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Antibiotic resistance reversal of multiple drug resistant bacteria using *Piper longum* fruit extract

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ABSTRACT

Present investigation was aimed to identify natural products of plant-origin as novel antibiotic resistance reversal agents. Aqueous and methanol extracts of *Piper longum* (fruits) were tested against multiple drug resistant (MDR) clinical isolates of *Enterococcus faecalis, Staphylococcus aureus, Salmonella typhi, Shigella sonnei*, as well as reference-plasmid-harboring strains of *Escherichia coli* (RP4) and *Bacillus subtilis* (pUB110). The crude methanol extract showed significant antibacterial activity with a minimal inhibitory concentration of 400 µg/mL against *Bacillus subtilis* (harboring *pUB110* plasmid). Methanol extract could reverse the antibiotic resistance in clinical isolates of *Shigella sonnei*, with a curing efficiency of 42%. In comparison with methanol extract, aqueous extract showed antibiotic resistance reversal efficiency against wider range of clinical isolates. Aqueous extract showed strong antibiotic resistance reversal activities against R-plasmid harboring strains of clinical origin- *Enterococcus faecalis, Staphylococcus aureus*, *Salmonella typhi* with curing efficiencies of 64%, 50% and 32% respectively. This antibiotic resistance reversal may be attributed to the elimination of R-plasmids as the multiple antibiotic resistance genes are usually located on R-plasmids. Active biomolecules from *P. longum* may prove to be a source to develop MDR reversal agents of natural origin to contain the development and spread of plasmid borne multiple antibiotic resistance.

INTRODUCTION

In recent past, emergence of ever-increasing number of multiple drug resistant (MDR) microbial strains has become a severe health threat to human-kind and one of the biggest challenges to global drug discovery programs (Alanis *et al.,* 2005; Shriram *et al.,* 2010).The inappropriate and over-use of antibiotics to treat microbial infections and consequent antibiotic selection pressure are thought to be the major causative factors contributing to the appearance of strains with reduced susceptibility to antibiotics (Selim, 2012; WHO, 2012).

The problem of explosive escalation of antimicrobial resistance has only been worsened by a steady decrease in the number of new antibiotics introduced in the last 10–15 years (Shriram *et al.,* 2008). Clinical isolates of *Staphylococcus aureus* and *Enterococcus* resistant to oxazolidinone linezolid have been reported, which is considered as a last line of defense against Vancomycin resistant bacterial infections.

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The genetic determinants that confer resistance to antibiotics are mostly located on plasmids (known as R-plasmids). These extra-chromosomal DNA sequences are often transferable to other bacteria in the environment and can be responsible for the emergence of resistance to multiple antibiotics (Schelz *et al.,* 2006).

Plasmid-mediated multidrug resistance is one of the most pressing problems in the treatment of infectious diseases. The use of plasmid-curing agents in combination with antibiotics may serve as a possible way to contain the development and spread of antibiotic resistance encoded by antibiotic resistant R-plasmids.

Looking at the severity of problem and present situation, the scientific community has advocated for the search for new antimicrobial agents (Nitha *et al.,* 2012). However, majority of the plasmid curing agents of synthetic origin such as acridine dyes, ethidium bromide and sodium dodecyl sulfate are unsuitable for therapeutic application due to their toxicity or mutagenic nature. Thus, there is a constant need of identifying novel curing agents that are more effective and non toxic. Herbal medicines have always been a rich source of drug discovery programs and many

plant derived compounds have shown promising activity against MDR bacteria and caused reversal of antibiotic resistance (Belofsky *et al.,* 2004; Beg and Ahmed, 2005; Marquez *et al.,* 2005; Khan *et al.,* 2009; Shriram *et al.,* 2008, 2010).

Piper longum, known as long pepper is a native of north-east India and an important traditional medicinal plant. It is found in various parts of India including evergreen forests from Konkan to Travancore regions of Western Ghats. The fruits of this plant are source of famous traditional drug *Pippali* (Sivarajan and Balachandran, 1994) besides being used as spice and in the manufacturing of pickle.

The plant has tremendous medicinal values and a known curing agent against cough, leprosy, diabetes, piles, cardiac diseases, chronic fever and to improve appetite to name a few (Manoj *et al.,* 2004). Various pharmacological activities including anti-allergy, antibacterial, anti-hepatitis and anti-tubercular have been reported from long pepper. However, we are reporting herein for the first time the reversal of antibiotic resistance by curing of bacterial plasmids containing resistant genes using methanol and aqueous extracts of *P. longum* fruits.

MATERIALS AND METHODS

Plant Materials

Plant material (mature fruits) was obtained from an Ayurvedic shop in Pune and samples were authenticated by Dr. Suresh Jagtap at the Medicinal Plants Conservation Centre (MPCC), Pune, India and a voucher specimen was deposited at MPCC Herbarium (No. MPCC2330) for future reference.

Extraction of Plant Material

Mature fruits were finely powdered with auto-mix blender. One hundred g dry powder of fruits was soaked in 250 mL methanol (Merck, Mumbai, India) and distilled water separately. The crude extract was prepared by cold percolation for 24 h at room temperature (26 ± 2°C). The filtrate was concentrated *in vacuo* at 40°C. This process was repeated three times to get total extract. Last traces of the solvent from the total methanol extract were removed under vacuum to get the black colored crude solid extract.

Table. 1: Bacterial strains and plasmid used.

Bacterial strain	Designation	Plasmid	Phenotype	Source
Enterococcus faecalis (VRE)	MCMB-812	pARI812	Va^r	MCM[a]
Staphylococcus aureus (VRSA)	MCMB-818	pARI818	Va^r, Ro^r, K^r, T^r, A^r	MCM[a]
Salmonella typhi	MCMB-814	pARI814	G^r	MCM[a]
Shigella sonnei	MCMB-815	pARI815	G^r	MCM[a]
Escherichia coli	MTCC-391	RP4	A^r, T^r, K^r	MTCC[b]
Bacillus subtilis	MTCC-1558	pUB110	K^r, N^r	MTCC[b]

[a]MACS Collection of Microorganisms, Agharkar Research Institute, G.G Agarkar Road, Pune 411 004, India; [b]Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh 160 036, India.

A – Ampicillin; G – Gentamycin; K – Kanamycin; N-Neomycin; Ro – Roxithromycin; T- tetracycline; Va- Vancomycin. The antibiotics followed by superscript letter r shows the resistance of bacterial strains to that particular antibiotic.

Bacterial strains

Bacillus subtilis and *E. coli* harboring reference plasmids pUB110 and RP4 were obtained from MACS Collection of Microorganisms (MCM) at Agharkar Research Institute, Pune and Microbial Type Culture Collection (MTCC) Chandigarh, India (Table 1). The clinical isolates were obtained from King Edward Memorial Hospital, Pune, India, and bacterial strains were identified as *E. faecalis, E. faecalis, S. aureus, S. sonnei* and *S. typhi* based on 16S rRNA gene sequence homology at Agharkar Research Institute, Pune (data not shown).

Determination of minimal inhibitory concentration (MIC) and sub-inhibitory concentration (SIC)

The MICs were determined by agar dilution method (Lorian, 1991). Brain heart infusion (BHI) medium (Himedia, Mumbai, India) was supplemented with specified concentration of antibiotic /curing agent. Test bacterial cultures were spot inoculated (10^5cells per spot) on these plates and incubated at 37°C for 24 h. The lowest concentration of antibiotic /plasmid curing agent that inhibited the growth was termed the MIC. The highest concentration of antibiotics / plasmid curing agent that allowed the growth of bacteria was considered as SIC. Ability of the curing agent to cure plasmid was evaluated at SIC.

Antibiotic resistance reversal activity

The curing of plasmid-mediated antibiotic resistance was performed by following Deshpande *et al.* (2001). In brief, the culture was grown in presence of a curing agent at specified concentration for 24 h at 37°C and then plated on BHI agar plates to obtain isolated colonies. Isolated colonies were then replica plated on to BHI agar and BHI agar containing antibiotics. The colonies which grew on BHI agar but failed to grow in presence of antibiotics were considered as putative cured derivatives. The percentage curing efficiency was expressed as number of colonies with cured phenotype per 100 colonies tested. The curing agent was tested up to 1200 µg per mL concentration.

Statistical analysis

All experiments were conducted in triplicate to check the reproducibility of the results obtained. The results are presented as means ± S.E. (standard error) and means were compared using Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$. All the statistical analyses were done by using MSTAT-C statistical software package.

RESULTS AND DISCUSSION

Microbial resistance to antimicrobial agents is usually mediated through resistant gene-coded bacterial plasmids. Plasmids are self-replicating extra-chromosomal DNA molecules found in Gram-negative and Gram-positive bacteria as well as in some yeast and other fungi. These plasmids, called R-plasmids, harbor a variety of genes encoding resistance to a wide spectrum of antimicrobial compounds. Resistant bacterial strains may

develop almost anywhere particularly in a pressurized environment containing previously non-resistant bacterial strains as contaminants. Antibiotic resistance causes great therapeutic and economic burden in the treatment of infectious diseases and it may threaten the success of antimicrobial chemotherapy. It is estimated that antibiotic resistance increase the hospital stay and morbidity rate two-fold (Schelz *et al.*, 2010). Apart from the public health threat, the search for newer microbial-sensitive treatments to overcome resistant microbes is usually very expensive and contributes to the higher costs of health care. Newer treatment regimes use more expensive pharmaceuticals and demand longer hospital stays for infected individuals (NIAID, 2011). The present piece of work may prove to be beneficial for searching novel potential phyto-therapeutic agents against multiple drug resistant bacterial strains and reversal of their plasmid-mediated-resistance.

In the present investigation, from 100 g fruits used each for methanol and aqueous extraction purpose, finally 25.9 g and 5.8 g black-color solid extracts were obtained from methanol and aqueous extracts respectively. These crude extracts were further tested for their potential against multiple antibiotic resistant bacterial strains. The antibacterial activity of both the extracts of *P. longum* was tested against clinical isolates as well as reference strains harboring R-plasmids. MIC and SIC for standard antibiotics (Table 2) as well as both the extracts were determined (Table 3 and Table 4). All the strains used in the present study shown resistance against both the extracts, at a concentration of 1200 µg/mL. However, striking results were observed against *B. subtilis* (harboring *pUB110* plasmid) as methanol extract showed significant antibacterial activity. The MIC for this strain was found to be at 400 µg/mL concentration of methanol extract (Table 3, Fig. 1). These results indicated that methanol extract of fruits of *P. longum* was a potent antibacterial agent against multiple drug resistant *B. subtilis*. Additionally, methanol extract of fruits cured R-plasmids in clinical strains of *S. sonnei*, with a noteworthy curing efficiency of 42% (Table 3, Fig. 2a). One of the most interesting findings of this investigation was that aqueous extract of *P. longum* fruits did not seem to be a very potent antibacterial agent. However, aqueous extract could cure R-plasmids in the strains of clinical origin and consequently reversed the antibiotic resistance of Vancomycin- resistan *E. faecalis* (VRE, 64% curing efficiency, Fig. 2b), Vancomycin-resistant *S. aureus*- pARI818 (VRSA, 50% curing efficiency, Fig. 2c), *S. typhi* -pARI814 (32% curing efficiency, Fig. 2d) (Table 4) at a concentration of 1200 µg/mL. VRE infections in hospitals are very difficult to treat. However, in this study the aqueous extract successfully reversed the plasmid-mediated Vancomycin resistance from clinical isolates of *E. faecalis* (VRE) and *S. aureus* (VRSA) and consequently making the antibiotic treatment significantly more effective (Table 4, Fig. 2). Present results have offered crude extracts of *P. longum* as a new source of safe plasmid curing agent which causes antibiotic resistance reversal. These findings indicated the possibility of a new type of combination between antibiotics and potential drugs effective against plasmid-encoded multiple antibiotic resistance. Identification of a novel curing agent derived from plant is significant, since majority of natural products are non-toxic to human and environment. Previous reports of plant derived curing agents are limited. Plumbagin from *Plumbago zeylanica* cured R-plasmids in *E. coli* (Lakshmi *et al.*, 1987). In another study, the alcoholic extract of *P. zeylanica* cured R plasmid harboring *E. coli* with 14 per cent efficiency (Beg and Ahmed, 2004). Anti-plasmid activity of essential oils was reported by Schelz *et al.* (2006). Our group has earlier reported potential plasmid curing agents from plants including *Dioscorea bulbifera* (Shriram *et al.*, 2008) and *Helicteres isora* (Shriram *et al.*, 2010). In the present investigation, we have shown that the *P. longum* fruit extracts could effectively eliminate the R-plasmids from bacterial as well as reference strains. Spontaneous loss of plasmid has been reported in literature (Trevers, 1986), however, the frequency of spontaneous loss for such plasmids has been known to be less than one in 10^6cells. In comparison, the antibiotic resistance curing efficiencies observed in present study were extremely high. The concentrations of the curing agents used in this study were sub inhibitory, since bacteria were already resistant to these concentrations of compound. It may further be assumed that bacteria are less likely to develop any mechanism to counter the plasmid curing property of crude extracts of *P. longum*. The findings of present study hold importance as there are many known antibiotics that are no longer effective owing to resistant strains of bacteria. Already ineffective antibiotics can be effectively used if R-plasmid-encoded antibiotic resistance is removed from the bacterial population, as proved from the current investigation.

Table. 2: MIC and SIC of antibiotics.

Bacterial strain	Antibiotic	MIC of antibiotic (µg/ml)	SIC of antibiotic (µg/ml)
Enterococcus faecalis(VRE)	Vancomycin	16	8
Staphylococcus aureus (VRSA)	Vancomycin	30	20
Salmonella typhi	Gentamycin	>400	400
Shigella sonnei	Gentamycin	25	15
Escherichia coli (RP4)	Kanamycin	>400	400
Bacillus subtilis (pUB 110)	Kanamycin	100	75

Table. 3: Curing of antibiotic resistance by methanol extract of *P. longum*.

Bacterial strain	MIC (µg/ml)	SIC (µg/ml)	% Curing efficiency (Mean ± S.E.)	Antibiotic resistance cured
Enterococcus faecalis (VRE)	> 1200	200	ND*	-
Staphylococcus aureus (VRSA)	> 1200	200	ND*	-
Salmonella typhi	> 1200	200	ND*	-
Shigella sonnei	> 1200	200	42 ± 1.5	Gentamycin
Escherichia coli (RP4)	> 1200	200	ND*	-
Bacillus subtilis (pUB 110)	400	200	ND*	-

ND: Not detected. *None of the 100 colonies tested showed phenotypic loss of antibiotic resistance. MIC: Minimal inhibitory concentration; SIC: Sub inhibitory concentration

Table. 4: Curing of antibiotic resistance by aqueous extract of *P. longum*.

Bacterial strain	MIC (µg/ml)	SIC (µg/ml)	% Curing efficiency (Mean ± S.E.)	Antibiotic resistance cured
Enterococcus faecalis (VRE)	> 1200	1200	64 ± 2.1^{c}	Vancomycin
Staphylococcus aureus (VRSA)	> 1200	1200	50 ± 1.8^{b}	Vancomycin
Salmonella typhi	> 1200	1200	32 ± 1.0^{a}	Gentamycin
Shigella sonnei	> 1200	1200	ND*	-
Escherichia coli (RP4)	> 1200	1200	ND*	-
Bacillus subtilis (pUB 110)	> 1200	1200	ND*	-

ND: Not detected. *None of the 100 colonies tested showed phenotypic loss of antibiotic resistance.
MIC: Minimal inhibitory concentration; SIC: Sub inhibitory concentration
Means within the column for curing efficiency followed by different superscript letters were significantly different from each other according to Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$.

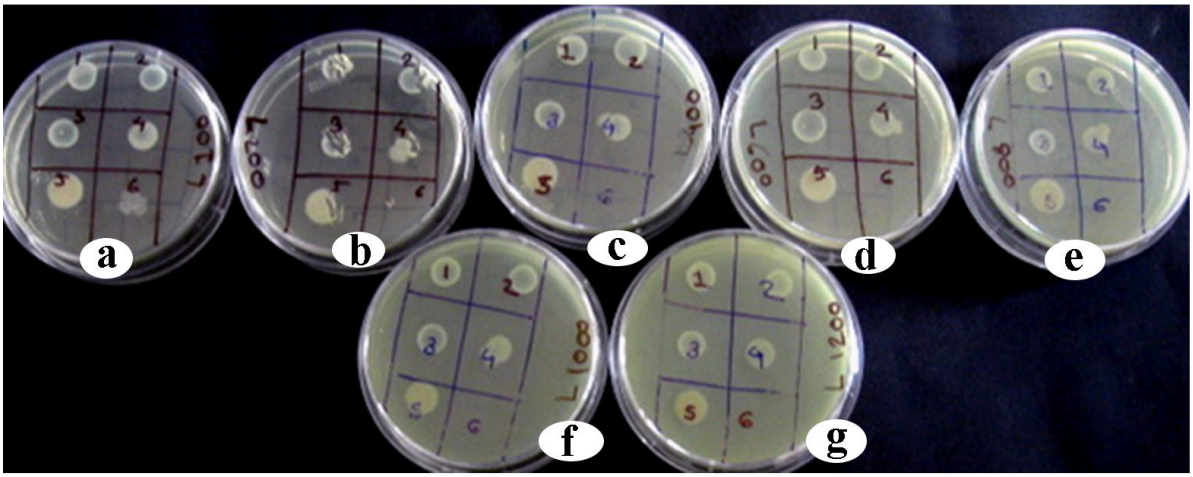

Fig. 1: MIC and SIC of methanol extract of *P. longum* fruits with varying concentrations of 100, 200, 400, 600, 800, 1000 and 1200 µg/mL (a-g).
Block 6 showing the MIC of *B. subtilis* pUB110 at 400 µg/Ml (c). The colonies were picked from plate showing in figure 1b and were used for further curing experiments.

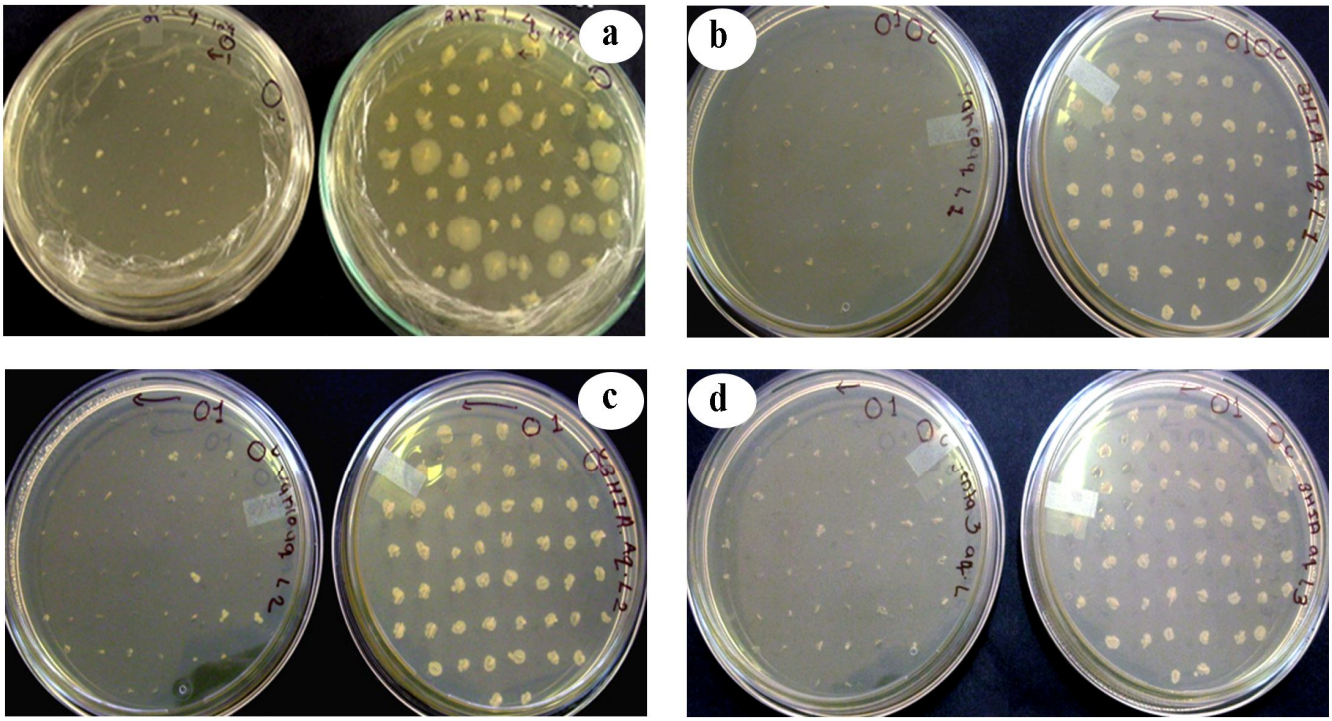

Fig. 2: Reversal of antibiotic resistance by methanol (a) and aqueous (b-d) extracts of *P. longum* fruits following replica plating. 2a. curing of S. sonnei resistance against Gentamycin; 2b. curing of VRE E. faecalis resistance against Vancomycin; 2c: curing of *S. aureus* resistance against Vancomycin and 2d. curing of *S. typhi* resistance against Gentamycin .

CONCLUSION

It can be concluded from the present results that aqueous and methanol extracts successfully reversed the multiple antibiotic resistance in cured derivatives making them sensitive to antibiotics. This antibiotic resistance reversal may be attributed to the curing of R-plasmids harbored by these MDR bacterial strains of clinical origin. These results indicate that *P. longum* may be a potential source to isolate pure compounds for containment of spread of plasmid-borne multiple antibiotic resistance**.**

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