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Research Paper

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

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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 12×10^8 *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), like norfloxacin, 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 antidiarrheic properties and could be a therapeutic alternative for diarrheas of bacterial etiology.

KEY WORDS: Antidiarrheic activity, *Euphorbia prostrate*, rat, *Shigella dysenteriae* type 1

Diarrhea is responsible for 3.1 million deaths monthly the world over, with more than 600,000 due to shigellosis.[1] *Shigella dysenteriae* type 1 (*Sd1*) is an endemic human pathogen, causing acute bacillary dysentery in regions with high population densities. Resistance to quinolone antibiotics has been reported for this organism which rapidly develops resistance to the current therapy.[2–4] Foreign multidrug-resistant *Sd1* strains might be introduced into new communities through travel. Dominant clones of ciprofloxacin-resistant *Sd1* were observed in Southeast Asia and in Canada.[5–7] In sub-Saharan Africa, factors like refugee camps, rainy season and hot climate favour shigellosis due to *Sd1*.[8] In Ngoela, East Cameroon, a 16.4% fatality rate of *Sd1-Escherichia coli* 0157-*Entamoeba histolytica*-induced diarrhea was recorded.[9] Many *Sd1* strains in this African region are resistant to most of the antibiotics generally available in rural health centres.[10] Effective medicinal molecules (fluoroquinolones, aminoglycosides, etc) are exceptional and less accessible (due to high cost). The resistance profile leaves few reliable and economical therapeutic options for *Sd1*. In Africa, some medicinal plants are currently used against bacillary dysentery.[11,12] In the central, eastern, southern and western rural parts of Cameroon, *Euphorbia prostrata* (Euphorbiaceae) is very often used for the treatment of dysentery. This plant contains flavonoid and phenolic compounds and was demonstrated to have anti-inflammatory and analgesic properties.[13,14] In the traditional use for diarrhea treatment, a handful of fresh plant is boiled in 500 ml water for 15-20 min. The decoction (supernatant) is drunk: a teaspoonful (≈5 ml) for infant and half a teacup (≈125 ml) for adults are recommended twice a day.

In the present work, we undertook a study to verify the claim of the medicinal value of *E. prostrata*, in a model of human bacillary dysentery induced in rats.

Materials and Methods

Animals used for the experiment

Three-month old albino mice (weighing 20-30 gm) and Wistar rats (weighing 170-210 gm) of either sex, bred in our animal house in the ambient environmental conditions, were used for toxicity and antimicrobial drug assessment, respectively. Animal housing and experiments *in vivo* were done according to the guidelines of the European Union on Animal Care (CEE Council 86/609)

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.[15,16] 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 12×10^8 (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 Cameroon at Yaoundé, 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 the powder was macerated for 4 days in 80% ethanol (5 L). The filtrate was evaporated to dryness in a rotary evaporator at 37˚C to yield 85 gm of dark green *E. prostrata* extract, 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, 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, jerkiness, sensitiveness to noise and touch, stools quality and frequency. The dose of the extract that would kill 50% of the animal population (LD_{50}) by the route given was estimated graphically or by using the following formula:[17]

$$LD_{50} = X_s - d(\sum p - ½)$$

X_s: 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:[18,19] Mayer and freshly prepared Dragendoff´s reagents (for alkaloids), Liebermann-Buchard test (for terpenoids and sterols), [$FeCl_3$ and $K_3Fe(CN)_6$] (for phenols and tannins), Shinoda test [(magnesium turnings and $K_3Fe(CN)_6$] (for flavonoids), Molish test (α-naphtol and H_2SO_4) (for polysaccharides), frothing test (for saponins), $FeCl_3$ and HCl (for phlobotannins) and NH_4OH (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.).[20] Bacterial suspensions were further diluted to obtain the 5×10^5 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 (∅; 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.[22,23] 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.[24]

Statistical analysis

The results are expressed as means ± S.E.M (standard error of the mean). Bacterial densities were $\log_{10}$ 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_{100} 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, phlobotannins, 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 (∅) began at 10 mg extract (∅ = 8.5 mm) and increased progressively up to ∅ = 22.5 mm for 120 mg of the extract; the inhibition diameter (∅) 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 μg/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 faeces was emitted within 24 h (the 2nd day) and thereafter the rats recovered their mobility progressively and presented greater aggressiveness, which decreased over the following days, except in the case of the diarrheic control and 10 mg/kg *E. prostrata* treated groups. Diarrheic stools were either soft or liquid, containing mucus or moulded and smooth and very often mucus-linked. At times, the faeces appeared molded and lumpy or presented blood marks. Stools of diarrheic rats emitted a fetid odour that attracted midges. By the 4th day of treatment, the stench vanished and by day 5, the midges stopped appearing. During the five days of therapy, no death was recorded with norfloxacin and *E. prostrata* extracts of 20 and 40 mg/kg, whereas 40 and 100% deaths were registered on day 6, respectively, in the 10 mg/kg *E. prostrata* and diarrheic control groups [Table 1].

Table 1

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

Day after treatment	Death rate (%)				
	Diarrheic control	Noroxine (20 mg/kg)	Euphorbia prostrata extract		
			10 mg/kg	20 mg/kg	30 mg/kg
1	0	0	0	0	0
2	0	0	0	0	0
3	0	0	20	0	0
4	40	0	20	0	0
5	80	0	20	0	0
6	100	0	40	0	0

***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* A_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; [a]*P* < 0.05, [b]*P* < 0.01 compared with diarrheic control

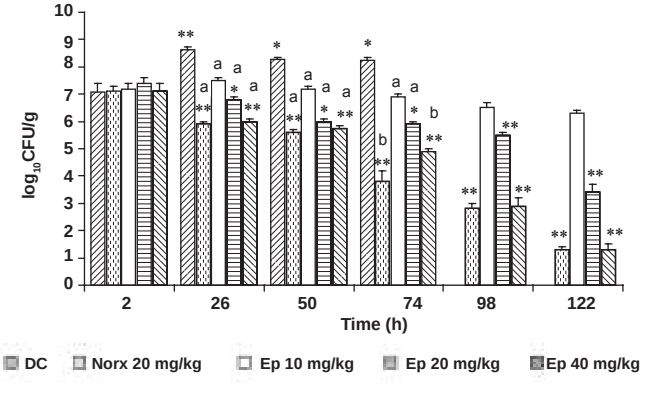

Figure 2: Total (A: number/day: nbr/day) and diarrheal (B: % of total faeces frequency TS) stool frequency during the treatment (twice per day) of *Shigella dysenteriae* type 1 diarrheic rats with aqueous ethanol extract of Euphorbia *prostrata* (Ep) and norfloxacin (Norx). 0-5 days after extract administration. Data are the mean ± SEM (n = 5 per group). *P < 0.05, **P < 0.01 compared with initial values at 2 h; [a]P < 0.05, [b]P < 0.01 compared with diarrheic control

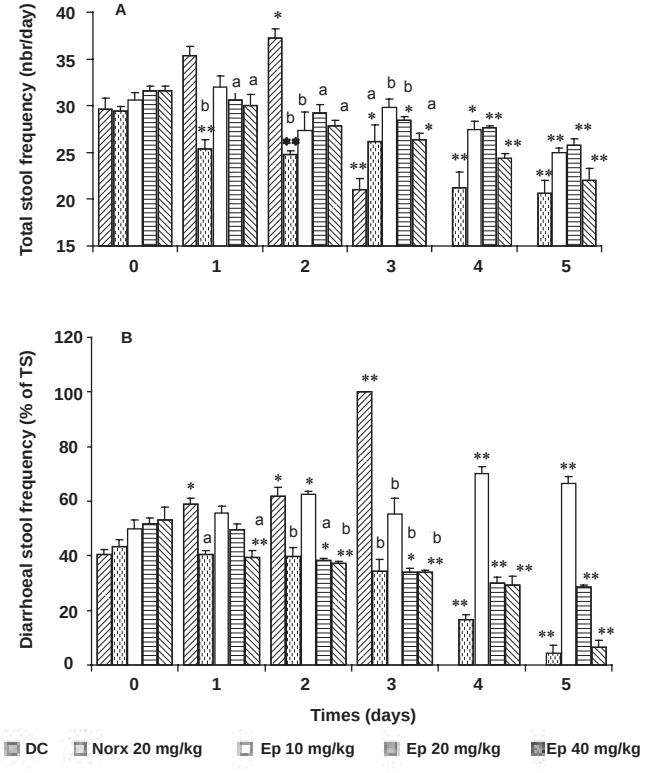

Table 2

Effect of *Euphorbia prostrate* extract (Ep) on rat intestinal propulsion. Data are the mean ± SEM (n = 5 per group)

	Témoin	*Ep 40 mg/kg*	*Ep 60 mg/kg*
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

*P < 0.05 and **P < 0.01, compared with control

The assayed doses represented 6×10^{-4}, 12×10^{-4} and 25×10^{-4} of the oral LD_{50}. 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_{50} 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.[25]

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 shigellocidal plants.[26] A ratio of MBC/MIC < 4 could indicate a bacterial activity for *E. prostrata*.[20] 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 diarrheic 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' diarrhea.[27] Intestinal fermentation in diarrheic rats, by reducing the pH and increasing faecal bulk,[28] 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 diarrhea.[29] 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[30] 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.[31] 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 behavioural changes, could lead to the reduction in aggressiveness as well as the reduction of mucus-coated stool and bloody diarrhea.[32,33] In addition to its use in diarrhea, *E.prostrata* is also traditionally used for the control of diabetes; the presence of tannins, polysaccharides, terpenoids, saponins, flavonoids (and alkaloids) in the extract probably explain its effect in this disease. *E. prostrata* extract progressively reduced the *Sd1* density in the faeces. By the 4th day of treatment, 40 mg/kg bw extract had reduced the *Sd1* population by 48.5% and by 83.5% by the 6th day. This progressive effect is of great importance because the toxin release that occurs with rapid destruction of microorganisms *in vivo* could lead to endotoxinic shock, which could result in high faeces frequency.[34] On the other hand, the rapid decrease in diarrheic stool (from the 4th day onwards) is responsible for the disappearance of midges. This is also of great significance, since flies are implicated in the transmission of shigellosis.[35] The *E. prostrata* extract also abolished all the disorders observed in the *Sd1*-induced diarrhea. It is therefore possible that the aqueous alcohol extract of *E. prostrata* is effective against infectious diarrhea

through antibacterial and/or antitoxin actions. These actions need to be elucidated with further investigations. Furthermore, the extract's antidiarrheic 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 antidiarrheic properties attest to the usefulness of *E. prostrata* in the treatment of diarrhea, especially infectious diarrheas such as *Sd1*-induced diarrhea.

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