Antibacterial and antidiarrhoeal effects of alkaloids of *Holarrhena antidysenterica* WALL

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The alkaloids from the ethanolic extract of *H. antidysenterica* seeds were evaluated for their antibacterial activity against clinical isolates of enteropathogenic *Escherichia coli (EPEC)* *in vitro*, and their antidiarrhoeal activity on castor oil-induced diarrhoea in rats, *in vivo*. The plasmid DNA, whole cell lysate and outer membrane protein profile of a clinical isolate of EPEC was determined in presence of alkaloids of *H.antidysenterica*. The disc diffusion and agar well diffusion methods were used to evaluate the antibacterial efficiency. The alkaloids showed strong antibacterial activity against EPEC strains. In castor oil-induced diarrhoea, alkaloids reduced the diarrhoea with decrease in the number of wet faeces in pretreated rats at a dose of 200-800 mg/kg. The loss of plasmid DNA and suppression of high molecular weight proteins were observed on alkaloids treatment.

Taking into account the multiple antibiotic resistance of EPEC, the results suggest usefulness of alkaloids of *H.antidysenterica* seeds as antibacterial and antidiarrhoeal agents.

**Keywords:** *Holarrhena antidysenterica*, alkaloids, EPEC, antidiarrhoeal.

**IPC Code:** Int CI A61K

Diarrhoea is a common symptom of intestinal disorders and it is a global threat to human health. It is a leading cause of morbidity and mortality, with over 1000 million episodes and over 4 million deaths annually in children under five year of age.

Enteropathogenic *Escherichia coli (EPEC)* is a member of the attaching and effacing (A/E) family of pathogens that induce diarrhoeal disease in a wide range of mammalian species. The A/E lesion is characterized by localized destruction (effacement) of brush border microvilli, intimate attachment of the bacillus to host cell membrane and the formation of an underlying pedestal-like structure on the host cell consisting of polymerized actin, α-actinin, ezrin, talin and myosin. The pathogenesis of EPEC infection encompasses 3 distinct stages: (i) Initial adherence: type IV fimbriae is associated with a high molecular weight plasmid (90 kb), present in most EPEC strains, (ii) signal transduction: the second stage consists of signaling in the host cells by the bacteria, their signaling requires type III secreted proteins (EspA, EspB, EspD, EspF and Tir), and (iii) intimate attachment: Tir functions as a receptor on the host cell membrane for intimate attachment of the outer membrane protein, intimin. The type III secretion system and type IV fimbriae in EPEC play principal role in the interaction between the bacteria and host epithelial cells, and can thus be exploited for therapy.

The WHO has constituted a diarrhoeal disease control program, which includes traditional medicinal practices together with the evaluation of health education and prevention approaches. *Holarrhena antidysenterica* WALL (Apocynaceae) commonly known as Tellicherry bark (English), Kurchi (Hindi), is a small deciduous tree with white flowers. This plant has many sanskrit names, the better known being Kutaja and Kalinga. It grows in the tropical Himalayas and is distributed throughout India.

Almost all the parts of the plant, viz bark, root, stem and seeds, are known to have various medicinal properties. The main ingredient of Kutajarishta, an antidiarrhoeal formulation, is *H. antidysenterica*, which has been reported to exhibit antimicrobial activity. The antimicrobial activity of *H. antidysenterica* bark extract has been reported against enteropathogens like enteroinvasive *Escherichia coli*, EPEC, *Salmonella typhimurium*, *Salmonella enteritidis*, *Shigella flexneri*, *Sh.boydii* and *Vibrio cholerae*. In addition, the plant has been reported to possess anthelmintic, appetising and astringent properties.

The seeds of *H. antidysenterica* are used in the treatment of dysentery, diarrhoea and fever. The
seeds contain many alkaloids but at low concentrations (1.82%)\(^\text{18}\). The multiple antibiotic resistance of \textit{EPEC}\(^\text{19,20}\) has promoted the search for new antibacterial agents from traditional medicinal plants. The present study has been undertaken to justify the antibacterial and castor oil-induced antidiarrhoeal activity of the alkaloids of \textit{H. antidisenterica}.

**Materials and Methods**

*Plant material*—The seeds of \textit{H. antidisenterica} were collected during August 2001, from trees growing in Tamil Nadu, India. Identification of the plant was established by the Centre for Advanced Studies in Botany, University of Madras, Chennai. After collection, the seeds were shade-dried and ground into coarse powder and stored in air-tight container for further use.

*Isolation of alkaloids from the seeds of \textit{H. antidisenterica}*—The dried pulverized powder of the seeds of \textit{H. antidisenterica} (1000 g) were defatted with petroleum ether (40\degree-60\degree C) in Soxhlet extraction apparatus for 2 hr. The plant material was dried under vacuum and mixed with 100 g calcium hydroxide and made into a slurry. The mixture was heated in a water bath for 30 min. Then the material was extracted with alcohol + 10% acetic acid mixture in a Soxhlet unit for 5 hr. After that the alcoholic extract was acidified with 1 N HCl (pH 3.2). After the acidification process, added 250 ml of ethyl acetate and mixing using a magnetic stirrer for 10 min. The aqueous solution was separated and the alkaloids precipitated by dropwise addition of concentrated ammonium hydroxide solution (pH 9). The precipitate was collected by centrifugation and the precipitated alkaloids was washed with 1% ammonium hydroxide solution. The precipitate was dissolved in 200 ml ethyl acetate and the ethyl acetate portion was separated and concentrated under vacuum. A yellowish brown residue was obtained which was re-crystallized with chloroform (Fig.1) The isolated alkaloid was confirmed by its melting point and by TLC.

The melting point of the isolated alkaloid was 123\degree-124\degree C. TLC was carried out on silica gel G pre-coated sheets using methanol:chloroform (3:17) as solvent system. The alkaloids were detected with Dragen dorf’s reagent (\(R_\text{f} \approx 3.0\)).

*Test Microorganisms*—The \textit{in vitro} antibacterial study was carried out against enteropathogenic \textit{Escherichia coli} (EPEC 1402fc; EPEC 1361fc). The clinical isolates were obtained from All India Institute of Medical Sciences, New Delhi, India. The bacteria were grown on nutrient agar plates supplemented with ampicillin (100 \mu g/ml) for 24 hr at 37\degree±2\degree C. An isolated colony was suspended in sterile saline and was used for further studies.

**Antibacterial activity**

*Disc diffusion method* (European pharmacopoeia)\(^\text{21}\)—Paper discs (\(q = 9\) mm) were impregnated with 1.25, 2.5 and 3.5 mg of the sample and allowed to air dry. Over the agar plate, the diluted bacterial culture was spread with a sterile swab. The discs impregnated with extract were placed over the agar plate and incubated at 37\degree ± 2\degree C for 24 hr. The antibacterial activity was measured as a diameter of inhibitory zone on the agar plate.

*Agar well diffusion method* (Hugo and Russel)\(^\text{22}\)—In this technique, bacterial culture was added aseptically to the agar medium at 45\degree C, mixed well and poured immediately onto sterile petri dishes. After solidification, wells of about 6 mm in diameter were cut into agar and 1.25, 2.5 and 3.5 mg of the alkaloid preparation were placed in the wells. The plates were incubated at 37\degree C and observations were made after 24 to 72 hr.

The experiments were repeated thrice and the results were expressed as the average of 3 experiments. Cefotaxine, chloramphenicol and ciprofloxin (Ranbaxy) were used as reference antibiotics for comparison.

*Microdilution method*—The method of Makane and Kanda\(^\text{23}\), was followed to determine the minimum inhibitory concentration (MIC). The total alkaloids were diluted to obtain concentrations ranging from 1.6-5 mg/ml. The test tubes containing 3 ml LB broth, 0.1 ml bacterial suspensions and different concentration of samples were incubated at 37\degree C for 24 hr. Bacterial turbidity was measured at 560 nm to determine bacterial inhibition. Chloramphenicol (100 \mu g/ml) was used as a reference antibiotic and tubes containing only the growth medium and organism were used as control.

*Isolation of whole cell lysate (WCL) and outer membrane proteins (OMP)* of \textit{EPEC} 1402fc—The method of Carlene et al.\(^\text{24}\), was followed for OMP and for SDS-PAGE the method of Laemmli\(^\text{25}\) was followed. OMP and WCL were isolated from an overnight LB broth culture of EPEC 1402fc and LB broth culture containing alkaloids (1 and 1.5 mg/ml). The isolated proteins were subjected to 12% SDS-PAGE and stained with Coomassie brilliant blue.
Dried pulverized *H. antidysenterica* seeds (1000 g)  

- Defatted with petroleum ether (40° - 60°C) in Soxhlet apparatus (2hr)  
- Dried under vacuum  
- Mixed with Ca(OH)$_2$ (100g)  
- Slurry  
- Heated in a water bath (30 min)  
- Material extracted with alcohol + 10% acetic acid in Soxhlet apparatus (5hr)  
- Alcoholic extract Concentrated  
- Acidified (1N HCl - pH 3.2)  
- Added ethyl acetate (250 ml) and stir (10 min)  

**Aqueous Phase**  
Aqueous solution ppted with dropwise addition of NH$_4$OH (pH 9)  
- ppt collected by centrifugation (3000 rpm/15 min)  
- ppt washed 1% NH$_4$OH and dissolved 200 ml ethyl acetate  

**Organic Phase**  
- evaporated (40°C)  
- Neutral & acidic material  

- Concentrated - vacuum  

Yellowish brown residue (alkaloid) crystallized with chloroform  
- Alkaloid - Melting point (123°-124°C) - identified by TLC using Dragendorff’s reagent

Fig. 1—Outline of the extraction procedure for alkaloids carried out for *H. antidysenterica*. 
Isolation of plasmid DNA—The method of Sambrook et al.,28 was followed. Plasmid DNA was isolated from an overnight LB broth culture of EPEC 1402fc and a culture containing the alkaloids (0.8, 1.6 and 3.2 mg/ml) and was subjected to 0.8% agarose gel electrophoresis.

Castor oil-induced diarrhoea in rats—The method followed was that of Awouter et al.,29 with modification by Das et al.30. Rats were fasted for 18 hr and were housed in steel cages containing six rats in each group. Group I received normal saline as negative control, group II received diphenoxylate hydrochloride (lomotil; 5 mg/kg) as positive control. Group III, IV, V and VI were administered, orally, with alkaloids at a dose of (200, 400, 600, 800 mg/kg). After one hour treatment all the rats received 1 ml castor oil, orally by gavage, and were observed for defecation for 4 hr after the castor oil challenge. The presence of characteristic diarrhoeal droppings was noted in transparent plastic dishes placed beneath the individual cages.

Results

Antibacterial activity

Disc diffusion method—The zone of inhibition exhibited by the alkaloids was observed for different concentrations (1.25, 2.5 and 3.5 mg) using disc diffusion method. It was found to be 12, 16 and 20 mm for the isolate 1402fc and 11, 12 and 17 mm for the isolate 1361fc (Table 1).

Agar well diffusion method—The effect of the alkaloids of H. antidyse lleti ca was evaluated against EPEC (1402fc and 1361fc) using this method. Inhibition zones were 15, 16 and 24 mm for 1402fc and 18, 19 and 21 mm for 1361fc (Table 1).

Microdilution method—In this technique, with increasing concentration of alkaloids, the optical density (OD) gradually decreased. This was compared with the standard antibiotic, chloramphenicol (100 µg/ml) (data not shown).

The antibacterial properties of H. antidyse lleti ca alkaloids were clearly evident and compared well with the antibacterial activity of the standard antibiotics (Tables 1).

Protein profile of OMP and WCL of 1402fc—The protein profiles of OMP and WCL of EPEC 1402fc culture grown in the presence and absence of alkaloids were compared. Suppression of high molecular weight proteins was revealed in alkaloids-treated culture (Fig. 2).

Plasmid profile of EPEC 1402fc—In this method, the plasmid DNA of EPEC 1402fc, which was grown in presence and absence of alkaloids were compared. Fig. 3 shows the loss of plasmid DNA bands in a concentration-dependent manner.

Castor Oil-induced diarrhoea—The alkaloids of H. antidyse lleti ca, like the standard anti diarrhoeal drug diphenoxylate, significantly inhibited the defecation when compared to untreated control rats in dose-dependent manner (Table 2).

Discussion

Research on natural sources has been actively encouraged by the WHO31. The present results clearly indicate that the ethanolic extract of H. antidyse lleti ca seed alkaloids strongly inhibit the

Table 1—Determination of antibacterial activity of alkaloids of H. antidyse lleti ca seeds and reference antibiotics by (A) disc diffusion and (B) agar well diffusion methods

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentration</th>
<th>Inhibition zone diameter (mm)</th>
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<tbody>
<tr>
<td></td>
<td>(mean value, n=3)</td>
<td></td>
</tr>
<tr>
<td>Alkaloids</td>
<td>1.25 mg</td>
<td>A 12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B 15</td>
</tr>
<tr>
<td></td>
<td>2.5 mg</td>
<td>A 16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B 16</td>
</tr>
<tr>
<td></td>
<td>3.5 mg</td>
<td>A 20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B 24</td>
</tr>
<tr>
<td>Ceftaxime</td>
<td>100 µg</td>
<td>A 23.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B 11.4</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>100 µg</td>
<td>A 20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B 19.8</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>100 µg</td>
<td>A 29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B 27</td>
</tr>
</tbody>
</table>

Fig.2—Protein profile of EPEC 1402fc on 12% SDS-PAGE. [OMP (Lane 2 - EPEC 1402 fc; Lane 3 and 4, EPEC grown in presence of alkaloids of H.antidyse lleti ca, 0.8 mg/ml and 1.6 mg/ml respectively) WCL (Lane 5-EPEC 1402fc; Lane 6 and 7 EPEC grown in presence of alkaloids of H. antidyse lleti ca, 0.8 mg/ml and 1.6 mg/ml respectively) Lane 1 indicates molecular weight (kDa)]
growth of EPEC strains. Most of the alkaloids having antibacterial, antidiarrhoeal, antimicrobial, anticancer, antimalarial activity and cure several infectious diseases. The present results are in agreement with the findings of Ballal et al., and Chakraborty et al.,. A total 29 different alkaloids are present in the crude extract of H. antidysenterica, coenzyme may be the component that inhibits the growth of bacteria.

The antidiarrhoeal effect of the alkaloids from H. antidysenterica was similar to the standard drug, diphenoxylate, by inhibiting the production of watery stools or fluid. The above observation suggests that the alkaloids of H. antidysenterica (in graded doses) reduced diarrhoea in castor oil induced rats. Tripathi reported that astringent properties of tannins of Punica granatum, are responsible for protein denaturation producing protein tannate, which reduces secretion from intestinal mucosa. Similarly, H. antidysenterica also contains the astringent properties of alkaloids which may produce antisecretory activity.

Abbanat et al. reported that the new antibacterial agents, Hongoquerins damage the membrane of E. coli imp strains, as the primary mode of their bactericidal action. Fukao et al. reported that the combination of hop resins sodium hexametaphosphate damage the cell membrane of the E. coli strains. The present observations, suggest that the suppression of high molecular weight outer membrane proteins (Fig. 2) may be due to the alkaloids of H. antidysenterica. The loss of plasmid DNA bands (Fig. 3) may be due to the involvement of alkaloids.

Further studies are necessary to confirm the role of alkaloids on plasmid and outer membrane proteins of EPEC. These findings have initiated search for a new drug target for the antidiarrhoeal activity of alkaloids. However, the antibacterial and antidiarrhoeal activity of alkaloids of H. antidysenterica look promising.

### Table 2—Effect of alkaloids of H. antidysenterica on castor oil-induced diarrhoea in rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Pretreatment</th>
<th>Defecation* (g) per group drops</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>Saline 5 ml/kg (Control)</td>
<td>2.632 ± 0.01</td>
</tr>
<tr>
<td>II</td>
<td>Diphenoxylate 5 mg/kg</td>
<td>0.557 ± 0.20</td>
</tr>
<tr>
<td>III</td>
<td>Alkaloid 200 mg/kg</td>
<td>2.021 ± 0.023</td>
</tr>
<tr>
<td>IV</td>
<td>400 mg/kg</td>
<td>1.257 ± 0.020</td>
</tr>
<tr>
<td>V</td>
<td>600 mg/kg</td>
<td>0.810 ± 0.026</td>
</tr>
<tr>
<td>VI</td>
<td>800 mg/kg</td>
<td>0.565 ± 0.35</td>
</tr>
</tbody>
</table>

*P < 0.001

![Fig. 3—Plasmid profile of EPEC 1402fc on 0.8% agarose gel. (Lane 1: EPEC 1402fc, Lane 2, 3 and 4 treated with alkaloids of H. antidysenterica, 0.8 mg, 1.6 mg and 3.2 mg/ml respectively)]](image-url)


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