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Ex-vivo Antispasmodic and *In vivo* Anti Salmonella Potential of *Rumex bequaertii* Leaves Extracts

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Authors' contributions

This work was carried out in collaboration among all authors. Authors CRMD, APA, RGBF, RTT, GTS and CNN collected plant material, performed pharmacological assays, statistical analysis and prepared the manuscript. Authors CRMD, APA, CNN, RGBF, AN, GTS, WP, JRK and PVT conceived, revised the manuscript and supervised the study. All authors read and approved the final manuscript.

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ABSTRACT

Objective: The present study aimed at investigating the acute toxicity, the *ex-vivo* antispasmodic potential of aqueous and ethanolic *Rumex bequaertii* leaves extracts in isolated ileum fragment and antimicrobial activity of *Rumex bequaertii* ethanolic leaves extract in animal models infected with *Salmonella typhi*.
Methods: Acute toxicity using a single dose of ethanolic extract of *Rumex bequaertii* at 2000 mg/kg was administered to female rats and effects were observed during 14 days. Different cumulative concentrations of the aqueous and ethanolic extracts of *Rumex bequaertii* leaves were tested for spontaneous contractions and potassium chloride-induced contractions in rat ileum fragment. Different doses of the ethanolic extract of *Rumex bequaertii* leaves were tested for antidiarrheal activity in Wistar rats infected with *Salmonella typhi*.
Results: Rats given the single dose of *Rumex bequaertii* ethanolic extract showed no significant changes in body and organs weights compared to control rats. Administration of extracts at all tested concentrations resulted to inhibition of ileum contractions. Inhibition of spontaneous contractions of ileum fragment by aqueous extract ranging from 24.48 to 90.19 %, for 2.91, to 15.25 mg/mL, respectively, while ethanolic extract was from 42.00 to 83.72 %, for 0.99, to 5.66 mg/mL, respectively. Concerning potassium chloride-induced contraction, aqueous extract concentrations (from 2.91 to 15.25 mg/mL) inhibited contractions from 31.46 to 67.23 %; the ethanolic extract (0.99 to 5.66 mg/mL) inhibited from 38.25, to 82.60 % and verapamil from 14.31 to 80.70 %, at 0.06 to 0.34 mg/mL. After administration of ethanolic extract, all tested doses resulted to reduction of *Salmonella typhi* load in stools and blood, with activities being duration and dose dependent.
Conclusion: The lethal dose 50 of the *Rumex bequaertii* ethanol extract is greater than 2000 mg/kg. Aqueous and ethanolic leaves' extracts of *Rumex bequaertii* possess *ex-vivo* antispasmodic and ethanolic leaves extract of *Rumex bequaertii* possess antimicrobial activity.

Keywords: Rumex bequaertii; Ex-vivo; antispasmodic; antimicrobial; wistar rats; Salmonella typhi.

1. INTRODUCTION

"Antispasmodics are muscular relaxants that are used to relieve cramps or spasms of the stomach, intestines and bladder" [1]. "They are commonly used for the treatment of different gastrointestinal disorders, including diarrhea and irritable bowel syndrome" [2]. "The gastrointestinal tract is under the control of the sympathetic and parasympathetic arms of the Autonomic nervous system" [3]. "Over activity of the parasympathetic arm causes increased peristalsis resulting in gastrointestinal cramps, gastritis, diarrhea and ulcers due to increased gastric secretions" [4]. "These disorders result from excessive involuntary muscle movement. Traditional treatment of gastrointestinal disorders by herbal plants is widely applied and has less side effects compared to synthetic drugs" [5]. Diarrhea is caused by several pathogens such as bacteria [6]. Potential enterobacteria that cause life-threatening diarrheal diseases worldwide include species of Salmonella, Shigella, Escherichia, Pseudomonas [7]. Among these, Salmonella and Shigella spp continue to be the leading cause of diarrhea in developing countries [8].

Salmonella typhi is a gram negative bacteria pathogen capable to cause severe diseases in humans such as diarrhea, vomiting and abdominal cramps 12 to 72 hours following infection [8]. Norfloxacin and ciprofloxacin have been developed to treat infectious diarrhea, however, there is nowadays an urgent need of new antimicrobial therapies [6]. For example, salmonellosis is now resistant to sulfonamides. In addition, the often-high cost of pharmaceutical products and inaccessibility of rural populations to health centers push them to resort to medicinal plants, which are widely available and display fewer side effects.

"Rumex bequaertii De Wild (Polygonaceae) is an herbaceous woody plant, reaching 1-2 m tall, with pale green or brown stem and very long and

narrow leaves of about 35 cm long. At maturity, it produces 3 mm red, shiny berries and trines" [9]. "In Cameroon, as well as in South and East Africa, *Rumex bequaertii* is used in traditional medicine for the treatment of rheumatism, stomach upset, diarrhea and abdominal pain, abscesses, malaria, cough, headache, and also as an anthelmintic and an antidote. Infusion of roots is used to treat pneumonia, dysentery, venereal diseases and as a purgative" [10,11,12]. *Rumex bequaertii* has antiviral, antiulcerogenic, antiulcer, antihistaminic and anticholinergic or antibradykinic properties [13,14,15]. Therefore, the current study investigated the ex-vivo antispasmodic potential of aqueous and ethanolic *Rumex bequaertii* leaves extracts in isolated ileum fragment and antimicrobial activity of *Rumex bequaertii* ethanolic leaves extract in animal infected with *Salmonella typhi*.

2. MATERIALS AND METHODS

2.1 Collection of Plant Materials

The fresh leaves of *Rumex bequaertii* were collected early in May 2019 in Foto, Dschang, West Region Cameroon and botanical identification was performed at the National Herbarium of Cameroon by comparison with the existing Voucher specimen nº 7665/SRFCam. These leaves were shade air-dried for two weeks and then crushed using a kitchen grinder. The powder obtained was used to prepare extracts.

2.2 Preparation of the Aqueous Extract

Five hundred grams (500 g) of powdered *Rumex bequaertii* leaves were boiled in 5 liters of distilled water for 30 minutes. The mixture obtained was filtered using Whatman filter paper (N° 3) and the filtrate was distributed into small portions of 250 mL and evaporated in an oven (Memmert) for 72 hours at 35 °C. The powder obtained was stored at room temperature for subsequent experiments.

2.3 Preparation of the Ethanolic Extract

Five hundred grams (500 g) of powder from the leaves of *Rumex bequaertii* were macerated in 3000 mL of ethanol for 48 hours. The solution obtained was filtered and the filtrate was concentrated using a rotative evaporator at 65 °C. The paste obtained was dried in an oven for complete evaporation of the solvent, then weighed and stored at room temperature.

2.4 Animals

Male and female *Wistar* rats weighing 140 -150 g and from 2.5 to 3 months old were used. These animals were obtained from the Animal House of the Animal Physiology Laboratory of the University of Dschang. Animals were kept at room temperature in clean cages with natural light/dark cycles and sufficient aeration. They were fed with a standard laboratory diet with free access to tap water. Diet composition per 1000 g was as follows: 668.7 g of cornmeal, 205.8 g of soybean meal, 102.7 g of fishmeal, 10.3 g of bone meal, 10.3 g of cooking salt, 1.1 g of cotton seed meal, 1.1 g of palm kernel meal and 1.1 g of vitamin complex. "The use, handling and careof animals were done in adherence to the European Convention for the protection of vertebrate animals used for experimental and other purposes (ETS-123), with particular attention to part III, articles 7, 8 and 9" [16].

2.5 Acute Toxicity

This study was carried out according to the modified protocol of the Organization of Corporation and Economic Development (OECD) guideline 423. Thus, 9 female rats aged 2.5 - 3 months (140 - 150 g) were divided into 3 groups of 3 animals each. They were fasted 14 hours before treatment, but with free access to drinking water. These animals treated by gavage as follow:

Group 1 -control: 5% of ethanol in water, for an administration volume of 1 mL/100 g body weight;
Group 2: 2000 mg / kg of the ethanol extract;
Groups 3 confirmation: 5% of ethanol in water, for an administration volume of 1 mL/100 g body weight with a delay of 48 hours from group 1 and 2;
Groups 4 confirmation: 2000 mg/kg of the ethanol extract with a delay of 48 hours from group 1 and 2.

After these treatments, the animals were observed individually for 4 hours and not received either food or water for the evaluation of toxicological manifestations. Behavior, body mass, signs of toxicity and mortality were evaluated during the first 24 hours after administration of the extract and once daily for 14 days [17].

2.6 Preparation of Ileum Fragments

Young rats, fasted for 18 h, were weighed and anesthetized with chloroform. The abdominal cavity was opened and fragments of ileum (2-3 cm) were removed from the mesenteries and oxygenated in Tyrode's solution (in mmol/L; 37° C): sodium chloride 136.9; KCl 2.7; $MgCl_2$ 1.1; NaH_2PO_4 0.4; $C6H_{12}O_6$ 5.6; $NaHCO_3$ 11.9 and $CaCl_2$ 1.8; (pH 7.4). The fragments were then mounted in an isolated organ tissue bath containing the same physiological solution at 37°C, continuously aerated with carbogen (95% O_2, 5% CO_2). A preload (1 g) was applied to each fragment and spontaneous contractions were recorded with an isotonic transducer coupled to a Power Lab system amplifier. It was connected to a computer equipped with graphics software. Organs were equilibrated within 30 minutes prior to the addition of any test substance. The tissue was stable when isotonic contractile responses were recorded. These experimental conditions made it possible to evaluate the spasmolytic and myorelaxant activities of the extracts in the absence of any agonist [18].

2.7 Spasmolytic Activity and Opening of Calcium Channels Induced by KCl

The spasmolytic activity was directly evaluated on the spontaneous contractions of the ileum fragments by cumulative addition of the concentrations of aqueous extract (2.91; 5.66, 8.25, 10.71; 13.04 and 15.25 mg/ml) or ethanolic extract (0.99; 1.96, 2.91, 3.85; 4.76 and 5.66 mg/mL).Muscle relaxant activity was achieved on precontracted ileum fragments with submaximal KCl concentration (80 mmol/L). Cumulative concentrations of each test substance were then added when a plateau was observed. The percentages of inhibition were calculated from the recorded contraction load (g) in the presence of aqueous extract (2.91; 5.66, 8.25, 10.71; 13.04 and 15.25 mg/ml) or ethanol extract (0.99; 1.96, 2.91, 3.85; 4.76 and 5.66 mg/mL) and verapamil (0.06; 0.12, 0 .17, 0.23; 0.29 and 0.34 mg/mL) used as standard, compared to control contractions considered as 100%.

2.8 Infectious Diarrhea Induced by *Salmonella typhi*

Diarrhea was induced using the method developed by Tsafack et al. [19] with some modifications. Four days after de-worming the rats with oxytetracycline (10 mg/kg), they received 1.5×10^8 CFU in 1 mL/100g of a *Salmonella typhi* suspension prepared in autoclaved 0.9% NaCl. After three days of observation, the bacterial loads as well as the appearance of the stools were evaluated. Animals were divided into 6 groups, with 6 rats per group. The groups were treated every day as follows: Group 1 (which was not infected) served as neutral control and received no treatment throughout the experiment; Group 2 (which was infected and received ethanol 5%) served as a negative control; Groups 3, 4 and 5 were treated after infection with ethanol extract at 120, 240 and 480 mg/kg, respectively; Group 6 served as positive control and rats of this group were treated with ciprofloxacin at 14 mg/kg.

All animals had free access to food and water. The blood and stools of each animal were collected every 48 hours during seven days of treatment and cultured in *Salmonella Shigella* Agar (SSA) prepared in Petri dishes. The inoculated dishes were incubated at 37 °C for 24 hours. To evaluate the efficacy of the treatment, the colonies were counted and converted to number of colonies (N) per mL of blood or per g of stool. Animals were weighed every day during treatment.

$$N = \frac{\text{Colonies count}}{\text{Volume}} \times \text{dilution factor}$$

2.9 Statistical Analysis

The statistical analysis was carried out using Graph Pad Prism version 5.0. The results obtained were expressed as Mean ± standard error of the mean (SEM). All groups were compared using one-way analysis of variances followed by the Turkey post-test or by the Bonferroni post-test for tests carried out on diarrhea induced by *Salmonella typhi*.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Acute toxicity

Rat given the single dose 2000 mg/kg-of *Rumex bequaertii* ethanolic extract showed no toxicological signs, no change in body and organs weights was observed. Behavior (grooming, coat, tremor mobility, reaction to noise) did not change. The body weight and organs weights did not varied during acute toxicity study of *Rumex bequaertii* ethanolic extract.

3.1.2 Spasmolytic activity and opening of calcium channels induced by KCl

Fig. 1 show the inhibitory effects of (A) aqueous and (B) ethanolic extracts on spontaneous contractions of isolated rat ileum. Aqueous extract reduced spontaneous contractions from 24.48 to 90.19%, respectively, for the concentrations 2.91 to 15.25 mg/mL, and ethanol extract from 42.00, to 83.72%, respectively, for the concentrations 0.99 to 5.66 mg/mL.

Fig. 2 show inhibitory effect of (A) aqueous and (B) ethanolic extracts; and (C) Verapamil on KCl-induced contractions of isolated rat ileum. It appears from this figure that the aqueous extract has reduced contractions from 31.46 to 67.23%, respectively for concentrations 2.91 and 15.25 mg/mL and the ethanol extract from 38.25 to 82.60%, for concentrations 0.99 to 5.66 mg/mL. Verapamil reduced at the percentages 14.31 to 80.70% respectively for 0.06 to 0.34mg/mL concentrations. The inhibitory effect was concentration dependent.

3.1.3 *In vivo* antibacterial activity of ethanol extract of *Rumex bequaertii* in rats

Rumex bequaertii ethanolic extract induced a significant reduction of the number of viable *S. Typhi* recovered from blood and feces as indicated in Figs. 3 and 4, respectively. Four days after administration of the ethanolic extract, all tested doses resulted in the reduction of *Salmonella typhi* load in stools and blood these effects being duration and dose dependent. From 120 to 240 mg/kg at day two, the number of colonies reduces on blood from 531UFC/g to 342 UFC/g respectively. Duration wise, at the concentration of 480mg/kg the number of colonies decreases from 531UFC/g to 9 UFC/g on stools; comparable to the reference drug where at day 2, 510 UFC/g were counted and 0 at day 8 of treatment. The same tendency of reduction was observed in the blood.

3.2 Discussion

Rumex bequaertii is found in Dschang-Cameroon, where its leaves are traditionally used to treat diarrhea and intestinal pain. *Rumex bequaertii* has antiviral, antiulcerogenic, antiulcer, antihistaminic and anticholinergic or antibradykinic properties [13,14,15]. Adult patients suffering from diarrhea are recommended by Cameroonian traditional practitioners to use leaves. Previous unpublished results obtained by authors showed that aqueous and ethanolic extracts of *Rumex bequaertii* inhibited misoprostol-induced intestinal motility and stools frequency in diarrheic rats, which suggested that extracts may have the potency to further regulate intestinal smooth muscle functionality.

Thus, the current study was undertaken to explore *ex-vivo* antispasmodic potential of aqueous and ethanolic *Rumex bequaertii* leaves extracts on isolated ileum fragments and antimicrobial activity of *Rumex bequaertii* ethanolic leaves extract in animal models infected with *Salmonella typhi*. Administration of a single dose of ethanolic extract of *Rumex bequaertii* (2000 mg/kg) in rat did not result in any deaths in the first stage. No animal death was recorded 48 hours after administration of the extract. After 14 days, grooming, coat, tremor, Mobility, reaction to noise, appearance of stool, breathing, convulsion, food, number of deaths, body weight and organs weight showed no significant variation. No convulsion and deaths were observed during the fourteen days of observation. These results suggest that the ethanol extract of *Rumex bequeartii* would have no influence on the behavior and physical appearance of the animals.

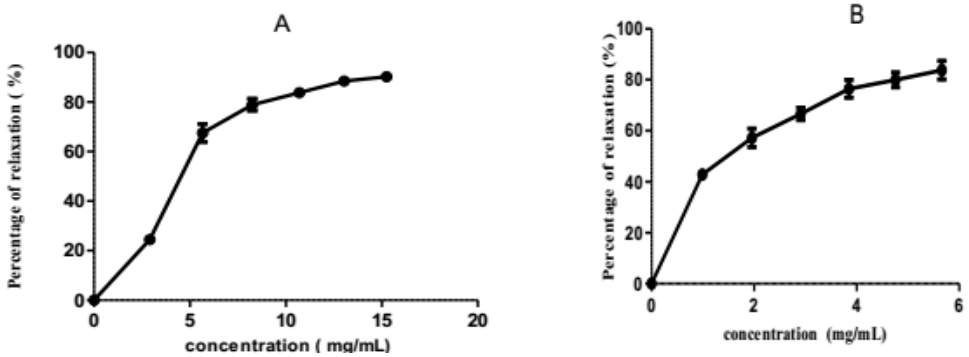

Fig. 1. Inhibitory effects of (A) aqueous and (B) ethanolic extracts on spontaneous contractions of isolated rat ileum

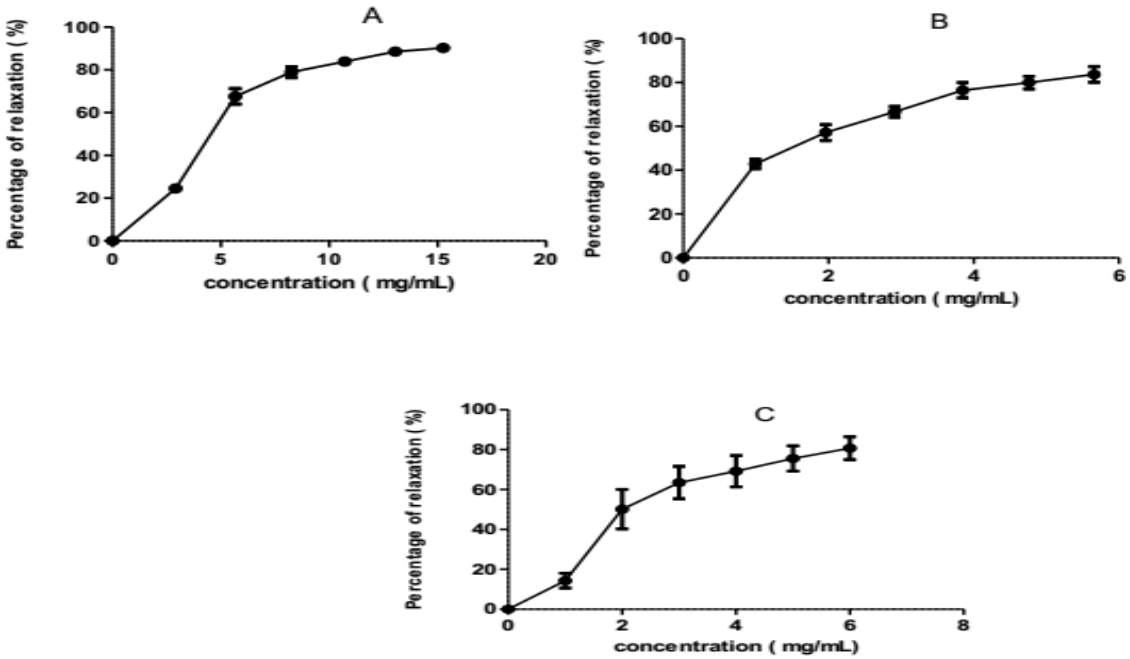

Fig. 2. Inhibitory effect of (A) aqueous and (B) ethanolic extracts; and (C) Verapamil on KCl-induced contractions of isolated rat ileum

NB: All the different concentrations are cumulative

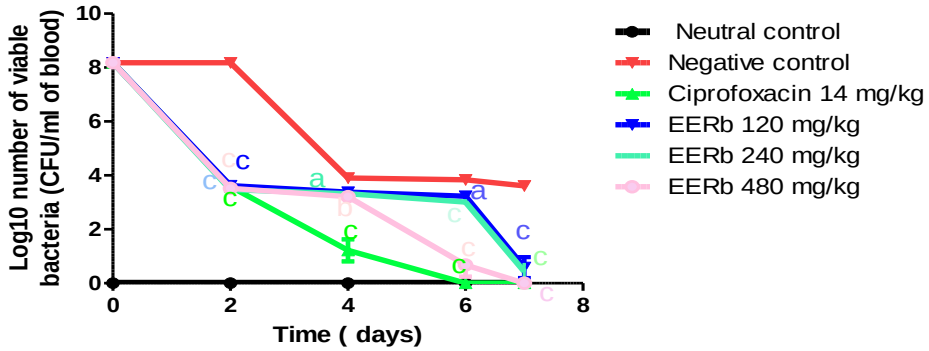

Fig. 3. Effects of ethanolic extract of *Rumex bequaertii (EERb)* on blood shedding of *S. typhi* (CFU/mL) in rats

Each value is expressed as mean ± SEM (n=6). [a]P=0.05, [b]P=0.01 and [c]P=0.001: significant differences compared to negative control

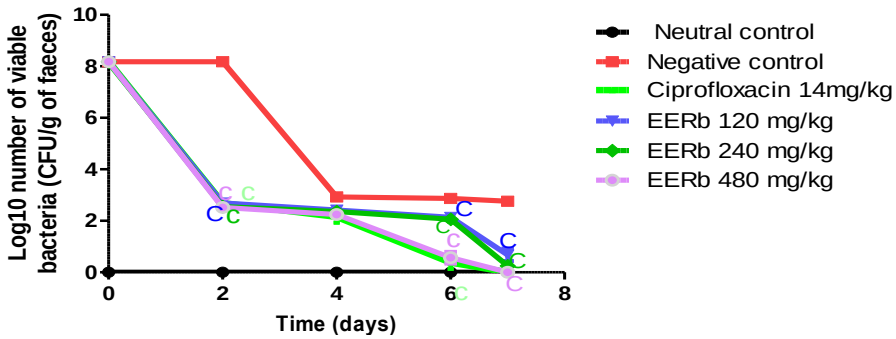

Fig. 4. Effects of ethanol extract of *Rumex bequaertii* (EERb) on fecal shedding of *Salmonella typhi* (CFU/g) in rats

Each value is expressed as mean ± SEM (n=6). [c]P= 0.001: significant differences compared to negative control

"The ethanolic extract of *Rumex bequaertii* is placed in category 5 which includes substances with LD50 greater than 2000 mg/kg according to OECD guideline 423, 2001" [17]. The absence of diarrhea indicates that the extract does not stimulate intestinalperistalsis [20]. Corporal weight changes are used as indicators of adverse effects of toxicsubstances [21,22]. "It has been shown that when plant extracts are able to inhibit spontaneous contractions of an isolated ileum fragment, this would indicate that they possess spasmolytic activity" [23]. "However, since several pathways are involved, it is necessary to look for the different mechanisms that can explain observed activity. This could be performed through an evaluation of calcium channels inhibitory effects" [24]. "Smooth muscles contraction depends on free cytoplasmic calcium, which promotes activation of contractile elements of smooth muscle cells" [23]. "The intracellular augmentation in free calcium would occur as a result of the L-type calcium channels opening, or the release from calcium stored in the sarcoplasmic reticulum" [25,26]. "It is known that high concentrations of extracellular potassium chloride (KCl) induce smooth muscles contractions, by activating L-type calcium channels opening, thus causing extracellular calcium influx, followed by an activation of contractile elements" [27,28]. "The concentration-dependent myorelaxant activity obtained with aqueous and ethanolic extracts on KCl-induced contractions shows that they inhibited the L-type calcium channels, thus preventing calcium influx. These myorelaxant effects were comparable to those expressed by verapamil, a known calcium channel inhibitor" [29].

As for the empirical use of this plant, *in vivo* study was undertaken to verify the therapeutic efficacy of the ethanol extract on *Salmonella* induced diarrhea. After administration of *Salmonella typhi,* the onset of diarrhea on day 3 in rats, associated with the morbidity observed in them, could be explained by the intestinal invasion of *S. typhi. Salmonella typhi* destroys enterocytes and mucosa by its combined action in the lumen of the digestive tract. It adheres to the apical pole of the enterocytes, then enters and causes lysis leading to the inflammatory response of the animal [30]. The results showed that administration of ethanolic extract of *Rumex bequaertii* inhibited the growth of *S. typhi*, and thus reduced the numbers of viable *S. Typhi* recovered from feces and blood samples. This reduction was dose-dependent in infected animals and their bacterial load was null at the dose 480 mg/kg within 5 to 7 days of treatment. The considerable decrease of the bacterial load in infected animals after beginning of the treatment could be due to the combined actions of the extract and the immune system. However, in negative controls, this decrease only occurred two to four days after that of the treated animals. The anti-salmonella property of *Rumex bequaertii* could also be attributed to the presence of secondary metabolites, such as flavonoids, tannins, which may have a synergistic action. The decrease in the bacterial load observed in all the treated animals could also be due to the polyphenolic compounds and alkaloids present in the extract. Arokiyaraj et al. [31] showed that polyphenolic compounds and alkaloids have immunostimulatory properties.

4. CONCLUSION

Antispasmodics are muscular relaxants that are used to relieve cramps or spasms of the stomach, intestines and bladder. The current study investigated the *ex-vivo* antispasmodic potential of aqueous and ethanolic *Rumex bequaertii* leaves extracts in isolated ileum fragment and antimicrobial activity of *Rumex bequaertii* ethanolic leaves extract in animal infected with *Salmonella typhi.* Based on the results of this study, we can conclude that the LD50 of the *R. bequaertii* ethanol extract is greater than 2000 mg/kg, and this extract was classified as poorly toxic. Aqueous and ethanolic leaves' extracts of *Rumex bequaertii possess ex-vivo* antispasmodic activity which may occur by inhibition of L-type calcium channels, thus preventing calcium influx. Ethanolic leaves extract of *Rumex bequaertii possess* antimicrobial activity.

ETHICAL APPROVAL

Prior authorization for use of laboratory animals in this study was obtained from the Cameroon National Ethics Committee (Registration number FWA-IRB00001954).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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