Antibacterial, antisecretory and antihemorrhagic activity of
Azadirachta indica used to treat cholera and diarrhea in India

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Abstract

Indigenous uses of Azadirachta indica A. juss (Maliaceae) (locally known as neem) leaves in different parts of India for curing gastrointestinal disorder such as diarrhea and cholera is wide spread. The objective of the present study was to evaluate the antibacterial and antisecretary activity of neem extract against Vibrio cholerae, a causative agent of watery diarrhea such as cholera. The methanol extract of neem leaf was tested for its antibacterial, antisecretory and antihemorrhagic activity against Vibrio cholerae. Azadirachta indica extract had significant antibacterial activity against the multi-drug-resistant Vibrio cholerae of serotypes O1, O139 and non-O1, non-O139. The minimum inhibitory concentration reached by 50% (MIC50) and 90% (MIC90), and minimum bactericidal concentration for the extract were 2.5, >5, and 10 mg/ml, respectively. Neem extract showed antisecretory activity on Vibrio cholerae induced fluid secretion in mouse intestine with inhibition values of 27.7%, 41.1%, 43.3%, 57.0%, and 77.9% at doses of 100, 200, 300, 450 and 1800 mg/kg, respectively. Oral administration of the extract inhibited hemorrhage induced by Vibrio cholerae in mouse intestine at a dose ≥ 300 mg/kg. The results obtained in this study give some scientific support to the uses of neem employed by the indigenous people in India employed for the treatment of diarrhea and dreadful disease cholera.
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Keywords: Antibacterial; Antihemorrhagic; Antisecretory; Azadirachta indica; Cholera; Indigenous

1. Introduction

Acute watery diarrhea accounts for 80% of the cases (death account for 50%) in the developing world (Tullock and Richards, 1993). Among the diarrheal diseases, cholera is a serious epidemic disease caused by the gram-negative bacterium Vibrio cholerae (Cholera Working Group, 1993; Nair et al., 1994). Vibrio cholerae, serotypes O1 and O139 has ability to produce an enterotoxin, cholera toxin (CT) that is a major determinant of virulence for cholera. Cholera toxin acts on mucosal cells and decreases the net flow of sodium ion into tissue producing a net flow of chloride (and water) out of tissue and into the lumen. This results in massive diarrhea and electrolyte imbalance. A person with full-blown cholera can lose 20 l of water daily (Lencer et al., 1992). Among the other virulence factors, ElTor hemolysin produced by Vibrio cholerae is also reportedly a potent toxin with both enterotoxic and cytotoxic activities (Honda and Finkelstein, 1979; Ichinose et al., 1987; Ramamurthy et al., 1993). Treatment with oral rehydration solution (ORS) has reduced the levels of mortality in children and adult by dehydration, but not morbidity for diarrhea (Amstrong and Cohen, 1999; Turvill et al., 2000). Some drugs such as racecadotril and loperamide are used to treat the secretary diarrhea. However, these drugs have side effects such as bronchospasm, vomiting and fever, and loperamide should not be administrated to children below 6 years of age, patients with constipation, and intestinal obstruction (Brown, 1979; Rogé et al., 1993; Salazar et al., 2000).

Different antibiotics are used to treat diarrheal patient. However, reports of drug-resistant Vibrio cholerae strains are appearing with increasing frequency (Mukhopadhyay et al., 1996). Emergence of resistance to multiple drugs is a serious clinical problem in the treatment and containment of the disease, as reflected by the increase in the fatality rate from 1% to 5.3% after the emergence of drug-resistant strains in Guinea-Bissau.
2. Materials and methods

2.1. Plant material and crude extract preparation

Leaves of Azadirachta indica A. juss (Maliaceae) were collected by the authors from local area (Kolkata, West Bengal, India). These leaves were thoroughly washed with distilled water to remove dirt. They were shade dried. The botanical identification of the leaves (with flower) was done by Prof. N.D. Paria (Department of Botany, University of Calcutta, Kolkata, India). The voucher specimen is conserved at Calcutta University Herbarium, Department of Botany, University of Calcutta, India, under the accession number CUH2395.

2.2. Crude extract preparation

Plant extracts were prepared by macerating air dried neem leaves (500 g) with 2500 ml of methanol in a Soxhlet apparatus for 18 h. The extract was then filtered through Whatman No. 42 filter paper and lyophilized yielding 21.84 g (4.36% yields) for neem leave extract. Lyophilized powder was stored at \(-20\) °C. Lyophilized powder was re-suspended in methanol to a concentration of 500 mg/ml and stored as stock sample at \(-20\) °C. Test samples were prepared by diluting further with methanol.

2.3. Bacterial strains

Among the clinical strains of Vibrio cholerae used in this study, strains NB2 and SG24 belonged to O1 and O139 serotypes, respectively (these strains were kindly provided by Dr. G. Balakrish Nair, NICED, Kolkata, present address ICD-DRB, Dhaka, Bangladesh). All these strains were able to produce cholera toxin and hemolysin. The other strains used in this study were Vibrio cholerae belonged to non-O1, non-O139 serotypes (strains PC4, PC9, PC11 and PC14) (these strains were isolated from aquatic environment and identified in our laboratory using API-20E system). These strains were positive for hemolysin production but negative for CT production.

2.4. Antibiotic susceptibility test

Antimicrobial susceptibility testing was performed by the disc diffusion method (Bauer et al., 1996) with commercially available disks (HiMedia, Mumbai, India) of ampicillin (10 \(\mu\)g); chloramphenicol (30 \(\mu\)g); co-trimoxazole (25 \(\mu\)g); ciprofloxacin (5 \(\mu\)g); furazolidone (100 \(\mu\)g); gentamicin (10 \(\mu\)g); neomycin (30 \(\mu\)g); nalidixic acid (30 \(\mu\)g); norfloxacin (10 \(\mu\)g); streptomycin (10 \(\mu\)g); tetracycline (30 \(\mu\)g). Culture suspensions were obtained after incubation at 37 °C in 5 ml Mueller–Hinton broth (MHB) (HiMedia) for 4–5 h and spread on Mueller–Hinton agar (MHA) (HiMedia) at 0.5 McFarland. The plates were incubated at 37 °C for 24 h. Isolates were considered susceptible, reduced susceptible, or resistant to a particular antimicrobial agent on the basis of the diameters of the inhibitory zones that matched the criteria of the manufacturer’s interpretive table, which followed the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS, 2002). The American Type Culture Collection (ATCC) strains Escherichia coli ATCC 25922 and Staphylococcus aureus ATCC 25923 were used for quality control.

2.5. Antibacterial activity

2.5.1. Screening for antimicrobial activity

Antibacterial activity of the extract was determined by agar-diffusion assay (Reeves, 1989). Bacterial strains were first grown in MHB under shaking condition for 4 h at 37 °C and after the incubation period 1 ml of culture were spread on MHA. The wells were made using sterile 6 mm cork borer in the inocu-
lated MHA plate. The wells were filled with 200 μl of the plants extracts (re-suspended in methanol) and blanks (methanol). The concentrations of extract employed were 10, 25, 50, 100, and 200 mg/ml. Tetracycline (150 μg/ml, 200 μl) was used as antibacterial positive control and the ATCC strain *Escherichia coli* ATCC 25922 was included for quality assurance. Zone diameter was measured after 24 h incubation at 37 °C. The photograph was taken in Gel documentation system (Vilber Lourmat, France).

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

MIC and MBC of neem extract were assessed using the broth microdilution method recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 1997, 1999). An inoculum of the microorganism was prepared from 24 h MHB cultures and suspensions were adjusted with a turbidity equivalent to that of a 0.5 McFarland standard. Suspensions were further diluted 1:10 in sterile MHB to obtain a final inoculum of 5 × 10^5 CFU/ml. The 96-well round bottom sterile plates were prepared by dispensing 180 μl of the inoculated broth into each well. A 20 μl aliquot of the plant extract was added. The concentrations of plant extract tested were 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20, and 40 mg/ml. Dilutions of tetracycline served as positive control, while broth with 20 μl of methanol was used as negative control. The ATCC strain *Escherichia coli* ATCC 25922 was included for quality assurance purposes. Plates were covered and incubated for 24 h in ambient air at 37 °C. After incubation, minimum inhibitory concentrations (MIC) were read visually; all wells were plated to nutrient agar (Hi-Media) and incubated. The minimal bactericidal concentration (MBC) was defined as a 99.9% reduction in CFU from the starting inoculum after 24 h incubation interval.

2.6. Toxicity assay

BalB/C mice (22–25 g body weight) were used in the present study. The animals kept in wire-mesh cages were acclimated to laboratory conditions (12 h dark:12 h light cycles; 24 ± 1 °C) and had been free access to food and water ad libitum. Male mice were treated with a dose of 1800 mg/kg of crude extract given orally (PO). The mortality rate within 72 h period was determined.

2.7. Antisecretory and antihemorrhage assay

The effect of the extract on fluid secretion and hemorrhage in intestine induced by *Vibrio cholerae* was studied using mouse model. In traditional medicine infusions or decoctions are usually taken three to four times (one cup of plant tea each time) per day when diarrhea occurs. Neem methanolic extract was tested at an oral dose of 450 mg/kg because the used dose is approximately three cups of plant tea which is recommended by Indian people to treat diarrheal diseases (Bhattacharya, 1977). In vivo activity was also tested at oral doses of 100, 200, 300, and 1800 mg/kg. Mice were fasted for 24 h before administration of bacterial inoculums and extract. For the preparation of bacterial inoculums, *Vibrio cholerae* strains were grown in MH broth overnight at 37 °C under shaking condition. After harvesting by centrifugation, cells were washed with 10 mM phosphate buffered saline (PBS), pH 7.2. The cells were then re-suspended in same buffer and cell numbers are adjusted to 2 × 10^9 CFU/ml. Each mouse was fed with 2% NaHCO₃ prior to administering either bacterial inoculums or extract or both. Mice were divided into two groups. Mice of each of groups 1 and 2 (n = 2 per group in duplicated) were administered 500 μl of bacterial inoculums (2 × 10^6 CFU/ml) orally. After 1 h mice were fed either with 200 μl of methanol (group 1) or crude extract (group 2; 100, 200, 300, 450, and 1800 mg/kg in 200 μl of methanol) orally. Mice were sacrificed after 24 h incubation. The fluid accumulation and hemolytic activity of *Vibrio cholerae* in intestine were observed. Photograph was taken, scanned with a Hewlett-Packard ScanJet 2400 scanner, and the image was arranged for the figure and labeled with Adobe Photoshop Version 6. Fluid accumulation ratio (the weight of intestine/rest of the body weight of mouse) was measured and the antisecretory activity was expressed in percentage of inhibition.

2.8. Statistical analysis

Values are expressed as mean ± S.D. Statistical significance was determined using Student’s t-test. Values with *p* < 0.05 were considered significant.

3. Results and discussion

We tested the strains of *Vibrio cholerae* belonged to serogroups O1, O139 and non-O1, non-O139 to evaluate the antibacterial activity of neem which used in Indian traditional medicine for the treatment of diarrhea and choler. Antibiotic susceptibility test was performed to confirm their multi-drug resistance patterns. All the *Vibrio cholerae* strains included in this study showed multi-drug resistance (Table 1). However, crude methanol extract from neem leaves showed inhibitory activity against *Vibrio cholerae* belonged to serotypes O1, O139 and non-O1, non-O139 by agar-diffusion assay with significance (*p* < 0.05) (Table 2 and Fig. 1). Thus the active plant extracts in this study showed antibacterial activity against multiply drug-resistant *Vibrio cholerae* strains. Furthermore, we also evaluated the antisecretory activity of extracts of neem against *Vibrio cholerae* model. In traditional medicine infusions or decoctions are usually taken three to four times (one cup of plant tea each time) per day when diarrhea occurs. Neem methanolic extract was tested at an oral dose of 450 mg/kg because the used dose is approximately three cups of plant tea which is recommended by Indian people to treat diarrheal diseases (Bhattacharya, 1977).

Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Resistant</th>
<th>Reduced susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB2</td>
<td>O1</td>
<td>A, T, Na, Fr, Co, S</td>
<td>–</td>
</tr>
<tr>
<td>SG24</td>
<td>O139</td>
<td>A, Co, S</td>
<td>–</td>
</tr>
<tr>
<td>PC4</td>
<td>Non-O1, non-O139</td>
<td>A</td>
<td>T</td>
</tr>
<tr>
<td>PC9</td>
<td>Non-O1, non-O139</td>
<td>A, T, Co</td>
<td>N, Fr</td>
</tr>
<tr>
<td>PC11</td>
<td>Non-O1, non-O139</td>
<td>A, T, Fr, Na</td>
<td>S</td>
</tr>
<tr>
<td>PC14</td>
<td>Non-O1, non-O139</td>
<td>A, T</td>
<td>Fr</td>
</tr>
</tbody>
</table>

A, ampicillin; C, chloramphenicol; Co, co-trimoxazole; Cf, ciprofloxacin; Fr, furazolidone; G, gentamicin; N, neomycin; Na, nalidixic acid; Ns, norfloxacin; S, streptomycin; T, tetracycline.
Table 2

<table>
<thead>
<tr>
<th>Strains of <em>Vibrio cholerae</em></th>
<th>Zone of inhibition diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude methanol extract (200 μl)/well</td>
</tr>
<tr>
<td></td>
<td>200 mg/ml</td>
</tr>
<tr>
<td>SG24 (O139)</td>
<td>16.5 ± 1.0</td>
</tr>
<tr>
<td>NB2 (O1)</td>
<td>16.7 ± 1.2</td>
</tr>
<tr>
<td>PC4(non-O1, non-O139)</td>
<td>18.0 ± 2.3</td>
</tr>
<tr>
<td>PC9(non-O1, non-O139)</td>
<td>15.3 ± 1.2</td>
</tr>
<tr>
<td>PC11(non-O1, non-O139)</td>
<td>16.7 ± 0.8</td>
</tr>
<tr>
<td>PC14(non-O1, non-O139)</td>
<td>14.5 ± 0.5</td>
</tr>
</tbody>
</table>

Antibacterial activity was expressed in terms of diameter of zone of inhibition (mean ± S.D., n = 3). *p* < 0.05 compared to control (methanol) is considered significant.

Antibacterial activity of *Azadirachta indica* leaves extract on *Vibrio cholerae* O1, O139 and non-O1, non-O139 strains of *Vibrio cholerae*. Zone of inhibition diameter (mm).

Fig. 1. Determination of effect of *Azadirachta indica* leaf methanol extract on *Vibrio cholerae* by agar-diffusion assay method. *Vibrio cholerae* strain PC14 (A) and strain NB2 (B) were spread on MHA. In each case, 200 μl of 200, 100, 50, and 25 mg/ml of neem extract (in methanol), 200 and 100 μl of methanol were added to the wells 1, 2, 3, 4, c1 and c2, respectively (Section 2).

resistant *Vibrio cholerae* that is a serious clinical problem in the treatment and containment of the disease.

For determination of MIC and MBC for the neem extract against *Vibrio cholerae*, the concentration ranges tested were from 0.025 to 40 mg/ml. The minimum inhibitory concentration reached by 50% (MIC₅₀) and 90% (MIC₉₀) of the strains for the extracts were 2.5 and >5 mg/ml, respectively. The minimum bactericidal concentration for the extract was 10 mg/ml.

Administration of leaves extract of *Azadirachta indica* (1800 mg/kg) did not produce any sign of toxicity in mice and none of the mice died. Mice (group 1) which were administered with *Vibrio cholerae* inoculums at a dose of 1 × 10⁹ CFU plus 200 μl of methanol (vehicle control) orally showed fluid accumulation and hemorrhage (Fig. 2) in the intestines. Fluid accumulation ratios in these control experiments were between 0.11 ± 0.01 and 0.16 ± 0.02 (n = 3 for each strain of bacteria). However, it was found that the extract at an oral dose of 450 mg/kg inhibited the fluid secretion (57.0% inhibition) and hemorrhage in the intestine induced by the strains of *Vibrio cholerae* for group 2 mice. In traditional medicine since infusions or decoctions are usually taken three to four times per day when diarrhea occurs, our results can be related with its traditional use because the used dose is approximately two to three cups of plant tea which is recommended by Indian people to treat diarrheal diseases (Bhattacharya, 1977). The antisecretory activity of extract tested is shown in Table 3.

Table 3

<table>
<thead>
<tr>
<th>Bacterial inoculum (CFU) administered</th>
<th>Extract administered at a dose (mg/kg)a</th>
<th>% inhibitionb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 × 10⁹</td>
<td>100</td>
<td>27.7 ± 7.8</td>
</tr>
<tr>
<td>1 × 10⁸</td>
<td>200</td>
<td>41.1 ± 3.4</td>
</tr>
<tr>
<td>1 × 10⁹</td>
<td>300</td>
<td>43.3 ± 1.3</td>
</tr>
<tr>
<td>1 × 10⁹</td>
<td>450</td>
<td>57.0 ± 5.9</td>
</tr>
<tr>
<td>1 × 10⁹</td>
<td>1800</td>
<td>77.9 ± 7.2</td>
</tr>
</tbody>
</table>

a Mouse was orally administered with bacterial inoculum followed by neem extract (Section 2).
b The antisecretory activity was expressed as the percentage inhibition (mean ± S.D., n = 4) compared to control (methanol), *p* < 0.05 is considered to be significant.
hemorrhage induced by the strains of *Vibrio cholerae* in mice intestines.

4. Conclusion

This study showed that methanolic extract of *Azadirachta indica* leaves was an effective antibacterial and antisecretory agent against *Vibrio cholerae*, the causative agent of dreadful disease cholera. Additionally, it was also found that neem extract had antihemorrhagic activity. The active extract found in this work may be employed for the treatment of the patient infected with *Vibrio cholerae* belonged to serotypes O1, O139 and non-O1, non-O139. Emergence of multiply drug-resistant *Vibrio cholerae* is a serious clinical problem in the treatment and containment of the disease. However, the active leaves extract in this work had bactericidal activity against different strains of multi-drug-resistant *Vibrio cholerae*. This could be suggested that the active *Azadirachta indica* leaves extract found in our work may be used as potential source to develop novel antimicrobial compound and antisecretory drug useful to treat cholera and diarrheal patients. The results obtained in this study give some scientific support to the indigenous uses of neem in India employed for the treatment of diarrhea and cholera. Further efforts should be directed at investigating the principle in this extract which has these antibacterial, antisecretory and antihemorrhagic activities against *Vibrio cholerae*.

Acknowledgements

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multiple antibiotic resistant and belong to heterogeneous non-O1, non-O139 O-serotypes. Epidemiology and Infection 122, 217–226.


