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Anti-bacterial Activity of the Extract of *Terminalia arjuna* Against Multi Antibiotic Resistant *Vibrio cholerae*

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Abstract

Morbidity and mortality due to diarrhoea continues to be a major problem in many developing countries. Water samples from different areas of Chittagong were collected and 22 *Vibrio cholerae* were isolated from the samples. In this experiment we found that 85% of the *Vibrio cholerae* isolated can grow at 6% NaCl whereas none of these can survive at 8% NaCl. Most of the isolates were resistant to at least 2 antibiotics. 95.45% were resistant to ampicillin, 50% to erythromycin, 63.63% to nalidixic acid, 13.63% to cephotaxine, 13.63% to ceftriaxone and 27.27% to cotrimoxazol. Arjun bark extract was used as a biological tool to resolve the antibiotic resistant *V. cholerae* problem. Arjun extract inhibited the growth of *V. cholerae* at all concentrations and zone diameter increased with the increase of concentrations. The regression coefficient of the relationship between concentration and zone diameter varies from 0.75 to 0.984 for most of the isolates which indicates that there exists a linear relationship. This study revealed that *Terminalia arjuna* would be a good antibacterial drug in the treatment of *Vibrio cholerae* infections, provided if found effective and nontoxic through *in vivo* studies.

Keywords: *Vibrio cholerae*; *Terminalia arjuna*; Antimicrobial; Drug resistance.

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1. Introduction

Morbidity and mortality due to diarrhoea continues to be a major problem especially amongst children in many developing countries, including India and Bangladesh [1]. Diarrhoeal disease in Bangladesh is estimated to be the fourth biggest killer of children [2]. The people of Bangladesh get diarrhoea through various sources and the most

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important is the lack of hygiene practice that causes severe sickness to them. *Vibrio cholerae* is the regular cause of cholera that most frequently necessitates hospitalization [3].

Historically, a number of large cholera epidemics have been associated with multiple-antibiotic-resistant (MAR) strains of *V. cholerae* [4, 5]. The antibiotic resistance pattern of epidemic strains has also changed frequently, and the emergence of *V. cholerae* O1 or O139 with different antibiograms has been documented. In many instances, the emergence of strains with altered antibiotic resistance has been associated with large epidemics of cholera [6].

Many plants in Bangladesh are used in traditional folklore medicine for the treatment of gastrointestinal disorders such as cholera, diarrhoea and dysentery. The potential benefits of herbal medicines could lie in their efficacy, safety, little or no side effects and relatively low costs [7]. In Bangladesh, there are several hundred plants belonging to the families Combretaceae such as *Terminalia arjuna* that have been reported to contain a wide range of secondary metabolites that have cytotoxic effects [8]. These cytotoxic effects have been demonstrated by antibacterial, anti-malarial, anti-fungal and anti-inflammatory activities [9, 10].

The present study was designed to examine the occurrence and emergence of multi-antibiotic resistant (MAR) *Vibrio* spp. with newer antibiotic-resistance profile in the aquatic environment of Chittagong, Bangladesh and to test antibacterial activity of *Terminalia arjuna* against isolated *Vibrio* spp. These findings have implications for understanding the antibiotic resistance pattern of *Vibrio* spp. and the feasibility of using *Terminalia arjuna* extract to prevent spread of pathogenic *Vibrio* strains before the initiation of cholera epidemics.

2. Materials and Methods

2.1. *Study area and sample collection*

Water samples from different drains, ponds and rivers of Bahadderhat, Rajakhali khal, Sirajoddullah road area, Karnophully, Patenga, Chakbazar, Arakan society and Rahomatgonj area, Chittagong were collected weekly during March to May, 2010. At each time of collection, precaution was taken to minimize cross-contamination of samples. Samples were aseptically collected in sterile bottles according to standard procedures and transported to the laboratory of Industrial Microbiology Research Division, BCSIR Laboratories Chittagong in an insulated box with ice to maintain a temperature ranging from 4 ^{o}C to 6 ^{o}C [11]. Samples were stored in ice for up to 6 h from the time of collection for transport and subsequent analysis in the laboratory.

2.2. *Enrichment of samples*

100 mL water was filtered through 0.22 µm membrane (Millipore Corp., Bedford, MA) and the filter paper was added to 50 mL alkaline peptone water (APW) for enrichment. Then the APW was incubated for 6-8 hour at 37 ^{0}C with shaking.

2.3. *Isolation of strains*

5 µL of enriched APW broth was streaked onto TCBS agar and incubated at 37 °C for 18 to 24 h. Typical *Vibrio*-like colonies were picked up from TCBS agar plate. Isolated colonies were streaked on Nutrient agar plates for pure culture and for presumptive identification. The identities of suspected *Vibrio cholerae*-like colonies were confirmed using biochemical tests.

2.4. *Identification of vibrio cholerae strains*

The shape and type of Gram reaction are microscopically studied using 18 hour culture from agar plate. The biochemical tests involved Kliger's Iron (KIA) Agar, Simmon's Citrate Slant, Motility Indole Urease (MIU), Lysine Iron agar (LIA), Urea broth, Peptone water, Methyl Red (MR), Voges Proskauer (VP), Nutrient Nitrate Broth (NB), carbohydrate fermentation test was done for lactose, sucrose, glucose and starch, Oxidase, and Catalase tests. Identification of isolates obtained in pure culture was based on Gram staining , biochemical characterisitcs and growth pattern on selective and differential media and; according to the procedures recommended in the Bergey's Manual of Determinative Bacteriology [12, 13].

2.5. *Salt tolerance of isolated vibrio cholerae strains*

9 ml of TSB containing various concentrations of NaCl at 0, 1, 3, 6, 8 or 10% NaCl were inoculated with 1 ml of culture grown in TSB and incubated for 18-24 h at 37 °C. Profused growth was considered as positive. Various species have different level in salt tolerance that could be used for their identification.

2.6. *Antibiotic susceptibility testing*

Twenty two strains were tested for antibiotic resistance by the standard agar disc diffusion technique [14] on Mueller Hinton agar using commercial discs (Oxoid, UK). The following antibiotics with the disc strength in parentheses were used: Ciprofloxacin (Cip, 5µg), cephotaxime (Cep, 30µg), ceftriaxone (Cef, 30µg), cotrimoxazoll (Cot, 25µg), ampicillin (Amp, 10µg), erythromycin (Ery, 15µg) and nalidixic acid (Nal, 30µg). A control strain of E. coli ATCC 25922 was included in each plate. Antimicrobial breakpoints and interpretation were taken from the CLSI standards [15].

2.7. *Collection and extraction of plant material*

The bark of *T. arjuna* was collected in fresh condition from Chittagong Hill tracts. The cleaned samples were cut into small pieces (1-2 cm), dried in air to make it suitable for grinding. The samples were ground to fine powder mechanically and the dried powder was kept steeped 72 hours in ethanol. The extract thus obtained was filtered and subjected to rotary vacuum evaporator at 50 ^{0}C and concentrated to gummy materials under reduced

pressure .The gummy materials were then collected in a small vial and then dried. Thus crude extracts were obtained.

2.8. *Determination of antibacterial activity of terminalia arjuna crude extracts*

The *in vitro* sensitivity of the 12 selected *Vibrio cholerae* isolates to the crude extracts of *T arjuna* was determined by disc diffusion method. Dried and sterilized paper discs were treated separately with desired concentration of previously prepared ethanolic solution of the crude extract using a micropipette dried in air under aseptic condition and placed at equidistance in a circle on the seeded plate. The concentrations of crude extract used were 1 mg/disc, 2 mg/disc, 3 mg/disc, 4 mg/disc and 5 mg/disc. These plates were kept for 4-6 hours at low temperature and the test materials diffuse from disc to the surrounding medium by this time. The plates were then incubated at 37 ^{o}C for 24 hours and zone diameter was measured in mm.

3. Results and Discussion

3.1. *Identification of the strains*

65 suspected *Vibrio cholerae* colonies have been isolated from the samples analyzed from TCBS agar. The isolates were purified by restreaking further on Nutrient agr (NA) and incubated for 18-24 hrs at 37 ^{0}C. Following overnight incubation in nutrient agar at 37 °C, the isolates were preserved in 30% glycerol at -20 °C. The shape and type of Gram reaction are microscopically studied using 18 hours culture from agar plate. Identification of isolates obtained from pure culture was based on Gram staining, biochemical characterisitcs and growth pattern on selective and differential media according to the procedures recommended in the Bergey's Manual of Determinative Bacteriology [12, 13]. Of the 65 suspected isolate 22 were identified as *Vibrio cholerae*. The percentage of *Vibrio cholerae* isolated from different sampling site is shown in Fig 1.

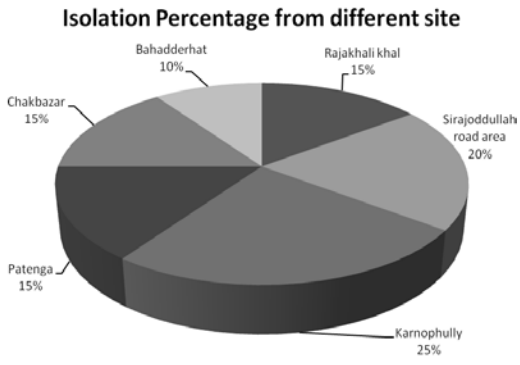

Fig. 1. Isolation percentage of *Vibrio cholerae* from different sampling site.

3.2. *Salt tolerance*

Various species of *Vibrio cholerae* have different characteristics in salt tolerances that could be used for identification. According to salt tolerance of *Vibrio* spp. [16], *Vibrio* cholerae can grow at 3% salt concentration and cannot grow at 6%, 8% salt concentration. But in this experiment we found that 85% of the *Vibrio cholerae* isolated can grow at 6% NaCl whereas about 45% can grow at 3% NaCl. None of the isolated *Vibrio cholerae* can survive at 8% NaCl. Of them 8 isolate can survive at 6% NaCl but cannot grow at 3% NaCl which indicates them to be obligate halophiles. 3 of the isolates showed no growth at any of the NaCl concentration which indicate that they are not halophilic. The salt tolerance of the isolates is shown in Fig. 2.

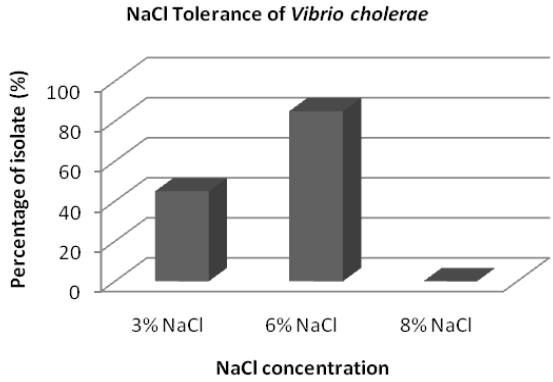

Fig. 2. Salt tolerance of isolated *Vibrio cholerae.*

3.3. *Antimicrobial resistance*

All the isolates were tested for antibiotic sensitivity against 7 commonly used antibiotics belonging to different groups. Out of the strains tested, most of them were resistant to at least 2 antibiotics. 95.45% were resistant to ampicillin, 50% were resistant to erythromycin, 63.63% were resistant to nalidixic acid, 13.63% were resistant to cephotaxine, 13.63% were resistant to ceftriaxone and 27.27% were resistant to cotrimoxazol. In this study ciprofloxacin, cephotaxine, ceftriaxone, cotrimoxazoll were found to be the most potent drug. The antibiotic resistance pattern of isolated *Vibrio cholerae* is shown in Fig. 3. These observations indicated that higher and newer resistance in *Vibrio cholerae* is occurring in natural environment. The results of the present antimicrobial resistant pattern clearly indicated that bacteria were more resistant to the antibiotics that were easily available and most frequently used. Less resistance was found to the antibiotics that were relatively expensive.

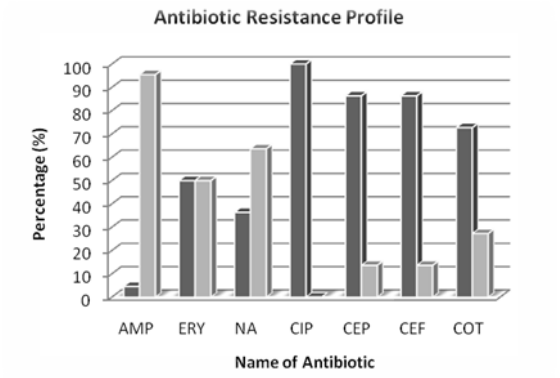

Fig. 3. Antibiotic resistance profile of isolated *Vibrio cholerae.*

3.4. *Susceptibility against T. arjuna extract*

The bark of the *Terminalia arjuna* constitutes an important crude drug, which contains tannins, triterpenoids saponins, flavonoids, sterols, calcium salts, alkaloidal and glycosidal substances, arjunine and arjunglyciside etc. It stops bleeding and pus formation in the gums and is useful in asthma, dysentery, menstrual problems, pains, leucorrhoea, wounds and skin eruptions [17]. Due to increased awareness of the importance of traditional medicine in human and animal health care, research into the efficacy of some of the herbs used in the treatment of some illness would be worthwhile **[**18**].**

Arjun bark extract was used as a biological tool to resolve the antibiotic resistant *V.cholerae* problem. Arjun extract showed promising effect against the isolated *Vibrio cholerae* at different concentrations (1 mg/L, 2 mg/L, 3mg/L, 4mg/L, 5 mg/L). The zone diameter at different concentration of extract is given in Table 1.

Table 1. Zone diameter at different concentration of *Terminalia arjuna* extract.

No. of Isolate	Zone of diameter (mm)					R^2 value
	1mg/disc	2mg/disc	3mg/disc	4mg/disc	5mg/disc	
12	8	8	10	12	12	0.9
13	-	-	-	12	12	0.75
16	-	-	-	-	12	0.5
22	10	10	14	17	17	0.896
36	-	-	-	8	9	0.775
37	-	13	14	15	17	0.709
54	8	10	10	14	14	0.888
55	9	11	13	16	17	0.984
56	-	-	8	10	11	0.876

'-` means no zone of inhibition.

Arjun extract inhibits the growth of *V. cholerae* at all concentrations and zone diameter increases with the increase of concentrations (Fig. 4).

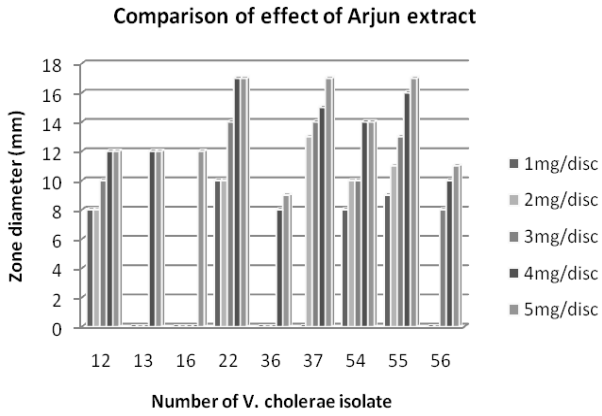

Fig. 4. Comparison of the effect of Arjun extract on *Vibrio cholerae* iaolates at different concentrations.

The regression value of the relationship between concentration and zone diameter is higher for most of the isolates (Table 1) which indicates there exist a linear relationship between them and better activity may be obtained by increasing the concentration of extract.

In case of isolate 22, which is resistant to ampicillin and erythromycin, the extract shows very promising activity with regression value 0.896 (Fig. 5). The zone diameter at 5 mg/disc is almost equivalent to the zone diameter in case of 10 µg/disc of ampicillin and 15 µg/disc of erythromycin.

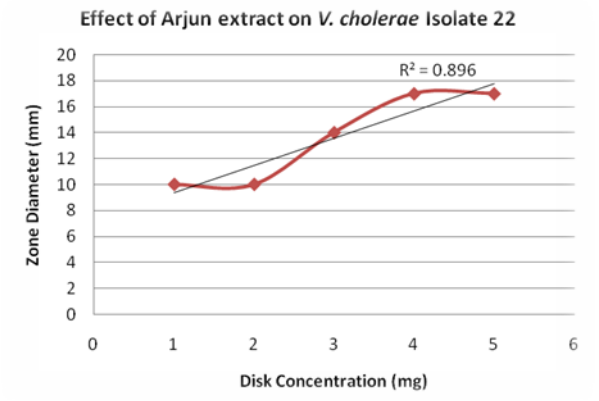

Fig. 5. Effect of Arjun extract on *Vibrio cholerae* isolate 22 at different concentrations.

In case of isolate 55, which is resistant to ampicillin, erythromycin, nalidixic acid, cephotaxime, ceftriaxone and cotrimoxazoll, the extract also shows very promising activity with regression value 0.984 (Fig. 6). The zone diameter at 5 mg/disc is almost equivalent to the zone diameter in case of 30 µg/disc of cephotaxime and 30 µg/disc of ceftriaxone.

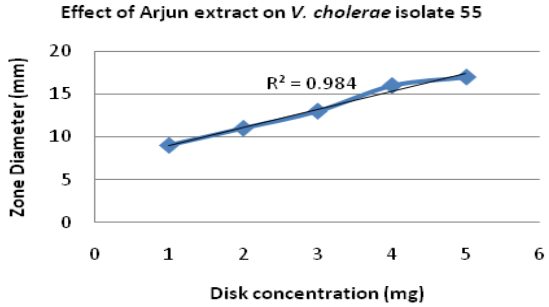

Fig. 6. Effect of Arjun extract on *Vibrio cholerae* iaolate 55 at different concentrations.

Similar results have been found for other *Vibrio cholerae* isolates tested. This study revealed that *Terminalia arjuna* would be a good antibacterial drug in the treatment of *Vibrio cholerae* infections, provided if it is found effective and nontoxic through *in vivo* study. Detailed study is needed to understand the molecular mechanism of *Terminalia arjuna* extract that may help us to make more effective therapeutics to combat Multi-Antibiotic Resistant (MAR) *Vibrio cholerae*. It is important to isolate the active antibacterial constituent(s) of *Terminalia arjuna*.

4. Conclusion

The additional antibiotic resistance might have contributed to the initial selection of the new strain, since these antibiotics are used to treat patients as well as livestock in Bangladesh. Antibiotics clearly influence the prevalence of novel antibiotic-resistant clones in cholera-endemic areas. It is hoped that this study would lead to the establishment of some compounds that could be used to formulate new and more potent anti-microbial drugs of natural origin against *Vibrio cholerae*. This is the first report on *Terminalia arjuna* for its antibacterial activity against the strains of *Vibrio cholerae*, especially against multiple antibiotic resistant isolates.

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