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Monitoring *in vitro* efficacy of *Holarrhena antidysenterica* against multidrug resistant enteropathogenic bacteria

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PEER REVIEW

ABSTRACT

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Comments

The novelty of the work is that the gamut of MDR bacteria was isolated from clinical samples in a hospital. Biochemical identifications were followed during the procedure, along with standard strains. Search of non-microbial antimicrobial is the call of the day for the avalanche of MDR pathogens. Secondly, Indian forest patches are the unique sources of tropical medicinal plants.

Details on Page S61

Objective: To assess antibacterial activities of leaf and bark extracts of *Holarrhena antidysenterica* (*H. antidysenterica*), used by an Indian aborigine for ailments of human gastrointestinal tract, against eight extended spectrum β -lactamase producing multidrug resistant enteropathogens.

Methods: Antibacterial activities of eight solvent-extracts of the plant were monitored by the agar-well diffusion method on lawns of all bacteria. Further, minimum inhibitory concentrations and minimum bactericidal concentrations of the best three solvent extracts were determined by the micro-broth dilution method. Preliminary phytochemical analysis of the active leaf and bark extracts were carried out.

Results: It was found that *Enterobacter aerogenes* was resistant to 14 of 16 antibiotics, likewise, *Escherichia coli* to 13, *Klebsiella* sp. to 14, *Salmonella paratyphi* to 7, *Salmonella typhi* to 15, *Shigella dysenteriae* and *Shigella sonnei* to 14, *Vibrio cholerae* to 4 of 16 antibiotics. It was found that plant-extracts with petroleum ether and *n*-hexane had the least antibacterial activity. Extracts of leaves with chloroform, methanol, and water registered moderate antibacterial activity, whereas bark-extracts with ethyl acetate, acetone, and ethanol had a comparatively higher antibacterial activity on all these strains. Maximum sizes of zone of inhibition due to leaf extracts with ethyl acetate, acetone, and ethanol, and on the other hand, bark extracts with ethyl acetate, acetone and methanol were recorded against these bacteria; minimum inhibitory concentration and minimum bactericidal concentration values of specifically these extracts were determined. Phytochemical analysis of the methanolic bark extract of *H. antidysenterica* confirmed the presence of alkaloids, terpenoids, reducing sugars, tannins, and flavonoids.

Conclusions: Data analysis revealed that leaves and bark of *H. antidysenterica* could serve as complementary/supplementary drugs along with suitable antibiotics to control the marauding multidrug resistant enteropathogens.

KEYWORDSEnteropathogens, Multidrug resistant, ESBL, *Holarrhena antidysenterica*, Phyto-extracts, MIC, MBC, Phytochemical analysis**1. Introduction**

Resistance to drugs/antibiotics in pathogenic bacteria is a growing clinical concern, and the spread of resistant strains worldwide has been discomobulating public health,

everywhere, exemplified with *Pseudomonas*, *Staphylococcus* and a few more pathogens^[1]. Particularly, β -lactam antibiotics were known to be equally active against both Gram-positive and Gram-negative bacteria. But, the range of β -lactam antibiotics – all penicillin derivatives,

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and all four generations of cephalosporins (cephalexin, cefazolin, cefuroxime, cefaclor, ceftazidime, ceftriaxone, cefoperazone, cefipime, ceftazopran, etc.) and monobactams, like aztreonam, have become notably ineffective when the resistance is afforded due to the production of extended spectrum β -lactamase (ESBL) that attacks the β -lactam ring with the eventual antibiotic inactivation^[2]. A strain of *Escherichia coli* (*E. coli*), the notable enteric pathogen producing CTX-M type cells (resistant to cefotaxime) with the ESBL activity was first recognized since 2 decades^[3,4]. Subsequently, *Klebsiella pneumoniae* (*K. pneumoniae*), another enteric pathogen of notoriety was also reported to produce β -lactamase^[5]. We have recently reported the prevalence of ESBL producing *Pseudomonas aeruginosa* embittering the clinical well-being of a hospital^[6].

Infections with ESBL producing bacteria mostly occur in gastrointestinal and urinary tracts, which often lead to complicated pyelonephritis or bacteremia^[7]. The extent of multidrug resistance in any marauding pathogen is the obvious obstacle, for the control *in vivo*. Frequently, ESBL producing enteropathogens are resistant to the preferentially used, trimethoprim-sulfamethoxazole or ciprofloxacin, and concomitantly to parenterally administered drugs, ceftriaxone, gentamicin or ampicillin/sulbactam^[8]. Thus, the interference with ESBL producing enteropathogens has become a commonplace in clinical managements today. Unfortunately, virulent enteric bacteria (*Klebsiella*, *Salmonella*, *Pseudomonas*, *Shigella*, *Enterococcus*, *Vibrio*, etc.) are active in non-hygienic dwellings; and those are often found as the unitary cause of high infant mortality and outbreaks of infrequent epidemics in developing countries; these pathogens are also multidrug resistant (MDR) ^[9,10], and obviously, due attention is needed. One burning example is the emergence of infections with *Clostridium difficile*, reaching epidemiologic proportions, as a result of non-prudent antimicrobial use in India^[10]. Further, many antimicrobials used in small animals (pets and food animals) are in the use for humans. Eventually, a number of bacteria have become MDR in animals as well as, are transmitted to their owners. For example, resistant strains of *Staphylococcus intermedius*, *Campylobacter* sp., *Salmonella* sp. and *E. coli* had been cited as arising from possible zoonotic sources^[11].

In this perspective, the search for more harmless drugs could be tried for MDR enteric bacterial strains with ESBL activity, concomitantly offering multidrug resistance. In such an endeavour, the medicinal plant, *Holarrhena antidysenterica* (Roxb. ex Fleming) Wall (family, Apocynaceae) (*H. antidysenterica*) (Figure 1), which is well-known for ethno-medicinal information active against enteric diseases, was selected to check its antibacterial property *in vitro* against eight clinically isolated ESBL producing enteropathogenic bacteria, *Enterobacter aerogenes* (*E. aerogenes*), *E. coli*, *Klebsiella* sp., *Salmonella paratyphi* (*S. paratyphi*), *S. typhi*, *Shigella dysenteriae* (*S. dysenteriae*), *Shigella sonnei* (*S. sonnei*) and *Vibrio cholerae* (*V. cholerae*). The specific name, antidysenterica stems from its ethnobotanical use against enteric diseases,

diarrhoea and dysentery^[12]. The purpose of the study was to check the control capacity of crude extracts of leaves and bark of the plant, against the whole spectrum of MDR strains of enteropathogens. Additionally, *H. antidysenterica* is used ethnobotanically for colic and fever; it is also used as carminative, astringent, lithontriptic tonic, aphrodisiac, cardio-suppressant, diuretic and antihypertensive drug, by tribal folk in India^[13,14]; seeds are also used as an anti-diabetic remedy in Asian countries^[15]. *In vitro* antimicrobial activity of *H. antidysenterica* has been well documented^[16,17], and *in vivo* abilities to control diarrhoea, dysentery, hemorrhage, hemorrhoids, amoebiasis and hepatitis have been also recorded^[18]. It is anticipated that phyto-drugs, preferably crude extracts, when scaled up clinically would be able to control resistant pathogens, as an inherent array of natural phytochemicals of non-microbial origin can never be breached by any pathogen; those are affordable too. Considering the role of crude phyto-extracts in traditional medicine used by ethnic people and elite mass both in developed and developing countries for hundreds of age-tested plants without any verification of their host toxicity with scientific exactitude, commercial formulations of plant-drugs are produced by several pharmaceutical companies now, generating millions of US \$. Indeed, it would not be felonious to use crude phyto-extracts in face of the precarious clinical consternations arising from MDR enteric pathogens, especially in developing countries to intimidate the disproportionate consternation of under-5 child mortality. It would not be out of place to mention here that with support of mandates from the World Health Organization (WHO) as well as local necessities, several drug manufacturing establishments have scaled up in all developing and developed countries worldwide to produce phyto-drugs against infectious ailments without any verification of the host toxicity^[1].



Figure 1. Photo of *H. antidysenterica*.

Complementary and alternative medicine (CAM) with well-known plants has been popular worldwide, and in the US particularly, those have been used for coagulation effects, lowering blood pressure, medicines for cardiac effects, sedative effects and many more including infective ailments and cancer^[19], but lesser-known plants were not yet used. For an eclectic effect, another system, integrative medicine has been followed, wherein the regular allopathic medicines are mixed up with phyto-drugs for treating acute diseases including the cancer^[20]. In Middle East countries, the use of crude plant-extracts in the form of infusions has been growing unusually popular, particularly with people above 40 years of age, consisting a 63% use of herbal products for acute diseases, cancer and heart problems – a concept now introduced, neoherbalism^[21]. Further, even for the treatment of osteoarthritis herbal medicines have been popular in the US^[22]. What's more, the value of plant-based prescribed drugs in 1990 was estimated at 15.5 billion US \$, which had been on the rise since then to 35 billion US \$ during the last decade^[1,23]. Such herbal trade markets and the use of herbal products must be developing in each country, unbeknownst to the systematically recorded databases. Indeed, herbal products are widely held today for health boosting as preventives by the WHO^[24], and in future it would be deeply held for more specific needs as CAM, if those could be scaled as remedial measures, without any dyslogistic prejudice, often seen with phyto-drugs. Obviously, host-toxicity testing of non-edible plant-products remains an essential corollary in CAM, too, for a scientific validation. *H. antidyenterica*, a non-toxic and an edible medicinal plant, for which host-toxicity testing is logistically redundant, could be used as a source of phyto-drug; and this paper presents a scientific basis of its antibacterial activity against MDR enteropathogenic bacteria.

2. Materials and methods

2.1. Extraction of plant extracts

For the hot extraction, a total of 40 g of powdered plant material (both leaves and barks of *H. antidyenterica*) was dissolved in 400 mL of an organic solvent in a soxhlet apparatus. The extraction was carried out at 40–60 °C, depending upon the boiling point of the organic solvent in use. About after 40 cycles or siphons, the liquid extract was collected in the bottom flask and was dried with a rotary evaporator till a semisolid mass was obtained, which was stored in 10% dimethyl sulfoxide at 4 °C until use. The left over plant material in the Soxhlet apparatus was further oven dried and was reused for getting more extracts using several organic solvents, in succession (successive extraction procedure), with non-polar to polar solvents: petroleum

ether, ethyl acetate, chloroform, *n*-hexane, acetone, methanol, ethanol and water.

2.2. Isolation and biochemical identification of bacterial strains

Eight enteropathogens (*E. aerogenes*, *E. coli*, *Klebsiella* sp., *S. paratyphi*, *S. typhi*, *S. dysenteriae*, *S. sonnei* and *V. cholerae*) were isolated from the in-house patients of IMS & Sum Hospital. Maintenance of clinically isolated bacteria on selected media and biochemical identifications of isolated bacterial strains were described previously^[25]. Bacterial strains were ascertained to taxa with results of colony characters and biochemical tests. Drug-sensitive strains of these bacteria obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India served as reference controls.

2.3. Determination of ESBL producers and antibiotic sensitivity patterns

Double-disc synergy test was used for the determination of ESBL producers^[6]. All bacterial strains were subjected to antibiotic sensitivity tests on Muller-Hinton agar by the disc diffusion of Kirby-Bauer's method by using 16 antibiotics of five different groups, as described previously^[25].

2.4. Antibacterial activity test, determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values

Antibacterial activities of plant-extracts (both leaves and bark) obtained with eight different solvents were recorded by the agar-well diffusion method as described previously^[25,26]. Chloramphenicol 30 µg/mL with an average size of zone of inhibition of 21 mm and dimethyl sulfoxide 10% with no antibacterial activity served as reference controls. Values of MIC and MBC of active plant extracts were determined as described previously^[27].

2.5. Phytochemical analyses

Phytochemical analyses of active extracts (both leaves and bark) were done to confirm the presence of phytochemicals, reducing sugars, saponins, flavonoids, steroids, terpenoids, tannins, alkaloids, resins, and glycosides as previously described^[25,26].

3. Results

E. aerogenes was identified basing on its colony characteristics on blood and MacConkey agar, along with

results of nine biochemical tests. White convex colonies with γ -haemolysis on blood agar, and on MacConkey agar pink coloured colonies (Figure 2) were noted because of lactose fermentation (Table 1).



Figure 2. Lactose fermenting pink-coloured colonies of *E. aerogenes* on MacConkey agar.

Further, it was found positive to catalase, Voges–Proskauer, citrate and nitrate reduction tests and negative to oxidase,

indole, methyl–red and urease tests (Table 2); with the triple sugar iron test, the bacterium was recorded to produce only acid, but no gas. Similarly, the rest seven bacterial isolates were identified based on their colony characteristics on suitable media and biochemical test results (Tables 1 and 2).

From, antibiograms of eight enteropathogens, it was found that *E. aerogenes* was resistant to 14 of 16 antibiotics, likewise, *E. coli* to 13, *Klebsiella* sp. to 14, *S. paratyphi* to 7, *S. typhi* to 15, *S. dysenteriae* and *S. sonnei* to 14, *V. cholerae* to 4 of 16 antibiotics. *E. aerogenes* was sensitive to ciprofloxacin and chloramphenicol; *E. coli* was sensitive to ciprofloxacin, co-trimoxazole, and chloramphenicol; *Klebsiella* sp. was sensitive to gentamicin and chloramphenicol, at specified levels of each antibiotic. Similarly, antibiotic sensitivity patterns of the rest other pathogens were recorded (Table 3). All the strains used in this study were ESBL producers, as confirmed by the double–disc synergy test.

Antibacterial activities of eight solvent extracts were monitored by the agar–well diffusion method on lawns of eight bacterial isolates. It was found that plant extracts (leaves and bark) with petroleum ether and *n*-hexane had

Table 1

Isolation and maintenance of clinically isolated enteropathogenic bacteria with colony characteristics.

Isolated bacteria	MTCC strain No.	Media	Colony characters
<i>E. aerogenes</i>	2990	Blood agar	white convex with gamma hemolysis
		MC agar	LF, mucoid
		Nutrient agar	flat dry, irregular
<i>E. coli</i>	443	MC agar	LF, flat dry pink irregular
		EMB agar	purple colour, flat dry, irregular colonies, with metallic green colour
<i>Klebsiella</i> sp.	4031	MC agar	LF, pink, mucoid
		CLED agar	yellow mucoid
<i>S. paratyphi</i>	3220	MC agar	NLF, colourless
		XLD agar	red colour, pinpoint colonies with black center
<i>S. typhi</i>	733	MC agar	NLF, colourless
		XLD agar	red colour, pinpoint colonies with black center
<i>S. dysenteriae</i>	–	MC agar	NLF circular, smooth translucent
<i>S. sonnei</i>	2957	MC agar	LLF, flat with jagged end
<i>V. cholerae</i>	3905	TCBS agar	Smooth, opaque: yellow colour

LF: lactose fermenting; NLF: non–lactose fermenting; LLF: late lactose fermenting; MC: MacConkey; EMB: Eosin Methylene Blue; CLED: cysteine lactose electrolyte deficient; XLD: Xylose–Lysine Deoxycholate; TCBS: Thio–Sulfate–Citrate–Bile Salts–Sucrose; –: not available.

Table 2

Biochemical identifications of the isolated MDR enteropathogenic bacteria with different tests.

Bacteria	Catalase	Oxidase	Indole	MR	VP	Citrate	Urease	TSI	Nitrate
<i>E. aerogenes</i>	+	–	–	–	+	+	–	A/A	+
<i>E. coli</i>	+	–	+	+	–	–	–	A/AG	+
<i>Klebsiella</i> sp.	+	–	–	–	+	+	+	A/AG	+
<i>S. paratyphi</i>	+	–	–	+	–	+	–	K/A	+
<i>S. typhi</i>	+	–	–	+	–	+	–	K/A/H ₂ S	+
<i>S. dysenteriae</i>	+	–	+	+	–	–	–	K/A	+
<i>S. sonnei</i>	+	–	–	+	–	–	–	K/A	+
<i>V. cholerae</i>	+	+	+	–	–	+	–	ND	+

MR: methyl red; VP: Voges–Proskauer; TSI: triple sugar iron. A/A H₂S: Acid in slant and butt with hydrogen sulfide gas production; K/A/H₂S: alkali/acid/ hydrogen sulfide gas production; A/A gas: acid and gas production; ND: not done; +: positive; –: negative.

the least antibacterial activity. Leaf extracts with chloroform, methanol, and water had a moderate antibacterial activity on all bacterial strains, whereas extracts with ethyl acetate, acetone, and ethanol had comparatively higher antibacterial activities (Table 4). Ethyl–acetate extract of leaves registered the maximum size of zone of inhibition against *E. aerogenes* (23.5 mm) and the least value against *V. cholerae* (14 mm). Further, acetone and ethanolic extracts of leaves had maximum zones of inhibition against *S. dysenteriae* (25 mm) and *Klebsiella* sp. (25 mm), and the least value against *S. sonnei* (18 mm) and *V. cholerae* (17 mm). Again, ethyl acetate, acetone and methanolic bark extracts of *H. antidysenterica* recorded the maximum antibacterial activity against these eight enteropathogens. Ethyl acetate bark extract registered the maximum size of zone of inhibition against *S. dysenteriae* (19 mm) and the least against *V. cholerae* (12 mm). Further, acetone and methanolic extracts of bark had the maximum zone of inhibition against *E. coli* (17 mm) and *S. paratyphi* (24 mm), and least values against *V. cholerae* (13 mm and 15 mm, respectively). Sizes of zones of inhibition of all solvent extracts against eight bacteria were recorded (Table 4).

Maximum zones of inhibition due to leaf extracts with ethyl acetate, acetone, and ethanol, and on the other hand, of

ethyl acetate, acetone and methanolic extracts of bark were recorded against eight MDR bacteria; MIC and MBC values of these extracts specifically were determined. The MIC value of ethyl acetate leaf extract was recorded against both *E. aerogenes* and *E. coli* as 3.125 mg/mL, and the maximum MIC value was recorded against *V. cholerae* as 25.000 mg/mL (Table 5).

Table 5

MIC and MBC of bioactive leaf extracts of *H. antidysenterica* against MDR enteropathogenic bacteria (mg/mL).

Bacteria	Ethyl acetate		Ethanol		Acetone	
	MIC	MBC	MIC	MBC	MIC	MBC
<i>E. aerogenes</i>	3.125	12.500	1.560	6.250	3.125	6.250
<i>E. coli</i>	3.125	12.500	12.500	50.000	3.125	12.500
<i>Klebsiella</i> sp.	6.250	25.000	1.560	12.500	3.125	25.000
<i>S. paratyphi</i>	12.500	25.000	12.500	25.000	6.250	25.000
<i>S. typhi</i>	6.250	25.000	12.500	25.000	3.125	12.500
<i>S. dysenteriae</i>	6.250	12.500	3.125	12.500	1.560	6.250
<i>S. sonnei</i>	12.500	25.000	12.500	50.000	1.560	12.500
<i>V. cholerae</i>	25.000	50.000	12.500	50.000	12.500	25.000

Similarly, for ethanolic leaf extracts, the MIC values were recorded against both *E. aerogenes* and *E. coli*, as 1.560 and 12.500 mg/mL, respectively, and the MIC values of acetone leaf extracts against both *S. dysenteriae* and *S. sonnei*

Table 3

Antibiotic susceptibility results of MDR enteropathogenic bacteria.

Bacteria	Amino–glycosides		β–lactams			Cephalo–sporins			Fluoroquinolones				Sulfon–amide		Stand–alones		
	Ac	Ge	Am	Ak	Pt	Ce	Cf	Ci	Gf	Na	No	Of	Co–t	Ch	Nf	Te	
<i>E. aerogenes</i>	R	R	R	R	R	R	R	S	R	R	R	R	R	S	R	I	
<i>E. coli</i>	R	R	R	R	R	R	R	S	R	R	R	R	S	S	R	R	
<i>Klebsiella</i> sp.	R	S	R	R	R	R	R	R	R	R	R	R	R	S	R	R	
<i>S. paratyphi</i>	S	S	R	R	S	R	R	S	R	S	S	S	R	S	I	S	
<i>S. typhi</i>	R	R	R	R	R	R	R	I	R	R	R	R	R	S	R	R	
<i>S. dysenteriae</i>	R	I	R	R	R	R	R	S	R	R	R	R	R	S	R	R	
<i>S. sonnei</i>	R	R	R	R	R	R	R	S	R	R	R	R	R	S	R	I	
<i>V. cholerae</i>	R	R	S	S	S	R	S	S	S	S	S	S	R	S	S	S	

R: resistant; S: sensitive; I: moderately sensitive. Ac: amikacin 30 µg/disc; Ge: gentamicin 10 µg/disc; Am: ampicillin 10 µg/disc; Ak: amoxycyclav 30 µg/disc; Pt: piperacillin/tazobactam 100/10 µg/disc; Ce: ceftriaxone 30 µg/disc; Cf: cefpodoxime 10 µg/disc; Ci: ciprofloxacin 5 µg/disc; Gf: gatifloxacin 5 µg/disc; Na: nalidixic acid 30 µg/disc; No: norfloxacin 10 µg/disc; Of: ofloxacin 5 µg/disc; Co–t: co–trimoxazole 25 µg/disc; Ch: chloramphenicol 30 µg/disc; Nf: nitrofurantoin 300 µg/disc; Te: tetracycline 30 µg/disc.

Table 4

Zone of inhibition in different hot solvent leaf extracts of *H. antidysenterica* against MDR enteropathogenic bacteria (mm) by agar–well diffusion method.

Bacteria	Petroleum ether	Ethyl acetate	Chloroform	<i>n</i> –hexane	Acetone	Ethanol	Methanol	Water	Chl
<i>E. aerogenes</i>	– (–)	23.5 (18.0)	13.0 (–)	7.0 (12.0)	23.0 (13.0)	24.0 (9.0)	11.0 (23.0)	15.0 (11.0)	21
<i>E. coli</i>	– (7.0)	23.0 (15.0)	14.0 (–)	6.0 (–)	24.0 (17.0)	21.0 (10.0)	12.0 (21.0)	13.0 (9.0)	20
<i>Klebsiella</i> sp.	8.0 (–)	21.6 (16.0)	12.0 (–)	10.0 (–)	23.0 (12.0)	25.0 (10.0)	10.0 (23.0)	13.0 (12.0)	21
<i>S. paratyphi</i>	11.0 (8.0)	21.0 (18.0)	14.0 (13.0)	9.0 (11.0)	21.0 (14.0)	19.0 (10.0)	11.0 (24.0)	13.0 (17.0)	20
<i>S. typhi</i>	10.0 (6.0)	22.0 (17.0)	14.0 (15.0)	– (6.0)	23.0 (15.0)	18.5 (9.0)	12.0 (19.0)	14.0 (11.0)	22
<i>S. dysenteriae</i>	– (–)	22.5 (19.0)	12.0 (–)	– (–)	25.0 (14.0)	22.0 (10.0)	12.0 (16.0)	12.0 (12.0)	21
<i>S. sonnei</i>	– (–)	22.0 (18.0)	13.0 (14.0)	– (8.0)	25.0 (14.0)	18.0 (9.0)	12.0 (21.0)	12.0 (15.0)	20
<i>V. cholerae</i>	– (–)	14.0 (12.0)	14.0 (–)	– (7.0)	17.0 (13.0)	19.0 (10.0)	10.0 (15.0)	13.0 (12.0)	21

Numbers in parenthesis represent those by bark. Chl: chloramphenicol 30 µg/mL. –: not done.

were 1.560 mg/mL. Further, the MBC values of ethyl acetate leaf extracts recorded against *E. aerogenes*, *E. coli* and *S. dysenteriae* as 12.500 mg/mL, and the MBC value was recorded against *V. cholerae* at 50.000 mg/mL (Table 5). Similarly, for acetone leaf extract, the MBC value was recorded against *E. aerogenes* at 6.250 mg/mL; and for ethanolic leaf extract the MBC value was 6.250 mg/mL against *E. aerogenes*. Moreover, MBC values of acetone and ethanol leaf extracts were 50.000 and 25.000 mg/mL, against *V. cholerae*, respectively (Table 5).

Further, the MIC value of ethyl acetate bark extract recorded against *S. dysenteriae* was 3.125 mg/mL, and the maximum value was 25.000 mg/mL against *V. cholerae* (Table 6).

Table 6

MIC and MBC of bioactive extracts of barks of *H. antidysenterica* against isolated strains enteropathogenic bacteria (mg/mL).

Bacteria	Methanol		Ethyl acetate		Acetone	
	MIC	MBC	MIC	MBC	MIC	MBC
<i>E. aerogenes</i>	3.125	25.000	6.250	25.000	12.500	25.000
<i>E. coli</i>	3.125	12.500	12.500	12.500	6.250	25.000
<i>Klebsiella</i> sp.	3.125	12.500	12.500	25.000	12.500	25.000
<i>S. paratyphi</i>	1.560	25.000	6.250	25.000	12.500	25.000
<i>S. typhi</i>	6.250	25.000	6.250	25.000	6.250	12.500
<i>S. dysenteriae</i>	12.500	25.000	3.125	12.500	12.500	25.000
<i>S. sonnei</i>	3.125	12.500	6.250	25.000	12.500	50.000
<i>V. cholerae</i>	25.000	50.000	25.000	50.000	6.250	12.500

Similarly, for acetone bark extracts, the MIC value was 6.250 mg/mL against *E. coli*, *S. typhi* and *V. cholerae*, while MIC of methanol bark extract against *S. paratyphi* was 1.560 mg/mL. In addition, the MBC values of ethyl acetate bark extract against both *E. coli* and *S. dysenteriae* were 12.500 mg/mL, and the MBC value was 50.000 mg/mL against *V. cholerae* (Table 6). Similarly, for acetone bark extract, the MBC values were 12.500 mg/mL against both *S. typhi* and *V. cholerae*; and for methanolic bark extract, the MBC value was 25.000 mg/mL, against *S. dysenteriae*.

Phytochemical analyses of ethanolic leaf extract of *H. antidysenterica* confirmed the presence of alkaloids, glycosides, terpenoids, saponins, tannins and flavonoids, whereas reducing sugars and steroids were absent. Similarly, the presence and absence of the phytochemicals in the ethyl acetate and acetone leaf extracts were recorded (Table 7).

Table 7

Phytochemical analysis of *H. antidysenterica*.

Plant part	Solvent extracts	Alkaloids	Glycosides	Terpenoids	Reducing sugars	Saponins	Tannins	Flavonoids	Steroids
Leaves	Ethyl acetate	+	–	+	+	–	+	+	–
	Ethanol	+	+	+	–	+	+	+	–
	Acetone	+	+	+	+	+	+	+	–
	Methanol	+	–	+	+	–	+	+	–
Bark	Ethyl acetate	+	–	+	–	+	+	+	–
	Acetone	+	–	+	+	+	+	+	–

+: present; – : absent.

Further, phytochemical analysis of methanolic bark extract of *H. antidysenterica* confirmed the presence of alkaloids, terpenoids, reducing sugars, tannins and flavonoids, whereas glycosides, saponins and steroids were absent. Similarly, the presence and absence of the phytochemicals in the ethyl acetate and acetone bark extracts were recorded (Table 7).

4. Discussion

β -lactamase enzymes are usually divided into four classes, A, B, C and D based on sequence homology. Enzyme classes, A, C and D utilize at the active site, serine for hydrolyzation of β -lactam antibiotics[28], while the enzyme class B utilizes zinc ions to catalyze the hydrolysis of the β -lactam moiety[29]. In fact, β -lactam encoding genes are transmitted by mobile genetic elements, such as transposons and plasmids to related or unrelated species of bacteria[30]. Eventually, community acquired infections caused by ESBL producing bacteria are too many[6,31]. Prevalence of ESBL strains among the clinical isolates depends on geographical areas. In an Indian report, figures of percentage of clinical isolates were 40% in *E. coli* and 45% in *K. pneumoniae*[32]; their prevalence was recorded even at higher values in European countries[33]. A global surveillance database collected from Europe, North and South America and Asia, recorded that ESBL producing *E. coli* and *K. pneumoniae* strains were recorded between 13.9% to 39%. In fact, the prevalence of ESBL isolates was higher in Asia than that of in other regions of the world[34]. Further, within 1970, most *E. coli* and *K. pneumoniae* strains contained plasmid-mediated ampicillin hydrolyzing β -lactamases, such as TEM-1 (temoneira), TEM-2 and SHV-1 (sulphydryl variable). However, those could be eliminated by the use of any third generation cephalosporin[35]. In Asia including Japan, multiple types of ESBLs, SHV, TEM and CTX-M type were detected only in *E. coli* and *K. pneumoniae* strains in epidemic patterns[36]. Most recently, carbapenamase producing *E. coli* and *K. pneumoniae* were NDM-1 (New Delhi metallo- β -lactamase-1) type that had expressed CTX-M-15 genes. ESBL producers were reported to co-exist with resistance to many other antibiotics, probably due to the activity encoded by plasmids, as demonstrated with *E.*

coli[37]. In a typical Indian study, a resistance pattern of ESBL producing *E. coli* was reported for co-trimoxazole (74%), gentamicin (75%) and chloroquinone (91%–96%)[38]. In *E. coli* and *K. pneumoniae*, TEM carrying genes, 1, 2 and SHV-1 produced β -lactamases; TEM-1 and TEM-2 are known to code for a broad spectrum resistance, but not to the extended spectrum, as they exhibit resistance simply to penicillin, but not to cephalosporins, the third generation β -lactams; and those genes were reported to be in ESBL *E. coli* since last 2 to 3 decades[5]. Thus, potentially superbugs of *E. coli* and *K. pneumoniae* strains are most likely to evolve worldwide that requires constant surveillance[39]. MDR *E. coli* strains caused bacteraemia approximately in 13% patients with cancer and neutropenia[40]. Incidentally, pyomyositis, an infective disease of the skeletal muscles occurring in tropical areas was due to ESBL-producing *E. coli* strains[41].

Hospital effluents in India have long been reported to have MDR enteropathogenic bacteria and those bacteria are drained to community sewage system without any scientific treatment[42]. Similarly from Nepal, MDR strains of *Shigella*, *S. typhi*, *Salmonella typhimurium* were reported from clinical samples[43]. It was reported from Singapore that all members of Enterobacteriaceae were resistant to the third generation cephalosporins with the domination of *K. pneumoniae*, followed by species of *Enterobacter* and *Citrobacter*. All these bacteria were reported to be resistant to a number of antibiotics[44]. In all these countries, no therapeutic strategies have been worked out for MDR enteropathogenic bacteria. Moreover, ESBL strains of *Enterobacter* sp. have been reported from dogs and this pathogen had been found as the determinant of neonatal sepsis carrying *bla* group of plasmids (*bla*_{CTX-M}, *bla*_{TEM} and *bla*_{SHV})[45,46]. From Malaysia, *Shigella* sp. was reported to be the third most common bacterium causing childhood diarrhoea, and it was found as resistant to seven antibiotics, including ceftriaxone, ciprofloxacin, nalidixic acid and trimethoprim/sulfamethoxazole[47]. From Kolkata, India, the increase of resistance in *Salmonella enterica*, serotype typhi to ampicillin, co-trimoxazole had been described[48]. A MDR *K. pneumoniae* strain, with the metallo- β -lactamase activity, first reported from Italy, was found to hydrolyze a carbapenem[49]. Pandrug resistance (resistance to all available antibiotics) of Gram-negative bacteria from Europe had been reported for *Klebsiella*, *Acinetobacter* and *Pseudomonas*, to state in short[50]. It had been demonstrated that during the evolution of an avirulent way of nutrition, a bacterium gains pathogenicity by the acquisition of foreign pieces of DNA that are incorporated into the main chromosome or those remain as plasmid(s)[51]. Concomitantly, during acquisitions and further spread of drug/antibiotic resistant genes among bacteria occurring in last 60 years approximately, several avirulent and virulent species have co-evolved to multidrug resistant avatars because of the

extensive antibiotic use in both man and animals. Initially, drug resistance and pathogenicity appears to be unlinked but, they are co-selected in the same bacterial replicon and a single determinant is involved for both virulence and resistance, as discussed extensively[52]; exchange of genetic materials is the most readily occurring event in bacteria. Further, pathogenic bacteria have their bases in the intracellular level in host tissues and produce virulence factors that are even not eliminated by drug/antibiotics, due to impermeability of the later many a times. Actually several virulent bacteria, induce inflammatory cytokines, necrotic or apoptotic factors, and the later is epitomized in apoptosis of macrophages of *Shigella*, eventually triggering inflammations of gut tissues. It was demonstrated that a MDR clone of *E. coli* as well as *Salmonella enterica* serotype typhimurium with *acrAB* system both extruded bile salts, which indicated that one DNA patch is involved in both virulence and resistance in these two genera. Compared to the non-pathogenic laboratory strain of *E. coli* K 12 clones, the enterohaemorrhagic strain of *E. coli* O157:H7 has an additional virulent plasmid similar to that of *Shigella* sp[53]. The adage, “one is what one has met in the life” is applicable to a bacterial strains too; *E. coli* was once a commensal and now its clonal nexuses have come into existence with a wide range of serotypes resistant to drugs/antibiotics rendering the status of an intractable pathogens, compatible to several mammalian hosts. A new concept of bio-terrorism has now held, which means that “antibiotic resistance and virulence” are grouped in a particular set of bacteria creating clinical concerns[54], often marauding aged/immunocompromised people. Additionally, enteropathogens cause destructive events in infants and under-5 children along with otherwise healthy adults. Thus, in the short course of 60 years or so, the pandrug resistance strains of Gram-negative bacteria, *E. coli*, *Acinetobacter*, *Pseudomonas* and *Klebsiella* as well as the Gram-positive *Staphylococcus aureus* have emerged[55].

The phytochemical, 2, 6-diisopropylphenol (propofol) was prepared by coupling with 9-hydroxy-11-Z-octadecenoic acid isolated from seed oil of *H. antidysenterica* with the C1- α -hydroxy function of 2, 6-diisopropylphenol. This compound was utilized for monitoring its anti-cancerous activity along with antibacterial properties against *E. coli* and other two Gram-positive bacterial[56]. From Bangladesh, antibacterial study of 16 plants including *H. antidysenterica* was reported against seven enteric bacteria, including the suppurative *Staphylococcus aureus*; this report described the preparation of crude medicines from plant extracts[57]. Further, the notorious enterohaemorrhagic *E. coli* (EHEC) O157:H7 was reported from Thailand to be controlled by 5 plants, including *H. antidysenterica*[58]. From North-India, two MDR strains of *V. cholerae*, O1 and O31 had been described and their control by plants, *Terminalia chebula*

and *Syzygium cumini* had been recorded. Simultaneously, these plants were also reported to have control over *Aeromonas hydrophila* and *Bacillus subtilis*[59]. Indeed, *H. antidysenterica* was reported to have a control over several food-borne enteropathogens[17]. Moreover, an Indian Ayurvedic preparation, Kutajarishta with 5 phyto-extracts including that of leaves of *H. antidysenterica* was recorded effective in controlling diarrhoea and dysentery[60]. Thus, it could be stated that leaves and barks of *H. antidysenterica* could be extensively used as complementary/supplementary medicine for MDR enteropathogens. As per WHO directives, non-committal opinion on phyto-drugs is now regarded as a pejorative attitude.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Enteropathogenic bacteria pose a great concern to public health, both in community and in hospital settings, as they are found frequently in all kinds of water bodies. They are responsible for precipitating fervent episodes of epidemics mainly in unhygienic areas of developing countries. Moreover, the MDR strains of these bacteria are uncontrollable. *H. antidysenterica*, a well-known Indian medicinal plant was tested for its antibacterial efficacy against clinically isolated eight MDR enteropathogenic bacteria. MIC and MBC values of crude extracts gave ideas on its efficacy. Phytochemical analysis was done to access the chemical potentiality of the plant.

Research frontiers

The work was done with clinically isolated MDR pathogens. Hence, this work has overriding importance on similar works, which were done with drug sensitive/standard strains here before. Results in the present study suggest that *H. antidysenterica* leaf and bark extracts could

be used in treating diseases caused by these eight MDR enteropathogenic bacteria. Apothecary could take up this plant for harnessing non-microbial antimicrobials after animal toxicity work.

Related reports

Ayurvedic preparation of this plant and its Pharmacological potential has been described in a review by Lather *et al.* Another study on antibacterial properties of medicinal plants against *E. coli* describes the antibacterial properties of the *H. antidysenterica*.

Innovations & breakthroughs

This plant could be a potential source of complementary and alternative source of medicine against intractable MDR enteropathogens. Since antibiotics are no longer effective in controlling these pathogens, the results obtained in this study promise for a new drugs that could help the pharmacy world to design a new molecule, which can control these pathogens attacking infants and under-5 children.

Applications

H. antidysenterica is an ethnomedicinal plant, which is used by aborigines in many part of India against diarrhoea and dysentery. This study provides a scientific validation of the medicinal/microbiological properties of this plant. In addition, it provides the details of the phyto-constituents, which may be responsible for the antibacterial activities of the plant. This plant is widely used as Ayurvedic medicine in the name of “Kutajarishta”, and this study will provide a scientific platform for further research on this plant to establish it as a frontline drug, as morphine or quinine.

Peer review

The novelty of the work is that the gamut of MDR bacteria was isolated from clinical samples in a hospital. Biochemical identifications were followed during the procedure, along with standard strains. Search of non-microbial antimicrobial is the call of the day for the avalanche of MDR pathogens. Secondly, Indian forest patches are the unique sources of tropical medicinal plants.

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