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In vitro and *in vivo* bactericidal activity of *Vitex negundo* leaf extract against diverse multidrug resistant enteric bacterial pathogens

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ABSTRACT

Objective: To investigate *in vitro* and *in vivo* antibacterial potentials of *Vitex negundo* (*V. negundo*) leaf extracts against diverse enteric pathogens. **Methods:** Water and methanol extracts of *V. negundo* leaves were evaluated against enteric bacterial pathogens by using standard disc diffusion, viable bacterial cell count methods, determination of minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC). **Results:** Methanol extract of *V. negundo* leaves showed potent antibacterial activity (inhibition zone: 9.9-22.6 mm, MIC: 200-3 200 μg/mL, MBC: 200-6 400 μg/mL) against all the pathogenic enteric bacteria (*Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio mimicus*, *Echerichia coli*, *Shigella* spps., and *Aeromonas* spps) tested. Methanol extract of *V. negundo* leaves showed potent bactericidal activity both *in vitro* laboratory conditions (MBC, 200-400 μg/mL) and in the intestinal environment (Dose, 1-2 mg/mL) of infant mice against pathogenic *Vibrio cholerae*, the major causative agent of cholera. Furthermore, assays using the mice cholera model showed that *V. negundo* methanol extract can protect mice from *Vibrio cholerae* infection and significantly decrease the mortality rate ($P<0.0001$). **Conclusions:** For the first time we showed that methanol extract of *V. negundo* leaves exhibited strong vibriocidal activity both *in vitro* and *in vivo* conditions. Therefore, it will be useful to identify and isolate the active compounds of this extract that could be a good alternative of antibiotics to treat cholera.

1. Introduction

Infectious disease is one of the leading causes of death worldwide, particularly, in developing countries. The treatment of most of the infectious diseases depends on the use of antibiotics or antimicrobial drugs. Global antimicrobial resistance is becoming an increasing public health predicament. Bacteria resistant to almost all available antibiotics have been identified[1]. Multiply resistant organisms render therapy more precarious and costly and sometimes unsuccessful. In developing countries, multidrug resistant enteric disease agents such as *Vibrio cholerae* (*V. cholerae*), *Escherichia coli* (*E. coli*) and *Shigella* spps. threaten and circumvent public health measures.

Diarrhoeal diseases are a major public health apprehension in Bangladesh. Every year a large number of people suffer from diarrhoea. *V. cholerae* is the major causative agent of cholera, the devastating diarrhoeal disease. In Bangladesh, *V. cholerae* O1 has developed antimicrobial resistance to trimethoprim, sulfamethoxazole, furazolidone and tetracyclin; and resistance is emerging to ciprofloxacin and azithromycin leaving few options for effective antimicrobial treatment for cholera[2,3]. Multidrug resistant *V. cholerae* have also emerged in African countries[4,5]. Besides, other enteric pathogens, including *Shigella* spp, diarrhoeagenic *E. coli*, and *Salmonella* spp., are rapidly growing resistance to currently available antibiotics[6]. Therefore, it is an urgent need to discover and develop more effective and new antimicrobial agents.

Natural products from the medicinal plants, spices

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and herbs were used worldwide and their antimicrobial potential has been recognized since antiquity[7]. A large number of modern medicines were derived originally from ancient herbal traditions. Medicinal plants have been used as remedies for human diseases for many years as they contain components of therapeutic value. There are numerous natural plant products which have antimicrobial activities that could be used either systemically or locally[8]. Antimicrobials of plant origin are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials[9]. Therefore, natural products from plants could be interesting alternatives to explore new antimicrobial agents.

Vitex negundo (*V. negundo*) belonging to the family Verbenaceae) is an important medicinal plant found throughout Bangladesh, India and some other tropical and temperate regions of the world. Even though almost all of its parts are used in Ayurvedic and Unani systems of medicine, the extracts from its leaves and roots are the most important in the field of medicine and drug[10]. Its leaves[11] and seeds[12] are widely used externally for rheumatism and inflammations of joints and are also reported to have insecticidal properties. The antimicrobial activity and chemical components of the essential oil isolated from *V. negundo* leaf[13-15] have been reported by several researchers in the past. In Bangladesh, leaves of *V. negundo* are widely used for different types of gastroenteritis as traditional medicine and it is immensely effective in different types of diarrhoea and dysentery. The leaves of *V. negundo* have been reported to possess pesticidal, antifungal and antibacterial properties[16]. However very little systemic information of *V. negundo* leaves are available to treat diarrhoeal diseases and its activity against enteric pathogenic bacteria. Our aim is to examine and evaluate bactericidal activity of *V. negundo* leaves extracts from Bangladesh against diverse multidrug resistant enteric pathogens in laboratory conditions as well as in animal model experiments.

2. Materials and methods

2.1. Collection of medicinal plants and spices

Leaves of *V. negundo* were collected from the Kishoreganj district, northern east part of Bangladesh and information about their traditional uses for gastroenteritis were collected from local people and also verified from two books i) *Traditional uses of ethnomedicinal plats of Chittagong hill tracts* published by Bangladesh National Herberium[17] and ii) *Useful plants of Bangladesh*[18].

2.2. Extraction procedure

Healthy and well grown *V. negundo* leaves were collected and immediately brought to the laboratory. The leaves were first washed with distilled water and then sterilized with 10% sodium hypochlorite to prevent the contamination of any microorganisms. They were then rinsed with distilled water again and dried the water. Ten grams of *V. negundo* leaves were cut into small pieces and were grinded to make paste with mortar and pestle. Appropriate solvents (water or methanol) were added to a concentration of 1 g/10 mL and extracted in a soxhlet apparatus for 72 hours. The extracts were pooled and the solvent was evaporated using a rotary evaporator under reduced pressure at 37 ℃. The crude extracts were kept at 4 ℃ until further use.

2.3. Bacterial strains and culture medium

Multidrug resistant and drug sensitive enteric pathogens used in this study were obtained from our laboratory collections. The details of the strains including their antibiotic resistance pattern are described in Table 1. All strains were grown in Luria broth (LB) broth or on LB agar at 37 ℃. Bacterial strains were maintained in nutrient agar slants at room temperature or in LB broth containing 15% glycerol at −80 ℃. Before use bacterial cultures were prepared by inoculating into a tube containing LB and

Table 1
List of drug resistant enteric bacterial pathogens examined in this study.

Microorganisms	Strain ID	Antibiotic resistant pattern	Source of isolation
V. cholerae O1	AY-1868921	STR, SXT, NA, ERY, TET	Patient
V. cholera O139	NIHCO270	STR, SXT, NA	Patient
V. cholera non O1 non O139	2006RV/86	STR, SXT, NA, AMP, KAN, GEN	Environmental water
E. coli O157:H7	M-885496	AMP, CHL, SXT, TET	Patient
S. dysenteriae	Vm110432	CHL	Patient
Shigella flexneri	M-12163	AMP, TET, CHL	Patient
Shigella boydi	M-297092	Sensitive	Patient
Shigella sonnei	M-275521	STR, SXT, NA, ERY, TET	Patient
V. parahaemolyticus (O3:K6) pandemic	AQ-3794	TETi	Patient
V. mimicus	MGL-2585	TETi	Environmental water
A. sobria	MGL-3585/1	NA, AMP	Environmental water
Aeromonas cavie	MGL-3615/1	AMP	Environmental water

i, intermediate resistant; STR, Streptomycin; AMP, ampicillin; TET, tetracycline; NA, nalidixic acid; SXT, Sulphamethoxazole trimethoprime; CHL, chloramphenicol; ERY, erythromycin; GEN, gentamicin; KAN, kanamycin.

subsequent incubation at 37 ℃ overnight.

2.4. Disc diffusion assay

The test solution was prepared with 10 mg/mL of crude extracts, dissolved in 5% dimethyl sulfoxide (DMSO). Whatman no.1 sterile filter paper discs (6 mm) were impregnated with 10 μL of the extract to obtain 100 μg/disc and allowed to dry at room temperature. Ciprofloxacin (5 μg/disc) was used as positive control and 5% DMSO was used as negative control. Bacterial growth inhibition was determined by standard disc diffusion assay[19]. Briefly, Petri plates were prepared by pouring 20 mL of Mueller Hinton agar and allowed to solidify. Plates were dried and 100 μL of bacterial suspension containing 1×10^6 cells/mL was poured in each plate and spread uniformly. The plates were allowed to dry for 5 minutes. The filter paper discs impregnated with extract (100 μg/disc) were then placed on the surface of the plate. Plates were kept for 2 hours in refrigerator to enable pre-diffusion of the extracts into the agar. Then the plates were incubated at 37 ℃ for 24 h and zone of inhibition was observed and measured in millimeters. Reported inhibition zones were the average values calculated from at least three replicate observations.

2.5. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The extract was dissolved in 5% DMSO to obtain 25.6 mg/mL and 0.5 mL of stock solution was incorporated into 0.5 mL of Mueller Hinton broth to get a concentration of 12 800, 6 400, 3 200, 1 600, 800, 400, 200, 100 and 50 μg/mL. Fifty microliter of standardized suspension of the test organism was transferred onto each tube. MIC of the crude extract was then tested in Mueller Hinton broth by broth micro-dilution method[20]. MBCs were determined by plating 100 μL of samples from each MIC assay tube with growth inhibition into freshly prepared Mueller Hinton agar plates and plates were incubated at 37 ℃ for 24 h. The MBC values were recorded as the lowest concentration of extract that did not permit any visible bacterial colony growth on the agar plates during the period of incubation[21]. These assays were repeated three times.

2.6. Bactericidal activity in vitro

Bactericidal activity was determined by the viable cell count method[22]. Five milliliter of nutrient broth was inoculated with the bacterial culture and incubated for 3 h at 37 ℃. Equal volumes of the bacterial suspension containing ~1×10^6 cells/mL (5 mL) and extract were mixed and incubated. At different time intervals viz. 0, 1, 2, 3, 4, 6, 16 h, 0.2 mL of the mixed suspension was spread on nutrient agar plates with different dilutions and were incubated for 24 h at 37 ℃. The mean number of colonies were counted and compared with that of the control in which the extract was replaced with DMSO. The experiment was repeated three times.

2.7. Laboratory animals

Swiss albino mice (5-7 days) of either sex used in this study were obtained from the Animal resources branch of ICDDR, B. The animals were housed in well-ventilated and hygienic compartments, maintained under standard environmental conditions and fed with standard rodent pellet and water *ad libitum*. All experimental procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals. This study was approved by the Animal Experimentation Ethics Committee of ICDDR, B (institutional).

2.8. Bacterticidal activity inside mice intestine

The assay of bactericidal activity of extracts inside mice intestine was similar to infant mouse colonization assays and were performed with modification of previously described method[23]. In brief, batches of 6-day-old infant CD-1 mice were inoculated intragastrically with a mixture of overnight cultures of *V. cholerae* strains with different concentrations of methanol extracts of *V. negundo* leaves. The extracts were mixed just before feeding of mice. Two control groups one was bacteria alone and another was bacteria and DMSO (5%) were fed to mice. Six mice were fed in each group. After 18 h of incubation, the mice were sacrificed, and the small intestines were removed and homogenized. The homogenates were plated on Luria agar plates, incubated overnight at 37 ℃ and the colonies were counted and compared with the controls and initial input. Each experiment was done at least on three different groups of mice.

2.9. Mice survival assay

The ability of the extracts to protect mice against pathogenic *V. cholerae* infection was determined by using groups of ten 5-day old mice with modifications of previously described methods[24]. Each mouse was given 0.05 mL bacterial suspension containing 1×10^{10} cfu/mL orally and after 1 hr incubation each mouse was given 0.05 mL of methanol extract of *V. negundo* leaves. Different concentrations of extract were administered to different groups of mice. Another control group was given bacteria and DMSO. All mice were kept at RT for 18 hrs and then live and dead mice were counted and compared with control. The experiments were done with three different groups of mice for each sample.

The mean values and the standard deviations were calculated from the data obtained with three separate experiments. The data on percentage of mice survival were statistically analyzed by using Student's t test for the significance, and a P value < 0.05 was considered statistically significant.

3. Results

3.1. Antibacterial activity of V. negundo leaf extract

Antibacterial activity for both methanol and water extracts of *V. negundo* leaves were examined against diverse enteric pathogens and most of them were resistant against one or more antimicrobial drugs (Table 1). Methanol extract exhibited potent antibacterial activity against *V. cholerae* O1, O139, non O1 non O139 strains; *E. coli* O157:H7 strain; *Vibrio parahaemolyticus* (*V. parahaemolyticus*) O3:K6 pandemic strain, and *Aeromonas* species (Table 2). The zone of inhibition ranged between (15.233±0.351) and (22.600±0.818) mm for above strains against 100 μg of extract. Moderate antibacterial activity was observed for other enteric pathogens tested (Table 2) and the zone of inhibition ranged between (9.966±0.702) and (13.466±0.288) mm. The water extract of *V. negundo* leaves showed moderate antibacterial activity against *V. cholerae* strains and *V. parahaemolyticus* strain only (Table 2).

3.2. MIC and MBC of methanol extract of V. negundo leaves

Between methanol extract and water extract of *V. negundo* leaves, methanol extract showed potent antibacterial activity against different enteric pathogens. Hence, we determined the MIC and MBC of methanol extract against different enteric pathogens. MIC value indicates the lowest concentration of the extract that exhibits the growth inhibition of particular bacteria and MBC value indicates the lowest concentration that can kill any particular organism. MIC value of the methanol extract of *V. negundo* leaf was 200 μg/mL for pathogenic *Vibrio* species and *Aeromonas sobria* (*A. sobria*), 800 μg/mL for *Vibrio mimicus* (*V. mimicus*) and all types of *Shigella* spp. except *Shigella dysenteriae* (*S. dysenteriae*), 1 600 μg/mL for pathogenic *E. coli*. The high MIC value was observed for *S. dysenteriae* strain (3 200 μg/mL). MBC value of *V. negundo* methanol extract was higher than the MIC. The MBC was 200 μg/mL for *V. cholerae* non O1 non O139 strains and for *A. sobria* strain, 400 μg/mL for *V. cholerae* O1, O139 strains and *V. parahaemolyticus* strain, 800 μg/mL for *V. mimicus*, different *Shigella* spps. except *S. dysenteriae*, 1 600 μg/mL for pathogenic *E. coli* strain.

3.3. Bactericidal activity of V. negundo leaves methanol extract in vitro

Since we found that methanol extract of *V. negundo* leaves showed potent bacterial killing activity, we examined the bactericidal activity of methanol extract against different enteric pathogens at different time intervals by using viable bacterial count methods under laboratory conditions. We used 500 μg/mL of methanol extract of *V. negundo* leaves, as this is the moderate concentration that can kill bacteria found from MBC results. The 500 μg/mL methanol extract of *V. negundo* leaves exhibited strong bactericidal activity

Table 2
Antibacterial activity of methanol extract and water extract of *V. negundo* leaf against pathogenic enteric bacterial strains (mean±SD).

Microorganisms	Mean zone of inhibition (mm)		
	Water extract (100 μg/disc)	Methanol extract (100 μg/disc)	Ciprofloxacin (5 μg/disc)
V. cholerae O1	10.033±0.404	21.133±0.503	33.833±0.416
V. cholera O139	9.466±0.665	19.700±0.529	32.166±0.832
V. cholera non O1 non O139	10.500±0.200	22.600±0.818	35.433±0.585
E. coli O157:H7	no zone	15.233±0.351	28.900±0.400
S. dysenteriae	no zone	10.566±0.568	28.033±0.808
Shigella flexneri	no zone	12.066±0.568	30.266±0.208
Shigella boydi	no zone	11.733±0.723	31.933±0.611
Shigella sonnei	no zone	13.466±0.288	29.033±0.305
V. parahaemolyticus (O3:K6) pandemic	10.133±0.251	18.466±0.472	34.633±0.642
V. mimicus	no zone	9.966±0.702	34.733±0.642
A. sobria	no zone	16.700±0.435	32.266±0.737
Aeromonas cavie	no zone	17.733±0.568	33.100±0.173

Table 3
Bactericidal activity of *V. negundo* leaf methanol extract (500 μg/mL) against different enteropathogenic bacterial strains in *in vitro* conditions.

Strains	Viable bacterial counts (cfu/mL) at different time intervals						
	Inoculums	1 h	2 h	3 h	4 h	6 h	16 h
V. cholerae O1	1.2×10^{7}	0	0	0	0	0	0
V. cholerae O139	2.2×10^{7}	1.8×10^{3}	0	0	0	0	0
V. cholerae non O1 non O139	7.8×10^{6}	0	0	0	0	0	0
V. parahaemolyticus O3:K6	9.8×10^{6}	0	0	0	0	0	0
E. coli O157:H7	7.9×10^{6}	7.4×10^{6}	6.3×10^{6}	2.1×10^{6}	5.4×10^{5}	1.2×10^{5}	8.6×10^{4}
S. dysenteriae	8.6×10^{6}	7.9×10^{6}	7.2×10^{6}	6.1×10^{6}	3.4×10^{6}	9.7×10^{5}	5.4×10^{5}
V. mimicus	4.3×10^{6}	3.7×10^{5}	3.1×10^{5}	2.7×10^{4}	1.3×10^{4}	9.5×10^{3}	9.0×10^{3}
A. sobria	7.2×10^{6}	0	0	0	0	0	0

against different enteric pathogens and this concentration was able to completely kill pathogenic *V. cholera* of different serotypes, *V. parahaemolyticus*, and *A. sobria* strains and were unable to kill *E. coli* O157:H7, *S. dysenteriae* and *V. mimicus* strains up to 16 hrs incubation period (Table 3). However, this concentration showed bacteriostatic activity against those strains.

3.4. Bactericidal activity of V. negundo leaf methanol extract in in vivo

The MBC value and *in vitro* bactericidal activity study clearly indicated that methanol extract of *V. negundo* leaves had potent antibacterial activity against *Vibrio* species, especially against all types of pathogenic *V. cholerae* strains. Therefore, these results convinced us to examine the bactericidal activity of these extracts in *in vivo* conditions. *V. cholerae* is a major causative agent of the devastating diarrhoeal disease cholera and these bacteria were able to colonize the infant mice intestine. Here we examined the ability of this extract to kill the bacteria in the mice intestinal environment. In this experiment we used pathogenic strains of *V. cholerae* O1 El Tor; O139; and non O1 non O139 strains. We administered ~1×10^8 live bacterial cells along with different concentrations of *V. negundo* leaves methanol extract and found that 2.0 mg/mL concentration of *V. negundo* leaf methanol extract killed all types of *V. cholerae* inside the mice intestinal environment and therefore we could not retrieve any viable bacteria from the homogenized mice intestine, whereas, 1.0 mg/mL concentration was enough to kill non O1 non O139 strain completely (Table 4). These results indicated that the active compound of *V. negundo* leaf which has bactericidal activity *in vitro* was found to retain the activity under *in vivo* conditions as well.

Table 4
Vibriocidal activity of *V. negundo* leaf methanol extract against different *V. cholerae* strains in mice intestinal environment.

Strains	Inoculums (cfu/mL)	*V. negundo* methanol extract (mg/mL)			
		0.5	1.0	2.0	3.0
V. cholerae O1	1.8×10^8	6.0×10^4	1.2×10^2	0	0
V. cholerae O139	8.2×10^7	1.2×10^3	2.2×10^2	0	0
V. cholerae non O1 non O139	2.3×10^8	3.4×10^3	0	0	0

3.5. V. negundo leaf extract can protect mice against V. cholerae infection

Pathogenic *V. cholerae* induce the mortality in infant mice[24]. Here we examined the ability of the *V. negundo* leaf methanol extract to protect mice challenged with a high dose of pathogenic *V. cholerae*. Mice have been used as a model for *V. cholerae* pathogenesis in which mice are ultimately killed by the bacteria. When we orally administered *V. cholerae* strains and then administered DMSO (5%) as control we observed that about 80% of mice died after 18 h of incubation. Whereas when we first administered cholera bacteria and then administered different concentrations of *V. negundo* leaf methanol extract, we found that the extracts had the ability to protect mice and increased their survival rate in a dose dependent manner (Figure 1). The 2 mg/mL and 3 mg/mL of *V. negundo* leaf methanol extract significantly increased ($P<0.001$ and $P<0.000\,1$) the survival rate up to 60% and 80% of mice respectively. On the other hand high mortality of mice was observed when 10 mg/mL of extract was used, suggesting that the higher dose might toxic to mice.

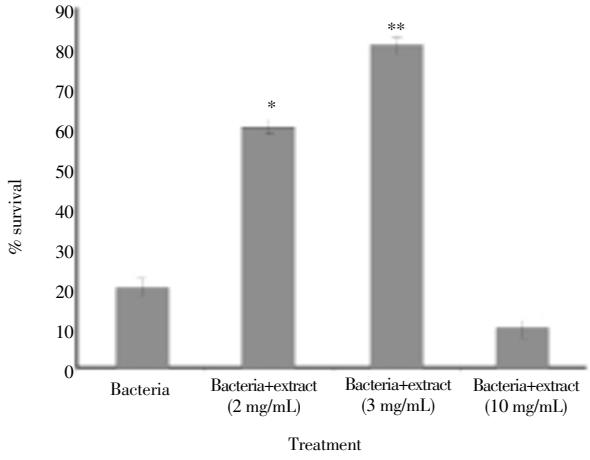

Figure 1. Effects of *V. negundo* leaf methanol extract on mice survival against *V. cholerae* infection.

4. Discussion

During the second half of the 20th century, the acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the available antibiotics led researchers to investigate the antimicrobial activity of medicinal plants.

In this study, we collected the *V. negundo* leaves from the north-east part of Bangladesh, where it is readily used to treat diarrhoea, dysentery and different types of gestroenteritidis. We examined the antibacterial activity of both methanol and water extracts against different drug sensitive and multi drug resistant pathogenic bacteria isolated from clinical and environmental sources. These bacteria are major causative agents of different kinds of enteric diseases. The extracts were examined for their antibacterial activity against various types of *V. cholerae* epidemic strains, *V. parahaemolyticus* O3:K6 pandemic strain, *E. coli* O157:H7 strain, different types of *Shigella* strains, toxigenic *V. mimicus* strain and strains of *Aeromonas* spps.

Toxigenic strains of *V. cholerae* belonging to the O1 and O139 serogroups cause cholera, a severe diarrheal disease

that occurs frequently as epidemics in many developing countries[25]. Strains belonging to other serogroups, collectively referred to as non-O1, non-139, have also been implicated as etiologic agents of moderate to severe human gastroenteritis[26,27]. *V. mimicus* has also been implicated in diarrheal disease, and some strains of *V. mimicus* have been demonstrated to carry virulence genes including cholera toxin gene and produce cholera toxin[27-29]. Pathogenic *V. parahaemolyticus* cause gastroenteritis through consumption of sea foods. Pandemic strains of *V. parahaemolyticus* mainly O3:K6 clone first emerged in Asia in 1995 and then rapidly spreads and causes outbreaks in north and south America, Europe and Africa[30-32]. Enterohaemorrhagic *E. coli* (EHEC) O157:H7 is a human pathogen that contributes to an average of 17 outbreaks and 75 000 cases of illness each year in the USA alone[33]. EHEC O157:H7 strains cause a range of clinical complications including diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome[34,35]. The largest outbreak caused by *E. coli* O157:H7 in 1996 in Sakai, Japan[36]. Bacillary dysentery caused by *Shigella* species is a public health problem in developing countries including Bangladesh. Shigellosis occurs as a disease endemic in Bangladesh, and at least three large epidemics caused by *S. dysenteriae* type 1 have occurred between 1972 and 1994, causing high morbidity and mortality, particularly in children[37,38].

V. negundo is a very well known medicinal plant for its wide range of uses traditionally. Almost all of its parts are used in Ayurvedic and Unani systems of medicine[10] and *V. negundo* leaf extracts had been reported to possess antibacterial, antifungal, pesticidal activity[13,39-42]. It also has antioxidant and anti-inflamatory and hepatoprotective activity[43-49]. However, effects of *V. negundo* leaf extracts on enteric pathogens are not well studied. In different parts of Bangladesh, *V. negundo* leaves are traditionally used for different types of gastroenteritis, fever, hyper acidity and leismoniasis[18].

We examined the methanol and water extract of *V. negundo* leaf by disc diffusion assay and found that methanol extract of *V. negundo* showed moderate to strong antibacterial activity against all types of enteric pathogens tested and exhibited very potent antibacterial activity against all types of *V. cholerae* strains, the major causative agent of cholera. The antibacterial activity of *V. negundo* leaf methanol extract was previously examined against other bacterial specieses[13,15,44].

As methanol extract exhibited potent antibacterial activity than water extract, we used methanol extract of *V. negundo* leaf for further experiments in this study. Methanol was widely used as a solvent to prepare medicinal plant extracts[50,51] and this is the solvent, well known for its nature to dissolve wide range of antimicrobials including tannins, polyphenols, terpenoids, saponins, lactones, flavones and phenones[7].

Since disc diffusion assay has some limitations and this assay only indicate the growth inhibition ability of the extracts against certain microorganisms, it could not tell whether the inhibition zone is for bactericidal or bacteriostatic activity of the certain extracts. Therefore, to confirm the nature of activity and specific dose against certain microorganisms, we determined the MIC and MBC of the extracts and found that MIC value was within the range of 200-800 μg/mL for all enteric bacteria tested except *E. coli* O157:H7 and *S. dysenteriae*. From MBC value it was found that methanol extract of *V. negundo* leaf could kill all types of enteric bacteria tested but different doses were needed for different bacteria. A range of 200-400 μg/mL of the extract was sufficient to kill the *V. cholerae*, *V. parahaemolyticus* and *A. sobria* strains. Hence, our data suggested that *V. negundo* leaf methanol extract has both growth inhibition as well as killing activity against all the bacteria tested and those activity is very potent on *V. cholerae* O1, O139, non O1 non O139 strains, *V. parahaemolyticus* pandemic strain, toxigenic *V. mimicus* and *Aeromonas* spps.

In vitro bactericidal activity of *V. negundo* leaf methanol extract (500 μg/mL) at different time interval suggested that it has very strong killing activity against all the *Vibrio* species and *Aeromonas* spps. tested and interestingly within one hour incubation period this extract was able to killed all the bacterial cells (~1×10^6 cfu/mL) incubated with the extract whereas this concentration of extract (500 μg/mL) was not able to kill *E. coli* O157:H7, *S. dysenteriae* and *V. mimicus* strains. However, this concentration can inhibit the growth of these microorganisms and act as potent bacteriostatic agent at lower dose. The microorganisms we examined in this study are Gram-negative bacteria. It was suggested that the lipopolysaccharides present in the outer membrane of Gram-negative bacteria gave them more resistance towards antibacterial agents than Gram-positive bacteria[52,53]. Nevertheless, we demonstrated here potent antibacterial activity of *V. negundo* leaf methanol extract against number of Gram-negative enteric pathogens.

As *V. negundo* leaf methanol extract exhibited bactericidal activity, especially on *V. cholerae*, which is called vibriocidal activity, *in vitro*, this result prompted us to examine the vibriocidal activity of this extract in *in vivo* conditions. *V. cholerae* can colonize in mice intestine and help to express virulence determinents and ultimately cause disease. Here 5-day old mice were administered high dose (~1×10^8 cfu/mL) of viable *V. cholerae* cells with different dose of *V. negundo* leaf methanol extracts (0.5 mm/mL to 10 mg/mL), as we do not know which dose will be able to kill bacteria inside mice intestinal environments. Very surprisingly we found that *V. negundo* leaf methanol extract was able to retain its activity in intestinal conditions and 2 mg/mL of concentrations were completely killed all the *V. cholerae*

bacteria administered whereas 1 mg/mL of extract was able to killed all the cells of *V. cholerae* non O1 non O139 strain. These results suggested that the compounds of *V. negundo* leaf responsible for antibacterial activity *in vitro* are effective to exert its activity *in vivo* also. This result convinced us to examine the potentiality of this extract whether it can be able to protect mice from the pathogenic *V. cholerae* infection.

Pathogenic *V. cholerae* can cause death of infant mice in cholera model experiments[24], therefore we administered the mice with high dose of pathogenic *V. cholerae* O1 strain orally and then challenged with different doses of *V. negundo* leaf methanolic extract to observe whether this extract can protect the mice from the disease and reduce the mortality rate. Here we also got motivating results that *V. negundo* leaf extract significantly induced the survival rate ($P<0.001$ and $P<0.000\,1$) of the mice in dose dependent manner and 3 mg/mL concentration was able to increase the survival rate up to 80%, whereas only 20% of mice were survived by bacteria without extract.

In this study, we have shown the antibacterial activity of *V. negundo* leaf extracts on a wide range of enteric pathogens *eg*., *V. cholerae*, *V. parahaemolyticus*, *V. mimicus*, *E. coli*, *Shigella* species and *Aeromonas* species. These organisms can cause severe diarrhoeal diseases and dysentery. Here for the first time we also carried out *in vivo* assay with extracts in mice cholera model and evidenced that *V. negundo* leaf methanol extract is equally potent both *in vitro* and *in vivo* conditions to kill the cholera causing bacteria and protect mice from disease and death. If we identified and isolate the active compounds from *V. negundo* leaf, responsible for vibriocidal acitivity *in vivo*, then it could be a good alternative of antibiotics to treat cholera to reduce the disease burden. Therefore, further studies should be undertaken to characterize the active compounds residing in *V. negundo* leaves. Additionally, evaluation of the effects of each individual compound on microorganisms as well as toxicological studies need to be performed.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Bax R, Mullan N, Verhoef J. The millennium bugs– the need for and development of new antibacterials. *Int J Antimicrob Agents* 2000; **16**: 51–59.

[2] Saha D, Karim MM, Khan WA, Ahmed S, Salam MA, Bennish ML. Single dose azithromycin for the treatment of cholera in adults. *N Engl J Med* 2006; **354**: 2452–2462.

[3] Faruque ASG, Alam K, Malek MA, Khan GY, Ahmed S, Saha D, et al. Emergence of multi-drug resistant strain of *V. cholerae* O1 in Bangladesh and reversal of their susceptibility to tetracycline after 2 years. *J Health Popul Nutr* 2007; **25**: 241–243.

[4] Mwansa JC, Mwaba J, Lukwesa C, Bhuiyan NA, Ansaruzzaman M, Ramamurthy T, et al. Multiply antibiotic-resistant *V. cholerae* O1 biotype El Tor strains emerge during cholera outbreaks in Zambia. *Epidemiol Infect* 2007; **135**: 847–853.

[5] Islam MS, Mahmud ZH, Ansaruzzaman M, Faruque SM, Talukder KA, Qadri F, et al. Phenotypic, genotyping and antibiotic sensitivity of strains isolated from cholera epidemic in Zimbabwe. *J Clin Microbiol* 2011; **49**: 2325–2327.

[6] Levy SB, Marshall B. Antibacterial resistant worldwide: causes, challenges and responses. *Nat Med Suppl* 2004; **10**: S122– S129.

[7] Cowan MM. Plant products as antimicrobial agents. *Clin Microbiol Rev* 1999; **12**: 564–582.

[8] Heinrich M, Barnes J, Gibbons S, Williamson EM. *Fundamentals of pharmacognosy and phytotherapy*. Edinburgh: Churchill Livingstone; 2004, p. 4–7.

[9] Iwu MW, Duncan AR, Okunji CO. New antimicrobials of plant origin. In: Janick J. *Perspectives on new crops and new uses*. VA: ASHS Press, Alexandria; 1999, p. 457–462.

[10] Lal S, Jain S, Prakash O, Jain N. Shambhalu: An important medicinal plant. *J Pharm Sci* 2006; **7**: 45–55.

[11] Dharmasiri MG, Jayakody JR, Galhena G, Liyanage SS, Ratnasooriya WD. Anti-inflammatory and analgesic activities of mature fresh leaves of *Vitex negundo*. *J Ethnopharmacol* 2003; **87**: 199–206.

[12] Chawla AS, Sharma AK, Handa SS, Dhar KL. Chemical investigation and anti-inflammatory activity of *Vitex negundo* seeds. *J Nat Prod* 1992; **55**: 163–167.

[13] Nagarsekar KS, Nagarsenker MS, Kulkarni SR. Evaluation of composition and antimicrobial activity of supercritical fluid extract of leaves of *Vitex negundo*. *Indian J Pharm Sci* 2010; **72**: 641–643.

[14] Taneja SC, Gupta RK, Dhar KL, Atal CK. The essential oil of *Vitex negundo* Linn. *Indian Perfumer* 1979; **22**: 162.

[15] Khokra SL, Prakash O, Jain S, Aneja KR, Dhingra Y. Essential oil composition and antibacterial studies of *Vitex negundo* Linn extracts. *Indian J Pharm Sci* 2008; **70**: 522–526.

[16] Rasaga CY, Morales E, Rideout JA. Antimicrobial compounds from *Vitex negundo*. *Philippine J Sci* 1999; **128**: 21–29.

[17] Uddin SN, Rahman MM. *Traditional uses of ethnomedicinal plants of the Chittagong hill tracts*. Bangladesh: National Herberium; 2006.

[18] Dey TK. *Useful plants of Bangladesh*. 1995.

[19] Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin pathol* 1966; **45**: 493–496.

[20] Ericsson HM, Sherris JC. Antibiotic sensitivity testing report of an international collaborative study. *Acta Pathol Microbiol Scand* 1971; **217**: 1–90.

[21] Suffredini IB, Sader HS, Goncalves AG, Reis AO, Gales AC,

Varella AD, et al. Screening of antibacterial activity extracts obtained from plants native to Brazilian amazon rain forest. *Braz J Med Ethnopharmacol* 2004; **62**: 183-193.

[22]Toda M, Okubo S, Hiyoshi R, Shimamura T. The bactericidal activity of tea and coffee. *Lett Appl Microbiol* 1989; **8**: 123-125.

[23]Gardel CL, Mekalanos JJ. Alterations in *V. cholerae* motility phenotypes correlate with changes in virulence factor expression. *Infect Immun* 1996; **64**: 2246-2255.

[24]Nishibuchi M, Seidler RJ, Rollins DM, Joseph SW. *Vibrio* factors cause rapid fluid accumulation in suckling mice. *Infect Immun* 1983; **40**: 1083-1091.

[25]Faruque SM, Albert MJ, Mekalanos JJ. Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. *Microbiol Mol Biol Rev* 1998; **62**: 1301-1314.

[26]Morris JG Jr, Black RE. Cholera and other vibrios in the United States. *N Engl J Med* 1985; **312**: 343-350.

[27]Janda JM, Powers C, Bryant RG, Abbott SL. Current perspective on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. *Clin Microbiol Rev* 1988; **1**: 245-267.

[28]Ramamurthy T, Albert MJ, Huq A, Colwell RR, Takeda Y, Takeda T, et al. *Vibrio mimicus* with multiple toxin types isolated from human and environmental sources. *J Med Microbiol* 1994; **40**: 194-196.

[29]Spira WM, Fedorka-Cray PJ. Purification of enterotoxins from *Vibrio mimicus* that appear to be identical to cholera toxin. *Infect Immun* 1984; **45**: 679-684.

[30]Okuda J, Ishibashi M, Hayakawa E, Nishino T, Takeda Y, Mukhopadhyay AK, et al. Emergence of a unique O3:K6 clone of *Vibrio parahaemolyticus* in Calcutta, India, and isolation of strains from the same clonal group from Southeast Asian travelers arriving in Japan. *J Clin Microbiol* 1997; **35**: 3150-3155.

[31]Matsumoto C, Okuda J, Ishibashi M, Iwanaga M, Garg P, Rammamurthy T, et al. Pandemic spread of an O3:K6 clone of *Vibrio parahaemolyticus* and emergence of related strains evidenced by arbitrarily primed PCR and toxRS sequence analyses. *J Clin Microbiol* 2000; **38**: 578-585.

[32]Nishioka T, Kamruzzaman M, Nishibuchi M, Satta Y. On the origin and function of an insertion element VpaI1 specific to post 1995, pandemic *V. parahaemolyticus* strains. *Genes Genet Syst* 2008; **83**: 101-110.

[33]Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, et al. Food-related illness and death in the United States. *Emerg Infect Dis* 1999; **5**: 607-625.

[34]Kaper JB, Nataro JP, Mobley HL. Pathogenic *Escherichia coli*. *Nat Rev Microbiol* 2004; **2**: 123-140.

[35]Nataro JP, Kaper JB. Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev* 1998; **11**: 142-201.

[36]Fukushima H, Hashizume T, Morita Y, Tanaka J, Azuma K, Mizumoto Y, et al. Clinical experiences in Sakai City Hospital during the massive outbreak of enterohemorrhagic *Escherichia coli* O157 infections in Sakai City, 1996. *Pediat Int* 1999; **41**: 213-217.

[37]Chen LC, Rahman M, Sarder AM. Epidemiology and causes of death among children in a rural area of Bangladesh. *Int J Epidemiol* 1980; **9**: 25-33.

[38]Ronsmans C, Bennish ML, Wierzba T. Diagnosis and management of dysentery by community health workers. *Lancet* 1988; **2**: 552-555.

[39]Zaidan MR, Noor RA, Badrul AR, Adlin A, Norazah A, Zakiah I. *In vitro* screening of five local medicinal plants for antibacterial activity using disc diffusion method. *Trop Biomed* 2005; **22**: 165-170.

[40]Perumal SR, Ignacimuthu S, Sen A. Screening of 34 Indian medicinal plants for antibacterial properties. *J Ethnopharmacol* 1998; **62**: 173-182.

[41]Kannathasan K, Senthilkumar A, Chandrasekaran M, Venkatesalu V. Differential larvicidal efficacy of four species of *Vitex* against *Culex quinquefasciatus* larvae. *Parasitol Res* 2007; **101**: 1721-1723.

[42]Sathiamoorthy B, Gupta P, Kumar M, Chaturvedi AK, Shukla PK, Maurya R. New antifungal flavonoid glycoside from *Vitex negundo*. *Bioorg Med Chem Lett* 2007; **17**: 239-242.

[43]Kulkarni RR, Virkar AD, D'mello P. Antioxidant and antiinflammatory activity of *Vitex negundo*. *Indian J Pharm Sci* 2008; **70**: 838-840.

[44]Vinuchakkaravarthy T, Kumaravel KP, Ravichandran S, Velmurugan D. Active compound from the leaves of *Vitex negundo* L. shows anti-inflammatory activity with evidence of inhibition for secretory Phospholipase A(2) through molecular docking. *Bioinformation* 2011; **7**: 199-206.

[45]Zheng CJ, Huang BK, Wang Y, Ye Q, Han T, Zhang QY, et al. Anti-inflammatory diterpenes from the seeds of *Vitex negundo*. *Bioorg Med Chem* 2010; **18**: 175-181.

[46]Tandon VR, Khajuria V, Kapoor B, Kour D, Gupta S. Hepatoprotective activity of *Vitex negundo* leaf extract against anti-tubercular drugs induced hepatotoxicity. *Fitoterapia* 2008; **79**: 533-538.

[47]Rahman MA, Islam MS. Antioxidant, antibacterial and cytotoxic effects of the phytochemicals of whole *Leucas aspera* extract. *Asian Pac J Trop Biomed* 2013; **3**(4): 273-279.

[48]Arokiyaraj S, Sripriya N, Bhagya R, Radhika B, Prameela L, Udayaprakash NK. Phytochemical screening, antibacterial and free radical scavenging effects of *Artemisia nilagirica*, *Mimosa pudica* and *Clerodendrum siphonanthus*-An *in-vitro* study. *Asian Pac J Trop Biomed* 2012; **2**(Suppl 2): S601-S604.

[49]Sahu MC, Padhy RN. *In vitro* antibacterial potency of Butea monosperma Lam. against 12 clinically isolated multidrug resistant bacteria. *Asian Pac J Trop Dis* 2013; **3**(3): 217-226.

[50]Devi PR, Kokilavani R, Poongothai SG. Anti microbial activity of the various leaf extracts of *Vitex negundo* Linn. *Anc Sci Life* 2008; **27**: 22-27.

[51]Parekh J, Karathia N, Chanda S. Screening of some traditionally used medicinal plants for potential antibacterial activity. *Indian J Pharm Sci* 2006; **68**: 832-834.

[52]Sawer IK, Berry MI, Ford JL. Effect of medium composition, agitation and presence of EDTA on the antimicrobial activity of cryptolepine. *Lett Appl Microbiol* 1997; **25**: 207-211.

[53]Gao Y, Belkum MJV, Stiles M. The outer membrane of Gram-negative bacteria inhibits antibacterial activity of brochocin C. *Appl Environ Microb* 1999; **65**: 4329-4333.