

International Journal of Pharmaceutical Sciences and Drug Research

2015; 7(1): 40-45



Research Article

ISSN: 0975-248X
CODEN (USA): IJPSPP

Activity of Aqueous Ethanol Extract of *Euphorbia scordifolia* on *Shigella dysenteriae* Type 1-Induced Diarrhea in Rats

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ABSTRACT

Euphorbia scordifolia is traditionally used in Cameroon for the treatment of many diseases, including diarrhea. We investigated the effect of the aqueous ethanol extract of *Euphorbia scordifolia* (EWES) on intestinal propulsion, bacterial *in vitro* growth and *in vivo* shigellosis. To value effects of EWES on intestinal propulsion, normal rats orally received 0, 50, or 75 mg/kg bw extract and the distance covered by the charcoal meal was measured. Shigellosis was induced by oral administration of 1.2×10^9 *Shigella dysenteriae* type A1 (Sd1) to the rats. Diarrheic rats were treated for 6 days with 50, 75 mg/kg EWES or 20 mg/kg norfloxacin. Nitric oxide (NO) production in blood and in colonic homogenate; blood parameters, diarrheal stool weight and Sd1 density were assessed, and death rate recorded. *In vitro*, the minimal inhibitory and minimal bactericidal concentrations of EWES were 4000 and 12000 µg/mL respectively. EWES significantly ($P < 0.01$) reduced intestinal transit, bacterial growth, diarrheal stool weight and decreased NO production in large intestine ($P < 0.01$). The results suggest that *Euphorbia scordifolia* possesses bactericidal and antidiarrheic properties, and could be a therapeutic alternative for diarrhea of bacterial etiology.

Keywords: Antidiarrheic activity, *Euphorbia scordifolia*, intestinal propulsion, *Shigella dysenteriae* type 1, rat.

INTRODUCTION

Diarrhea is characterized by the alteration of the bowel movement and the increase in the water content, volume or frequency of stools, and abdominal pain. [1] Diarrheal diseases are a major problem in the developing countries and are responsible for million people death each year, with more than 600 000 deaths due to shigellosis. [2]

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Received: 15 October, 2014; Accepted: 31 October, 2014

Shigella dysenteriae type 1 (Sd1) is an endemic human pathogen, causing acute bacillary dysentery in regions with high population densities. [3] *S. dysenteriae* is spread by contaminated water and food and is the cause of most severe dysentery because of its potent and deadly Shiga toxin. Many *Shigella dysenteriae* strains in sub-Saharan Africa are resistant to most of the antibiotics generally available in rural health centers. [4] like diphenoxylate, loperamide, atropine, and many antibiotics are available for the treatment of diarrhea,

but they have some side effects. ^[5] In Africa, some medicinal plants are currently used against bacillary dysentery. ^[6] The whole plant of *Euphorbia scordifolia* is traditionally used by Cameroon inhabitants (Central Africa) for diarrhea cure. The present study was undertaken to evaluate the antidiarrheal potential of aqueous ethanol extract of *Euphorbia scordifolia* in *Sd1*-induced diarrhea in rats.

MATERIALS AND METHODS

Extract

The whole plant of *Euphorbia scordifolia* (Euphorbiaceae) was collected from Batié (West Region of Cameroon) in September and was compared by the National Herbarium of Yaoundé, Cameroon, to the voucher specimen N° 20631/SFR Cam. The plant was washed thoroughly with water, shade-dried and grounded. The powder of *E. scordifolia* (1 kg) was macerated in 5 L of 20 % ethanol for 72 hours in a percolator. The obtained filtrate was evaporated to dryness in a rotavapor and yielded 55.8 g (5.58%) of dark green *E. scordifolia* extract. For oral administration, extract was dissolved in distilled water so that each animal received less than 1 mL/100 g bw solution.

Bacterial strain

The bacterial strain, *Shigella dysenteriae* type 1 (*Sd1*), isolated from local hospitals patients was provided by the Centre Pasteur of Yaoundé, Cameroon, for the study.

In vitro antimicrobial susceptibility

Disc diffusion method

To determine the minimal inhibitory concentration (MIC) we employed the paper disc diffusion method that we adapted to E-test. ^[7] Sterile 6 mm Ø filter paper discs (Schleicher & Schul, No. 2668, Dassel, Germany) were impregnated with 50µL of the plant extract at various concentrations. 500µL *Sd1* inoculum (5×10^5 CFU) were flooded respectively in *Salmonella Shigella* (SS) and in Mueller Hinton (MH) agar (Oxoid) and incubated for 15 min at 37°C. After the incubation, the impregnated paper discs were placed on the plates according to decreasing extract concentrations (50000 to 24.4µg/mL). The petri dishes were incubated at 37°C for 24 h, after when the growth inhibition and the MIC (first disc which did not present the growth inhibition) were observed on the petri dishes. The test was performed under sterile conditions in duplicate and repeated three times.

Macrodilution method

The *Sd1* strains were adjusted to 0.5 McFarland and diluted (1:1000) in Brain Heart infusion (Oxoid). A dilution series of the extract, ranging from 50,000 to 24.4µg/mL, were prepared and then transferred to the broth in 11 tubes. The tubes were inoculated with *Sd1* suspension at a final density of 10^5 cells/mL. The tubes were incubated at 37°C for 24 h. The lowest concentration of the tube which did not show any visible growth after macroscopic evaluation was considered as the MIC. ^[8] This was confirmed

spectrophotometrically and by viable cells count in SS agar. The Minimal Bactericidal Concentration (MBC: concentration producing 99.99 % reduction of CFU in the initial inoculum) was determined by subculture on nutrient agar. The tubes without growth after 24 h of incubation were subcultured on Mueller Hinton agar and on SS agar for 24 h. MBC was determined as the lowest concentration that showed no bacterial growth in the subcultures. ^[9]

In vivo anti-diarrheic activity

Diarrhea induction

Prior to the study, Wistar albino rats (60-98 g) were allowed to be acclimatizing for 1 week in our laboratory environment (temperature and day/light cycle). Animal housing and *in vivo* experiments were done according to the guidelines of the European Union on Animal Care (CEE Council 86/609) ^[10] that was adopted by the Institutional Committee of the Ministry of Scientific Research and Innovation of Cameroon. The animals were fed with standard rat diet: carbohydrates 50-55%, fats 15-20% and proteins 25-30% and water *ad libitum*. Rats were kept singly in metabolic cages. To induce diarrhea, after verifying that they were not *Sd1* carrier, each rat was orally administered a solution of 1.2×10^9 saline diluted *Sd1* cells. ^[3, 11-12]

Antibacteria activity

When diarrhea appeared, the rats were randomly divided into 4 groups of 5 animals each. Group 1, diarrheic control (DC), didn't received any treatment; the three other groups: Nor20, EWES50, and EWES75, were respectively administered (p.o.) twice daily (6:00 AM and 6:00 PM) for six consecutive days 20 mg/kg bw antibiotic norfloxacin (A-320 Norfen 400 mg tablet; cadila pharmaceuticals Ltd), 50 mg/kg bw and 75 mg/kg bw extract. To make sure that the food given to the animals was not implicated in the diarrhea induction, a group of five normal rats (Normal control: NC) received food and water and neither bacterial inoculums nor drug. The stools were collected daily using a sterile stool pot of the metabolic cage. The weight and feces quality were evaluated for seven consecutive days following bacterial administration. The enumeration of *Sd1* in feces was performed before induction and for 7 consecutive days following the appearance of the diarrhea. For this purpose, 0.5 g of feces was homogenized in 4.5 mL of sterile saline; serial dilutions were made and 500µL of each dilution was spread over SS agar plate. After incubation (24 h at 37°C), the number of CFU was determined. ^[13] After six days of treatment, all survival animals were sacrificed, their blood and their colon were collected for blood cells count using manual method and/or for nitric oxide (NO) determination using the modified Griess method. ^[14-15]

NO dosage

NO concentration was evaluated in serum and in colon homogenates. To obtain Griess solution, 250µL of Griess 1 (0.8 g sulfanilic acid + 250 mL acetic acid 30%)

were added to 250 μ L of Griess 2 (0.05 g of α -naphthylamine + 100 mL acetic acid 30%). 500 μ L of serum or homogenate sample were added to 500 μ L of Griess solution and the total solution obtained were incubated for 20 minutes at room temperature. After incubation, the absorbance was recorded with a spectrophotometer (T60-1611ESW) at 553 nm. [15]

Blood cells count [14]

Red blood cells (RBCs), white blood cells (WBCs), and platelet cells (PCs) were counted using microscope (MOTIC 1820 LED: SM7432-MC1ST-RPIWFM). One drop of blood diluted in Marciano solution (1/200 for RBC count), in Lazarus solution (1/20 for WBC count) and in Plette solution (1/100 for PC count). One drop of each solution was deposited on Mallassez count cell and was kept for 10 minutes in a dark condition. The blood cells were then counted on the microscope. For WBC, the total number was calculated using the formulae:

$$\text{WBCs } (/mm^3) = \frac{\text{Average number of chamber WBC counted} \times \text{Dilution}}{\text{Volume}}$$

The Hematocrit (Ht) was determined using a microhematocrit tube. The Hemoglobin (Hb) concentration was measured by spectrophotometer method. The blood was diluted in Drabking solution (1/250) and the absorbance was evaluated at 510 nm.

Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were determined respectively by the formulae:

$$\text{MCV} = \frac{\text{Ht}}{\text{RBC}} \times 10; \quad \text{MCH} = \frac{\text{Hb}}{\text{RBC}} \times 10;$$

$$\text{MCHC} = \frac{\text{Hb}}{\text{Ht}} \times 10;$$

Where Ht = hematocrit value; RBC = red blood cell number; Hb = hemoglobin number

Intestinal propulsion

Four groups of five normal rats each were fasted for 18 h with free access to water. The first group (normal control: NC) was administered distilled water (1 mL/100 g bw). The three other groups respectively received *E. scordifolia* aqueous ethanol extract 50 mg/kg (EWES50), 75 mg/kg (EWES75) and the standard drug (atropine sulphate: Gland Pharma. Pally. Dundigal. Post, Hyderabad, India), 0.2 mg/kg bw i.p. Thirty minutes later each animal was orally given 2 mL of charcoal meal (10% activated charcoal by 5% gum acacia) as died marker. Each animal was sacrificed thirty minutes after administration of charcoal and the distance covered by the charcoal in the intestine was expressed as percentage of the total distance traveled from the pylorus to the caecum. [5, 16-17]

Statistical analysis

Data were expressed as mean ($\bar{x}$) $\pm$ standard error of mean (SEM) in the table. Data were analyzed by one-way ANOVA followed by Dunnett's T-test and Tukey

test using computerized GraphPad InStat version 3.05 (Graph Pad software, U.S.A.). Differences between groups were considered significant at $P < 0.05$.

RESULTS

Susceptibility of *Sd1* to *E. scordifolia* aqueous ethanol extract

In vitro, *E. scordifolia* inhibited *Sd1* growth; the MIC and MBC values were 4,000 and 12,000 μ g/mL respectively. The ratio MBC/MIC value was 3.

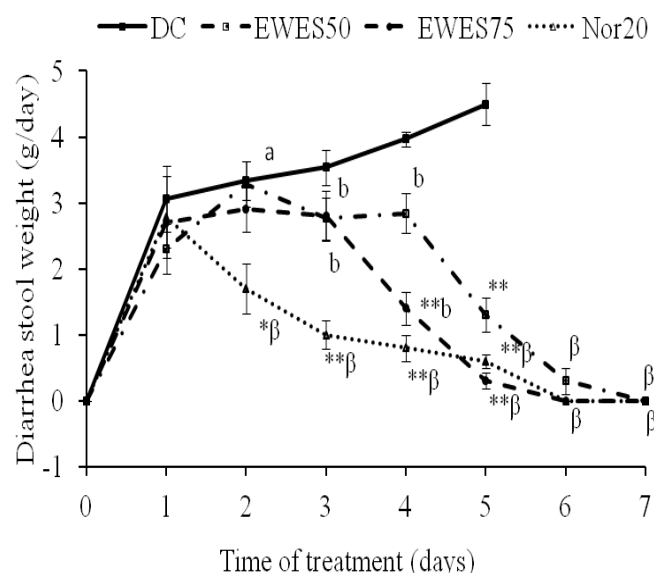


Fig. 1: Diarrheal stool weight during the treatment of *Shigella dysenteriae* A1 (*Sd1*) diarrheic rats with aqueous ethanol extract of *Euphorbia scordifolia* 50 mg/kg bw (EWES50), 75 mg/kg bw and norfloxacin 20 mg/kg bw (Nor20).

Data are the mean $\pm$ SEM, (n=5). Significant difference: * $P < 0.05$, ** $P < 0.01$ compared with diarrheic control rats (DC); $^aP < 0.05$, $^bP < 0.01$ compared with Nor20; $^{\beta}P < 0.01$ compared with initial values (d1: diarrhea appearance and treatment start). d0: *Sd1* administration.

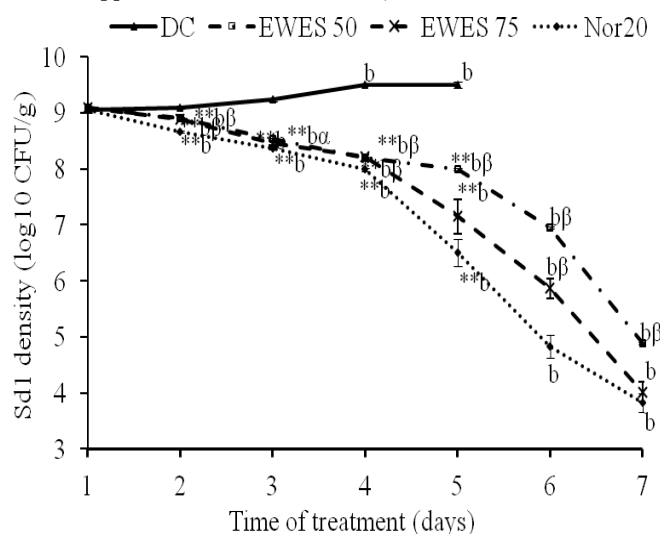


Fig. 2: *Shigella dysenteriae* A1 density ($\log_{10}$ transformed) in diarrheic rat stools over 6 days treatment with 50 mg/kg bw (EWES50) and 75 mg/kg bw *Euphorbia scordifolia* aqueous ethanol extract and norfloxacin 20 mg/kg bw (Nor20).

Data are the mean $\pm$ SEM (n=5 per group). Significant difference: * $P < 0.05$, ** $P < 0.01$ compared with diarrheic control rats (DC); $^aP < 0.05$, $^bP < 0.01$ compared with Nor20; $^{\beta}P < 0.01$ compared with initial values. d1: diarrhea appearance and treatment start.

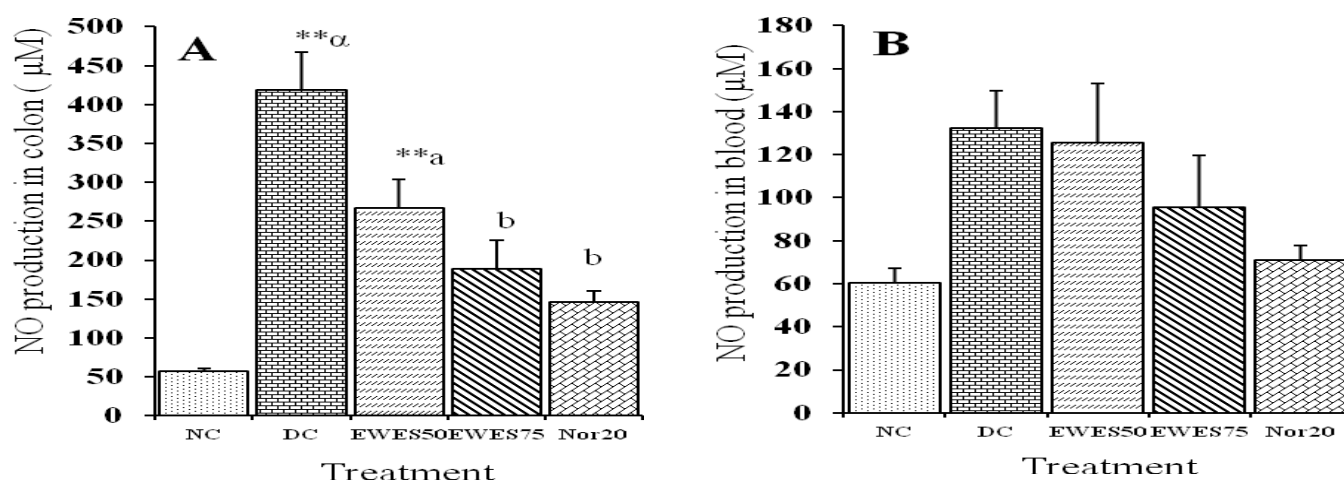


Fig. 3: Nitric oxide (NO²⁻) production in the colon (A) and blood (B) of *Shigella dysenteriae* A1 (Sd1) diarrheic rats after 6 days treatment with *Euphorbia scordifolia* aqueous ethanol extract 50 mg/kg bw (EWES50), 75 mg/kg bw and norfloxacin 20 mg/kg bw (Nor20). Data are mean $\pm$ SEM, (n=5). Significant difference: ** P <0.01 compared with normal control (NC); ^a P <0.05, ^b P <0.01 compared with diarrheic control (DC).

Table 1: Blood cells rate in *Shigella dysenteriae* A1 diarrheic rats after 6 days treatment with *Euphorbia scordifolia* aqueous ethanol extract 50 mg/kg bw (EWES50), 75 mg/kg bw and norfloxacin 20 mg/kg bw (Nor20).

Groups	WBC (10 ³ /mm ³)	Hb (g/dL)	Ht (%)	RBC (x10 ⁶ /mm ³)	PC (x10 ⁵ /mm ³)	MCV (µm ³)	MCH (pg)	MCHC (pg/dL)
NC	10.39 $\pm$ 0.39	17.65 $\pm$ 0.73	49.65 $\pm$ 2.40	8.04 $\pm$ 0.34	8.46 $\pm$ 0.54	61.7 $\pm$ 1.2	21.9 $\pm$ 0.1	35.6 $\pm$ 0.8
DC	6.5 $\pm$ 0.4**	14.6 $\pm$ 0.7	41.7 $\pm$ 2.8	7.1 $\pm$ 0.2	8.5 $\pm$ 0.5	58.9 $\pm$ 3.5	20.6 $\pm$ 0.8	35.2 $\pm$ 0.8
EWES50	10.4 $\pm$ 1.0 ^b	17.8 $\pm$ 0.3 ^b	53.0 $\pm$ 1.4	7.7 $\pm$ 0.1	6.7 $\pm$ 0.7	68.0 $\pm$ 1.2	23.2 $\pm$ 0.1	34.2 $\pm$ 0.5
EWES75	10.1 $\pm$ 0.9 ^b	14.7 $\pm$ 0.7	41.3 $\pm$ 3.4	6.8 $\pm$ 0.2	10.1 $\pm$ 0.5	60.1 $\pm$ 3.5	21.5 $\pm$ 0.6	36.1 $\pm$ 1.3
Nor20	10.4 $\pm$ 0.4 ^b	16.2 $\pm$ 0.5	46.3 $\pm$ 2.0	7.6 $\pm$ 0.1	8.1 $\pm$ 0.1	60.6 $\pm$ 2.6	21.3 $\pm$ 0.8	35.2 $\pm$ 0.9

Data are the mean $\pm$ SEM (n=5 per group). Significant difference: ** P <0.01 compared with normal control (NC); ^b P <0.01 compared with diarrheic control (DC). WBC: white blood cells, Hb: hemoglobin, RBC: red blood cells, PC: platelet cells, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration.

Table 2: Effect of *Euphorbia scordifolia* aqueous ethanol extract 50 mg/kg bw (EWES50) and 75 mg/kg bw (EWES75) and atropine 0.2 mg/kg (AT0.2) on intestine transit in normal rats.

Treatment	ITL (cm)	CCL (cm)	RTLCL (%)	Inhibition rate (%)
NC	112.40 $\pm$ 3.14	67.20 $\pm$ 4.00	59.67 $\pm$ 2.63	-
AT0.2	120.60 $\pm$ 2.46	37.07 $\pm$ 4.28	30.55 $\pm$ 2.98**	48.80
EWES50	93.20 $\pm$ 4.02	38.40 $\pm$ 1.69	41.26 $\pm$ 1.11**	30.85
EWES75	98.40 $\pm$ 3.64	36.47 $\pm$ 37.36	37.36 $\pm$ 3.71**	37.39

Values are expressed as mean $\pm$ S.E.M. (n=5). Significant difference: ** P <0.01 compared with control. ITL: Intestine total length; CCL: Charcoal covered length; RTLCL: Rate of total length covered

Antidiarrheic activity

Normal rats receiving only the food did not exhibit any diarrheic sign. Four hours after *Sd1* inoculum administration, the animals became calm, less mobile and curled up. The first diarrheic feces were emitted 25 h after inoculum administration, and thereafter, the rats recovered their mobility progressively and presented greater aggressiveness which decreased over the following days. Diarrheic stools were either soft or liquid containing mucus or molded, presented blood marks at times. Diarrheic stools emitted a fetid odor that attracted midges and that vanished after three days of treatment. During the six days therapy, no death was recorded in all treated groups, whereas we recorded 60 % death in diarrheic control group. The first day of the diarrhea onset, stools emission were: 3.06 $\pm$ 0.49 g, 2.78 $\pm$ 0.62 g, 2.30 $\pm$ 0.37 g and 2.70 $\pm$ 0.37 respectively for TD, Nor20, EWES50 and EWES75. These values increased in the untreated group over

time. They decreased significantly (P <0.01) from the second day in the 20 mg/kg norfloxacin treated group (Nor20) and for the third day in extract treated groups (Figure 1). The diarrhea was canceled by the sixth day of treatment.

After the diarrhea onset in rats, the stool *Sd1* density was about 1.2×10^9 in all groups. These values increased significantly (P <0.05) from the third day in the untreated group. In all treated groups, these values decreased significantly (P <0.01) to 0.9×10^4 , 7.6×10^4 , 1.5×10^4 CFU respectively for Nor20, EWES50 and EWES75 by the 6th day of treatment (Figure 2).

Nitric oxide production

In diarrheic control (DC) rats, nitric oxide (NO) production in colon was markedly high compared to normal control: 418.15 $\pm$ 48.65 μ M vs 57.34 $\pm$ 3.76 μ M (P <0.01). 50 mg/kg and 75 mg/kg bw *E. scordifolia* extract, as norfloxacin, have significantly (P <0.01) reduced nitric oxide (NO) production in diarrheic rats colon: 267.09 $\pm$ 36.82 μ M and 188.67 $\pm$ 36.58 μ M, 146.52 $\pm$ 14.25 μ M respectively (Fig. 3A).

The blood level of the NO was higher in DC than in normal rats: 132.42 $\pm$ 17.26 μ M vs 60.59 $\pm$ 6.61 μ M (Fig. 3B). After six day therapy, neither the norfloxacin nor the *E. scordifolia* extract significantly reduced NO production in diarrheic rats blood.

Blood parameters

RBC, Ht, PC, MCV, MCH, and MCHC values did not change significantly in untreated and treated diarrheic

rats. WBC and Hb level were reduced in DC compared to normal control (NC) rats: $6.55 \pm 0.38 \times 10^3/\text{mm}^3$ vs $10.39 \pm 0.39 \times 10^3/\text{mm}^3$ for WBC and $14.63 \pm 0.67 \text{ g/dL}$ vs $17.65 \pm 0.73 \text{ g/dL}$ for Hb. Excepted the 75 mg/kg bw extract, the norfloxacin and the *E. scordifolia* extract have prevented the blood WBC and Hb decrease in diarrheic animals (Table 1). The different blood parameters values in diarrheic treated animals were comparable to the normal control values.

Intestinal transit test

Treatment with atropine, 50 and 75 mg/kg bw *E. scordifolia* extract significantly ($P < 0.01$) inhibited the intestine normal propulsion by 48.80, 30.86 % and 37.39 % respectively (Table 2).

DISCUSSION

The purpose of the present work was to establish the scientific claim of the traditional use of *Euphorbia scordifolia* extract to treat diarrhea. Anti-diarrheic effects of the aqueous ethanol extract of *E. scordifolia* were investigated using *in vitro* bacterial growth inhibition and *in vivo* activity against *Shigella dysenteriae* A1-induced diarrhea in rats.

In vitro antimicrobial study showed an inhibitory activity of extract against *Sd1* growth. The extract MIC and MBC values were seemingly high. These high values are certainly due to the fact that we used crude extract. [18] For aqueous ethanol extract of *E. scordifolia* the ratio MBC/MIC was lower than 4 and could thus indicate a bactericidal activity. [9] This bactericidal property was confirmed by the *Sd1* count in stools, where the decrease of bacterial population was similar to that obtained with animals treated with norfloxacin. Many diarrheic rats developed signs such as curling up, soft stools, glary, bloody or mucus-linked lumpy feces and feces with a fetid odor that likely expressed the presence of pus. These signs are typical of infectious or "invasive" diarrhea. [19] The pathological phenomena characterizing *shigella* infection are linked to two particular aspects: the invasive power of the germ and its capacity to secrete a verotoxin (VT). This results in serious tissue damage, predominantly in the sigmoid colon and rectum, responsible for a severe dysentery syndrome. [19]

Diarrheic rat colon tissue and blood produced high quantity of the mediator nitric oxide (NO). The NO is a gaseous signaling molecule that regulates various physiological and pathophysiological responses in the human body. These include circulation and blood pressure, platelet function, host defense, and neurotransmission in central nervous system and in peripheral nerves. [20] The NO synthesis is catalyzed by a family of three nitric oxide synthase (NOS) enzymes: [20-21] the neuronal NOS (nNOS, NOS I) predominantly expressed in neurons in brain and peripheral nervous system; the endothelial NOS (eNOS, NOS III) mainly expressed in endothelial cells. Both nNOS and eNOS are constitutively expressed and are inactive in resting

cells. Increase in free intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) stabilizes the binding of calmodulin to eNOS and nNOS, and activates the enzyme to produce NO; and when the $[\text{Ca}^{2+}]_i$ decreases, the NO production ceases. The third isoform of the NOS family is the inducible NOS (iNOS, NOS II). Exposure to microbial products, such as lipopolysaccharide (LPS) or proinflammatory cytokines such as interleukin-1 ($\text{IL-1}\beta$), tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) induces the expression of iNOS gene in various inflammatory and tissue cells. Binding of calmodulin to iNOS is tight even at low $[\text{Ca}^{2+}]_i$, and therefore, iNOS is also called as a calcium-independent NOS, and it can constantly produce high levels of NO for prolonged periods. [20-21] High levels of NO in diarrheic control rats might result from the lipopolysaccharides (LPS) or the VT (or shiga toxin) produced by *Sd1* and which is very often implicated in the inflammation associated with diarrhea. [22] After six day treatment, diarrheic rats NO concentration significantly decreased in colon homogenate and in blood. Because nNOS and eNOS are activated by increased intracellular Ca^{2+} , the extract could therefore act by blocking calcium channels or by inhibiting the production of IP_3 , which reduce the NO production in blood. As for iNOS, activated by bacterial lipopolysaccharides (LPS), the high rate of NO in diarrheic rats could result from the high bacterial load, which increases LPS concentration and in the treated groups, the low bacterial load (low LPS concentration) would lead to decrease NO production by leukocytes (macrophages and neutrophils).

Blood parameters revealed low values of red blood cells and white blood cells in diarrheic control rats. Shigellosis induced anemia by lost blood (RBC and WBC) in the feces. [23] The NO has also shown to regulate leukocyte recruitment into the inflammatory focus. [20] *Shigella* induce macrophage apoptosis, releasing $\text{IL-1}\beta$ and thereby recruiting neutrophils to and across the intestinal epithelial layer [24] that could lead to decrease the total white blood cell number in blood circulation. The low rate of RBC, Ht and Hb in diarrheic control rats could be explained by the lost of blood in diarrheic stools. Rats treated with 75 mg/kg *E. scordifolia* extract presented equally low rate of WBC, RBC, Ht and Hb. This could be explained by the presence of potential toxic compounds in the crude extract (such as saponin).

Ethanol aqueous extract of *E. scordifolia* also significantly reduced intestinal transit, but less than atropine. Atropine decreased intestinal transit through its anti-cholinergic effect which blocks the muscarinic receptor. [25] *E. scordifolia* as atropine might act on muscarinic receptors, or on opioid receptors located on gut smooth muscle and probably by other mechanisms that lead to the inhibition of intracellular calcium mobilization such as inhibition of IP_3 and prostaglandin

synthesis, calcium channel blocking, which would reduce the intracellular calcium concentration and consequently decrease eNOS and nNOS.

E. scordifolia extract was found to be bactericidal, inhibited intestinal propulsion and nitric oxide production. Furthermore *E. scordifolia* prevented death in diarrheic rats. These results attest the antidiarrheal properties of *Euphorbia scordifolia*, especially on infectious diarrhea such as shigellosis.

ACKNOWLEDGEMENT

The authors wish to thank the Centre Pasteur of Yaoundé-Cameroon, for providing the *Shigella dysenteriae* type 1 (088-1A) strain, the Institute of Medical Research and Medicinal Plants studies (IMPM) for the financial support.

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Source of Support: Nil, Conflict of Interest: None declared.