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Original Research Article

In vivo antibacterial activity and biochemical effects of methanol extract of *Annona muricata* leaves against multidrug-resistant *Salmonella* Typhimurium in Wistar rats

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ABSTRACT

Background: The increasing antibiotic resistance and paucity of new antibiotics has contributed greatly to the high morbidity and mortality of salmonellosis necessitating the search for alternative treatments. *Annona muricata* has shown promising activity against multidrug-resistant (MDR) *Salmonella* in vitro but in vivo studies are rare. This study evaluated the activity of *A. muricata*, against MDR *Salmonella* typhimurium in vivo.

Methods: The minimum inhibitory concentration (MIC) of a methanol crude extract of *A. muricata* was determined by micro-dilution assay against a characterized MDR clinical *S. Typhimurium* strain. Wistar rats infected with 3×10^8 CFUs/mL of the MDR strain were treated with 50-200 mg/kg body weight of extract for 10 days. Faecal load of *S. Typhimurium* colonies was determined by the direct plate count technique on days 1, 5 and 10. Animals were sacrificed and blood was collected for biochemical analyses. Data were analysed using GraphPad Prism Software.

Results: The *S. Typhimurium* strain was multidrug-resistant and the extract recorded a MIC of 2 mg/ml. The extract produced a significant ($p < 0.001$) dose-dependent reduction in *Salmonella* colonies in faeces of treated rats with a 100% inhibition recorded at 200 mg/kg body weight on day 10. Liver and renal function tests did not indicate any abnormalities ($p < 0.05$).

Conclusions: This is the first report of in vivo activity of *A. muricata* leaves against multidrug-resistant *Salmonella*. The high activity and lack of adverse toxicity supports its use in traditional medicine and hence is a potential treatment for resistant *Salmonella* infections.

Keywords: *Annona muricata*, Antibacterial, Multidrug-resistant, *Salmonella*, Wistar rats

INTRODUCTION

The global burden of salmonellosis remains high with up to a billion cases and over 150,000 deaths annually.¹ It is essentially a foodborne infection transmitted through contaminated food and water, largely due to poor hygiene. There are two main species; *Salmonella enterica* and

Salmonella bongori and six subspecies with over 2600 serovars but only a few serovars cause disease in humans.² In terms of clinical characteristics, the serovars are grouped into typhoidal (*S. typhi* and *S. paratyphi* A, B and C) which infect only humans causing life-threatening enteric fever and non-typhoidal *Salmonella* (*S. Enteritidis*, *S. Typhimurium* etc) which infect both humans and

animals and cause mild self-limiting disease in humans.³ Meanwhile, invasive non-typhoidal *Salmonella* mainly due to *S typhimurium* which is more virulent has emerged in sub-Saharan Africa with a high morbidity and mortality.^{1,4} Initially, salmonellosis was treated with antibiotic (penicillins, chloramphenicol and trimethoprim/sulfamethoxazole). Emergence of resistance led to the use of fluoroquinolones, but with increasing resistance to fluoroquinolones, cephalosporins like ceftriaxone and macrolides like azithromycin are presently being used.¹ However, multidrug resistance, emerging extensive and pan-drug resistance has been reported for various serovars of *Salmonella*.^{1,4,5} The resistance threat has triggered efforts in search of new efficacious therapies. New approaches in this search include exploration of probiotics, prebiotics, antimicrobial peptides, bacteriophages, small molecules from medicinal chemistry and other compound libraries, combination therapy and natural products such as essential oils and other secondary metabolites and extracts from medicinal plants.^{1,6}

With respect to medicinal plants, the antimicrobial properties of a vast number of plants have been reported with a good number of antibacterial herbal products derived from them and some undergoing development into herbal medicines.^{7,8} Of interest is *Annona muricata* (Annonaceae), a tree plant widely distributed in the world in both tropical and non-tropical areas. It is commonly known as sour sop (Cameroon), graviola (in Portuguese), guanabana (in Latin America). *Annona muricata* leaves are used to treat headaches, insomnia, cystitis, bacterial infections and cancer, diabetes, hypertension, respiratory illnesses, fever; the seeds are used to treat parasitic infections, and the fruit is used to treat diarrhoea and neuralgia, increase milk production in lactating women among several other uses.^{9,10} Pharmacological studies have demonstrated a wide range of activities in this plant including cytotoxic anticancer, antiprotozoal, antibacterial, antiviral and antioxidant activities in vitro and anticancer, anti-hypoglycaemic, hypotensive, wound healing activities in vivo among others.¹⁰

Decoctions of the leaves and bark are used to treat typhoid fever in western Cameroon.¹¹ The antibacterial activity of *A. muricata* has been reported in a number of studies. Low to moderate antibacterial activity was reported for the aqueous extract of the leaves against oral pathogens.¹² Moderate to high activity has been recorded for *A. muricata* against *Staphylococcus aureus* and *Escherichia coli*.¹³ However, studies conducted exclusively in leaf extracts were limited to disc diffusion method only, the findings of which may not be considered reliable since the disc method gives semi qualitative results and is affected by several factors.¹⁴ In particular, studies on multidrug-resistant (MDR) strains of *Salmonella* are very rare. Furthermore, in vivo studies of the antibacterial activity of *A. muricata* are very rare. A recent in vitro study, recorded high bacteriostatic activity for leaf extracts of *A. muricata* against MDR *Salmonella*.¹⁵ This study further explored

this activity of *A. muricata* leaves in vivo in a rat model of MDR *S Typhimurium* infection.

METHODS

Collection and preparation of plant extract

The leaves were harvested in Buea, Southwest Cameroon and a voucher specimen was authenticated by Mr. Peter Njimba, a botanist in the Limbe Botanic Garden in the South West Region of Cameroon and assigned the number SCA519. The leaves of *Annona muricata* were air dried to a constant weight under shade for three weeks, then ground to powder and weighed. The powder (230 g) was macerated by submerging completely in 5 l of methanol in a closed bottle and kept for 72 hrs with occasional shaking.

The macerate was filtered using a Whatman filter paper No. 1 and the filtrate concentrated using rotary evaporator (BUCHI Rotavapor R-200, Switzerland), under reduced pressure at 40 °C. The crude extract was recovered from the flask using a small volume of methanol and was left on the shelf at room temperature with weighing until constant mass indicating complete evaporation of residual solvent. The mass of the dried crude extract was recorded and then stored at -20 °C until use.

Antibiotic susceptibility testing of *Salmonella Typhimurium*

A clinical strain of *S. Typhimurium* was isolated at the Microbiology laboratory of University of Yaoundé Teaching Hospital, a tertiary hospital in Cameroon. The isolate was sub-cultured twice on *Salmonella shigella* (SS) agar to obtain pure colonies. Antibiotic susceptibility testing was carried out on the isolate using recommended anti-*Salmonella* antibiotics (ampicillin, chloramphenicol, sulfamethoxazole / trimethoprim and ciprofloxacin), to determine its resistance profile.¹⁶ A McFarland 0.5 suspension of the bacterium (100 µl) was uniformly spread on a freshly prepared Mueller Hinton agar plate and allowed for 15 minutes to dry. Discs of the selected antibiotics were embedded on the plate and then incubated at 37 °C for 24 hours. Then the plates were examined and the diameter of clear zones of inhibition were measured in millimetres.

Determination of minimum inhibitory concentration of extract

The minimum inhibitory concentration (MIC) of the extract was determined as previously described.¹⁷ Briefly, a 20 mg/ml of a stock solution of the extract was prepared in 10% dimethyl sulfoxide (DMSO) by adding 100 µl of DMSO to the weighed extract, this was vortexed to dissolve completely then 900 µl of Mueller Hinton broth was added and further vortexed. The stock solution was double diluted in Mueller Hinton broth to obtain eleven concentrations (0.125, 0.25, 0.5, 1, 2, 4, 8, 10, 12, 16 and to 20 mg/ml). Then 100 µl of each solution was added in

duplicate in a 96-wells microtitre plate in serial order. The bacterial suspension (100 µl of 1×10^6 CFUs/ml) was added to all the wells containing the extract giving final extract concentrations of 0.0625 to 10 mg/ml and a bacterial density of 1×10^5 CFUs/ml. Negative control (bacteria in broth) and positive control (bacterial suspension and 50 µg/ml of Gentamycin) wells were included. The optical density (OD) of the wells was read at 595 nm and then incubated at 37 °C for 24 hrs. After incubation, the optical density was read again. The percentage inhibition was calculated using the following formula:

$$\% \text{Inhibition} = [(\Delta \text{OD NC} - \Delta \text{OD E}) / \Delta \text{OD NC}] \times 100$$

Where NC=negative control, E=extract. The lowest concentration which produced at least 50% inhibition was considered as the MIC.

In vivo antibacterial assay

Ethical clearance for this work was obtained from the University of Buea Institutional Animal Care and Use Committee (UB-IACUC N^o 18/2024). Male and female Wistar rats (18 each) weighing between 70 and 200 g body weight were purchased from an animal house and acclimatized in the study laboratory for 10 days in aseptic conditions. The animals were maintained at room temperature with a 12-hour light-dark cycle and fed normally with a standard diet and water given *ad libitum*. All cages were cleaned before the start of the experiment and the faeces of all animals were checked for the presence of Salmonella by culturing on *Salmonella Shigella* (SS) agar (Liofilchen, Italy). Animals confirmed positive for Salmonella were excluded from the experiment.

The test was done as described, and in accordance with guidelines of the Organization for Economic Cooperation and Development version 423.¹⁸⁻²⁰ Briefly, 30 test animals (15 males and 15 females) were inoculated orally using a 22G gavage with the characterized MDR clinical strain of *S. Typhimurium* at a cell density of 3×10^8 CFUs/ml in a volume of 10 ml/kg. The faeces of the infected animals were cultured on SS agar two hours following infection to check for the presence of *S. Typhimurium* colonies.

This was done for three consecutive days and an increase in number of colonies over this period indicated establishment of infection. Clinical signs and symptoms such as rectal temperature, frequent defecation and texture of stool further confirmed infection. Infected animals were placed in 6 groups of 6 animals each (3 males and 3 females). The groups and their treatments were as follows: T1, T2 and T3 infected and given 50, 100 and 200 mg/kg body weight of extract respectively, NC (negative control, infected, untreated), PC (positive control, infected, treated with 8 mg/kg ciprofloxacin) and NG (normal group, uninfected, untreated). Daily treatments with the methanol extract of *A. muricata* lasted for 10 days, negative control and normal groups received distilled water throughout the treatment period. The faecal load of Salmonella for each

animal was determined as described.²¹ A stool sample (0.5 g) was dispersed in 1 ml of saline (0.9 % NaCl), centrifuged at 3000 rpm for 5 mins and aliquots (100 µl) serially diluted and plated on SS agar. The plates were then incubated at 37 °C for 24 hrs and colonies of *S. Typhimurium* counted as colony forming units (CFUs) per ml. Faecal bacterial load was determined on days 1, 5 and 10 of treatment and thereafter the animals were fasted over-night and then anaesthetized with ketamine/Xylazine (90/10 mg/kg).²² Blood was collected by cardiac puncture for biochemical analysis.

Biochemical analysis

Blood specimens were allowed to coagulate, centrifuged at 2000 rpm for 15 minutes to obtain serum. The serum obtained was used to quantify liver enzymes (alanine transaminase (ALT), aspartate transaminase (AST) and kidney function parameters (urea and creatinine) using diagnostic test kits (Chronolab, Switzerland), following manufacturer's instructions.

Statistical analyses

The zones of inhibition were interpreted based on reference values of the Clinical and Laboratory Standard Institute for antibiotic susceptibility.²³ The mean ($\pm$ standard deviation) of all data were calculated. Mean faecal loads were analysed using one-way ANOVA. Means of biochemical parameters were compared using un-paired *t* test. A *p* value <0.5 was considered statistically significant.

RESULTS

Antibiotic susceptibility and activity of extract

The antibiotic susceptibility profile of *S. Typhimurium* showed diameter of zones of inhibition ranging from 6-12 mm. Based on reference values of zone diameters of the clinical and laboratory standards institute, the isolate showed resistance to antibiotics (ampicillin, chloramphenicol, ciprofloxacin and Ceftriaxone), belonging to four distinct classes and intermediate sensitivity to a folate pathway inhibitor (sulfamethoxazole-trimethoprim). This shows that the *S. Typhimurium* strain was multidrug-resistant (Table 1).²⁴

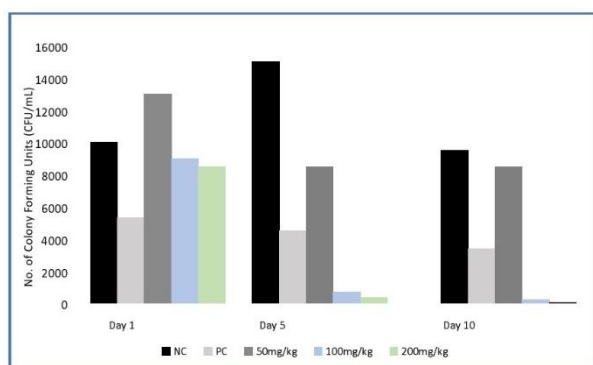
The micro-dilution assay for the methanol crude extract of *A. muricata* leaves gave a MIC value of 2 mg/ml with a bacterial percentage inhibition of 63.8%. This MIC of 2 mg/ml was considered as high antibacterial activity.²⁵

In vivo antibacterial activity of extract

S. Typhimurium colonies were seen in cultured faeces of the infected rats following inoculation. The number of colonies increased in the first three days and clinical features or signs and symptoms such as soft and semi-formed faeces, frequent defecation and high rectal

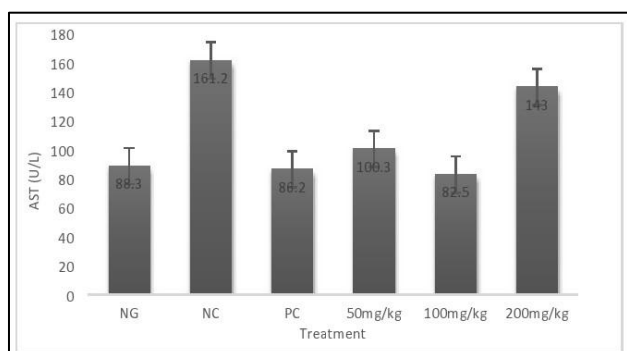
temperatures were observed. These features persisted in the negative control animals up to day 5 then gradually subsided while in treated animals they started subsiding on day 2 of treatment. The extract produced a dose-dependent decrease in the number of colonies recovered.

On the last day of treatment (day 10), there was a 100% inhibition of the *Salmonella* cells with no colonies in the faeces of animals treated with the 200 mg/kg body weight of extract. The mean faecal load of animals treated with the extract was significantly lower than those of the negative control group ($p < 0.001$). The positive control animals had significantly lower colonies compared to the 50 mg/kg groups ($p < 0.001$) while there was no significant difference with the 200 ($p = 0.099$) and 100 mg/kg ($p = 0.226$) groups, (Figure 1). There was also no significant difference in faecal load between animals treated with 200 and 100 mg/kg body weight of crude extract ($p = 0.991$). No mortality was recorded during the study.



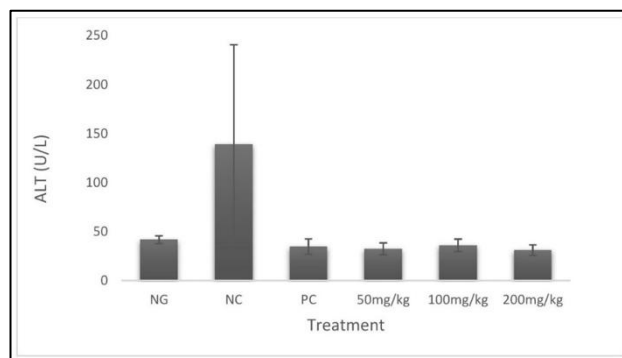
NC=negative control, PC=positive control. Significant difference between treated animals compared to NC, p value < 0.001

Figure 1: Faecal *S. typhimurium* colonies of Wistar rats treated with methanol extract of *Annona muricata* leaves.



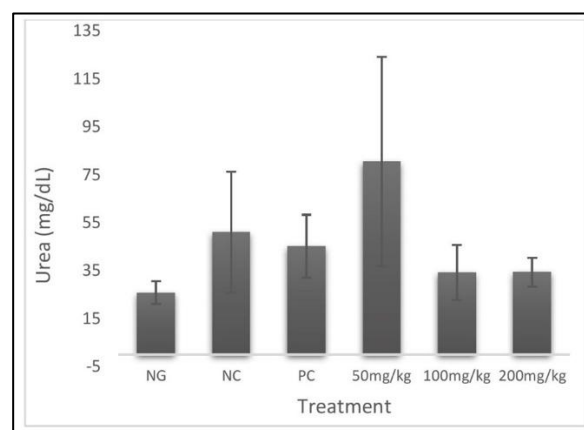
$p = 0.023$ and 0.011 for animals treated with 50 and 100 mg/kg respectively compared to negative control (NC)

Figure 2: Effect of methanol extract of *Annona muricata* leaves on Aspartate aminotransferase (AST), activity in rats infected with multidrug resistant *S. Typhimurium*.



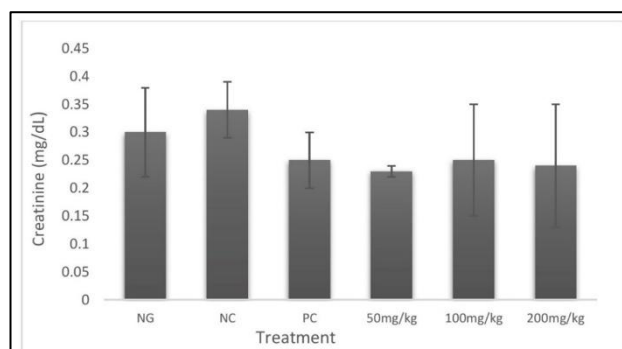
$p > 0.05$ for all treated animal groups compared to negative control (NC)

Figure 3: Effect of methanol extract of *Annona muricata* leaves on Alanine aminotransferase (ALT) activity in rats infected with multidrug resistant *S. Typhimurium*.



$p > 0.05$ for all treated animal groups compared to negative control (NC).

Figure 4: Effect of methanol extract of *Annona muricata* leaves on serum urea levels in rats infected with multidrug resistant *S. Typhimurium*.



$p > 0.05$ for all treated animal groups compared to negative control (NC)

Figure 5: Effect of methanol extract of *Annona muricata* leaves on serum creatinine levels in rats infected with multidrug resistant *S. Typhimurium*.

Effect of extract on biochemical parameters

Aspartate aminotransferase

There was no dose-dependent trend in AST activity among animals treated with the crude extract. Generally, the AST values of all treatment groups were lower (83.3 ± 2.39 to 143.0 ± 19.76 U/l) than those of the negative control animals, with values for 50 and 100 mg/kg treatment groups significantly lower ($p=0.023$ and 0.011 respectively) (Figure 2).

Alanine aminotransferase

The ALT of negative control animals was quite high compared to all other experimental groups. However, it was not significant compared to the treated groups ($p \leq 0.080$ to 0.088) (Figure 3). Furthermore, it was

observed that mean ALT had a high standard deviation (139.2 ± 101.3).

Urea

Urea analysis showed no dose related trend among treatment groups. Infection with *S. Typhimurium* resulted in an increase in urea concentration as seen in the negative control compared to the normal group.

Treatment with the extract decreased the urea concentration except for the 50 mg/kg body weight group which had a much higher level but was not significantly higher than the negative control ($p=0.370$) (Figure 4). Higher doses of extract (100 and 200 mg/kg body weight) significantly decreased urea concentration compared to animals treated with the lowest dose (50 mg/kg body weight) of the extract ($p=0.001$).

Table 1: Antibiotic susceptibility of clinical *Salmonella* Typhimurium strain shows multidrug resistance.

Antibiotic class	Antibiotic	Zone of inhibition (Cut-off value) (mm)	Interpretation
Penicillin	Ampicillin	6 (≤ 13)	Resistant
Folate pathway inhibitors	Sulfamethoxazole-Trimethoprim	11 (11-15)	Intermediate sensitivity
Phenicol	Chloramphenicol	10 (≤ 12)	Resistant
Fluoroquinolones	Ciprofloxacin	12 (≤ 15)	Resistant
Cephems	Ceftriaxone	9 (≤ 19)	Resistant

Creatinine

The creatinine level of treated groups was generally lower than those of the negative control (Figure 5). No dose related changes in mean creatinine levels were seen in rats treated with the crude extract. There was no significant difference in mean creatinine levels between the negative control and all treatment groups ($p=0.088$ to 0.181).

DISCUSSION

This study was motivated by the high activity recorded against MDR *Salmonella* strains and low risk of toxicity for the crude extract of the leaves of *A. muricata*, in view of finding an alternative treatment to resistant *Salmonella* infections.¹⁵ This in vivo study of the methanol extract of the leaves of *A. muricata* demonstrated significant activity against a MDR *S. Typhimurium* with no mortality or adverse toxicity in the experimental animals at relatively low doses of 50 to 200 mg/kg body weight. To the best of our knowledge, this is the first report of high activity of *A. muricata* against MDR *Salmonella* in vivo. This finding indicates that of *A. muricata* leaves are a potential alternative treatment for resistant *Salmonella* infections.

The antibiotic susceptibility profile of the clinical isolate showed that the isolate was resistant to four classes of antibiotics tested which confirms that the strain was multidrug -resistant (Table 1). A MIC of 2 mg/ml was

recorded for this strain in the microdilution assay which is within the range (0.0625 to 8 mg/ml), recorded for the hexane and methanol extracts of *A. muricata* leaves against 16 strains of MDR *Salmonella* in the cited in vitro work.¹⁵ This MIC (2 mg/ml) is high activity according to published criteria for extract activity and therefore confirms the activity of *A. muricata* leaves against MDR *Salmonella*.²⁵ The MIC was determined in order to serve as a guide in choosing the doses to use in the in vivo study. The MIC value of 2 mg/ml is equivalent to 2000 mg/kg but the doses used in the in vivo study were much lower because the higher dose of 2000 mg/kg resulted in mortality of mice in the acute toxicity test in a previous work.¹⁵ Hence, the in vivo doses (50 to 200mg/kg), were then chosen according to the OECD guidelines whereby the maximum should not exceed 300 mg/kg if toxicity occurs at 2000 mg/kg.²⁰

The extract produced a dose-dependent decrease in the number of *S. Typhimurium* colonies in the faeces of the treated rats with a significant difference compared to the untreated negative control ($p < 0.05$). Optimum efficacy was recorded at 200 mg/kg which produced 100% clearance while only 30% inhibition was achieved in animals treated with 50 mg/kg body weight of crude extract on day 10. In the negative control animals, the colony counts increased by day 5 and decreased on day 10, this decrease is likely due to immune clearance. This implies in the treated animals; bacterial inhibition was as a result of both the crude extract and the effect of the immune system of the

rats.¹⁹ In vivo studies on the methanol extract of leaves of *A. muricata* against MDR *S. Typhimurium* in rats are rare. A study of an aqueous extract of the leaves in broiler chickens reported a dose-related elimination and significant reduction of *Salmonella* in the ileum and caecum respectively; a similar effect was recorded for coliform, *Escherichia coli* and *Lactobacillus*.²⁶ However, in vivo studies of other plants in a mammal have reported activity against MDR *Salmonella*. For instance, an in vivo study reported significant activity against MDR *S. Typhimurium* for the methanol extract of the stem bark of *Voacanga Africana* in Wistar rats.¹⁹

In the pathogenesis of *Salmonella*, the pathogen passes from the lymphatic system into the blood stream to other parts of the body such as the liver, spleen, kidney which may result in organ damage and dysfunction.¹⁸ These changes are reflected in increased liver enzyme (AST and ALT) activity in *S. Typhimurium* infected rats (Figures 2 and 3). This effect was reversed in treated rats with significantly lower levels of the enzymes ($p < 0.05$). In the case of ALT there was no significant difference between other treatment groups compared to the negative control; this is likely due to the high variability in the ALT values of the individual animals as seen from the high standard deviation of the negative control (139.2 ± 101.3) Figure 3.

Abnormally high urea and creatinine indicate renal damage. The concentrations of these metabolites in the negative control were significantly higher ($p < 0.05$), than in all other groups of animals, except the animals treated with 50 mg/kg of crude extract which had a significantly high urea (Figure 4). A close look at the raw data shows that one rat in this group had an unexpected high urea which was at least 60% higher than the values of all the other rats used in the study also resulting in a high standard deviation. This outlier could have been induced by trauma during the study.

CONCLUSION

This is the first report of in vivo activity of *A. muricata* leaves extract against multidrug-resistant *Salmonella*. The high activity coupled with lack of adverse toxicity supports the use of the leaves in traditional medicine and indicate that the leaves of *A. muricata* is a potential treatment for resistant *Salmonella* infections. Further in vivo studies should be conducted using different organic extracts and fractions other than methanol; and equally the secondary metabolites should be isolated and tested.

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Conflict of interest: None declared

Ethical approval: The study was approved by the Institutional Ethics Committee

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