

In vitro* studies on Efflux pump Inhibition of *Catharanthus roseus* and piperine against ofloxacin resistant *M.tuberculosis

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ABSTRACT

Background: Tuberculosis is an infectious bacterial disease caused by *M.tuberculosis*, which commonly infect lungs and treatable with a six month courses of antibiotics. Misuse of antibiotic leads to develop resistance demands to develop novel antimycobacterial agent. With this context, *Catharanthus roseus* flower extract and *p.nigrum* extract has been evaluated against *M.tuberculosis*.

Methods: Drug resistance of *M.tuberculosis* was evaluated as per the CLSI guidelines. Phytochemical of *C.roseus* flower was analyzed by GCMS and Piperine was extracted from *P.nigrum* by methanol extraction. Antibacterial efficiency against *M.tuberculosis* was initially done by Kirby-Bauer method. Alamar blue assay was used to detect the MIC and efflux pump inhibition activity. Synergistic activity of phytochemicals was done by checker board assay.

Results: Out of 32 *M.tuberculosis* isolates, 6 of them were resistant to Ofloxacin and 12 were resistant to Streptomycin. GC-MS analysis of *C.roseus* confirms the presence 14 components which includes Furfural, 2(5H) Furanone and Hydrastininic acid as major constituent. Antibacterial study on *C.roseus* and piperine showed potent antimycobacterial activity against OFL resistant *M.tuberculosis*. The percentage of relative inhibitory zone of *C.roseus* was 133 % and piperine was found to be 111 %. Both the plant extracts were synergistically effective at 6.25 and 3.1 µg/ml. Of these tested phytochemicals, piperine was found to be potent efflux pump inhibitor.

Conclusion; Antimycobacterial activity of *C.roseus rosea* and Piperine was evaluated against Ofloxacin resistant *M. tuberculosis*. The present study concludes both the piperine act as efflux pump inhibitor and synergistically more active with *C.rosues*. This is the first report on Synergistic bioactivity of *C.roseus* and piperine fractionation against Pathogenic *M.tuberculosis*.

Keywords: Fluoroquinolones; Mycobacterium; Efflux pump; piperine; synergistic

I. Introduction

Management of TB/MDR-TB patient requires intense multi-chemotherapy for at least six months to two years. It is very hurtful to a patient's health due to high levels of drug toxicity and its adverse effect [1]. The increase in the number of multi-drug resistant *M. tuberculosis* isolates has drawn the attention towards the developments of novel drugs like fluoroquinolones (FQs) for the treatment of TB. The principal cellular target of the FQs is the DNA gyrase encoded by *gyrA* and *gyrB* genes. Recent investigations confirm the resistant of FQs mediated by decrease the intracellular concentration of antibiotics, reducing their clinical efficacy upregulation due efflux system of *M.tuberculosis* [2]. Medicinal plants are the foundation of many important drugs of the modern world. The primary reasons for choosing medicinal plants, besides their known antibacterial properties, were their use in respiratory ailments widely reported in ethnobotanical surveys. *Catharanthus roseus* L (apocyanaceae) is an important ornamental medicinal plant cultivated largely for its anticancer activity [3] but not explored against *M.tuberculosis*. Medicinal importance of this plant has increased considerably because of the discovery of six anti-cancerous activity containing alkaloids [4] namely vincristine and vinblastine active [5]. The credit of discovery of these two alkaloids goes to Nobel et al [6]. Black pepper (*Piper nigrum*) is a flowering vine in the family Piperaceae. Piperine is the major plant alkaloid present in black pepper *Piper nigrum* and long pepper *Piper longum*, is reported to have bioavailability enhancing activity for some drugs. Antimicrobial activity of piperine against gram positive and gram negative bacterial strains has been studied and reported by few workers [7]. The potential effect of piperine with ciprofloxacin combination

against *S. aureus* has proved it's an efflux pump inhibitor [8]. More than 230 plants from various families have been used to treat tuberculosis, including several piper species. Based on several literatures, this work is aimed to screen the antimycobacterial activity of *C.roseus* and *P.nigrum* as well as to investigate the efflux pump inhibition and synergistic effects of the active fraction on the growth of mycobacterial cells.

II. Objective:

The current study is aimed to find out the effective phytochemical from *C.roseus* and *P.nigrum* against *M.tuberculosis*.

III. Material and Method

3.1. Processing of plant material

About 20 g of finely powdered *Catharanthus roseus L* was kept in 500 ml Erlenmeyer flask and mixed with 100 ml of Methanol and incubated for 48 h under 15° C. solvent phase was filtered through NCF paper and air dried under vacuum evaporator and re dissolved in distilled water (1mg/ml). Phytochemical analysis of *C.roseus* was done by GCMS analysis.

3.2. Extraction of Piperine from black pepper [9]

To 10 mL of a 10% KOH in 95% ethanol in a 125mL Erlenmeyer flask concentrated pepper extract (20%) was added and stirred well. The sample heated at water bath and water drop wise added until no more solid appears to form and then allowed the mixture to stand at least overnight to collect the solid yellow crystal by filtration and re dissolved in distilled water (1 mg/ml). The presence of piperine was detected by Vanillin reagent.

3.3. Test pathogen [10]

Mycobacterium tuberculosis resistant to ofloxacin (OFL) was isolated from clinical sputum samples collected from local hospitals of Karur district. Totally 68 Sputum sample was collected and inoculated on LJ media. Plates were incubated under anaerobic jar for three weeks and isolates were identified by AFB staining and nitrate reduction test. Antibiotic resistant pattern against streptomycin and ofloxacin was done by stroke method [11]. Resistant to ofloxacin was detected by two fold dilution of ofloxacin with final concentration of 0.25, 0.5, 1, 2, 4, 8, 16, 32 and 64 mg/l. MIC of ≥ 4 mg/l was selected for further studies.

3.4. Preliminary anti Mycobacterial activity

A suspension of 0.5 McFarland standard of *M.tuberculosis* resistant to ofloxacin was prepared using normal saline and was introduced onto the surface of sterile Dubos oleic albumin agar plates. A sterile disc previously soaked in known concentration of extracts (100 µg/disc) was carefully placed at the centre of the seeded and labeled Dubose oleic agar (HiMedia). Sterile discs containing methanol alone was served as negative control and ofloxacin used as positive control. For each test solution, three replicates were maintained. The antimicrobial activity in terms of percentage relative inhibition zone diameter (RIZD) was also calculated by applying the expression

$$\%RIZD = \frac{IZD \text{ sample} - IZD \text{ negative control} \times 100}{IZD \text{ antibiotic standard}}$$

3.5. Determination of Minimal inhibitory concentration [12]

The antimicrobial activity of the plant extract was determined by 2-fold dilution method. The test was performed in 96-well sterile micro plates by diluting 100µl of 2× working solution of plant extracts serially diluted to obtain final concentrations of 100 - 0.75 µg/ml. Then, 10µl of 0.5 McFarland *M. tuberculosis* inoculum was added to all the wells and wells in columns one served as drug-free controls. Micro plate was incubated for 20 days at 37°C under anaerobic jar. Following incubation, 3 µl of Resazurin blue solution was added to all well and was re-incubated at 37°C for 24 h. After this incubation, the oxidation of resazurin indicator were recorded and tabulated.

3.6. Synergistic effect piperine and *C.roseus* by checkerboard assay [13]

The crude Methanol extract of *C.roseus* and Piperine one mg per ml was prepared. For comparison, individual plant extracts were used as control. Two fold serial dilutions of piperine and two fold serial dilutions of *C.roseus* were prepared for every combination tested (100–0.75µg/ml) and 100 µl aliquots of each component was placed into the wells of the sterile 96-well microtiter plate along with 10µl *M.tuberculosis*. The microtiter plates were then incubated at 35°C and MIC was determined after One week of incubation followed by the addition of 3µl of resazurin indicator. The Fractional inhibitory concentration (FIC index) for all the combinations was determined using the following formula,

$$\text{FIC index} = \text{FICA} + \text{FICB} = [\text{A}]/\text{MICA} + [\text{B}]/\text{MICB}$$

3.7. Effect of efflux inhibitors activity of plant extract [14]

To determine the extent of the efflux pump mediated ofloxacin resistance *M. tuberculosis*, MIC levels of ofloxacin and plant extracts were determined using resazurin microtitre assay in the presence or absence of efflux pump inhibitors DNP and verapamil. Stock solution of DNP was prepared in DMSO while verapamil was dissolved in distilled water. The final concentration used in resazurin microtiter assay of verapamil was (5 mg/l) and DNP was (20 mg/l). All experiments were made in replicate.

IV. Results

4.1. Prevalence of ofloxacin resistant of *M.tuberculosis*

Of the 68 suspected samples, 32 samples were found to be AFB positive. *M.tuberculosis* was isolated from the AFB positive samples on LJ media. All the 32 plates showed fried egg colonies. Antibacterial susceptibility of 32 isolates revealed that 18 of them were found to be antibiotic resistant. Among them, 8.75 % resistant to streptomycin and 37.5% were showed high degree of resistance to Ofloxacin (Fig 1).

All these drug resistant isolates were isolated from the samples of age group between 40 to 50. *M.tuberculosis* is frequently isolated pathogenic bacteria from clinical sputum sample. The frequency of *M.tuberculosis* in this study was 47 percentages. Among the isolated *M.tuberculosis* 43.75% were sensitive to ofloxacin and Streptomycin. The use of fluoroquinolones needs to be controlled to sustain the management of MDR TB. Eventhough, development of FQ resistance prior to use of antibiotic has been widely reported [15]. In order to minimize the quinolones and to control MDR TB it's necessary to screen new phytochemical which is most suitable for prophylaxis of TB

4.2. Plant sample analysis

GCMS analysis of methanol extract of *C.roseus flower* showed presence of 14 different components were compared with NIST library. The active principles with their retention time (RT), molecular formula, molecular weight (MW) are represented in Table 1. Furfural, Furfural, 2H-Pyran-2,6 (3H)-dione, Dihydro-3-(2H)-thiophenone are found to be a major constituents. The presence of various bioactive compounds justifies the use of the flower extract for various ailments and isolation of individual phytochemical constituents subjected it to pharmacological biological activity will definitely give fruitful results. *C.roseus rosea* have very rich of phytochemicals specially 130 alkaloids which are potential sources of antimicrobial agents [16]. Piperine (1-piperoyl piperidine) is an amide alkaloid found in plants of Piperaceae family like Piper longum (long pepper), Piper nigrum (blackpepper). piperine was extracted as yellow crystals from *P.nigrum* and confirmed by the addition of vanillin reagent.

4.3. Antibacterial activity of *C.roseus rosea* and Piperine

Studies on antimicrobial activity of tested phytochemicals showed bactericidal activity against *M.tuberculosis*. Antibiogram of *C.roseus* (SJCBO2049) on *M.tuberculosis* showed potent antimycobacterial activity and the maximum zone of inhibition was 22 ± 1.63 mm. Piperine extracted from the *P.nigrum* showed significant antimycobacterial with 20 ± 1.08 mm (Table 2). The MIC of the natural products was determined so that sub inhibitory concentrations could be used to check for efflux pump inhibition. Both the extracted compounds have had minimum inhibitory concentration at same concentration level.

The efficacy on antitubercle activity of phytochemicals have analyzed by calculating relative inhibitory zone of diameter (RIZD). Of these tested compound *C.roseus* showed 133% of RIZD. Survey of literature on *C.roseus* extracts indicted that information on antibiogram against mycobacterium studies is very scarce. Lot of work has been done on *C. roseus* regarding its anticancer and antidiabetic activity, but only a few reports are there for its antitumor [17], antioxidant [18], antimicrobial properties [19]. As an important medicinal plant, antibacterial potential of aqueous extracts of *C.roseus* against *Xanthomonas campestris* also documented [20]. Antimycobacterial activity of Acetone extract of *P.nigrum* was evaluated and reported earlier by Grange and Davey [21]. Similarly potent antimicrobial activity of piperine from *P.nigrum* by acetone and dichloromethane against Gram positive pathogens also been reported [22]

Among different concentrations (100 to 0.75 µg/ml) of tested phytochemicals, The MIC of both tested compounds were found to be 50 µg/ml showed positive inhibitory activity against OFL resistant *M.tuberculosis* (table 3)

4.4. Efflux pump inhibitor assay

Results of the present study conclude that efflux mechanism of ofloxacin resistance in *M. tuberculosis* isolates inhibited by Piperine. The MIC of 16 mg/l ofloxacin was reduced in to 4 mg/l by DNP, 2 mg/l for verapamil and piperine. *C.roseus* did not have any effect on Efflux pump inhibition (table 4) and there are no

changes in the MIC of ofloxacin. It was observed that two fold reduction of ofloxacin by DNP and four fold reduction by verapamil and Piperine. The *in vitro* studies on piperine strongly provide evidence for synergistic and effective efflux pump inhibitor activity against *M.tuberculosis*. Rifampicin in combination with piperine, a *trans-trans* isomer of 1-piperoyl-piperidine, reduced the MIC and mutation prevention concentration (MPC) of rifampicin for *M. tuberculosis* [23]. Inshad et al [24] reported that the piperine activity may be comes from its ability to interfere with DNA and protein synthesis. Efflux pump inhibition activity of phytochemicals were widely studied with different antibiotics [25].in this study, Piperine showed 4 fold reduction of ofloxacin and synergistic activity at 12.5µg/ml. DNP is the proton motive force inhibitors whereas verapamil is a calcium channel blocker for ABC transporters. Gupta et al [26] have also reported the reversal of resistance to all major anti-tubercle drugs in presence of efflux pump inhibitors CCCP and verapamil. But these inhibitors do not change the MICs of FQ for reference strain H37Rv *M.tuberculosis* [27]. According to Jin et al, piperine present in the plant leaves was reported to have inhibition action toward the intrinsic efflux pump system of mycobacteria [28-29]. Piperine increases the membrane permeability [30] and thus, regulates the uptake of drugs across the cell and ensures accumulation of drug in the cells [31].

4.5. Synergistic activity of Piperine and *C.roseus*

Results of checkerboard assay between *C.roseus* and piperine was given in table 5. The Fractional inhibitory concentration (FIC) of piperine was 0.125 and 0.0632 for *C.roseus*. The Fractional inhibitory concentration index (FICI) followed by combinations of piperine and *C.roseus* was 0.06. FICI < 0.5 revealed that both the phytochemicals have synergistic activity against Mycobacterial strain. This concludes both piperine and *C.roseus* extracts were most effective in combination than alone. The bioenhancing property of piperine was first studied against *M.tuberculosis* along with *C.roseus*. Piperine was found to increase the bioavailability of rifampicin by about 60% and hence reduce the dose from 450 to 200mg [32]. Several *in vivo* studies on piperine have shown promising results in bioenhancing capacity of piperine for various drugs [33]. This is the first study on biological synergistic effect of piperine and *C.roseus* combination against *M.tuberculosis*.

V. Conclusion

The bioassay of piperine and *C.roseus* showed potent anti TB activity. In addition, promising new concepts such as the efflux pump inhibitors and synergy between piperine and *C.roseus* has been studied. The current finding encourages us to develop new alternative medicine that includes piperine alone and/or in combination with *C.roseus* to fight against the drug resistance among Drug resistant *M.tuberculosis* strains.

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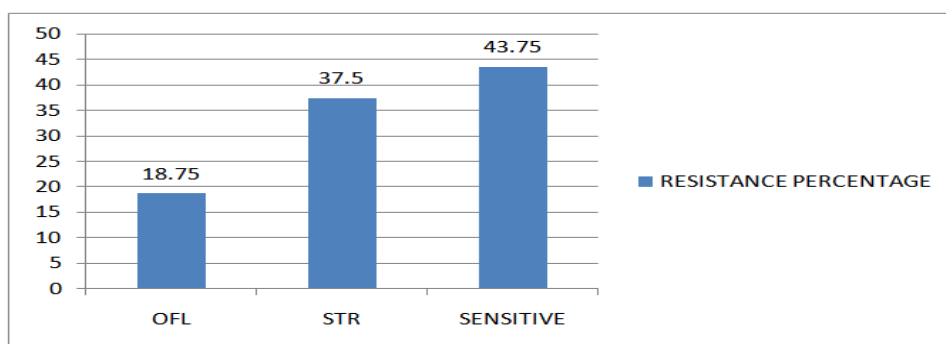


Fig 1. Prevalence of Drug resistance of isolated *M.tuberculosis*

Table 1. GCMS analysis of *C.roseus* flower extract

Fraction no	Peak value/RT/MWT	Library match
	234	Acetamide, 2-diethylamino-N-(1-phenylethyl)-
	86	Butane, 1,2:3,4-diepoxy-,
	96	Furfural
	144	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one
	110	2-Furancarboxaldehyde, 5-methyl-
	98	2-Furanmethanol
	98	6-Oxa-bicyclo[3.1.0]hexan-3-one
	102	Dihydro-3-(2H)-thiophenone
	112	2H-Pyran-2,6(3H)-dione
	90	Dihydroxyacetone
	251	Hydrastininic acid
	205	2-Bromo-4-chloroaniline
	142	2-Furanacetic acid, .alpha.-hydroxy

Table 2. Antimycobacterial activity of *C.roseus* and piperine

Sample	Zone of Inhibition (mm) by stroke method (1 mg/ ml)			
	Methanol extract	Ofloxacin	Methanol	RIZD
<i>Piperine</i>	20 ± 1.08 mm	9 ± 0. 12 mm	10± 0. 15 mm	111
<i>C.roseus</i>	22±1.63 mm	9 ± 0. 12 mm	10± 0. 15 mm	133

Table 3. Microplate Alamar Blue assay for MIC

Concentration(µg/ml)	Blank	100	50	25	12.5	6.25	3.16	1.5	0.75
<i>C.roseus L</i>	-	-	-	+	+	+	+	+	+
<i>Piperine</i>	-	-	-	+	+	+	+	+	+

+, Positive growth -;No growth

Table 4. Efflux pump inhibition of plant extracts

Drug	Concentration of ofloxacin mg/ml									
	64	32	16	8	4	2	1	0.5	0.25	Blank
Ofloxacin	-	-	-	+	+	+	+	+	+	+
Ofloxacin and DNP	-	-	-	-	-	+	+	+	+	+
Ofloxacin and verapamil	-	-	-	-	-	-	+	+	+	+
Ofloxacin and Piperine (50µg/ml)	-	-	-	-	-	-	+	+	+	+
Ofloxacin and <i>C.roseus</i> (50µg/ml)	-	-	-	+	+	+	+	+	+	+

Table 5. Checkerboard assay of *Catharanthus roseus* and Piperine against *M.tuberculosis*

Piperine µg/ml	100									
	50									
	25									
	12.5									
	6.25				0.06*					
	3.16									
	1.5									
	0.75									
	0									
		0	0.75	1.5	3.16	6.25	12.5	25	50	100
	<i>C.roseus</i> µg/ml									

* FICI