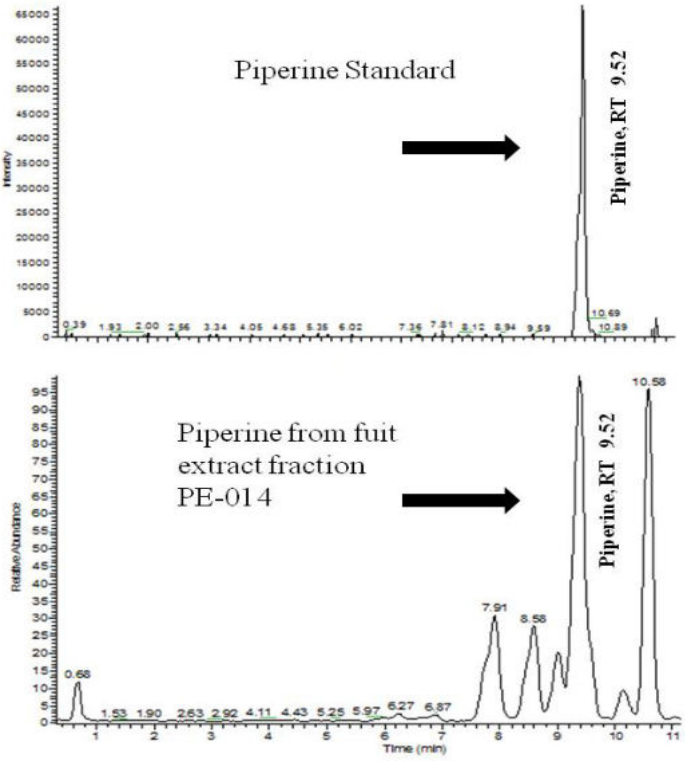

Figure 2 HPLC chromatogram of fraction PE-014 shows peak on retention time 9.52, whichcorrespond to the authentic reference standard of piperine.

At R_f value 0.26, a fraction PE-014 and standard piperine shows equal bands, this confirms the presence of piperine in the fraction PE-014, from piper fruits. It was observed that the purified sub-fraction PE-014 contains piperine crystals (8µg/mL) as most active ingredient. It was also widely available in the literature that piper fruit contains this alkaloid which is responsible for the pungency of this spice. After identification of this compound, further spectroscopic confirmation was performed by doing NMR, IR and other methods. The H^1 NMR of the piperine represent δ 1.48 (4H, m, C-3"and C-5"), 1.65 (2H, m, C-4"), 3.57 (4H, m, C-2"and C-6"), 5.95 (2H, s, OCH_2O), 6.52 (1H, d, J=16 Hz, C-2), 6.82 (2H, m, C-3 and C4), 7.71 (1H, m, C-5), 7.23 (1H, br, s, C-2'), 6.99 (1H, m, C-6'), 6.77 (1H, m, C-5') (Table 3).

Table 3 H^1 NMR data for the bioactive piperine from the fruits of *Piper longum.*

δ_H	^1H	Position	HMBC
1.48	4H	m	C-3" and C-5"
1.65	2H	m	C-4"
3.57	4H	m	C-2" and C-6"
5.95	2H	s	OCH_2O
6.52	1H	d	C-2
6.77	1H	m	C-5"
6.82	2H	m	C-3 and C-4
6.99	1H	m	C-6
7.23	1H	br, s	C-2'
7.71	1H	m	C-5

While C^{13} NMR of this extracted piperine represent δ 23.44, 24.52 and 25.25 (C-3", C-4", C-5"), 41.95 and 45.64 (C-2", C-6"), 100.19 (OCH_2O), 104.48 (C-2)107.31(C-3), 119.24 (C-2'), 121.34 (C-5'), 124.32 (C-6'), 129.74 (C-1'), 136.83 (C-4), 141.02 (C-5), 147.00 (C-3'), 147.08 (C-4'), 163.97 (C-1) (Table 4).

Table 4 C^{13} NMR data for the bioactive piperine from the fruits of *Piper longum*.

^{13}C	HMBC
23.44	C-3”
24.52	C-4”
25.25	C-5”
41.95	C-2”
45.64	C-6”
100.19	OCH_2O
104.48	C-2
107.31	C-3
119.24	C-2’
121.34	C-5’
124.32	C-6’
129.74	C-1’
136.83	C-4
141.02	C-5
147	C-3’
147.08	C-4’
163.97	C-1

The IR spectra represent (KBr) 2934, 1634 (CO), 1611.9, 1583.5, 1490, 1443.5, 1364.3, 1251.3, 1194.3, 1131.4, 1026.3, 998.1, 926.4, 847.9, 805.9, 701.3, 608.0 cm^{-1}. Even the compound is known from the plant since long back, but this is first ever report about its potential activity against multidrug resistant *Mycobacterium* strain. Many reports available about the anti-bacterial potential of piperine and sometime even the same compound (piperine) has been isolated from other plant sources like *Ludwigia hyssopifolia* L. and reported to be active against several pathogenic bacteria [32-33].

Conclusion

Our bioassay guided fractionation based phytochemical analysis of *Piper longum* fruit extract, thus revealed that piperine is the most active ingredient of fruit extract of *Piper longum* plant that has significant activity against an array of pathogenic bacterial strains. This is the first report on bioactivity guided fractionation for lead molecule having maximum antimycobacterial potential in *Piper longum* fruit. The interesting part of this work is the finding related to the activity of piperine against the multidrug resistant strains of *Mycobacterium*, especially *M. tuberculosis*. This report is first of its kind, and has significant biomedical importance in treatment of tuberculosis. This finding also encouraged us to develop new alternative therapy that includes piperine alone or with the combination of available drugs to cope with increasing drug resistant among many strains of *Mycobacterium* specifically. In our further study we will study the performance of several drug combinations with piperine and evaluate its potential in treatment of tuberculosis.

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