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Antibacterial & antiplasmid activities of *Helicteres isora* L.

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***Background & objectives*: The multiple drug resistance (MDR) is a serious health problem and major challenge to the global drug discovery programmes. Most of the genetic determinants that confer resistance to antibiotics are located on R-plasmids in bacteria. The present investigation was undertaken to investigate the ability of organic extract of the fruits of *Helicteres isora* to cure R-plasmids from certain clinical isolates.**

***Methods*: Active fractions demonstrating antibacterial and antiplasmid activities were isolated from the acetone extracts of shade dried fruits of *H. isora* by bioassay guided fractionation. Minimal inhibitory concentration (MIC) of antibiotics and organic extracts was determined by agar dilution method. Plasmid curing activity of organic fractions was determined by evaluating the ability of bacterial colonies (pre treated with organic fraction for 18 h) to grow in the presence of antibiotics. The physical loss of plasmid DNA in the cured derivatives was further confirmed by agarose gel electrophoresis.**

***Results*: The active fraction did not inhibit the growth of either the clinical isolates or the strains harbouring reference plasmids even at a concentration of 400 µg/ml. However, the same fraction could cure plasmids from *Enterococcus faecalis*, *Escherichia coli, Bacillus cereus* and *E. coli (RP4)* at curing efficiencies of 14, 26, 22 and 2 per cent respectively. The active fraction mediated plasmid curing resulted in the subsequent loss of antibiotic resistance encoded in the plasmids as revealed by antibiotic resistance profile of cured strains. The physical loss of plasmid was also confirmed by agarose gel electrophoresis.**

***Interpretation & conclusions*: The active fraction of acetone extract of *H. isora* fruits cured R-plasmids from Gram-positive and Gram-negative clinical isolates as well as reference strains. Such plasmid loss reversed the multiple antibiotic resistance in cured derivatives making them sensitive to low concentrations of antibiotics. Acetone fractions of *H. isora* may be a source to develop antiplasmid agents of natural origin to contain the development and spread of plasmid borne multiple antibiotic resistance.**

Key words Antibacterial activity - antibiotic resistance - antiplasmid activity - *Helicteres isora* - multiple drug resistance - plasmid-curing

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Helicteres isora L. (family: *Sterculiaceae*) distributed widely in forests throughout India and commonly known as East Indian screw tree, is a medicinally important sub-deciduous shrub or a small tree. Almost all parts of the plant are used in traditional medicinal system for curing various diseases[1-3]. Water extract of the fruits exhibits anti-HIV activity[4] and reported to possess antispasmodic activity[5]. The roots showed significant hypolipidaemic and hypoglycaemic[6-8], while bark has shown significant hypoglycaemic activity[2].

The emergence of multiple drug resistant (MDR) bacteria to commonly used antibiotics is a severe health problem and major challenge to the global drug discovery programmes[9]. The problem of explosive escalation of antimicrobial resistance has only been worsened by steady decrease of the number of new antibiotics introduced in the last 10 to 15 years[10]. After the discovery of quinolones, only one class of antibiotics, the oxazolidinones has been introduced to the market[11]. All the other antibacterial agents entered the market during this period were structural modification of existing molecules. The need for new antibiotics is more pressing than ever[12]. Clinical isolates of *Staphylococcus aureus* and *Enterococcus* resistant to oxazolidinone linezolid, which is considered as a last line of defence against vancomycin resistant bacterial infections have been reported[13,14]. Most of the genetic determinants that confer resistance to antibiotics are located on plasmids. These extra-chromosomal DNA sequences are often transferable to other bacteria in the environment and can be responsible for the emergence of multiple resistances to antibiotics[15]. The use of antiplasmid agents in combination with antibiotics may serve as a possible way to combat this resistance encoded by plasmids[16]. However, majority of the known plasmid curing agents including acridine orange, ethidium bromide and sodium dodecyl sulphate are toxic or mutagenic and hence unsuitable for therapeutic applications. Also, no single curing agent can cure all plasmids from different hosts. Thus, there is a constant need to develop new curing agents with high efficacy and safety. Traditional herbal medicines have always been a rich source of drug discovery programmes[17] and many plant derived compounds have shown promising activity against MDR bacteria[12,15,18-21]. The present study was planned to investigate the antibacterial and antiplasmid activities of active fraction purified from the acetone extract of the fruits of *H. isora* against the bacterial strains *S. aureus* and *Escherichia coli* harbouring reference plasmids pUC18 and RP4, and resistant R plasmids from clinical isolates of *Enterococcus faecalis, Bacillus cereus, E. coli* as well as from reference plasmid RP4.

Material & Methods

Plant materials: The fruits of *H. isora* were collected from forests in Satara region of Western Ghats, Maharashtra, India, and samples were authenticated by Dr Suresh Jagtap, at the Medicinal Plants Conservation Centre (MPCC), Pune, India. A voucher specimen was deposited at MPCC Herbarium (No. MPCC2310), Pune, India.

Plant extraction: Shade dried pods (fruits) of *H. isora* L. were finely powdered with auto-mix blender. One kg dry powder of pods was soaked in 3 litre acetone. The crude acetone extract was prepared by cold percolation for 12 h at room temperature (26±2°C). The filtrate was concentrated *in vacuo* at 40°C. The recovered solvent was reused for further extraction. This process was repeated three times to get total acetone extract. Last traces of the solvent from the total acetone extract were removed under vacuum to get the crude extract as a dark green coloured semi solid (gel like) residue of 32 g (3.2%).

Column chromatography: Acetone extract (30 g) was fractionated by step wise gradient of hexane and acetone (99:1, 96:4, 92:8, 90:10, 85:15, 75:25, 70:30, 60:40, 50:50, 0:100) on Si-gel (60-120, Merck, India) glass column (45 cm *l* :5 cm *d*) giving 10 fractions of 3.09, 2.50, 3.20, 4.21, 2.11 3.25, 4.05, 4.23, 2.18, 1.03 g respectively. The column chromatographic fraction obtained by 8: 92 (acetone: hexane) solvent system was further purified by repeated column chromatography to obtain 60 mg of the active fraction.

Bacterial strains and plasmids: Bacterial strains *S. aureus* and *E. coli* harbouring reference plasmids pUC18 and RP4 were procured from MACS Collection of Microorganisms at Agharkar Research Institute, Pune and Microbial Type Culture Collection (MTCC) Chandigarh, India (Table I). The clinical isolates were obtained from King Edward Memorial Hospital, Pune, India, and bacterial strains were identified as *E. faecalis, B. cereus, E. coli* based on 16S rRNA gene sequence homology at Agharkar Research Institute, Pune. The resistant R plasmids were isolated from these clinical isolates (strains) as well as from reference plasmid RP4.

Table I. Bacterial strains and plasmids used in the study

Bacterial strains	Designation	Plasmid	Phenotype*	Source
Bacillus cereus	MCMB -817	pAR187	Km^r	MCM[a]
Bacillus subtilis	MTCC-1558	pUB110	Km^r, Nm^r	MTCC[b]
Enterococcus faecalis (VRE)	MCMB-812	pARI812	Va^r	MCM[a]
Escherichia coli	MTCC-391	RP4	AP^r, Tc^r, Km^r	MTCC[b]
Escherichia coli	MCMB-813	pARI813	Gm^r	MCM[a]
Escherichia coli	MTCC-1601	pUC18	Ap^r	MTCC[b]
Escherichia coli	MTCC-837	R751	Tb^r	MTCC[b]
Pseudomonas aeruginosa	MCMB-816	pARI816	Gm^r	MCM[b]
Salmonella Typhi	MCMB-814	pARI814	Gm^r	MCM[a]
Shigella sonei	MCMB-815	pARI815	Gm^r	MCM[a]
Staphylococcus aureus (VRSA)	MCMB-818	pARI818	Va^r, Ro^r, Km^r, Tc^r, Ap^r	MCM[a]

[a] MACS Collection of Microorganisms, Agarkar Research Institute, G.G Agharkar Road, Pune 411 004, India; [b] Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh 160 036, India. *Ap, ampicillin; Gm, gentamycin; Km, kanamycin; Ro, roxithromycin; Tb, tobramycin; Tc, tetracyclin, Va, vancomycin. The antibiotics followed by superscript letter r shows the resistance of bacterial strains to that particular antibiotic.

Determination of resistance to antibiotics: Antibiotic resistance profile was determined by disc diffusion method[22]. In brief, the antibiotic discs were placed on brain heart infusion agar plate on which test bacterial cultures (~10^6 cells) were spread. The plates were incubated at 6°C for 2 h to allow pre diffusion of antibiotics and then at 37°C for 24 h after which the zone of inhibition around the antibiotic discs was measured. The cultures were then assessed as resistant, intermediate or sensitive according to the interpretation table provided by the manufacturer (Don Whitely Scientific Equipments/Hi media, Mumbai, India).

Determination of minimal inhibitory concentration (MIC) and sub-inhibitory concentration (SIC): The MICs was determined by agar dilution method[23]. Brain heart infusion medium was supplemented with specified concentration of antibiotic /curing agent. Test bacterial cultures were spot inoculated (10^5 cells 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.

Antiplasmid testing: The plasmid curing was performed by method described by Deshpande *et al*[24]. 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 Luria agar plates to obtain isolated colonies. Isolated colonies were then replica plated on to Luria agar and Luria agar containing antibiotics. The colonies which grew on Luria 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 at 25, 50, 100, 200 and 400 µg per ml concentrations. Dimethyl sulphoxide (DMSO) was used as negative control in plasmid curing experiment. The loss of plasmid DNA in the cured derivatives was further confirmed by agarose gel electrophoresis of plasmid DNA preparation of cured derivatives.

Statistical analysis: All experiments were conducted in triplicate to check the reproducibility of the results obtained. The results are presented as means ± SE (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

The crude acetone extract showed antiplasmid activity (data not shown), which was further fractionated to separate the fraction containing active principle responsible for the plasmid curing activity. The (92:8, v/v) hexane:acetone fraction of the column chromatography exhibited the optimum plasmid curing activity and hence was taken for further studies.

The purified fraction inhibited the growth of *S. aureus, S. Typhi* and *E. coli* harbouring reference plasmids pUC18 and RP4 at minimal inhibitory concentration of 800 µg. The same fraction could cure R-plasmids in clinical isolates of *E. faecalis*,

Table II. Curing of antibiotics resistance by *H. isora*

Bacterial strain	MIC (µg/ml)	SIC (µg/ml)	% Curing efficiency Mean ± SE (n=3)	AB^r cured
Enterococcus faecalis (VRE)	>800	800	14 ± 1.0	Vancomycin, Ampicillin
Staphylococcus aureus (VRSA)	800	400	ND	--
Escherichia coli	>800	800	26 ± 1.5	Gentamycin
Bacillus cereus	>800	800	22 ± 0.9	Kanamycin
Salmonella Typhi	800	400	ND	--
Shigella sonei	>800	800	ND	--
Pseudomonas aeruginosa	>800	800	ND	--
Bacillus subtilis (pUB110)	>800	800	ND	--
Escherichia coli (pUC18)	800	400	ND	--
Escherichia coli (RP4)	800	400	2 ± 0.4	Ampicillin, Kanamycin, Tertracyclin
Escherichia coli (R751)	>800	800	ND	--

None of the 100 colonies tested showed phenotypic loss of antibiotic resistance.
MIC, minimal inhibitory concentration; SIC, sub inhibitory concentration; AB^r, resistant to particular antibiotics

E. coli as well as *B. cereus* with curing efficiency ranging from 2 to 26 per cent (Table II). The loss of plasmid in cured derivatives was confirmed by agarose gel electrophoresis of plasmid DNA preparation of cured derivatives (Fig.). The comparison of antibiotic resistance profile of original hosts and their cured derivatives revealed that *E. coli* isolates originally resistant to ciprofloxacin, cefoperazone, ceftazidime and roxithromycin became sensitive to each of these antibiotics as a result of plasmid curing (Table III). Similarly, resistance to tobramycin, ampicillin, cloxacillin and vancomycin was eliminated in the cured derivatives of *E. faecalis* as a result of plasmid curing. Resistance to gentamicin, tobramycin and roxithromycin was eliminated subsequent to plasmid curing in *S.* Typhi.

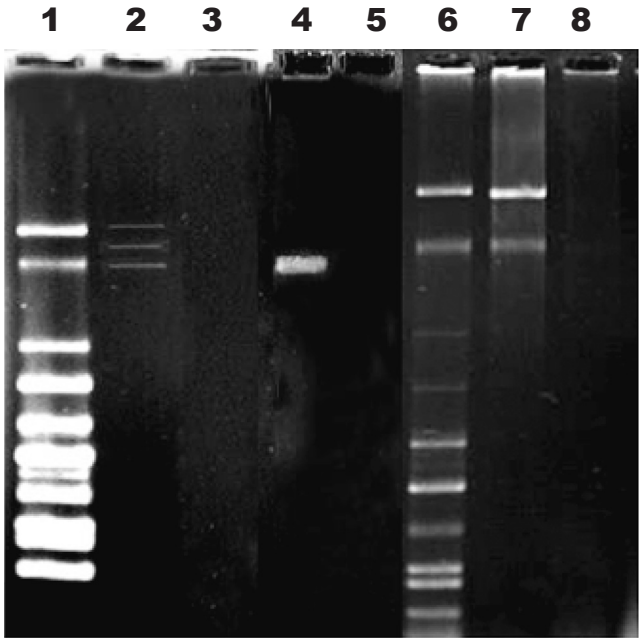

Fig. Plasmid DNA profile of bacterial strains and cured derivatives by agarose (0.8%) gel electrophoresis for confirming the antiplasmid activity of acetone extract of the fruits of *H. isora*. Lanes 1 & 6: V 517 (*E. coli* multiple plasmid standard), 2: *E. faecalis* VRE, 3: *E. faecalis* VRE CH, 4: *E. coli,* 5: *E. coli* CH, 7: *E. coli* RP4, 8: *E. coli* RP4 CH. CH: plasmid cured by *H. isora.*

Discussion

Majority of the plasmid curing agents reported earlier such as acridine dyes, ethidium bromide and sodium dodecyl sulphate are unsuitable for therapeutic application due to their toxicity or mutagenic nature[25,26]. Also each of the known curing agents is effective against only a limited number of plasmids in a limited number of hosts. Thus, there is a constant need of identifying novel curing agents that are more effective and non toxic. The present results have offered organic extracts of *H. isora* as a new and safe plasmid curing agent. 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 non toxic to human and environment. Previous reports of plant derived curing agents are limited. Plumbagin, 5-hydroxy-2-methyl-1, 4-nepthoquinone isolated from *Plumbago zeylanica* cured R-plasmids in *E. coli*[18]. This was

Table III. Antibiotic resistance pattern of plasmid cured bacterial strains

	Radius of inhibition zone (mm)												
AB	Ap	G	K	N	S	T	Va	Ax	As	Ro	Cf	Cs	Ca
Conc. (µg)	25	30	30	30	25	30	30	10	10	30	30	75	30
Cultures													
E. coli	22 ± 1.0^{b}	20 ± 1.1^{a}	21 ± 1.1^{cd}	18 ± 1.0^{c}	16 ± 0.5^{a}	24 ± 1.0^{de}	---	22 ± 1.1^{b}	19 ± 0.4^{b}	---	---	---	---
E. coli CH	26 ± 0.8^{e}	20 ± 1.0^{a}	15 ± 0.5^{a}	14 ± 0.5^{a}	18 ± 1.0^{b}	23 ± 0.9^{d}	---	25 ± 0.7^{c}	20 ± 0.6^{bc}	10 ± 0.2^{a}	33 ± 1.5^{de}	30 ± 1.3^{de}	25 ± 1.0^{a}
VRE	32 ± 1.2^{d}	---	---	---	---	10 ± 0.8^{b}	18 ± 1.0^{a}	22 ± 0.5^{b}	20 ± 0.7^{bc}	23 ± 0.8^{c}	15 ± 1.0^{a}	18 ± 1.2^{a}	---
VRE CH	35 ± 2.0^{e}	---	---	---	---	15 ± 0.7^{c}	21 ± 1.0^{b}	32 ± 1.0^{d}	30 ± 1.0^{d}	23 ± 1.0^{c}	17 ± 0.6^{b}	19 ± 0.6^{ab}	---
S. Typhi	20 ± 1.0^{a}	21 ± 1.0^{ab}	18 ± 0.6^{b}	15 ± 0.5^{ab}	---	---	---	10 ± 0.3^{a}	14 ± 0.5^{a}	---	30 ± 1.0^{c}	23 ± 0.9^{c}	25 ± 1.0^{a}
S. Typhi CH	20 ± 1.0^{a}	27 ± 0.8^{c}	20 ± 1.0^{c}	18 ± 0.6^{c}	---	8 ± 0.4^{a}	---	10 ± 0.4^{a}	14 ± 0.5^{a}	12 ± 0.5^{b}	32 ± 1.2^{d}	29 ± 1.0^{d}	30 ± 1.5^{b}

The values are expressed as mean ± SE (n=3)
AB, antibiotics used (µg); Ap, ampicillin; G, gentamycin; K, kanamycin; N, neomycin; S, streptomycin; T, tetracyclin; Va, vancomycin; Ax, amp/cloxacillin; As, amp/sulbactum; Ro, roxithromycin; Cf, ciprofloxacin; Cs, cefoperazone; Ca, ceftazidime. CH, plasmid cured strain by *H. isora*
Means within columns followed by different letters in superscript are significantly different from each other according to Duncan's Multiple Range Test at $P \leq 0.05$

probably the first report in which herbal compound had cured/ eliminated plasmid encoding antibiotic resistance. In another study[20], the alcoholic extract of *P. zeylanica* cured R plasmid harbouring *E. coli* with 14 per cent. Recently antiplasmid activity of essential oils was reported by Schelz *et al*[15]. However, in the present investigation, we have shown that the fraction from *H. isora* fruits could effectively eliminate R plasmids from reference strains as well as Gram-positive and Gram-negative strain of clinical isolates. Spontaneous loss of plasmid has been reported in literature[27]. 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 high (10^6 times higher). The antibiotic resistance may occur due to mutations. Mutagenic activity of the compound can be harmful especially in clinical applications. It is necessary to ensure that antibiotic resistance curing 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 curing agents used in this study were sub inhibitory, since bacteria were already resistant to these concentrations of compound. It can be assumed that bacteria are less likely to develop any mechanism to counter the plasmid curing property of the acetone extract of *H. isora*.

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