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Activity of aqueous ethanol extract of *Euphorbia prostrata* ait on *Shigella dysenteriae* type 1-induced diarrhea in rats

Kamgang René, Gonsu Kamga Hortense¹, Wafo Pascal², Mbungni N. J ean Alexis, Pouokam Ervice Vidal, Fokam Tagne Michel Archange, Fonkoua Marie Christine³

ABSTRACT

Aim: *Euphorbia prostrata* (Euphorbiaceae) is traditionally used in Cameroon for the treatment of many diseases, including diarrhea. We investigated the acute toxicity and effect of the aqueous ethanol extract of the plant on gastrointestinal propulsion, *in vitro* bacterial growth and *in vivo* bacillary dysentery.

Materials and Methods: Diarrhea was induced by oral administration of $12 \times 10^8$ *Shigella dysenteriae* type 1 (Sd1) cells. Diarrheic rats were treated for 5 days with 10, 20 or 40 mg/kg extract or 20 mg/kg norfloxacin. The faeces frequencies and the number of Sd1 were assessed and the death rate recorded.

Results: The aqueous ethanol extract of *E. prostrata* was not toxic. *In vitro*, the minimal inhibitory and minimal bactericidal concentrations of the extract were 3,500 and 12,000 µg/ml, respectively. *In vivo*, diarrhea went along with increase in faeces frequency ($P < 0.01$ by the 3rd day), increase in the bacterial population to a maximum on the 2nd day after infection ($P < 0.01$). The death rate in diarrheic control group was 100% by day 6. *E. prostrata* extracts (20 and 40 mg/kg) reduced the bacterial growth ($P < 0.01$), so that by the 6th day Sd1 density was <100 and no death was recorded. There was a significant ($P < 0.01$) reduction in faeces frequencies. The extract exhibited notable ($P < 0.01$) inhibition of intestinal propulsion.

Conclusion: The results suggest that *E. prostrata* possesses bactericidal and anti diarrheic properties and could be a therapeutic alternative for diarrheas of bacterial etiology.

KEY WORDS: Antidiarrheic activity, *Euphorbia prostrata*, rat, *Shigella dysenteriae* type 1
that was adopted by the Institutional Committee of the Ministry of Scientific Research and Innovation of Cameroon. These animals were allowed water and food ad libitum. Rats were kept singly in metabolic cages. Diarrhea was induced in rats according to the method described earlier, using Shigella dysenteriae type 1 (Sd1) strain provided by the Centre Pasteur of Yaoundé, Cameroon. For this purpose, after verifying that the rats were not Sd1 carriers, each rat in the 5 groups (of 5 animals each) was orally administered 12 × 10^6 (the 4 McFarland standard) saline-diluted Sd1 cells. Plant material and extract

The whole plant of Euphorbia prostrata Ait was collected in Yaoundé, Cameroon and identified by the National Herbarium of Yaoundé, Cameroon, where a voucher specimen was deposited (Reference 65 596/HNC). The whole aerial part was washed thoroughly with water, shade dried and ground; 1.5 kg of Cameroon at Yaoundé, where a voucher specimen was Yaoundé, Cameroon and identified by the National Herbarium. Biological extracts were prepared by using the method described earlier.[15,16] A 1 kg sample of plant material was chopped, washed thoroughly with water, shade dried and ground; 1.5 kg of ethanol was added, and the mixture was kept for 7 days. The filtrate was evaporated to dryness in a rotary evaporator at 37 °C. Extractions were performed three times. The alcoholic extract, which was obtained after evaporation, was redissolved in 1% DMSO. The filtrate was evaporated to dryness in a rotary evaporator at 37 °C. For this purpose, after verifying that the rats were not Sd1 carriers, each rat in the 5 groups (of 5 animals each) was orally administered 12 × 10^6 (the 4 McFarland standard) saline-diluted Sd1 cells, which was subsequently dissolved in 1% DMSO.

Acute toxicity

The acute toxicity of the extract was evaluated in 56 normal albino mice separated into 7 groups of 8 mice each (4 males and 4 females). Each group was fasted for 24 h, after which they were treated once orally with one of the increasing doses of the extract: 0, 1, 2, 5, 10, 15, 20, 25 or 30 gm/kg bw; the volume of each administered dose did not exceed 1 ml. The mice were then observed for at least 48 h and up to 7 days, for death, lethargy, jerking, sensitivity to noise and touch, stools quality and frequency. The dose of the extract that would kill 50% of the animal population (LD50) by the route given was estimated graphically or by using the following formula:[17]

\[
LD_{50} = X_c - d(N_p - \frac{1}{2})
\]

\(X_c\): 100% lethal dose; \(d\): interval between 2 successive doses; \(p\): death ratio per group.

Preliminary phytochemical screening test

Screening of the phytochemical properties of the aqueous ethanol extract of E. prostrata was done using the following chemicals and reagents: Mayer and freshly prepared Dragendorff’s reagents (for alkaloids), Liebermann-Buchard test (for terpenoids and steroids), [FeCl₃ and K₃Fe(CN)₆]ₙ (for phenols and tannins), Shinoda test [(magnesium turnings and HCl), Molish test (α-napthol and H₂SO₄) (for polysaccharides), frothing test (for saponins), FeCl₃ and HCl (for phenols and tannins) and NH₄OH (for anthraquinones).

In vitro antimicrobial activity:
The antimicrobial activities of aqueous ethanol extracts of E. prostrata were tested using both serial dilution in broth and agar diffusion methods. Sd1 inocula were standardized by matching the turbidity of the culture to the 0.5 McFarland standard, as recommended by the National Committee of Clinical and Laboratory Standards (Indiana, U.S.A.).[28] Bacterial suspensions were further diluted to obtain the 5 × 10⁷ CFU inoculum. In the agar diffusion method, 500 µl Sd1 inocula was seeded over Mueller-Hinton II (Boetec) agar. The well technique was used and each well was filled with 200 µl of each drug solution: standard antibiotic for control (norfloxacin 5 µg), increasing doses of E. prostrata extract (0.01-200 mg) and 1% DMSO as negative control. After 18 h incubation at 37°C, the inhibition diameters (\(\varnothing\); in mm) against Sd1 were determined using a caliper square. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of antimicrobial extract that prevented visible growth. This was confirmed spectrophotometrically and by a count of viable cells on Hektoen (Becton Dickinson and Company) agar.[21,22] The minimal bactericidal concentration (MBC) was determined as the lowest concentration of extract allowing only 0.01% survival for Sd1. Each test was repeated three times.

In vivo antidiarrheic activity

Antimicrobial activity: Diarrheic rats were randomly divided into 5 groups, each containing 5 animals. When diarrhea appeared, animals were administered the antidiarrheic drugs twice daily by the oral route (using an orogastric tube) for 5 consecutive days: the first group (diarrheic control) received vehicle (1% DMSO); the second group received the antibiotic norfloxacin (Noroxine, Laboratory Merck Sharp and Dohme-Chibret MSD, Paris) 20 mg/kg; and groups 3-5 received 10, 20 and 40 mg/kg E. prostrata extract, respectively, with the aim to be as close as possible to the doses of the extract used traditionally (estimated at 20 mg/kg bw of extract for 60 kg adult). To make sure that the food given to the animals was not implicated as the cause of diarrhea, a group of 5 normal rats was used as negative control, receiving neither bacterial inoculum nor drug but only food and water. Stools were collected using a white cloth fixed under the bars supporting the animals in the metabolic cage. The frequencies of faeces were evaluated for 6 consecutive days following Sd1 administration. Enumeration of Sd1 in faeces was performed before the induction of diarrhea and after the appearance of diarrhea at 2, 26, 50, 74, 98 and 122 h. For this purpose, 0.5 gm faeces was homogenized in 4.5 ml sterile saline; serial dilutions were made and 500 µl of each dilution was seeded over Hektoen agar. After 24 h incubation at 37°C, the number of CFU was determined. Animals were observed for 7 days from the day on which diarrhea was induced and the death rate was recorded.

Gastrointestinal propulsion: Rats in groups of 5 each were fasted for 48 h with free access to water. Each animal was administered per os 0.5 ml of a charcoal suspension (5% charcoal powder in 10% Arabic gum) in water. Each rat received orally, 30 min prior to the administration of the charcoal meal, one of the two doses of E. prostrata extract: 40 or 60 mg/kg bw. The control group received an equal volume of 1% DMSO. Half an hour after the meal, the rats were sacrificed and the percentage length of intestine traversed by the charcoal solution from the pylorus to the caecum was determined.[29]

Statistical analysis

The results are expressed as means ± S.E.M (standard error of the mean). Bacterial densities were log₁₀ transformed before analysis of the means. Data were statistically evaluated using the analysis of variance (ANOVA) with post hoc Dunnet’s t-test. Differences between groups were considered significant at P < 0.05.

Results

Acute toxicity

Single doses of E. prostrata extract elicited overt signs of toxicity from 15 gm/kg bw, progressively reducing different
behavioural parameters—movement, sensitiveness to touch and noise and aversive behaviour—over a period of 48 h. After this time, animal behaviour returned to normal. No significant change was observed in the quality and frequency of the faeces. The first animal death was observed with an extract dose of 10 gm/kg bw and the LD_{50} was found to be 25 gm/kg bw. Graphical and estimated LD_{50} were 16.25 gm/kg bw.

Phytochemical properties
In the water ethanol extract of E. prostrata, anthraquinones, flavonoids, phenols, phlobatannins, polysaccharides, saponins, tannins and terpenoids were identified. Alkaloids were not present in very high amounts.

Susceptibility to Sd1
In the agar diffusion method, the E. prostrata extracts were active against Sd1. The inhibition diameter (Æ) began at 10 mg extract (Æ = 8.5 mm) and increased progressively up to Æ = 22.5 mm for 120 mg of the extract; the inhibition diameter (Æ) with norfloxacin was 22.5 mm. The susceptibility of Sd1 to E. prostrata was determined, with the MIC and MBC values being 3500 and 12,000 g/ml, respectively.

Antidiarrheic activity
Animal behaviour, stool quality and mortality: Normal rats receiving only the food did not exhibit any diarrheic sign. Four hours after inoculum administration the animals became calm, less mobile and curled up. The first diarrheic sign. Four hours after inoculum administration the animals were active against E. prostrata (twice/day) on death rate of Shigella dysenteriae type 1 diarrheic rats (n = 5 per group). *

<table>
<thead>
<tr>
<th>Day after</th>
<th>Diarrheic control</th>
<th>Noroxine (20 mg/kg)</th>
<th>Euphorbia prostrata extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0 0</td>
<td>0 0 0 0</td>
<td>10 mg/kg 20 mg/kg 30 mg/kg</td>
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<tr>
<td>2</td>
<td>0 0</td>
<td>0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0 0</td>
<td>20 0 0 0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>40 0</td>
<td>20 0 0 0</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>80 0</td>
<td>20 0 0 0</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>100 0</td>
<td>40 0 0 0</td>
<td></td>
</tr>
</tbody>
</table>

Stool bacterial density and frequency: In the stools of diarrheic control rats, Sd1 density increased significantly (P < 0.01) from day 1 after diarrhea appeared; 3.9 × 10^9 and 2.6 × 10^9 by the 2nd and 3rd days, respectively, vs 1.2 × 10^9 administered. Compared with the diarrheic control and the initial value, the well-known antibiotic (norfloxacin) significantly (P < 0.01) reduced Sd1 growth from the 1st up to 6th day after start of therapy; it showed bactericidal effects. Similar to norfloxacin, E. prostrata inhibited bacterial growth in a dose-dependant manner [Figure 1]. E. prostrata extract at 10 mg/kg inhibited bacterial growth by the 3rd day and beyond, maintaining the Sd1 density at a level similar to that administered. The extract, at the doses of 20 and 40 mg/kg, effectively (P < 0.01) reduced Sd1 density from 26 h of therapy and beyond. Similar to norfloxacin (20 mg/kg), the extract (40 mg/kg) reduced the bacterial density by 82%. From the 2nd day of treatment, the total faeces frequency, while increasing in control diarrheic rats, decreased in all the treated rats and significantly so from the 3rd day [Figure 2A]. The extract at the doses of 20 and 40 mg/kg, as well as the well-known drug norfloxacin, markedly reduced the diarrheic stool rate and frequency so that by the 5th day diarrheic faeces frequencies were less than 10% of total stool, both in norfloxacin and 40 mg/kg extract treated rats [Figure 2B].

Gastrointestinal relaxant activity in vivo: Aqueous alcohol extract of E. prostrata significantly reduced the gastrointestinal propulsion of the charcoal solution meal. At oral doses 40 and 60 mg/kg there was 10% (P < 0.05) and 17% (P < 0.01) inhibition, respectively [Table 2].

Discussion
The purpose of the present work was to establish the scientific rationale for the traditional use of E. prostrata extracts in treating infectious diarrhea. Antidiarrheic effects of an 80% ethanol extract were investigated using in vitro bacterial inhibition and in vivo activity against Shigella dysenteriae type 1 induced diarrhea in rats. Acute toxicity (LD_{50} and overt signs) and phytochemical properties were also studied.

Figure 1: Shigella dysenteriae type 1 density (log_{10} transformed) in diarrheic rat stools over 5 days of treatment (twice a day beginning 2 h after the appearance of diarrhea) with aqueous ethanol extract of Euphorbia prostrata (Ep) and norfloxacin (Norx). Data are the mean ± SEM (n = 5 per group). *P < 0.05, **P < 0.01 compared with initial values at 2 h; *P < 0.05, **P < 0.01 compared with diarrheic control

Table 1
Effect of treatment with aqueous ethanol extract of Euphorbia prostrata (twice/day) on death rate of Shigella dysenteriae type 1 diarrheic rats (n = 5 per group)

<table>
<thead>
<tr>
<th>Day after treatment</th>
<th>Diarrheic control</th>
<th>Noroxine (20 mg/kg)</th>
<th>Euphorbia prostrata extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 mg/kg 20 mg/kg 30 mg/kg</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0 0</td>
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<td>20 0 0</td>
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<td>5</td>
<td>80 0</td>
<td>20 0 0</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>100 0</td>
<td>40 0 0</td>
<td></td>
</tr>
</tbody>
</table>
The assayed doses represented $6 \times 10^{-4}$, $12 \times 10^{-4}$ and $25 \times 10^{-4}$ of the oral LD$_{so}$ Since aqueous ethanol extract of E. prostrata at doses below 10 gm/kg did not provoke any change in the behaviour of normal animals and because the LD$_{so}$ value was much higher than 5 gm/kg, the extract can be considered safe for all practical purposes in the laboratory and for all medicinal uses, according to the WHO criteria.

The in vitro study showed the extract’s inhibitory activity against Sd1, which was equiactive to norfloxacin. The extract’s MIC and MBC values are seemingly high. These high values are almost certainly due to the fact that we used crude extract, since other studies have shown high MIC and MBC for the crude extracts of some shigellacial plants. A ratio of MBC/MIC < 4 could indicate a bacterial activity for E. prostrata. This was further confirmed by the Sd1 count in stools, where the decrease of bacterial population was similar to that obtained with norfloxacin treatment. By the 5th day, the bactericidal actions of both E. prostrata extract and the reference antibiotic norfloxacin were quite evident, with Sd1 density in the stool becoming less than a hundred.

Many diarrheal rats developed signs such as curling up, soft stools, glairy/bloody or mucus-linked lumpy faeces, and faeces with a fetid odour that likely expressed the presence of pus. These signs are typical of infectious or ‘invasive’ diarrheaa. Intestinal fermentation in diarrheal rats, by reducing the pH and increasing faecal bulk, raised the faeces frequency. Sd1 swarming is responsible for shiga toxin production, which induces the production of an important reactive oxygen metabolite, the mediator nitric oxide (NO), which is implicated in the inflammation associated with diarrheaa. Toxin resulting from the bacterial swarming was suspected of being responsible for the limb weakness that occurred within 48 h in animals that did not pass stool and also for animal deaths. Norfloxacin (noroxin, 20 mg/kg) and E. prostrata extract (20 and 40 mg/kg) reduced the bacterial population and at the same time may have slowed down the intestinal propulsion. Decrease in intestinal transit time could have led to exacerbation of enteroinvasive infections, but this might have been prevented by the bactericidal property of the extract and thereby resulted in the decrease in stool frequency.

Phytochemical assessment revealed the presence of phenols, saponins, flavonoids and tannins (and alkaloids) which possess antioxidant and anti-inflammatory activities. Flavonoids are responsible for the inhibition of intestinal motility and secretion, which could lead to a decrease in the frequency of wet faeces. Infections may be prevented by the antimicrobial properties of some compounds, such as anthraquinones, saponins and phenols. In the course of its intestinal anti-inflammatory action, some flavonoids inhibit inducible NO synthase. This action, since NO is responsible for the limb weakness that occurred within 48 h in animals that did not pass stool and also for animal deaths. Norfloxacin (noroxin, 20 mg/kg) and E. prostrata extract (20 and 40 mg/kg) reduced the bacterial population and at the same time may have slowed down the intestinal propulsion. Decrease in intestinal transit time could have led to exacerbation of enteroinvasive infections, but this might have been prevented by the bactericidal property of the extract and thereby resulted in the decrease in stool frequency.

Table 2

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Effect of Euphorbia prostrata extract (Ep) on rat intestinal propulsion. Data are the mean ± SEM ($n = 5$ per group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Témoin</td>
<td>Ep 40 mg/kg</td>
</tr>
<tr>
<td>Ep 60 mg/kg</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Total length (cm)</td>
<td>44.63 ± 1.89</td>
</tr>
<tr>
<td>Charcoal covered length (cm)</td>
<td>34.27 ± 1.32</td>
</tr>
<tr>
<td>Progression rate (%)</td>
<td>76.79 ± 1.67</td>
</tr>
<tr>
<td>Inhibition rate (%)</td>
<td>10.1</td>
</tr>
</tbody>
</table>

$^*P < 0.05$ and $^{**}P < 0.01$, compared with control

Figure 2: Total (A: number/day: nbr/day) and diarrheal (B: % of total faeces frequency TS) stool frequency during the treatment (twice per day) of Shigella dysenteriae type 1 diarrheic rats with aqueous ethanol extract of Euphorbia prostrata (Ep) and norfloxacin (Norx). 0-5 days after extract administration. Data are the mean ± SEM ($n = 5$ per group).

* $P < 0.05$, $^{**}P < 0.01$ compared with initial values at 2 h; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with diarrheic control.

\begin{table}
\begin{tabular}{|l|c|c|c|}
\hline
 & Témoin & Ep 40 mg/kg & Ep 60 mg/kg \\
\hline
Total length (cm) & 44.63 ± 1.89 & 47.28 ± 2.73 & 46.42 ± 1.68 \\
Charcoal covered length (cm) & 34.27 ± 1.32 & 32.65 ± 2.86 & 29.59 ± 1.43 \\
Progression rate (%) & 76.79 ± 1.67 & 69.06 ± 2.7* & 63.74 ± 1.52** \\
Inhibition rate (%) & 10.1 & 17 & \\
\hline
\end{tabular}
\end{table}
through antibacterial and/or antitoxic actions. These actions need to be elucidated with further investigations. Furthermore, the extract’s anti-diarrheal effect could also result from the combined slowing down of stool ejection.

In this study, the S. dysenteriae type 1 strain was not resistant to the fluoroquinolone norfloxacin; the E. prostrata extract was found to be bactericidal; it inhibited behavioural changes, decreased stool frequency and prevented death in diarrheic animals. The bactericidal effect was comparable to that of the fluoroquinolone norfloxacin and was less sudden.

All these anti-diarrheal properties attest to the usefulness of E. prostrata in the treatment of diarrhea, especially infectious diarrheas such as Sd1-induced diarrhea.

Acknowledgments

We acknowledge the Centre Pasteur of Yaoundé - Cameroon, for providing us Shigella dysenteriae 1 strain and the University of Yaoundé I for the university research support fund (URSF2000-2002).

References


