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ANTIDIARRHOEAL AND ANTIMICROBIAL ACTIVITIES OF THE *PENTADESMA BUTYRACEA* STEM BARK METHANOLIC EXTRACT

Sylvie Lea Wansi[1*], Idrice Kamtchouing[1], Jules Roger Kuiate[2], Telesphore Benoît Nguelefack[1], Hippolyte K. wabo[3], Albert Kamanyi[1]

[1]Laboratory of Animal Physiology and Phytopharmacology, Department of Animal Biology, Faculty of Science, University of Dschang, PO Box 67 Dschang, Cameroon
[2]Laboratory of Microbiology and Antimicrobial Substances, Department of Biochemistry, Faculty of Science, University of Dschang, PO Box 67 Dschang, Cameroon
[3]Laboratory of Natural Product Chemistry, Department of Chemistry, Faculty of Science, University of Dschang, P O Box 67 Dschang, Cameroon

***Corresponding author e-mail:** wansylvie@yahoo.fr

ABSTRACT

Pentadesma butyracea Sabine (Clusiaceae) is a medicinal plant used in Cameroon to cure ailments such as gastrointestinal disorders. This study was aimed at investigating the antidiarrhoeal and antimicrobial activities of the methanolic extract from the stem bark of *P. butyracea* (MEPB). Plant extract was prepared by maceration in methanol. Its oral antidiarrhoeal effects were evaluated *in vivo* on castor oil (10ml/kg bw) and magnesium sulfate (3g/kg bw) induced diarrhea. Diarrheic rats were treated with 125, 250, and 500mg/kg extract or loperamide 2.5mg/kg. The frequency of defecation, the latency time and water content of stool were assessed. The broth microdilution method was used to evaluate the *in vitro* antibacterial activity against 10 bacterial strains: *Enterococcus feacalis, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, Shigella flexneri, Enterobacter cloacae, Staphylococcus epidermidis, Salmonella thiphymurium* et *Proteus mirabilis*. *In vivo* antimicrobial activity of the extract was assessed using an *S. flexneri*-infected rat model. *P. butyracea* extract significantly and dose dependently increased the time of appearance of the first stools and decreased the frequency of defecation and the water content of stool. It also showed relatively good antimicrobial activity against all the tested microorganisms (MIC 64 – 256 µg/ml) and MBC (256 – 1024 µg/ml); with *Enterococcus feacalis* being the most sensitive. At dose 500 mg/ml, *P. butyracea* significantly reduced *shigella flexneri* density (80.37%). Results suggest that *Pentadesma butyracea* possesses antidiarrheic and antimicrobial properties and attest its usefulness in traditional treatment of gastrointestinal disorders such as diarrhea, and the treatment of some bacterial infections as shigellosis.

Key words: *Pentadesma butyracea,* Antimicrobial, Antidiarrhoeal, Gastrointestinal disorder, *Shigella flexneri.*

INTRODUCTION

Diarrhea, characterized by a situation in which daily stools exceeds 300 g and contains 60-95% water, is still one of the major health threats to population in tropical and subtropical countries [1]. Moreover, human infections constitute a serious problem and the most frequent pathogens are microorganisms such as bacteria. Diarrhea can be cause by infectious, chemical and organic agents. For example, Shigellosis is associated with 5-15 % of cases of diarrhea and 30-50% of cases of dysentery worldwide [2]. In recent year, multidrug resistance in human pathogenic microorganism has been developed due to indiscriminate use of commercial antimicrobial drugs commonly apply in the treatment of infectious diseases [3]. In Cameroun and other African countries, diarrhea remains the number one killer disease

among children under five years, while babies between the ages of 7-12 months remain susceptible [4-5]. In addition, many rural populations leave very far away from health centers, limiting their access to medication. Thus, dependency on plants as medicine in controlling diseases is common among rural population in Cameroun because of their relative safety and affordability compared to the cost of modern medicines. More so, plants are natural sources of antimicrobial agents primarily because of the large biodiversity of such plants and the relatively large quantity of metabolites that can be extracted from these plants [6]. Therefore, there is the need to provide scientific basis of justification on the therapeutic uses of medicinal plants against infectious diseases and stimulates the research for new chemotherapeutic agents in medicinal plants [7]. *Pentadesma butyracea* is a perinea plant that can attain the height of 35 m, with a diameter of 100 – 150 cm. This plant is commonly found in tropical rain forest, humid and swampy areas [8]. In Tropical Africa, *Pentadesma butyracea* is used to treat bronchitis, fiver, viral infections and venereal diseases [9-10], whereas in Cameroon, it is traditionally used in the treatment of intestinal parasites, stomach ache, fiver and also as an aphrodisiac [11-12]. Besides, the different xanthones and benzophenones isolated from the roots of this plant by Wabo [10], are renowned for their anti-carcinogenic properties. Meanwhile, the maceration of the stem bark is used like an antiseptic body lotion and as an anti-diarrheic [9]. Phytochemical analysis of *P. butyracea* parts (roots, stem bark and leaves) revealed the present of 1,3,5-trihydroxy-2-methoxyxanthone; triterpenes, tannins, epicathechin as well as lupeol; fatty acids and steroid [10-11-12]. The leaves contain mucilaginous substances, free anthraquinons, coumarines, leucoanthraquinones, saponines, sterols and triterpenes [13]. Majority of sesquiterpenes hydrocarbons, especially α-caryophyllene, have been discovered in essential oils extracted from different parts of *P. butyracea* [13]. This study reports the antidiarrheic properties of the *P. butyracea* on castor oil and Magnesium sulphate induced diarrhea as well as the evaluation of the antimicrobial activity on 10 human pathogenic bacteria species in rat's model, in order to establish the claimed biological activities of this plant.

MATERIALS AND METHODS

Plant material

The stem bark of *P. butyracea* was collected in Mount Bamboutos (West Region of Cameroon) in October 2008. The plant was identified in the Department of Plant Biology, University of Dschang and confirmed at the National Herbarium in Yaoundé - Cameroon (reference code N° 6861/SRF/Cam). The stem bark was then dried at room temperature under shade. The dried plant material was ground into a fine powder and used in the preparation of the methanolic extract.

Preparation of plant extract

The powdered plant (2.3 kg) was macerated in 5 liters of methanol for 72 hours. The filtrate was evaporated to dryness in a rotary evaporator (Bushi R-200) at 60°C. 94.6g of the dried extract was obtained, giving a percentage yield of 4.11 %.

Animals

A Shigella flexneri infected rat model was used for the *in vivo* antibacterial assay. Wistar albino rats of both sex, weighing 150-180 g and 11 to 13 weeks old. For castor oil and magnesium sulphate induced diarrheal essay rats used were 6 weeks old and weighed 100 g. These animals were raised in the animal house of the Laboratory of Animal Physiology and Phytopharmacology of the University of Dschang (Cameroon). They were submitted to 12 hours light-dark cycle with free access to food and water. *In vivo* experiments were done according to the European guidelines of the European Union on Animal Care (CEE council 86/609) [14] that was adopted by the Institutional Committee of the Ministry of Scientific Research and Innovation of Cameroon.

Microorganisms

The microorganisms used in this study consisted of common strains implicated in gastro-intestinal disorder (including diarrhea and dysentery) in human and animals. They comprise 5 bacteria strains (*Enterococcus feacalis* ATCC 10541, *Pseudomonas aeruginosa* ATCC 27853, *E coli* ATCC 11775, *Staphylococcus aureus* ATCC 25922, and *Klebsiella pneumonieae* ATCC 13883) and five bacterial clinical isolate (*Shigella flexneri*, *Enterobacter cloacae*, Staphylococcus *epidermidis*, *Salmonella thiphymurium* et *Proteus mirabilis*). The clinical isolates were obtained from "Centre Pasteur" of Yaoundé-Cameroon.

Culture media

Müller Hilton Agar (MHA) (Conda, Madrid, Spain) and Müller Hilton Broth (MHB) were used for the anti-bacterial studies. The strains were maintained at + 4°C on MHA slants.

Castor oil and Magnesium sulphate-induced diarrhea

Castor oil and magnesium sulphate were used to induce respectively a secretory and an osmotic

diarrhea respectively. This was done according to the method described by Teke *et al.* [15] with slight modifications. The rats were all screen initially with 0.5 ml of castor oil, one week before the actual experiment. Only those showing diarrhea were selected for the final experiment. They were fasted for 24 h prior to the treatment, but had free access to water. For each experiment, 30 rats were randomly assigned into five groups of six animals each: Group 1 served as negative control and received distilled water (10ml/Kg), Group 2 received the standard drug, Loperamide (2.5 mg/kg bw) *per os* (positive control). Groups 3, 4 and 5 received the methanolic extract of *P. butyracea* at the doses of 125, 250, 500 mg/kg bw *per os* respectively. One hour after administration of the extract, all animals received 10 ml/kg body weight of castor oil or 3 g/kg bw of magnesium sulphate. They were then kept in separate metabolic cages, the floor of which was lined with a transparent absorbent paper to collect faces. Following castor oil administration/magnesium sulphate, the following parameters were measured for 6 hours: latency time, frequency of defecation and water content of stool. The water content of stool was determined using the following formula:

$$\text{Water content} \quad \frac{Ww - Dw}{Ww} \times$$

Ww = Wet weight of stool.
Dw = Dry weight of stool.

Antimicrobial activity

In vitro antibacterial assay

The *in vitro* antibacterial activity of the extract was performed by determining the minimum inhibitory concentrations (MICs) and Minimum Bactericidal Concentration (MBCs) using broth microdilution method [16]. For this purpose, stock solutions of the extract and standard drug were prepared at a concentration of 4096 µg/ml and 64 µg/ml respectively; successive dilutions permit to obtain the tested concentrations that varied from 1024 to 8 µg/ml in a 96 micro-well plates containing 95 µl of MHB and 5 µl of inoculum (standardized at 2.0 x 10^6 CFU/ml by adjusting the optical density to 0.1 at 600 nm SHIMADZU UV-120-01spectrophotometer) [17]. The negative control well consisted of 195 µl of MHB and 5µl of the standard inoculums. The plates were covered with a sterile plate sealer, then agitated to mix the content of the wells using a plate shaker and incubated at 37°C for 24h. Each concentration was tested in triplicate and the experiment repeated three times.

The plates were incubated at 35°C for 18 h. Growth was monitored using iodotetrazolium chloride (INT). All concentrations at which no visible pink color changes was observed were considered as inhibitory concentrations and the lowest of these concentrations was considered as the MIC. The bactericidal concentrations were determined by adding 50 µl aliquots of the preparations (without INT), which did not show any visible colour change after incubation during MIC. The determination of the MBC was done by culturing 50µl of liquid from each well that showed no change in color on MHA and incubated for 24h at 37°C. The lowest concentration that yielded no growth after this sub-culturing was taken as the MBC [18].

In vivo anti-shigellosis in rat model

Twelve weeks old deparsited albino's rats were starved for 18 hours with free access to water. The inoculum was prepared at 9 × 10^8CFU/ml (McFarland 3 standard). This inoculum (2 ml) was administered through gavages to each rat. Food was given to the rats *ad libitum* as from the end of the third hour to the end of the assay that lasted 6 hours. Only infected rats showing signs of diarrhea were selected for the rest of the experiment. These rats (36) were randomly assigned into six groups of six each and treated as follow: Group 1, composed with non infected rats received distilled water (neutral control), Group 2 composed of infected rats received distilled water only (diarrheic control); Group 3 composed of infected rats received Ciprofloxacin (2.5 mg/kg) (positive control); Group 4, 5 and 6 composed of infected rats received the methanolic extract of *P. butyracea* at the doses 125 mg/kg, 250 mg/kg and 500 mg/kg respectively. In order to minimize reinfection from fecal matter, each animal was placed in a cage whose bottom was lined with a gauge that allowed the feces to pass through.

The stools of infected animals collected in sterile containers for the evaluation of the bacterial load. The faeces (0.5 g) were completely dissolved in 5 ml of 0.9 % NaCl solution. Then, 250 µl of the suspension obtained were diluted in 9,750 ml of 0.9 % NaCl. Fifty µl of the final suspension were cultured on a *Salmonella-Shigella* agar for each stool sample. After 18 hours of incubation at 37°C the bacterial load was assessed and expressed in terms of number of Colony Forming Units (CFU) per gram of faeces per animal. The relative body weight of the animals was also evaluated during the treatment period.

Statistical analysis

Data were subjected to the one way analysis of variance (ANOVA) and recorded as mean ± SD. Where differences exist between treatments, means were compared using Turkey posttest for anti-diarrheal analysis and ANOVA two ways followed by Bonferroni test for anti-microbial analysis of variance, where $p < 0.05$ was considered statistically significant. The data were analyzed using Graph pad prism version 5.0.

RESULTS

In vivo antidiarrhoeal activity of the MEPB on Castor oil induced diarrhea

Effect on the frequency of defecation, the latency time and the water content of stool

P. butyracea was found to be effective in a dose dependent manner against castor oil induced diarrhea on experimental rats at all tested doses. At the dose of 500 mg/kg body weight, the extract produced a significant decrease (P 0.001) in the severity of diarrhea, by reducing the frequency of defecation and water content of stool in rats, of 76.02% and 35.70% compared to the negative control. However, these effects were lower compared to that of Loperamide (positive control) (Table 1). At the same dose of the extract, a significant increase (P 0.001) of 374% in the latency time comparable to that of the negative control was observed.

In vivo antidiarrhoeal activity on osmotic diarrhea induced by Magnesium sulphate

Effect on the frequency of defecation, latency time and water content of stool

As shown in Table 1, independently of the dose administered, the extract significantly (P 0.001) decreased the frequency of defecation with respect to the negative control. At the dose of 500 mg/kg the extract showed a comparable effect to that of Loperamide. In addition, the latency time was increased in a dose dependent manner compared to the negative control. The highly significant (P 0.001) increase in the latency time caused by Loperamide at the dose of 2.5 mg/kg was comparable to that of the extract at the dose of 500 mg/kg. Whereas, the water content of stool did not vary significantly at all doses of the extract in magnesium sulphate induced diarrhea compared to the negative control.

Antimicrobial properties of MEPB

In vitro anti-microbial activity

Table 2 shows the anti-bacterial effects of the MEPB on the different bacterial strains used in the study. Considering the crude nature of the extract, values of the MIC of 8 mg/ml or below against any microorganism tested was considered as active. The best MIC and MBC values for the microorganisms tested were 64 and 256 µg/ml respectively. The greatest and remarkable antimicrobial activity of the extract was recorded with *Enterococcus feacalis.* In general, the ratio MIC/MBC ≤ 4 shows a bactericidal effect of the extract.

In vivo antibacterial activity of the MEPB on S. flexneri induced diarrhea

In the stools of treated rats, *Shigella Flexneri* density decreased significant from the first day after induction to the last day of the treatment compared with the initial value administered. Compared with the diarrheic control, the antibiotic ciprofloxacin significantly reduced the density of *Shigella flexneri* in stools from the first to the last day of the treatment. Similar to ciprofloxacin, MEPB inhibited the bacterial growth in a dose dependent manner. The extract at the dose of 125, 250 and 500 mg/kg effectively reduced shigella density from the seventh day of therapy and beyond; the percentage of reduction of *shigella flexneri* density was respectively 69.03%, 75.54% and 80.37% compared to the value administered (Figure 1). Thus, the dose 500 mg/kg of the extract appeared to be more active than ciprofloxacin (70.63%) compared to the negative control.

Effect of the MEPB on body weight during the treatment period

No variation in body weight was observed in the animals during the treatment period (18 days) with the MEPB and ciprofloxacin, compared to the negative control group (Figure 2).

DISCUSSION

Antidiarrheic properties of the methanolic extract of P.butyracea

Diarrhea originates from an imbalance between the absorptive and secretory mechanisms in the intestinal tract accompanied by hurry, resulting in an excess loss of fluid in faeces [19]. We focused our study on three main etiologies of diarrhea: the secretory, induced by castor oil; the osmotic induced by magnesium sulphate and infectious by *Shigella flexneri*. From the results, it appears that the extracts of *P. butyracea* possess both antisecretory and antiosmotic effects associate to antibacterial properties. In fact, the extract significantly reduces the osmotic imbalance induced in rats by magnesium sulphate. Indeed, magnesium sulphate is known to increases the permeability of electrolytes at the level of the intestinal mucosa, alongside, the secretion of

cholecystokin in the duodenum, leading to hyper secretion which inhibits fluids' reabsorption [20-21].

The tested extract may content active substances that have increased the absorption of water and electrolytes from the gastrointestinal tract of rats.

In contrast , the active principle found in castor oil, ricinoleic acid, irritates the intestinal mucosa and this results in the biosynthesis of inflammatory mediators such as Prostaglandins (PgE2) and Histamine and consequently an increase in intestinal motility and hyper secretion [22-23-24]. It is obvious that the extract may have the capacity of inhibiting the effect of ricinoleic acid on the muscosa of the intestine. In castor oil induced diarrhea, the anti-diarrheic effect of the *P.butyracea* could result from inhibition of prostaglandin/histamine synthesis or by installing an anti-secretory mechanism [25].

Loperamide used in this study as reference drug act by inhibiting the peristaltic activity, through indirect effect on circular and longitudinal muscle of the intestinal wall, also by stimulating the absorption of water and electrolytes by enterocytes by increasing the intestinal transit time (anti-spasmodic) of the bowel content [26]. It is then possible that the *P.butyracea* methanolic extract act in the same manner. Moreover, the anti-diarrhea activity of the extract could be due to the presence of flavonoids and tannins which have the capacities to inhibit intestinal motility and hydroelectrolytes' secretion [27]. Otherwise, tannins could act by reducing intracellular calcium ion concentration or by activating the calcium pomp which would lead to muscle relaxation [28]. Indeed, Noudogbessi et al. [13] have described the presence of gallic tannin, flavonoids and terpenoids in methanolic extract of this plant.

Antibacterial properties of the extract

Although less effective than the reference drug, *P.butyracea* showed relatively good antibacterial properties both *in vitro* and *in vivo*. Secondary metabolites groups that have previously been described in this plant species including flavonoids, tannins, triterpens, steroids and fatty acids [13] are known to possess antimicrobial properties [10-11-12-29]. The census of pathogens revealed that E. coli entero-invasive (EIEC) and entero-hemorragic (EHEC), Salmonella, Shigella, *Vibrio cholerae* are the major bacterial pathogens most often responsible for pandemic and epidemic diarrheal infectious diseases in developing countries [30]. *Shigella flexneri* is known to secrete verotoxins which helps it to colonize the colon mucosa and penetrates the epithelial cells, inducing inflammation and degeneration of the lamina propria. Consequently, the desquamation and ulceration of the mucosa, which leads to the loss of blood and mucus in the intestinal lumen, which in turns hinders reabsorption [2-31]

Tested on shigellosis-induce rat model, the dose 500 mg/kg of the extract was more active than the reference drug Ciprofloxacin at the dose 2.5 mg/kg. This result is interesting since in the *in vitro* test the MIC of ciprofloxacin (0.25µg/ml) was 1024 time lower than that of the extract (256 µg/ml). So, it seems as the immune system may have play a role in the final antimicrobial effect of the plant extract in infected rats. Also, some substances administered orally can undergo transformations during absorption, transport and distribution processes (pharmacokinetics), thereby, producing metabolites which could either be more active or less active than the original compound [32]. In the case of our extract, the metabolite would be more active, thus, could explain this result.

The infected rats did not show any variation in body weight after treatment, compared to the control. This could be due to the loss of appetite, possibly induced by the anorexic properties of the plant extract. Indeed, the extract could stimulate the secretion of Leptine which is capable of inhibiting Y-neuropeptid, the most powerful appetite stimulator to the best of our knowledge or through the inhibition of hunger and thirst receptors in the stomach by the MEPB [33].

CONCLUSION

This study has demonstrated the antidiarrheic properties of *P. butyracea* methanolic extract on secretory and osmotic diarrhea, associated to relatively good and large antibacterial activities. With these properties, *P.butyracea* is a good candidate for the development of an antidiarrheic phytomedicine.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

Table 1: **Effect of the different doses of the extract and loperamide in castor oil and magnesium sulphate induced diarrhea in rats after 6 hours of treatment.**

	Treatments	Frequency of defecation in 6H	Percentage inhibition of the frequency of defecation	Latency time (min)	Percentage of water content of stool
Castor oil (10ml/kg bw)	Distilled water (10ml/kg)	4.17 ± 0.31	0 %	54.50 ± 2.03	84.18 ± 0.96
	Loperamide (2.5 mg/kg)	0.17 ± 0.17^{z}	95.92 %	340.33 ± 19.67^{z}	12.47 ± 12.47^{z}
	MEPB 125 mg/kg	2.00 ± 0.26^{zc}	52.04 %	79.67 ± 14.08^{c}	70.30 ± 10.40^{b}
	MEPB 250 mg/kg	1.33 ± 0.21^{zb}	68.11 %	165.33 ± 22.41^{zc}	61.60 ± 8.81
	MEPB 500 mg/kg	1.00 ± 0.31^{za}	76.02 %	258.33 ± 39.16^{zb}	35.70 ± 11.55^{y}
Magnesium sulphate (3g/kg bw)	Distilled water (10ml/kg)	3.33 ± 0.21	0 %	219.83 ± 15.07	72.44 ± 2.74
	Loperamide (2.5 mg/kg)	0.50 ± 0.2^{z}	84.98 %	334.17 ± 14.01^{z}	35.60 ± 16.46^{x}
	MEPB 125 mg/kg	1.67 ± 0.21^{zb}	49.85 %	234.83 ± 7.65^{b}	69.68 ± 2.65
	MEPB 250 mg/kg	1.33 ± 0.33^{za}	60.06 %	283.67 ± 16.78^{x}	61.72 ± 12.56
	MEPB 500 mg/kg	0.83 ± 0.31^{z}	75.08 %	326.67 ± 16.00^{z}	37.94 ± 14.63

Each value represent the mean ± SEM. $^{a}P < 0.05$; $^{b}P< 0.01$; $^{c}P < 0.001$ *significant differences compared to the positive control.* $^{x}P < 0.05$; $^{y}P < 0.01$; $^{z}P<0.001$ *significant differences compared to the negative control*.

Table 2: **Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values of MEPB and Ciprofloxacine on bacterial strains and isolates**

Bacteria	MEPB (µg/ml)			Ciprofloxacine (µg/ml)		
	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
Staphylococcus epidermidis	256	512	2	0.25	0.50	2
Enterococcus feacalis	64	256	4	0.25	0.25	1
Pseudomonas aeruginosa	128	512	4	1	2	2
Shigella flexneri	256	512	2	0.25	1	4
Proteus mirabilis	128	256	2	0.25	0.50	2
Echerichia coli	128	512	4	0.25	1	1
Salmonella thiphymurium	128	256	2	1	1	1
Staphylococcus aureus	256	1024	4	0.25	0.50	2
Enterobacter cloacae	256	512	2	1	4	4
Klebsiella pneumoniae	128	256	2	1	2	2

Values are the mean of three independent determinations. MIC: minimal inhibition concentration (µg/ml). MBC: minimal bactericidal concentration (µg/ml).

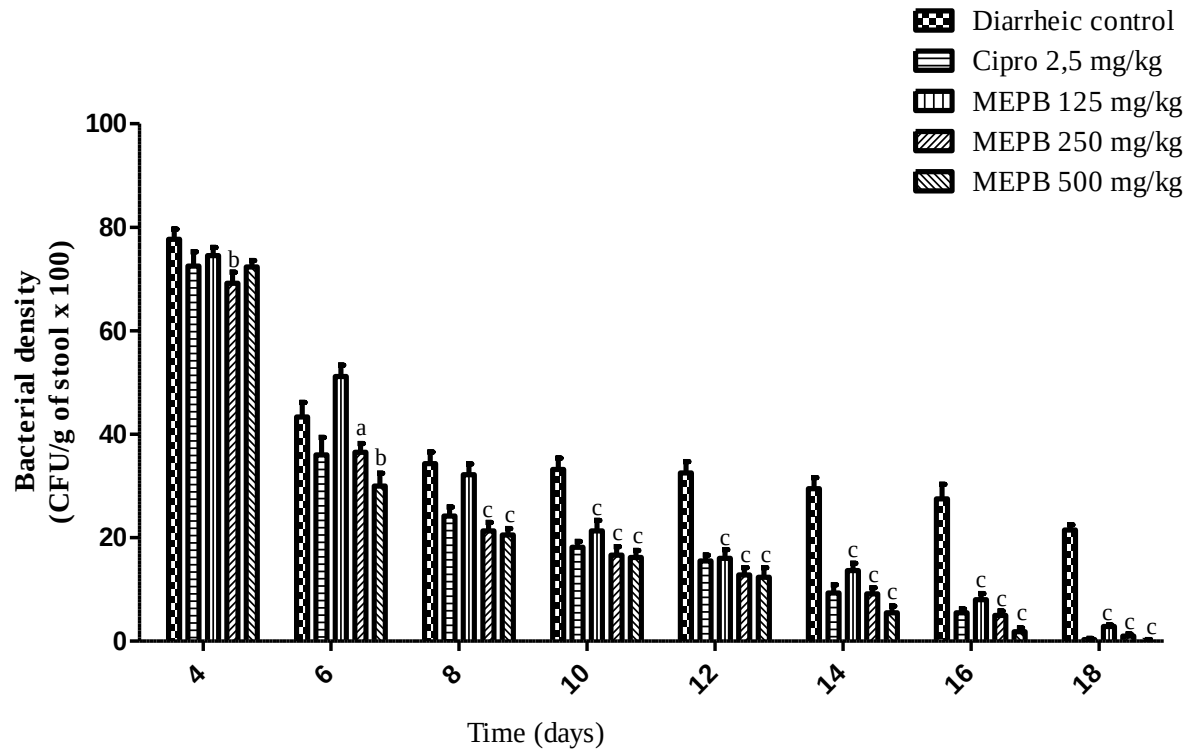

Figure 1: ***Shigella flexneri*** **density in infected rat stool over 18 days of treatment (beginning from the fourth day, after induction of diarrhea in rats), with MEPB and ciprofloxacin (Cipro). Data are mean ± SEM (n=6 per group).** $^{a}P \leq 0.05$, $^{b}P \leq 0.01$, $^{c}P \leq 0.001$, **compared with the diarrheic control.**

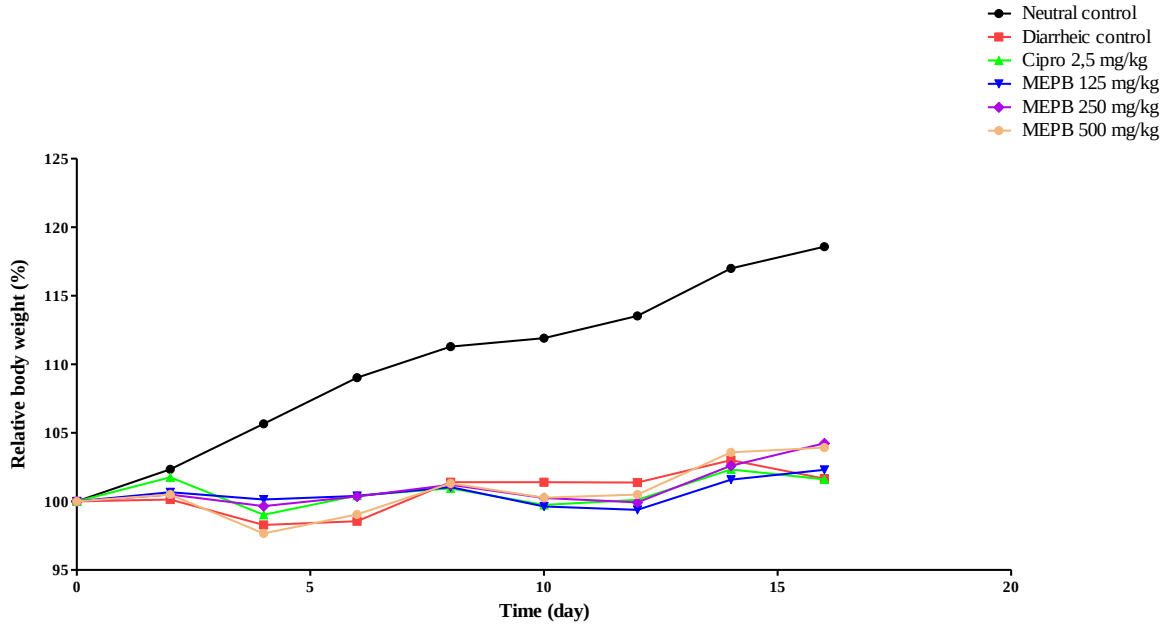

Figure 2: Variation of the relative body weight of ***Shigella flexneri*** **diarrheic rats treated with MEPB and Ciprofloxacin (Cipro) with respect to time (day). Each value represents the mean ± SEM (n= 6 per group).**

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