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Original Article

IN VITRO PLASMID CURING ACTIVITY OF AQUEOUS EXTRACT OF *TERMINALIA CHEBULA* FRUIT AGAINST PLASMIDS OF *BACILLUS SUBTILIS* AND *SHIGELLA SONNEI*

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ABSTRACT

Objective: The aim of this investigation was to evaluate *in vitro* plasmid curing activity of the aqueous extract from *Terminalia chebula* fruit against multi-drug resistance bacteria.

Methods: The aqueous extract of *Terminalia chebula* fruit was prepared in citrate phosphate buffer, pH 6.8. The extract was partially purified through gel chromatography. Antibacterial and antiplasmid activity against *Salmonella typhi, Bacillus subtilis, Escherichia coli* and *Shigella sonnei* was checked using the broth dilution method. The contents of crude extract were estimated.

Results: The crude extracts exhibited anti plasmid activity against plasmids of *B. subtilis* and *Sh. sonnei*. Loss of plasmid band was confirmed by agarose gel electrophoresis. The curing efficiency of aqueous extract was found to be 100% and 44% against plasmids of *B. subtilis* (pUB110) and *Sh. sonnei*(pARI-815) respectively. The results were compared with conventional curing agent such as ethidium bromide and acridine.

Conclusion: The aqueous extract of *T. chebula* fruit has the potential to inhibit growth of strains which are resistant to many antibiotics. Results could provide new insight into the treatment of infectious diseases which are facing limitation of antibiotics.

Keywords: *Terminalia chebula* fruit**,** Aqueous extract, Antiplasmid activity, Antibiotic resistance.

INTRODUCTION

Microorganisms have developed resistance to many antibiotics [1] due to indiscriminate use of antimicrobial drugs, which have created the immense clinical problem in treatment of infectious diseases [2]. Shortly after the introduction of each antimicrobial compound, an emergence of antimicrobial resistance is observed because most of the antibiotics available in the market are slightly different in structure from the preexisting drugs [3]. Currently, oxazolidinone linezolid is used as a last line of defense against vancomycin-resistant bacterial infections, but bacteria also showed resistance to linezolid and resistant clinical isolates of *Staphylococcus aureus* and *Enterococcus* have been obtained [4, 5].

The ability of bacteria to expand their ecological niche, also in the presence of antibiotics, can be explained by accumulation of point mutations leading to the modification of existing genes or by the acquisition of resistance genes by horizontal gene transfer. Genes responsible for antibiotic resistance are usually located on extrachromosomal genetic determinants known as plasmids. These extrachromosomal DNA sequences are often transferable to other bacteria in the environment and can be responsible for the emergence of resistance to multiple antibiotics [6]. Plasmid-mediated multidrug resistance (MDR) is one of the most imperative tribulations in the treatment of infectious diseases. The use of plasmid curing agents in association with antibiotics may serve as a feasible way to control the development and spread of antibiotic resistance encoded by antibiotic resistance plasmids (R-plasmids) [7,8]. However, majority of the known conventional plasmid-curing agents, such as acridine orange, ethidium bromide and sodium dodecyl sulphate, are toxic or mutagenic hence not recommended for *in vivo* testing and therapeutic applications [9]. Therefore, in the present scenario, there is a need to investigate new plasmid curing agents from natural sources such as plants for the treatment of infectious diseases with high efficiency and safety.

From decades, plants are used for treatment of diseases, and they do not lose their importance and luster with the laps of time. They are the copious source of bioactive molecules such as proteins, peptides and small organic molecules with therapeutic use [10, 11]. Many plant-derived compounds have shown promising activity against MDR bacteria [12-15]. Recently plasmid curing agents such as 8-epidiosbulbin E acetate (EEA) and 1'-acetoxychavicol acetate were reported from bulbs of *Dioscorea bulbifera* [16] and rhizomes of *Alpinia galanga* [17] respectively. In continuation with search of plasmid curing activity in plants, we have tested the partially purified aqueous extract of *Terminalia chebula* fruit (hirda). *T. chebula* Retz. is a plant from *Combretaceae* family, commonly known as the king of medicine and is used in many Ayurvedic preparations. The tree is abundant throughout India and Southeast Asia, especially in deciduous forests and areas of scanty rainfall. Its yellowish-brown fruit is included in the Indian Pharmacopoeia under the category 'astringent'. Medicinal properties of *T. chebula* have been amply documented in ancient Indian literature [18]. It is used to treat digestive tract diseases, urinary diseases, heart diseases, parasitic infections, fever, flatulence, constipation [19] and is an important constituent of 'triphala' formulation, a composite mixture of *T. chebula*, *T. bellerica* and *Emblica officinalis* [20]. This is the first time report of plasmid curing activity of hirda fruit.

MATERIALS AND METHODS

Plant material

Fruit of *T. chebula* was procured from the market and authenticated by Botany group of Agharkar Research Institute, Pune.

Bacterial strains and growth conditions

Salmonella typhi (pARI-814), *Shigella sonnei* (pARI-815), are clinical isolates obtained from KEM hospital, Pune. These strains were identified by 16s rRNA sequence homology. *Escherichia coli* (RP4), *Bacillus subtilis* (pUB 110) were obtained from MTCC, Chandigarh. Stock cultures were stored at-80 °C using glycerol stock method and cultures were revived twice before the experiments using brain heart infusion broth (Hi Media, Mumbai) at 37 °C for 16 h.

Preparation of extract

Hirda fruit powder (20 g) was suspended in 100 ml of 20 mM citrate phosphate buffer of pH 6.8 and 800 mg of polyvinyl pyrrolidone was added to remove polyphenols. The mixture was kept at 4°C for 24 h. The homogenate was filtered through Whatman filter paper No.1 and

centrifuged at 10,000 rpm at 4 °C for 15 min. The supernatant was lyophilized up to 40 ml and loaded on Sephadex G-100 column (3 × 50 cm) pre-equilibrated with 0.1 M NaCl and eluted in same solution.

Phytochemical estimations

The aqueous extract of hirda fruit after gel chromatography was estimated for proteins, amino acids, sulphydryl group, fatty acids, sugars, chlorophyll and phenolic compounds. The protein content was determined by Bradford's method [21]. The ninhydrin test for amino acid analysis was performed according to the method described by Sadashivam and Manickam [22]. Sulphydryl group was determined by Ellman's method [23]. The presence of fatty acid derivatives was estimated by Folch lipid extraction method and analyzed by thin layer chromatography [24]. Total sugar was estimated by phenol-sulfuric acid method [25], total phenolic content was determined by Folin-Coicalteau reagent [26] and chlorophyll content was estimated according to the method described by Sadashivam and Manickam [27].

HPLC analysis of crude extract

HPLC profile of aqueous extract of hirda fruit after gel chromatography was developed using analytical HPLC (Waters) with UV detector at 280 nm. The stationary phase was X Bridge C18 column and the mobile phase was acetonitrile and water (5: 95) containing 0.1 % TFA. Flow rate was maintained at 1.5 ml/min.

SDS-PAGE of crude extract

The homogeneity and the molecular weight of proteins present in aqueous extract of hirda fruit were assessed according to the method of Laemmli [28]. SDS-PAGE was performed with 15% resolving and 4% stacking gels. A set of molecular weight marker proteins (6-205 kDa) was run in the gel to determine the molecular weight of proteins present in crude extract.

MALDI/MS analysis

Mass spectral analysis was performed on a Voyager-De-STR (Applied Biosystems) MALDI-TOF. A nitrogen laser (337 nm) was used for desorption and ionization. A spectrum was acquired in the range of 10 to 100 kDa, in linear mode with delayed ion extraction and with an accelerating voltage of 25 kV. The low mass ion gate was set at 4500 Da. All the analyses were performed in four replications. The instrument was calibrated with myoglobulin and bovine serum albumin.

Antibacterial and plasmid curing activity

Initially, the antibacterial activity of aqueous extract of hirda fruit after gel chromatography was tested against *S. typhi, Sh. sonnei, B. subtilis* and *E. coli* using agar well method. The zone of inhibition was measured in millimeters following incubation at 37 °C for 24 h. Reference strains as well as clinical isolates harboring R plasmids used in the present investigation are listed in table 1. The minimal inhibitory concentration and sub inhibitory concentration of *T. chebula* extract (TCE) were determined by the broth dilution method. Plasmid curing [29] was performed as described previously by Desphande *et al.* [30]. In brief, pathogenic bacterial cultures were grown in the presence of TCE at specified concentrations (10-200 μg ml^{-1}) for 24 h at 37 °C and then plated on brain heart infusion agar (BHIA) plates to obtain isolated colonies. The isolated colonies were then replica plated onto BHIA and BHIA containing antibiotics. Colonies that failed to grow in the presence of antibiotics were considered as putative cured derivatives. Physical loss of the plasmid in the cured derivative was confirmed by agarose gel electrophoresis of the plasmid DNA preparation of respective cured cultures. The percentage curing efficiency was expressed as number of colonies with cured phenotype per 100 colonies tested.

Table 1: Bacterial strains and their antibiotic resistance

Bacterial strain (Clinical isolates)	Designation	Reference plasmid	Antibiotic Resistance	MIC (μg ml^{-1})
Salmonella typhi	[a]MCMB-814	pARI-814	G	200
Shigella sonnei	[a]MCMB-815	pARI-815	G	50
Bacillus subtilis	[b]MTCC-1558	pUB110	K	>200
Escherichia coli	[b]MTCC-391	RP4	A, K, T	>200

[a]MCM: MACS Collection of Microorganisms (Agharkar Research Institute, Pune, India); [b]MTCC: Microbial Type Culture Collection (Institute of Microbial Technology, Chandigarh, India), G-Gentamicin, A-Amikacin, K-Kanamycin, T-Tetracycline

RESULTS

Purification and estimation of aqueous extract of hirda fruit

Polyvinyl pyrrolidone was added to aqueous extract of hirda fruit to remove polyphenols and extract was passed through Sephadex G-100 column to sequestrate small organic molecules. Phytochemical analysis of crude extract unveils the presence of sugars, amino acids, proteins and sulphydryl group. The data is presented in table 2. The HPLC chromatogram showed three prominent peaks at 280 nm (fig. 1a). The SDS-PAGE analyses supported presence of protein content in aqueous extract (Fig.1b) due to the presence of three bands at 16, 31 and 39 KDa. This was also confirmed by MALDI-MS analysis (fig. (1c).

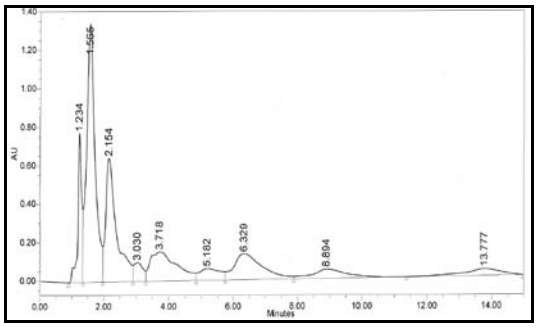

Fig. 1a: HPLC profile of crude aqueous extract of *T. chebula* fruit

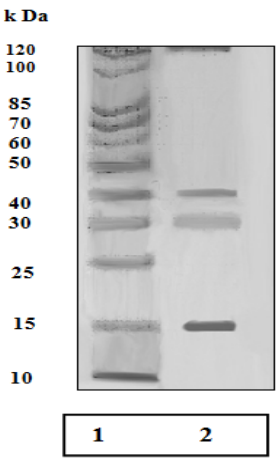

Fig. 1b: SDS-PAGE of crude aqueous extract of *T. chebula* fruit; Lane No. 1: Standard molecular markers (6-205 kDa) Lane No. 2: Aqueous extract of *T. chebula*

Antibacterial and plasmid curing activity

The antibacterial activity of aqueous extract of hirda fruit was tested against clinical isolates as well as reference strains harboring R-

plasmids. All the strains tested were resistant to 100 µg ml^{-1} concentration of aqueous extract. These results indicated that aqueous extract of *T. chebula* did not contain any significant antibacterial agent at 100 µg ml^{-1} concentration. However, the same extract cured R-plasmids in clinical strains of *Sh. sonnei.* Aqueous extract also cured reference R-plasmid of *B. subtilis* (pUB110) at a curing efficiency of almost 100% (table 3). Antibiotic resistance may occur due to mutations and mutagenic activity of the compound, which can be harmful, especially in clinical applications. To ensure that reversal of antibiotic resistance was due to loss of a plasmid and not due to mutations, agarose gel electrophoresis (0.7 %) of plasmid DNA preparation from original hosts as well as their respective cured derivatives was performed. Loss of plasmid bands in cured derivatives of *Sh. sonnei* was the physical confirmation of plasmid curing efficiency of TCE (fig. 2).

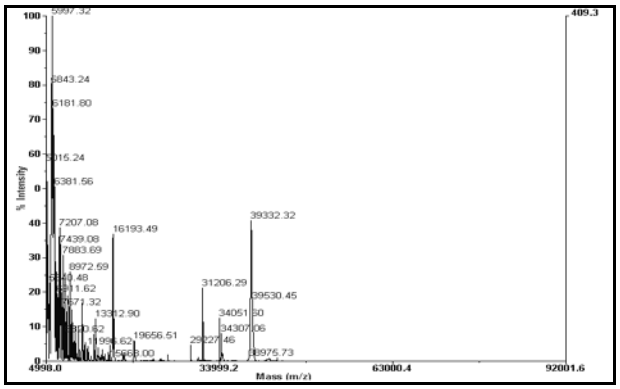

Fig. 1c: MALDI-MS analysis of crude aqueous extract of *T. chebula* fruit

Table 2: Phytochemical estimation of aqueous extract of hirda fruit

S. No.	Analysis	Results
1	Amino acids	Positive
2	Sulphydryl groups	Positive
3	Lipid residue	Negative
4	Polyphenols	Negative
5	Total sugars	Positive
6	Chlorophyll	Negative

Test for the presence of amino acids, lipid residue, polyphenols, chlorophyll, total sugars and sulphydryl groups were carried out as described in methods

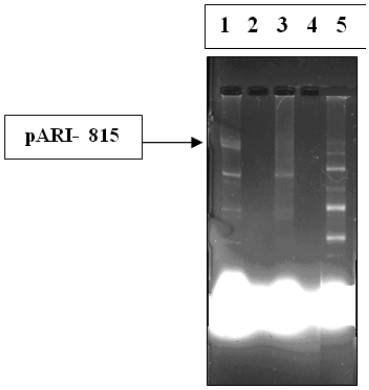

Fig. 2: Plasmid DNA profile of *Shigella sonnei* and its cured derivative on 0.7 % agarose gel; Lane No. 1: *Sh. sonnei*, lane 3: *Sh. sonnei* cured one, lane 5: *E. coli* MTCC 131 multiple plasmid standard (V517)

Table 3: Plasmid curing activity of crude extract of hirda fruit

Clinical isolates/plasmids	Plasmid curing activity					
	MIC (µg ml^{-1})	SIC (µg ml^{-1})	Curing efficiency	Resistance* cured	Acridine	EtBr
Salmonella typhi (pARI-814)	>100	100	N. D.	G	14	18
Shigella sonnei (pARI-815)	>100	100	44	G	8	10
Bacillus subtilis (pUB110)	>100	100	100	K	4	4
Escherichia coli (RP4)	>100	100	N. D.	A, K,T	10	6

* G-Gentamicin, A-Amikacin, K-Kanamycin, T-Tetracycline, EtBr-Ethidium bromide, N. D. – Below detection limit (none of the 100 colonies tested showed elimination of antibiotic resistance). The results are shown as means ± SD (n = 3).

DISCUSSION

Curing of plasmid-mediated drug resistance in pathogenic strains of bacteria is of great practical significance both in treatment of bacterial infection and in microbial genetics to identify the phenotype encoded by plasmids. Compounds of natural origin, including proteins have been reported to have antimicrobial activity [31,32] and have also been reported to reverse resistance to antimicrobial drugs [33]. However, there is a paucity of information describing plasmid curing agents of plant origin. Lakshmi *et al.* [15] reported that plumbagin (5-hydroxy-2-methyl-1, 4-napthoquinone) from *Plumbago zeylanica* was capable of eliminating reference plasmids of *E. coli*. Essential oils showing antiplasmid activities were reported by Schelz et al. [6]. Considering the antibacterial activity [34] of *T. chebula* fruit, we have tried to cure the drug resistance plasmids (pARI-815) and (pUB-110) of *Sh. Sonnei* and *B. subtilis* strains respectively, which are resistance to antibiotics. Data obtained were found better than the conventional curing agents such as acridine orange and ethidium bromide.

It is quite noticeable that aqueous extract of hirda fruit did not show any significant antibacterial activity against *S. typhi, B. subtilis, Sh. Sonnei* and *E. coli*. However, the extract cured plasmids pUB 110 and pARI-815 at curing efficiency of 100% and 44% respectively. Most of the plasmid curing agents such as ethidium bromide, acridine orange, acriflavin, etc. has been reported to be mutagenic and carcinogenic in nature. Mutagenic activity of a compound can be harmful, especially in therapeutic applications. It was, therefore, of interest to confirm that reversal of antibiotic resistance in cured derivatives was indeed due to loss of plasmid and not due to mutations. Hence, agarose gel electrophoresis of plasmid DNA preparation from original hosts as well as their respective cured derivatives were performed. Loss of DNA band in cured derivatives corresponding to the plasmid band in wild host practically confirmed plasmid curing effected by partially purified aqueous extract of *T. chebula*.

Phytochemical analysis of partially purified aqueous extract indicated the absence of fatty acids, lipids, total phenolics and chlorophyll (table 2). However, proteins were detected in the same extract. HPLC analysis of the partially purified extract revealed three prominent peaks (detected at 280 nm). Denaturing polyacrylamide gel electrophoresis of same extract revealed the presence of three bands at 16, 31 and 39 kDa molecular wt. Hence, it may be speculated that one or more of these proteins could be responsible for plasmid curing activity exhibited by *T. chebula* aqueous extract.

CONCLUSION

Development of bacterial resistance to multiple antibiotics has made treatment of infectious diseases increasingly difficult over the past

few years. Many known antibiotics have now become ineffective owing to the development and spread of plasmid encoded high level of resistance in bacteria. However, such ineffective antibiotics can be rendered effective if R-plasmids encoding antibiotic resistance are eliminated from the bacterial population. Unfortunately, plasmid curing agent can cure only a limited number of plasmids from a limited range of hosts. Hence, there is a continuing need of finding newer plasmid curing agents who are effective and less toxic. Partially purified aqueous extract of *T. chebula* showing plasmid curing properties reported in the present investigation offers a new potential candidate for potential therapeutic applications. Aqueous extract of *T. chebula* has been in use in Ayurvedic medicines over last hundreds of years without any reported toxic effects. The present investigation also revealed the presence of three protein bands in partially purified aqueous extract that showed plasmid curing activity. Further purification and characterization of these individual proteins will aid an investigation related to the mode of action as well as toxicity studies.

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CONFLICT OF INTERESTS

The authors have declared that there is no conflict of interest

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