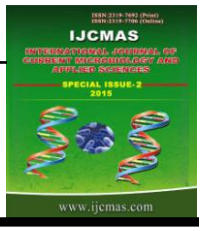
Int.J.Curr.Microbiol.App.Sci (2015) Special Issue-2: 187-198

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Special Issue-2 (May-2015) pp. 187-198**
http://www.ijcmas.com



Original Research Article

Reversal of Plasmid Encoded Antibiotic Resistance from Nosocomial Pathogens by Using *Plumbago auriculata* Root Extracts

R. B. Patwardhan[1]*, P.S. Shinde[2], K. R.Chavan[2] and Anushka Devale[2]

[1]Department of Microbiology, Haribhai. V. Desai college of Commerce, Arts and Science, (Affiliated to Pune University), Maharashtra State, Pune-411002, India
[2]Department of Microbiology, Modern College of Commerce, Arts and Science, Shivajinagar, Maharashtra State, Pune-411005, India
**Corresponding author*

Keywords

Nosocomial pathogens, Multiple Drug Resistance, *Plumbago auriculata*, Plasmid curing

A B S T R A C T

Multiple drug resistance (MDR) is a serious and emerging problem in treatment of diseases caused by nosocomial pathogens. Most of the bacteria harbour plasmids and show resistance to multiple antibiotics. Plasmid elimination can be one of the ways to make the antibiotic resistant clinical strains sensitive. In the present investigation 23 nosocomial pathogens like *Pseudomonas aeruginosa, Proteus vulgaris, Escherichia coli, Klebsiella pneumoniae* were isolated and identified from clinical samples from Bharati Hospital, Pune, Maharashtra. Root extracts of *Plumbago auriculata* were used to cure plasmid mediated antibiotic resistance. The plasmid curing activity of *Plumbago* extracts was determined by growth of organisms in presence of root extract. The physical loss of plasmid DNA was confirmed by agarose gel electrophoresis. The present investigation revealed the broad spectrum antibacterial activity and plasmid curing potential (13–32 %) of *Plumbago auriculata* root extracts. So, that *Plumbago auriculata* plant root extract can be used in combination with antibiotics to prevent the development and spread of antibiotic resistance in treatment of nosocomial infections.

Introduction

Plasmid-mediated multidrug resistance is one of the most upcoming problems in the treatment of infectious diseases, as bacteria have reached the resistance to most of the antibiotics that are available for treatment. Plasmids are extrachromosomal pieces of double stranded DNA which multiply independently inside the bacteria (Trevors *et al.,* 1985). Antibiotic resistance in bacteria may be an inherent trait of the organism that renders it naturally resistant, or it may be acquired by means of mutation in its own DNA or acquisition of resistance conferring DNA from another source (Nafiseh *et al.,* 2010). The nosocomial infections are caused by a variety of organisms, including methicillin resistant *Staphylococcus aureus,* penicillin resistant *Pneumococci*, vancomycin resistant *Enterococci*, and many antibiotic resistant Gram negative bacteria.

One of the ways to minimize plasmid transmission of antibiotic resistance is to eliminate the plasmids. This process is known as plasmid curing and many compounds have been shown to be capable of causing this effect (Jayasimha *et al.,* 2014). Majority of the plasmid curing agents are of synthetic origin includes acridine dyes, ethidium bromide and sodium dodecyl sulphate, acridine orange, acriflavine are unsuitable due to their toxicity or mutagenic nature (Shriram, *et al.,* 2010). There is constant need of developing novel curing agents which are more effective and at the same time non toxic. So, the solution to this problem would be the use of plant derived secondary compounds which would show promising activity against multiple-drug resistant bacteria and cause reversal of antibiotic resistance the naturally occurring quinones constitute a major class of bioactive compounds which can serve as potential plasmid eliminating agents (Shriram *et al.,* 2010). Various herbal extracts from plants like *Cinnamomum verum, Zingiber officinale, Nigella sativa, Piper nigrum, Plumbago zeylenica,* etc. containing phenol (eugenol), tannins, flavonoids, terpenoids, napthoquinones, alkaloid, saponin could be used for plasmid curing (Ibtisam *et al.*, 2011).

The family *plumbaginaceae* is distributed as a weed in before tropical tropical and subtropical countries of the world. It consists of 10 genera and 280 species. The genus *Plumbago* includes 3 species, namely *Plumbago indica L., P. auriculata* (*P. capensis L.*), and *P. zeylanica* which are distributed in India. *P. auriculata* belongs to *Plumbaginaceae* family present in all over India. Plumbagin is naturally occurring yellow coloured pigment (2-methyl-5-hydroxy-1, 4 napthoquinone) mainly present in roots of *Plumbago* which possess antimicrobial activity (Patwardhan *et al.,* 2015). The use of plasmid-curing herbal extract of *Plumbago* plant in combination with antibiotics could be used to inhibit the development and spread of antibiotic resistance encoded by antibiotic resistant R-plasmids.

Materials and Methods

Successive extraction by Soxheletion

Plumbago auriculata roots (in powder form) were extracted by successive extraction by using Soxhelet apparatus with petroleum ether, chloroform, acetone and ethanol at boiling temperature for the treatment of 24 hours with each solvent.

Solvents were used according to eluotropic series based on polarity. The extracts were filtered and concentrated to dryness under reduced pressure on a rotary evaporator. After evaporation of the solvents, extracts were dissolved in 10 ml DMSO and evaluated for their antibacterial potential.

Microbial strains and culture conditions used

Nosocomial pathogens from clinical samples (urine, pus) were obtained from the Department of Microbiology, Bharti Hospital, Pune (India) and identified on the basis of morphological, cultural and biochemical characteristics according to the Bergey's Manual of Systematic Bacteriology (Kloos and Schleifer, 1986).

Chemicals and media

Antibiotic powders were obtained from Parke-Davis, Ltd. Mumbai, India. Antibiotic discs, Luria agar/broth, Muller Hinton agar/broth were purchased from Hi-Media Lab Ltd, Mumbai, India.

Determination of antibacterial activity by disc diffusion

Antibacterial susceptibility testing was performed by the Kirby - Bauer disc diffusion method according to clinical laboratory standards Institute guidelines (CLSI). About 10^5 cells from overnight grown culture were spread on Luria agar plates. Polydiscs containing antibiotics (Don Whitley Scientific Equipments, Mumbai, India) were placed on the plates. The zones of inhibition around the antibiotic discs were measured after incubation at 37°C for 24 hr. The cultures were assigned as resistant or sensitive by using CLSI standards..

Minimal inhibitory concentration (MIC) of antibiotics

MIC of each culture was performed at different antibiotic concentrations employing double dilution agar plate method and concentration range for each antibiotic used was 1–1024 µg / ml (Dhakephalkar and Chopade *et al.,* 1994). Plates of different antibiotic concentration were prepared using 20 ml of sterile molten Mueller Hinton agar. 2µl of 16 hrs old culture, O.D. of which was adjusted to 0.5 McFarland units (10^5 cells) was spot inoculated on the plates which were incubated at 37°C for 16–18 hrs. The development of colonies on the plates indicated resistance to the antibiotic added. MIC was interpreted as the lowest concentration of the antibiotic showing complete inhibition of growth.

Antibacterial activity MIC by E test

The Modified E-test (Ingroff *et al.,* 1996) has been developed to provide a direct quantification of antibacterial susceptibility by using single plate. It consists of a predefined, continuous, and exponential gradient of antimicrobial agent concentrations on the discs. A modified E test was used to determine MIC of active compound. Bacterial strains of *S. aureus, E. coli, P. aeruginosa* and *K. pneumoniae* were tested against the active compound by modified E test on Mueller Hinton Agar in petri plates. Bacterial isolates were grown overnight on nutrient agar at 37°C. O.D. was adjusted to 0.5 McFarland unit (10^5 cells/ml). The plates were inoculated by dipping a sterile swab into the cell suspension and spreading it across the surface of the agar in all three directions. The discs containing varying concentrations of active compound (250–1600 µg / ml) were placed on the plate in ascending order. The plates were incubated at 37°C and read after 48 h. The MIC of active compound root extracts was recorded as the drug concentration at the point where dense colonial growth intersected the disc.

Plasmid isolation was performed by Kado & Liu (1981) method and plasmid DNA isolation was detected by horizontal agarose gel electrophoresis by Sambrook & Maniatis (1989) method.

Curing of antibiotic resistance genes on plasmid

The plasmid curing was performed by the method given by Deshpande *et al.* (2001). The culture was grown in the presence of a curing agent at the specified concentration (250-4000 µg / ml) for 24 hr at 37°C and then plated on Luria agar plates to obtain well isolated colonies. The isolated colonies were inoculated on Luria agar plate to get the master plate. The colonies from master plate were then replica plated on Luria agar containing antibiotics. The colonies formed on Luria agar master plate and failed to grow in presence of a particular antibiotic were considered as putative cured

derivatives. Plasmid curing was performed in strains *P. aeruginosa*, *E. coli*, *K. pneumonia* and *P. vulgaris*. The percentage curing efficiency was expressed as number of colonies with cured phenotype per 100 colonies tested. The physical loss of plasmid in the cured derivative was confirmed by agarose gel electrophoresis of the plasmid DNA preparation of respective cultures. Antibiotic sensitive cured colonies were also tested for loss of resistance to antibiotics by using polydiscs. The experiment was performed in duplicate.

Results and Discussion

Twenty three bacterial strains were isolated from clinical specimens like urine and pus. These strains were identified as *Escherichia coli* (6), *Klebsiella pneumoniae* (5), *Pseudomonas aeruginosa* (6), *Staphylococcus aureus* (4) *Proteus vulgaris* (1) and *Enterobacter cloacae* (1) (Table 1) Maximum strains (18) were isolated from urine sample. Antibiotic resistance profile studied by polydisc diffusion method revealed that majority of bacterial isolates were found to be resistant to multiple antibiotics (Table 2).

Root extracts of *Plumbago auriculata* used extensively in alternative medicines were tested for their ability to inhibit bacterial strains of clinical origin. Table 3 describes broad spectrum antibacterial activity of organic extracts of *Plumbago auriculata* roots in different solvents like ethanol, chloroform, acetone and petroleum ether. Strong antibacterial activity of *Plumbago* extracts was observed against *Proteus vulgaris* and *Klebsiella pneumoniae* followed by *E. coli* in ethanol extract. However, Plumbago root extracts showed less activity against *Pseudomonas aeruginosa*. Crude ethanol root extract demonstrated higher antibacterial activity compared to chloroform, acetone or petroleum ether root extracts. Thus, the present study demonstrates broad spectrum antibacterial activity of *Plumbago auriculata* root extracts.

P. auriculata root extract contains a wide range of naphthoquinones including plumbagin (Table 4). Antimicrobial activity of plumbagin had been previously reported (Hook et al., 2014).

Plasmid curing activity of these extracts in different solvents was tested against R-plasmids in between multi resistant clinical isolates (Table 6). Among all four extracts tested, *P. auriculata* ethanol root extract demonstrated maximum plasmid curing activity than petroleum ether, chloroform and acetone extracts against all multidrug resistant clinical isolates tested. *P. auriculata* root extracts cured plasmids in *P. aeruginosa* with 13%, E. coli with 15%, *P. vulgaris* with 32% and *K. pneumoniae* with 30% curing efficiencies. Plasmid curing was also performed with acridine orange a conventional plasmid curing agent and plumbagin; which is one of the main naphthoquinone of *P. auriculata* (Table 7). It was found that plasmid curing efficiency in all the clinical nosocomial pathogens by using crude root extract of *P. auriculata* was found to be more as compared to plumbagin and acridine orange.

Maximum curing efficiency was found in *P. vulgaris* (32%). Acridine orange was unable to eliminate plasmid from *P. aeruginosa*. After plasmid elimination all cured nosocomial strains were tested for their antibiotic susceptibility pattern by using antibiotic polydiscs. They were found to be more sensitive to the different antibiotics tested than wild type non cured strains. It was confirmed by results of replica plate technique (Table 5; Fig. 1). Agarose gel

electrophoresis showed absence of plasmids in the cured derivatives.

Today huge emergence of multi-drug resistant (MDR) nosocomial pathogenic bacteria is major problem throughout the world. There is an urgent need to discover new therapeutics that would be effective against MDR nosocomial pathogens. The present research indorsed that herbs with unique secondary metabolites like naphthoquinones could be considered as potential candidates for developing new antimicrobial drugs.

Microbial resistance to antibiotics is usually mediated through resistant gene encoded bacterial plasmids. Majority of conventional plasmid curing agents of synthetic origin 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. (Khderet.al, 239010) The present results have offered *Plumbago auriculata* as a new and safe plasmid curing agent.

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). The present piece of work may prove to be beneficial for searching novel potential phyto-therapeutic plasmid curing agents against multiple drug resistant bacterial strains and reversal of their plasmid-mediated-resistance.

There are previous reports of anti-inflammatory and antioxidant activity of *Plumbago auriculata* (Dorni *et al.,* 2006). Antimicrobial activity of *P. zeylanica* root extracts has been reported (Patwardhan *et al.,* 2015) but there are no previous reports on antibacterial activity of this *P. auriculata*. We report here for the first time the broad spectrum antibacterial activity of *Plumbago auriculata* against pathogenic Gram negative bacteria *Proteus vulgaris, Klebsiella pneumoniae, E. coli* and *Pseudomonas aeruginosa*. Few reports have appeared in recent years about the reversal of antibiotic resistance in bacteria after treatment with certain plant extracts (Lee *et al.,* 1998). However, such activity for root extracts of *P. auriculata* is reported for the first time. In the present study plasmid curing by *P. auriculata* root extracts is at much higher frequency (13 – 32 %), therefore it can be a genuine plasmid curing agent. Cured derivatives were found to be more sensitive than wild type strains which was proved by replica plate technique. Agarose gel electrophoresis revealed presence of plasmids in wild type strain but absence of plasmids in the cured derivative which was the physical confirmation of plasmid curing effected by *P. auriculata* root extracts.

These finding resulted in 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 nontoxic to humans and environment.

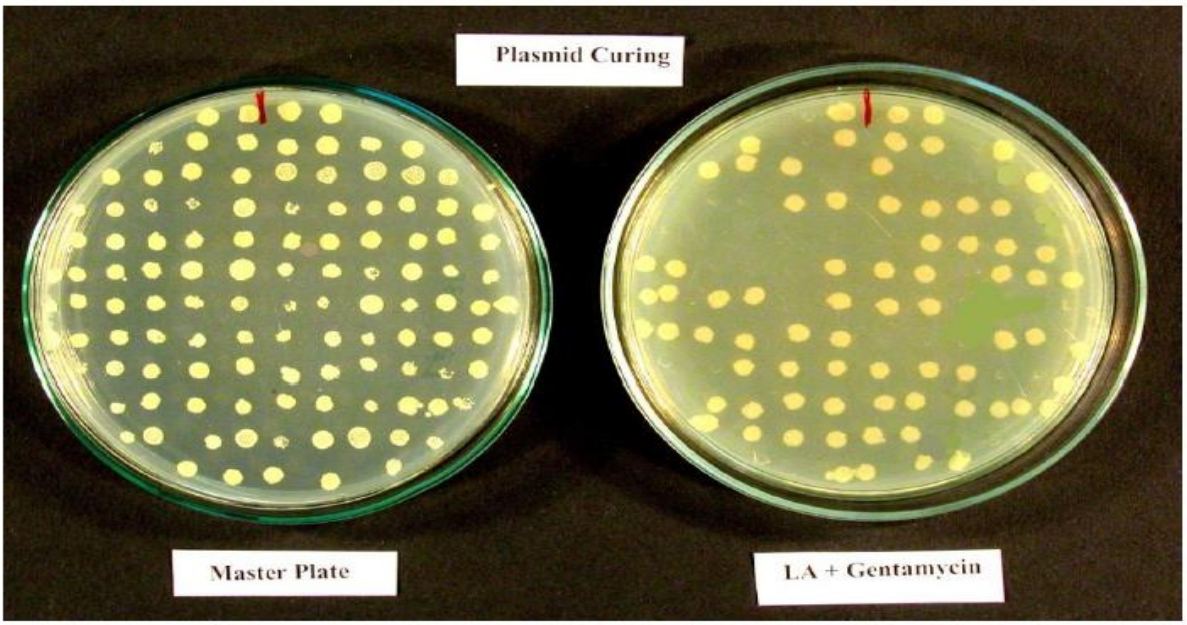
Previous reports of plant derived curing agents are limited. *Plumbago auriculata* plant contains terpenoids, napthoquinones and other secondary metabolites. In the present investigation, we have shown that the crude extract of *Plumbago auriculata* roots could effectively eliminate R-plasmids from Gram-negative strains of clinical origin. The frequency of spontaneous loss for such plasmids has been known to be less

than one in 10^6 cells. In comparison, the antibiotic resistance curing efficiencies observed in present study were extremely (13-32 %) after high. Plumbagin from *Plumbago auriculata* cured R-plasmids in *E. coli* (Lakshmi *et al.*, 1987).

Table.1 Bacterial strains used

Strain	Source
Pseudomonas aeruginosa (6)	Urine
Proteus vulgaris (1)	urine
Escherichia coli (6)	Pus
Klebsiella pneumoniae (5)	Urine
Enterobacter cloacae (1)	Urine
Staphylococcus aureus (4)	Urine

Fig.1 Plasmid curing in *Proteus vulgaris* with *P. auriculata* ethanol root extract: Replica Plate technique



A:Master plate-Luria agar B :Replica plate-Luria agar with gentamycin

Table.2 Determination of degree of antibiotic resistance in clinical pathogenic bacteria

Antibiotic	**% strains showing antibiotic resistance**					
	Enterobacter	***Pseudomonas aeruginosa***	***Escherichia coli***	***Klebsiella pneumoniae***	***Proteus vulgaris***	***Staphylococcus aureus***
β lactam						
Penicillin	100%	25%	25%	25%	25%	25%
Ampicillin	100%	25%	75%	80%	75%	50%
Amoxicillin	100%	75%	100%	75%	75%	25%
Piperacillin	100%	25%	100%	50%	75%	75%
Cefotaxime	100%	100%	100%	75%	50%	25%
Ceftazidime	100%	100%	100%	100%	100%	25%
Ceftriazone	100%	100%	100%	80%	100%	25%
Cefuroxime	100%	100%	100%	100%	100%	25%
Aminoglycosides						
Amikacin	100%	25%	60%	40%	100%	25%
Gentamycin	100%	25%	25%	40%	100%	25%
Streptomycin	100%	25%	25%	25%	100%	50%
Tobramycin	100%	25%	40%	60%	100%	25%
Quinolones						
Ciprofloxacin	100%	75%	60%	80%	100%	25%
Lomefloxacin	100%	25%	25%	25%	100%	25%
Nalidixic acid	100%	25%	25%	25%	100%	25%
Norfloxacin	100%	50%	60%	40%	100%	25%
Ofloxacin	100%	25%	25%	25%	100%	25%
Sparfloxacin	100%	25%	25%	25%	100%	50%
Tetracyclines						
Doxycycline	100%	25%	25%	50%	100%	25%
Tetracycline	100%	25%	25%	25%	100%	50%
Phenolics						
Chloramphenicol	100%	25%	25%	25%	100%	25%
Others						
Erythromycin	100%	25%	25%	25%	100%	25%
Rifampicin	100%	25%	50%	25%	100%	50%
Polymyxin B	—	—	20%	25%	100%	—

Zone diameter interpretive standards were used as per National Committee for Clinical Laboratory Standards (NCCLS, 2002) CLSI.

-: Culture sensitive to the antibiotic

Table.3 Antibacterial activity of *P. auriculata* root extract

Test organism	Inhibition zone diameter (mm) of *P. auriculata* root extract in																			
	Ethanol (µg/ml)					Chloroform (µg/ml)					Acetone (µg/ml)					Petroleum ether (µg/ml)				
	250	500	1000	2000	4000	250	500	1000	2000	4000	250	500	1000	2000	4000	250	500	1000	2000	4000
P. aeruginosa	—	—	—	7.4	9.5	—	—	—	5.4	7.2	—	—	—	3.6	8.3	—	—	—	6.2	8.9
P. vulgaris	—	14	17.6	19	22.4	—	12.8	15.4	17.3	20.4	—	13.3	14.6	18	20.8	—	15.1	16.6	17.1	19.3
E. coli	—	—	11	12.4	13.6	—	—	10.3	11.7	12.8	—	—	9.7	12.7	13.2	—	—	10.2	11.4	12.7
K. pneumoniae	—	15	16.8	18.6	21.7	—	14	14.8	15.6	16.9	—	12.3	12.8	14.6	17.5	—	13	15.8	17.6	20.7

-: No zone of inhibition at the bottom of table

Table.4 Chemical constituents of *Plumbago auriculata*

Class	Name of the compound	Reference
Napthoquinones	Plumbagin, 3-O- methyldroseron, Isoshinalone,6- hydroxyl plumbagin, Chitranone, Maritinone epi-isoshinanolone, 5 hydroxy 1,4 naphthoquinone	Hook. et al (2014)
Steroids	Sitosterol and 3-*O*-glucosylsitosterol, Plumbagic and Palmitic acids	De Paiva et al (2005)
Terpenoids	Lupeol, Lupeol acetate	De Paiva et al (2005)

Table.5 Antibiotic resistance pattern of bacterial strains before and after curing by *Plumbago auriculata*

Antibiotics	Zone of diameter (mm) Before And After Curing							
	Pseudomonas		*Proteus vulgaris*		*Escherichia coli*		*Klebsiella pneumoniae*	
	Before Curing	After Curing	Before Curing	After Curing	Before Curing	After Curing	Before Curing	After Curing
GEN	R	R	R	29	R	9	R	R
CL	18	23	R	R	16	29	R	R
NA	R	R	R	R	R	17	R	R
LE	R	R	22	36	11	22	13	33
COT	16	30	R	R	R	R	R	R
CTR	17	32	25	38	R	R	R	29
SPX	15	32	R	R	R	R	R	32
AK	R	R	R	27	R	12	15	27
CIP	24	39	R	R	R	R	R	33
IPM	R	R	21	36	R	R	R	R
TOB	R	R	26	42	R	R	R	R
K	R	R	23	31	R	R	R	25
S	R	R	19	34	R	R	R	R
CPD	R	R	R	20	R	R	R	R
TI	R	R	R	20	R	R	R	R
MO	R	R	R	R	R	R	15	34
NX	R	R	R	R	R	R	15	35
GAT	R	R	R	R	R	R	15	34

R : Resistant at the bottom of table

Table.6 Plasmid cuing by root extracts of *Plumbago auriculata* by employing successive extraction

Strain	Antibiotic resistance markers	Ethanol extract of *P. auriculata*	
		Conc. (µg/ml)	Efficiency of curing (%)
P. aeruginosa	CL, COT, CTR, SPX, CIP	>1000	13
P. vulgaris	IPM, TOB, LE, K, GEN, S, CTR, CPD, TI	500	32
E. coli	GEN, CL, NA, LE, AK	1000	15
K. pneumoniae	CIP, MO, SPX, LE, NX, K, GAT, AK, CTR	500	30

CL: Colistin, COT: Co-trimoxazole, CTR: Ceftriaxone, SPX: Sparfloxacin, CIP: Ciprofloxacin, AK: Amikacin, GEN: Gentamicin, LE: Levofloxacin, NA: Nalidixic acid, IPM: Impinem, TOB: Tobramycin, K: Kanamycin, S: Streptomycin, CPD: Cefpodoxime, TI: Ticarcillin, MO: Moxifloxacin, NX: Norfloxacin, GAT: Gatifloxacin. Successive extraction of *Plumbago auriculata* roots powder with solvents of increasing polarity was performed. Growth from SIC was serially diluted and plated on Luria agar to get isolated colonies. 100 colonies were replicated on Luria agar and Luria agar containing antibiotic (10µg/ml). Colonies that grew on Luria agar but not on Luria agar containing antibiotic were considered as cured colonies.

Table.7 Curing of R-plasmids from clinical isolates by acridine orange, plumbagin and *Plumbago auriculata* root extracts

Bacterial Strain	Acridine Orange		Plumbagin		Ethanol root extract	
	SIC (µg/ml)	% curing efficiency	SIC (µg/ml)	% curing efficiency	SIC (µg/ml)	% curing efficiency
P. aeruginosa	1000	—	500	5	>1000	13
P. vulgaris	250	19	125	30	500	32
E. coli	250	8	250	11	1000	15
K. pneumoniae	500	18	250	26	500	30

* SIC _ Subinhibitory concentration (µg/ml) used in plasmid curing
Total number of clones tested was 100.

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 made effective if R-plasmid-encoded antibiotic resistance is removed from the bacterial population, as proved from the current investigation. The antibiotic resistance may occur due to mutations. Mutagenic activity of the compound can be harmful especially in clinical applications. The loss of antibiotic resistance was due to loss of plasmid-encoded genes and not due to mutations, which was confirmed by the physical loss of plasmid observed in agarose gel electrophoresis. The concentrations of the crude extract used in this study were sub inhibitory. Since bacteria were already resistant at these concentrations they are less likely to develop resistance against it.

The antibiotic resistance reversal by *P. auriculata* would be attributed to the curing of R-plasmids harbored by these MDR nosocomial bacterial strains of clinical origin. The root extracts of *P. auriculata* would be a possible source to cure antibiotic resistance in multi-drug resistant strains of microorganisms from community as well as hospital settings. Many of the ineffective antibiotics could be used again if used in combination with such curing agent. *P. auriculata* is used in traditional system of Indian medicine in the treatment of neurological disorders like Parkinson's disease, Alzheimer and Huntington's disease. So it cannot be toxic to humans. Therefore *P. auriculata* extracts as curing agent would be better than hazardous curing agents like ethidium bromide, acridine orange, SDS etc. It can be concluded from the present investigation that root extracts of *P. auriculata* successfully reversed the multiple antibiotic resistance in cured derivatives making them sensitive to antibiotics. Thus, such root extracts as plasmid curing agents have tremendous advantage in the treatment of nosocomial infections.

References

Adel. K. Khder, and Srwa. A. Muhammed. 2010. Potential of Aqueous and Alcohol Extracts of Quercus infectoria, Linusmus itatissium and Cinnamomum zeylanicium as Antimicrobials and Curing of Antibiotic Resistance in E. Coli Current Research Journal of Biological Sciences 2(5): 333-337, 2010 ISSN: 2041-0778

Akter, S., Rafiq-Un-Nabi, F. Ahmed. Rupa, Md. L. Bariand and M. A. Hossain. Antibiotic resistance and plasmid profiles in bacteria isolated from market-fresh vegetables. Agriculture, Food and Analytical Bacteriology.

De Paiva Selma Ribeiro, Maria Raquel Figueiredo. and Maria Auxiliadora Coelho Kaplan 2005. Isolation of secondary metabolites from roots of *Plumbago auriculata* by counter current chromatography Phytochemical Analysis Volume 16, Issue 4, (2005)

Deshpande., NM, Dhakephalkar. PK, and Kanekar PP.2001. Plasmid mediated dimethoate degradation in Pseudomonas aeruginosa MCMB-427. Lett App Microbiol 2001 ; 33 : 275-279.

Dhakephalkar., P.K. & Chopade, B.A. 1994 High levels of multiple metal resistance and its correlation to antibiotic resistance in environmental isolates of Acinetobacter. BioMetals 7, 67-74.

Dorni, C.A., Vidyalakshmi, K.S. Vasanthi, H.R, Rajamanickam, G.V and Dubey,G.P. 2006. Anti-inflammatory activity of Plumbago capensis. Pharmacognosy magazine, 2(8):239-243.

Gabriella. Spengler., Annamária. Molnár, Zsuzsanna. Schelz, Leonard. Amaral, Derek. Sharples, and Joseph. Molnár. 2006. The Mechanism of plasmid curing in bacteria. Current Drug Targets.

Gamal Mohamed Elsherbiny., Curing of Plasmid - Mediated Antibiotics Resistance in Multi-Drug Resistant Staphylococcus sp. The international journal of research publications.

Hayfaa. M. Elias., Marwan. Khalil. Qader and Wasan. M. Salih. 2013. Determination of plasmid DNA role in multidrug resistant Pseudomonas aeruginosa clinical isolates. International Journal of Microbiology and Immunology Research Vol. 1(8), pp. 080-086.

Hêro. F., and Salah. Akrayi. 2012. Antibacterial effect of seed extracts of Cardamom (Elettaria Cardamomum) against Staphylococcus aureus And Proteus mirabilis. Tikrit Journal Of Pure Science 17 (2) ISSN: 1813 – 1662.

Hook., C. Mills and H.Sheridan.2014. studies in natural products chemistry. Volume 41.

Ingroff AE., Pfaller M, Erwin ME and Jones RN. (1996). Interlaboratory evaluation of E test for testing antifungal susceptibilities of pathogenic yeasts to five antifungal agents by using Casitone agar and solidified RPMI 1640 medium with 2% glucose. Journal of Clinical Microbiology .34(4): 848-852.

John. S. Wolfson., and David. c. Hooper.1985. The fluoroquinolones: structures, mechanisms of action and Resistance, and spectra of activity in vitro. Antimicrobial Agents And Chemotherapy, P. 581-586 Vol. 28, No. 4

Kado C. L., S. T. Liu. 1981: Rapid procedure for detection and isolation of large and small plasmids. Journal of Bacteriology, Mar. 1981, P. 1365-1373 Vol. 145, No. 3.

Kloos W. E., and Schleifer. K. H. Genus IV Staphylococcus Rosenbach. 1884, 18AL. In Bergey's Manual of Systematic Bacteriology, Sneath PHA, Mair NS, Sharpe ME, Holt JG, Editors. Baltimore: Williams and Wilkins. U.S.A, 1986 p 1013-1035.

Lee CK., Kin KH, Moon. KH and Shun. K. H. 1998. Screening and isolation of antibiotic resistance inhibitors from herb materials – resistance inhibition of

volatile components of Korean aromatic herbs. Arch Pharm Res; 21 : 62-66.

Michael. A. Kohanski., Daniel. J. Dwyer and James. J. Collins. 2010. How antibiotics kill bacteria: from targets to networks. Nat Rev Microbiol. 8(6): 423–435. doi:10.1038/nrmicro2333

Nafiseh. Karoubi., G. Sairamalinga. Reddy, K. Ramesh. Jayasimha and Rayalu. Daddam. Analysis of antibiotic resistant genes in bacteria. International Journal of Analytical, Pharmaceutical and Biomedical Sciences.

National Committee for Clinical Laboratory Standards (now Clinical and Laboratory Standards Institute, CLSI). 1997. Methods for dilution antimicrobial susceptibility testing for bacteria that grows aerobically. Approved Standards M7-A4. Villanova, PA: NCCLS.

Nisha. Sharma., and Purshotam. Kaushik. Medicinal. 2014. Biological and Pharmacological Aspects of Plumbago zeylanica (Linn.). Journal of Pharmacognosy and PhytochemistryS 3(4): 117-120

Patwardhan, R. B., P. K. Dhakephalkar and B.A. Chopade. 2015. Antibacterial and plasmid curing activities of root extracts of Plumbago zeylanica. International Journal of Herbo Medica Vol. - 2 (1) 13 - 25.

Renuga, G., and A. Babuth and Apani. Evaluation on anti-microbial potential of Root extracts Plumbago zeylanica L against Human intestinal microflora. International Journal of Pharmaceutical and Biological Research (IJPBR).

Ruiz-Barba, J. L., J. C. Piard, and R. Jimhez. Diaz. 1991. Plasmid profiles and curing of plasmids in Lactobacillus plantarums trains isolated from green olive fermentations. Journal of Applied Bacteriology, 71, 417-421.

Sambrook J., Fritsch EF and Maniatis T. 1989 Plasmid Vectors. In: Molecular Cloning: A Laboratory Manual 2nd Edition. Cold Spring Harbour: CSH Press. p.1.1-1.109.

Sanjana. Dattaand. Mishra., and R. N. Plumbago zeylinica Linn. (Chitrak) - Review as Rasayan (Rejuvenator / Antiaging). International Journal of Research in Pharmaceutical and Biomedical Sciences ISSN: 2229-3701

Schelz Z., Hohmann. J and Molnar J. 2010. Recent advances in research of antimicrobial effects of essential oils and plant derived compounds on bacteria. Ethnomedicine: A Source of Complementary Therapeutics,; 6:179-201

Trevors, J. K., 1986. Plasmid curing in bacteria. FEMS Microbiology Reviews 32: 149-157.

Varsha. Shrirama., Sheetal. Jahagirdarb, C. Lathac, Vinay. Kumard, Prashant. Dhakephalkar ,Supada. Rojatkare and Mahadeo. G. Shitolea. 2010 Antibacterial & antiplasmid activities of Helicteres isora L. Indian J Med Res 132, pp 94-99.

Vinay. Kumar., Varsha. Shriram and Javed. Mulla. 2013 Antibiotic resistance reversal of multiple drug resistant bacteria using Piper longumfruit extract Journal of Applied Pharmaceutical Science Vol. 3 (03), pp. 112-116.

Zaman, M. A., M. H. Pasha and MZ Akhter. 2010. Plasmid curing of Escherichia coli cells with ethidium bromide, sodium dodecyl sulfate and acridine orange. Bangladesh J Microbiol, Volume 27, Number 1, pp 28-31