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In-vitro and *in-vivo* Activities of the Ethanol Pulp Extract of *Annona muricata* (Linn) Fruit in Albino Rats Infected with *Salmonella typhi*

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Authors' contributions

This work was carried out in collaboration between both authors. Author EOD designed the protocol for the study and revised the manuscript. Author OSF carried out the experiments, analyze the data and managed the literature search. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: To investigate the *in-vitro* and *in-vivo* activities of the crude ethanolic extract of unripe *Anonna muricata* fruits in albino rats infected with *Salmonella typhi.*
Methodology: Matured unripe fruits of *A. muricata* were collected, dried, powdered and extracted using 70% ethanol. The extract was constituted with 30% dimethylsulphoxide (DMSO) to make varying concentrations. For the *in vitro* examination, clinical and typed isolates of *S. typhi* were obtained from Don Bosco Catholic Hospital, Akure and Department of Pharmacognosy, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria. After which the ethanolic crude extract was assayed against the isolates using agar well diffusion method while the comparative study with standard antibiotics was done by disc diffusion method. Meanwhile, eighteen healthy albino rats of same age between 170-220g in weight were selected and divided into six groups containing three each. The infectivity dose (ID) was determined with the clinical *S. typhi*. After which the rats were infected and orally administered a standard dose of the *A. muricata* fruit extract (400 mg/kg) accordingly for 7 days. During the treatment period, the fecal samples were collected to monitor the

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ability of the extract to reduce the fecal shedding of *S. typhi.* Also, the rats were weighed daily to establish the effect of treatment on their metabolism.
Results: Ethanol extract of pulp of *A. muricata,* at concentrations of 50 mg/ml – 200 mg/ml, could not produce zone of inhibition (ZI) on cultures of clinical and typed *S. typhi* isolates but at concentrations of 250 mg/ml and 500 mg/ml it produced ZI of 4.93 – 11.37 mm($P< 0.05$) on the clinical isolate. Minimum inhibitory concentration of the extract on typed *S. typhi* and on the clinical isolate was 150 mg/ml and 250 mg/ml, respectively. The MBC for the two *S. typhi* isolates were 350 mg/ml and 400 mg/ml respectively. *S. typhi* colony forming units per ml (cfu/ml) of suspensions of faeces of infected rats, treated with the ethanol extract of *A. muricata pulp* decreased significantly ($P<0.05$) as days of the treatment increased while the cfu/ml of the control group increased($P<0.05$). There was no significant ($P> 0.05$) difference between weights of *S. typhi*-infected rats treated with any of the regimes but weight of the untreated group (control) significantly ($P<0.05$) decreased.
Conclusion: Ethanol extract of pulp of unripe *A. muricata* appears to have inhibitory effects against *Salmonella typhi*.

Keywords: In-vitro; In-vivo; Annona muricata; ethanol; unripe fruit.

1. INTRODUCTION

In addition to the anti-infectious properties of antibiotics, they also possess the ability to modulate the immune system in order to improve the long term outcome of patients with chronic diseases and/or disorders [1]. However, their side effects and prohibitive cost of these conventional drugs necessitated the search for an alternative. A number of trees and shrubs have been claimed to possess medicinal benefits especially in the treatment of infectious and non-infectious human and animal ailments [1]. Some of these plants are believed to also enhance positive health and maintain organic resistance against infection by ensuring body equilibrium and conditioning the body tissues [2,3].

Annona muricata tree is a slender, low-branching but bushy plant and it is about 25 or 30 feet (7.5-9 m) in height. The fruit is covered with a leather-like, reticulated but tender, inedible and bitter skin from which protrude few or many stubby spines. Similarly, the skin appears dark-green in the immature fruit and it becomes slightly yellowish-green before the mature fruit is soft to the touch [2,4]. The plant has been reported to have a rich history of use in alternative medicine. Generally, the fruit and its juice are used for worms and parasites, to reduce fevers, to increase mother's milk production after childbirth and as an astringent in the treatment of diarrhea and dysentery. The crushed seeds of *A. muricata* are used in the treatment of head lice, worms, internal and external parasites. The plant leaf extract contains a wide spectrum of activity against a group of bacteria that are responsible for the most common bacterial diseases [5,6].

Salmonella typhi is a gram-negative, flagellated, non-encapsulated, non-sporulating and facultative anaerobic bacillus. It causes typhoid fever where the bacteria move from the small intestine into blood via the lymphatic system. Typhoid fever perforation incidence has been reported to be high in West Africa in contrast to other countries and it is endemic only in areas where the standard of sanitation is low. Both sexes and all ages are susceptible through contamination of water. The cause of major outbreaks can occur through cross-connection of a man with polluted water supply, faecal contamination of wells or poultry purification [7]. Every year, the disease affects more than 21 million persons globally and 216,510 deaths [8]. Evidence suggests that the fluoroquinolones are the optimal choice for combating typhoid fever in adults and that they may also be used in children. However, the recent emergence of resistance to fluoroquinolones, due to indiscriminate and widespread use in primary health care settings is threatening. In addition to resistance problem, the safety of antibiotics remains an enormous global issue. It was estimated that 2.22 million hospitalized patients had serious Adverse Drug Reactions (ADR) and 106,000 died in a single year in the USA. Hence, the need for alternatives therapies for the treatment of *Salmonella* infections [9].

One of the criteria used for better understanding of the plant derived medicine involves the proof to show that the formulated medicine does what it is claimed to do [3]. Previous studies on the *in-vitro* antibacterial activities of the aqueous, methanolic and ethanolic extract of various parts of *A. muricata* especially the leaves has been

reported against *S. aureus, V. cholerae, E. coli*, *Salmonella typhi* and *Salmonella enteridis* [10,11]. However, little attention has been paid to the *in-vitro* and *in-vivo* antibacterial study of the pulp of unripe *A. muricata* fruit. Hence, the present study was aimed at investigating the *in-vitro* and *in-vivo* anti-typhoid activities of ethanolic pulp extract of unripe *A. muricata* fruit against *Salmonella typhi*.

2. MATERIALS AND METHODS

2.1 Plant Collection and Identification

Fresh unripe fruits of *Annona muricata* (Linn) were collected from a garden in Arimoro area of Ilesha, Osun State, Nigeria. The fruits were identified and authenticated by using the herbarium specimens of the Department of Crop, Soil and Pest management, Federal University of Technology, Akure (FUTA).

2.2 Source and Preservation of Bacteria

Pure clinical and typed isolates of *Salmonella typhi* were obtained from the stock culture of Don Bosco Catholic Hospital, Akure, Ondo State and Department of Pharmacognsy, Obafemi Awolowo University, Ile-Ife, Osun State respectively. The clinical *S. typhi* was isolated from a typhoid patient.

2.3 Extraction of the Bioactive Constituents of Unripe *A. muricata* Fruit

The methods of [12] and [13] were employed. Unripe *A. muricata* fruits were washed with sterile water. The peels and seeds were separated from the pulp and then cut into smaller pieces. The fruits were oven dried at 50℃ for 4 days. The dried fruits were then pulverized into fine powder by blending in a high-speed Philips Model blender. They were separately kept in an airtight container to avoid the absorption of moisture. Three hundred grams (300 g) of the powdered sample was soaked in two thousand, five hundred millimeters (2000 ml) of 70% ethanol as solvents to extract the bioactive compounds. The container was labeled appropriately and left for 72 hours (3 days). After which it was sieved using muslin cloth and then filtered using 0.45 µm micropore filter. The filtrates were vaporized to dryness using rotary evaporator and subsequently lyophilized to remove the extracting solvent. Then, the ethanol extract was reconstituted with 30% dimethlysulphoxide (DMSO) to obtain varying concentrations [14].

2.4 Preparation of Standard Inoculums of *Salmonella typhi* for *In vitro* Assay

Three isolated overnight colonies were transferred to a tube of sterile saline. The bacterial suspension was compared to the 0.5 McFarland standards against a sheet of white paper on which sharp black lines were drawn. The bacterial suspension was adjusted to be the proper density as the 0.5 McFarland by adding sterile saline or more bacterial growth. Then bacterial suspension was diluted to obtain 10^6 cfu/ml [11,15].

2.5 Antibacterial Sensitivity Testing of the *A. muricata* Extract

Using a sterile pipette, 0.1 ml of the bacterial suspension was drawn out and was aseptically introduced into sterile Petri dishes. Sterilized Mueller Hinton Agar (MHA, Difco, USA) that had been cooled to about 45^{O}C was aseptically poured into the Petri dishes containing 0.1 ml of *S. typhi*. Each Petri dish was gently swirled in a clockwise direction in order to ensure that the bacterium is homogeneously distributed with MHA. The plates were allowed to stand for 40 minutes for the inoculated bacteria to be established in the medium. After which three wells were aseptically bored on each agar plate using a sterile cork borer (7 mm) at allowance of 30 mm between opposite wells and the edges of the petri dishes. A 0.1 ml of the different concentrations of the extracts was then introduced into each well in the plates using sterile pipette. A control well was in the centre with 0.1 ml of the reconstituting reagent (30% DMSO). The plates were incubated at 37℃ for 24 hours. The resulting zones of inhibition were measured using a caliper. The experiment was done in triplicates and the average reading was taken to be the zone of inhibition of the bacterial isolate at different concentrations of the extract [16].

2.6 Antibiotic Assay

Standard antibiotics such as gentamycin -30 µg, chloramphenicol – 30 µg, ciprofloxacin – 10 µg, tetracycline – 30 µg, amoxicillin – 30 µg and nalixidic acid – 30 µg were compared against the clinical and typed *S. typhi* isolates. With the aid

of a sterile pipette, 0.1 ml of the bacterial suspension was drawn out and was aseptically introduced into sterile Petri dishes. Sterilized Mueller Hinton Agar (MHA) that had been cooled to about 45℃ was aseptically poured into the Petri dishes containing 0.1 ml of *S. typhi*. Each Petri dish was gently swirled in a clockwise direction in order to ensure that the bacterium was evenly distributed. The plates were allowed to stand for 40 minutes for the inoculated bacteria to be established in the medium. The standard discs were gently laid aseptically on the plates using a sterile pair of forceps. The plates were incubated at 37℃ for 24 hours. After the incubation period, petri dishes were observed for zones of inhibition around the disc to which the bacteria is sensitive. The diameter of the clear zones was measured in millimetres (mm) using a caliper [15,16].

2.7 Determination of Minimum Inhibitory Concentration (MIC) and Determination of Minimum Bactericidal Concentration (MBC)

About 5 ml of freshly prepared Mueller Hinton Broth (MHB) was drawn out with sterile pipette into test tubes, then 0.1 ml of inoculum of *Salmonella typhi* (1 x 10^6 cell/ml) was inoculated into each test tube and mixed thoroughly. With the aid of sterile pipette, one millimeter of the different concentrations of plant extracts was withdrawn into each test tube containing the broth culture of *S. typhi*. The tests were then incubated at 37℃ for 24 hrs. Growth in each tube was checked for by turbidity measurement using a spectrophotometer (Beckman model 35). Growth inhibition was indicated by low turbidity while growth was indicated by high turbidity. The concentration at which there was no growth as indicated by clear broth is taken as the minimum inhibitory concentration while the MBC was determined by taking a loopful from each tube that showed no growth during MIC determination and streaked onto extract free agar plates, incubated at 37℃ for 24 hours. The least concentration at which no growth was observed was noted as the MBC [15].

2.8 Experimental Animals

Forty-two healthy Swiss albino rats (170-220 g) between 5-7 weeks old were obtained from Department of Animal Production and Health, Federal University of Technology, Akure, Ondo State, Nigeria. The rats were fed with standard rat pellets (Livestock Feeds, Ikeja, Lagos State) and water *ad libitum*. The animals were housed under standard laboratory conditions and were acclimatized for 7 days before the treatment started. The experimental procedures involving animals were conducted in conformity with international, national and institutional guidelines as described by [16].

2.8.1 Preparation of Standard Inoculum of *S. typhi* for *In vivo* Assay

A 0.238 g of sodium hydrogen phosphate was dissolved in 0.019 g of potassium dihydrogen phosphate and sodium chloride respectively. The mixture was made up to 100 mL with distilled water and pH was adjusted to neutral pH (7). Then standard inoculum of *S. typhi* was inoculated into 1000 mL of nutrient broth and incubated for 24 hrs. After incubation, the cells were centrifuged at 2000 rpm for 10 minutes and the supernatant was discarded. Pellets were re-suspended in PBS and centrifuged again for four times. The final cell button was resuspended in PBS and serially diluted 10^1 to 10^6 [14].

2.8.2 Determination of Infectious Dose (ID)

The method described by [18] was adopted. Twenty-four healthy Swiss albino rats were used to determine the ID of *S. typhi*. The rats were divided into six groups of four rats. Each group was infected with different concentrations of *S. typhi* suspension. The groups were closely monitored for seven days. The concentration of *S. typhi* suspension that produces the signs like unformed stool, weak, scattered fur, falling of hairs, stool with mucous and weight loss in animals given is taken as the infectivity dose (ID) of *S. typhi*. Also, corresponding colony-forming units per millilitres (cfu/mL) of the bacterial dilutions was determined using plate count method on *Salmonella-Shigella* agar (SSA).

2.8.3 *In-vivo* bioassay

A total number of eighteen rats were used and divided into six groups; Group I was infected with infective dose of *S. typhi* but was not treated with the extract while Group II was infected with the infective dose and then treated with 400 mg/ml ethanolic extract of unripe *A. muricata* fruit after the infection had set in. Group III was given prophylactic dose 400 mg/ml extract of *A. muricata* for three days before being infected with *S. typhi.* Then Group IV was administered only extract of *A. muricata*; Group V was infected

and treated with ciprofloxacin; while Group VI was fed with basal meal and water only. All the experimental rats in groups I, II, IV and V were observed for signs of infection before being treated [18,19].

2.8.4 Effect of the extract on faecal shedding of *S. typhi* in Swiss albino rats

Faecal samples were collected before and during infection for monitoring. One gram (1 g) of the sample was serially diluted in physiological saline, plated in triplicates on *Salmonella Shigella* Agar (Difco, USA), and then incubated at 37℃ for 24 hours. Typical colonies of *S. typhi* (clear colonies with pinpoint black centres) were then counted on plates that contained between 30 and 300 colonies [20].

2.8.5 Quantity of stool passed out by swiss albino mice

The stool passed out by the mice in each group was collected and measured before and during the infection using the sensitive digital weighing balance.

2.8.6 Determination of rat's body weight

The method of [18] was employed. Each group of rats was fed two times a day with standard rat chow (Livestock Feeds, Ikeja) and given 20 ml of water ad libitum two times daily. The body weight of each rat was weighed using a sensitive weighing balance before and during the period of infection and treatment to determine the effect of treatment on body weight. They were also observed daily for general signs of toxicity and mortality.

2.8.7 Statistical analysis

Mean values of replicates were reported with their standard deviations using SPSS 16.0. Analyses of Variance (ANOVA) were achieved to calculate significant differences in the treatment means, and the mean separations were achieved by Duncan's Multiple Range Test ($p \leq 0.05$).

3. RESULTS AND DISCUSSION

3.1 Results

The sensitivity pattern of clinical and typed isolates of *S. typhi* to ethanolic extract of unripe *A. muricata* fruits showed that the degree of susceptibility as indicated by zones of inhibition varied from one concentration to the other. In the case of clinical isolate, no inhibition was observed at concentrations of 50-200 mg/ml but at higher concentrations of 250-500 mg/ml used, a significant increase in zones of inhibition which ranged from 4.93-11.37 mm were observed. Also, there was no inhibition up to concentration of 100 mg/ml for typed isolates. However, zones of inhibition which ranged from 3.03-14.03 mm were observed at concentrations of 150-500 mg/ml (Table 1). The MIC values for typed and clinical *S. typhi* were 150 and 250 mg/ml respectively while the MBCs were 350 and 400 mg/ml respectively.

Table 1. Inhibition zone diameters (mm) of the ethanol extract of pulp of *Annona muricata* against clinical and typed *Salmonella typhi*

Extract (mg/ml)	*S. typhi* clinical	*S. typhi* Typed
50	0.00^{a}±0.00	0.00^{a}±0.00
100	0.00^{a}±0.00	0.00^{a}±0.00
150	0.00^{a}±0.00	3.03^{b}±0.09
200	0.00^{a}±0.00	5.13^{cd}±0.03
250	4.93^{b}±0.07	4.93^{c}±0.09
300	5.53^{c}±0.09	7.20^{e}±0.15
350	7.03^{d}±0.15	8.10^{f}±0.10
400	10.03^{e}±0.03	11.97^{g}±0.88
450	10.93^{e}±0.07	12.37^{g}±0.88
500	11.37^{f}±0.09	14.03^{h}±0.03

Data are presented as Mean±S.E (n=3). Values with the same superscript letter(s) along the same column are not significantly different (P<0.05)

The result of the sensitivity of clinical and typed *S. typhi* to conventional antibiotics revealed that of all the antibiotics used in this study, ciprofloxacin (10 µg) had the highest antimicrobial activity against the clinical (11.00 mm) and typed (16.00 mm) isolates. This was followed by Gentamycin (10.33-12.67 mm), Tetracycline (8.67-12.33 mm) and Nalixidic acid (4.67-12.00 mm). Clinical *S. typhi* was resistant to chloramphenicol (30 µg) while the typed was susceptible (3.50 mm). However, both isolates of *S. typhi* were resistant to amoxicillin (30 µg) (Table 2).

The dose of *S. typhi* that produced infection signs like unformed stool, weakness, scattered fur, falling hairs, stool with mucous and weight loss in the experimental rats (Infectious Dose - ID) was 6.8×10^{6} cfu/ml. The result of faecal shedding of *S. typhi* before and during treatment revealed that all groups did not shed *S. typhi* in their faeces on day 0 (before treatment).

Similarly, *S. typhi* was not isolated in stool samples of groups IV and VI examined throughout the treatment period. However, group I (infected rats) showed increasing *Salmonella* shed as against decreasing shed in groups treated with extract and conventional antibiotics (group II and V respectively) while the treated-infected group started shedding *S. typhi* on day 4 (Table 3).

The result of the effect of treatment on the quantity of stool passed out by experimental rats showed a decrease was observed in the quantity of stool passed in group I till the fourth day. However, from the 4th day, a significant increase was observed. Also, quantity of stool passed by group treated with extract (II) decreased. Stool passed in group III decreased till 2nd day and increased accordingly from day 3. However, no significant change was observed in group 6 (control) (Fig. 1).

In this study, the weights (g) of rats in their respective treatment groups were monitored. There was no significant change in weight of rats in all the groups before and after treatment except group I (only infected) where there was significant decrease in weight till the fifth day. However, significant increase was observed on the sixth and seventh day (Table 4).

Table 2. Antibiotic sensitivity pattern of the *Salmonella typhi* to the ethanol extract of *Annona muricata* fruit

Standard antibiotics	Clinical *S. typhi* (mm)	Typed *S. typhi* (mm)
GEN (30 µg)	10.33^{d}±0.88	12.67^{c}±0.88
NAL (30 µg)	4.67^{b}±0.33	12.00^{c}±2.52
CHL (30 µg)	0.00^{a}±0.00	3.50^{b}±0.50
AMO (30 µg)	0.00^{a}±0.00	0.00^{a}±0.00
TET (30 µg)	8.67^{c}±0.88	12.33^{c}±1.20
CIP (10 µg)	11.00^{e}±0.58	16.00^{d}±1.00

Data are presented as Mean±S.E (n=3). Values with the same superscript letter(s) along the same column are not significantly different (P<0.05).
Key: GEN – Gentamycin, NAL – Nalixidic acid, CHL – Chloramphenicol, AMO – Amoxicillin, TET – Tetracycline, CIP – Ciprofloxacin

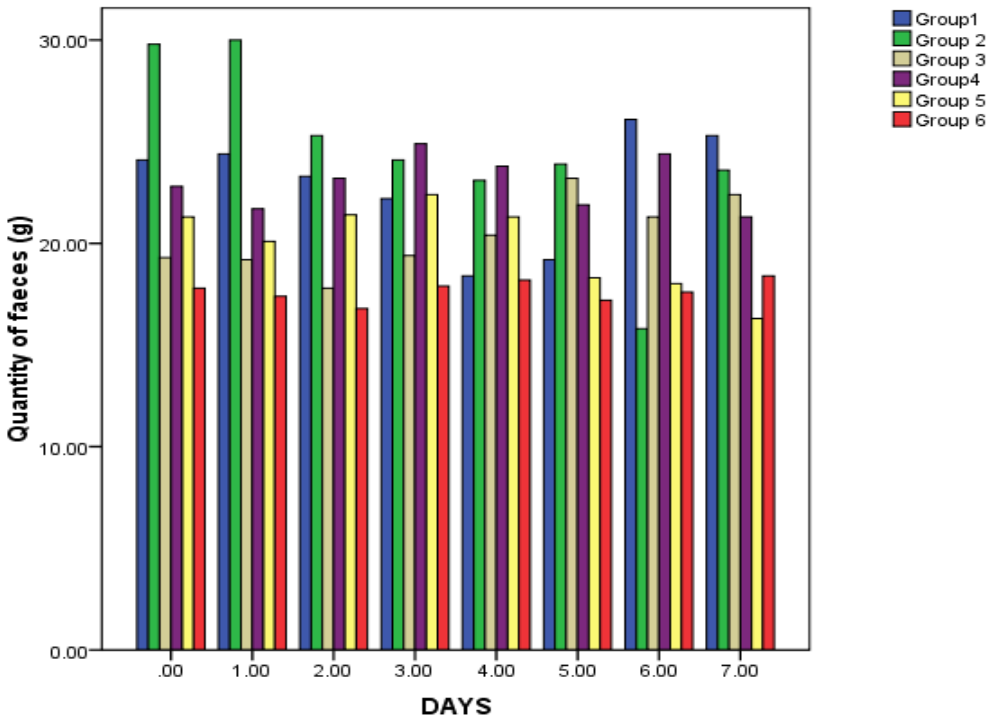

Fig. 1. Effect of treatment on the quantity of stool passed out by experimental rats infected with *Salmonella typhi*
00 day – Before treatment, 1-7 days = Treatment

Table 3. Effect of treatment on faecal shedding of *Salmonella typhi* (cfu/ml) using pour plating method

Days	*Salmonella typhi* count (x10^3) (cfu/ml)					
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
0	0.00^a±0.00	0.00^a±0.00	0.00^a±0.00	0.00^a±0.00	0.00^a±0.00	0.00^a±0.00
1	59.67^b±0.88	65.67^h±1.20	0.00^a±0.00	0.00^a±0.00	58.00^g±1.15	0.00^a±0.00
2	64.67^b±1.20	62.00^g±1.15	0.00^a±0.00	0.00^a±0.00	44.00^f±3.05	0.00^a±0.00
3	76.67^c±0.88	39.33^f±1.76	0.00^a±0.00	0.00^a±0.00	25.33^e±0.33	0.00^a±0.00
4	100.67^d±1.76	36.67^e±1.33	46.00^b±1.15	0.00^a±0.00	19.33^d±0.67	0.00^a±0.00
5	86.67^e±1.76	23.67^d±0.88	52.00^c±1.15	0.00^a±0.00	12.00^c±1.54	0.00^a±0.00
6	108.67^e±1.76	18.00^c±1.15	85.00^d±1.15	0.00^a±0.00	5.67^b±1.20	0.00^a±0.00
7	160.00^e±3.464	4.67^b±0.88	96.00^e±1.15	0.00^a±0.00	2.00^{ab}±0.00	0.00^a±0.00

Data are presented as Mean±S.E (n=3). Values with the same superscript letter(s) along the same column are not significantly different (P<0.05). Group 1 = Infected & untreated, Group 2 = Infected & treated with extract, Group 3= Given extract before infection, Group 4 = Given extract only, Group 5= Infected & treated with Ciprofloxacin, Group 6 = Basal diet and water (Control), 0 day – Before treatment, 1-7 days = Treatment.

Table 4. Effect of treatment on weight of experimental rats (g) before and after infection with *Salmonella typhi*

Days	Weight of rats (g)					
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
0	183.37^b±5.57	221.60^a±4.00	167.63^a±3.73	181.30^a±8.79	190.00^a±11.12	212.20^a±11.77
1	181.67^{ab}±5.12	220.03^a±3.37	167.50^a±3.76	181.67^a±8.71	189.67^a±10.77	212.60^a±11.69
2	179.97^{ab}±4.12	217.50^a±2.44	168.10^a±3.45	181.83^a±8.67	189.17^a±10.82	212.67^a±11.73
3	176.27^{ab}±5.78	221.94^a±5.40	165.18^a±3.18	179.53^a±9.54	187.07^a±11.24	212.41^a±12.04
4	166.55^{ab}±4.67	218.96^a±4.04	159.75^a±3.67	181.29^a±9.43	182.99^a±11.51	196.31^a±24.96
5	159.97^a±13.73	217.75^a±3.76	160.58^a±2.69	181.93^a±8.84	183.20^a±11.41	198.23^a±21.85
6	182.20^{ab}±1.96	216.27^a±3.71	160.70^a±2.57	182.30^a±8.82	184.37^a±10.89	199.03^a±21.73
7	175.73^{ab}±5.73	211.30^a±5.13	170.20^a±0.58	183.77^a±9.78	183.93^a±10.59	185.85^a±15.91

Data are presented as Mean±S.E (n=3). Values with the same superscript letter(s) along the same column are not significantly different (P<0.05). Group 1 = Infected & untreated, Group 2 = Infected & treated with extract, Group 3= Given extract before infection, Group 4 = Given extract only, Group 5= Infected & treated with Ciprofloxacin, Group 6 = Basal diet and water (Control), 0 day – Before treatment, 1-7 days = Treatment

3.2 DISCUSSION

The result of the *in-vitro* susceptibility test (Table 1) revealed that the extract of the unripe *A. muricata* fruits possesses antimicrobial activity against the clinical and typed *S. typhi* used in this study though at higher concentration. This agreed with the findings of [21] who reported that the ethanolic fruit extract of *Tamarindus indica* inhibited *Staphylococcus aureus* at a concentration of 250 mg/ml and above. Similarly, it was reported that the aqueous leaf extract of *A. muricata* had inhibitory effects on the growth of *E. coli, S. aureus, B. subtilis* and *S. typhi* at concentration of 200 mg/ml. Also, [22] reported that *Eugenia caryophyllata* was found to effectively inhibit the growth of *S. typhi*. However, this result differed from the works of [23] who reported that leaf extract of *Aloe vera* had no inhibition on various gram positive (*S. aureus*, *S. epidermidis*, *Streptococcus pyogenes*) and gram negative (*P. aeruginosa*) isolates used in their study.

In this study, there were variations in the inhibitory activities demonstrated by the extract of *A. muricata* against the clinical and typed isolates. The lack of inhibition observed at concentrations of 50-200 mg/ml for clinical *S. typhi* and 50-100 mg/ml for typed *S. typhi* could be due to the absence of alkaloid in the extract as reported by [24]. This is corroborated with the findings of [25] who reported alkaloids to be the most efficient therapeutically significant plant substance. However, the inhibition observed at higher concentrations might be accounted for by the presence of other bioactive components such as tannin, saponin and flavonoid in the ethanolic extract [24]. This agreed with study carried out by [26] who attributed the antimicrobial action of leaf and stem extracts of *Kigelia africana* and *Strophanthus hispidus* to the astringent nature of

the phenolic constituents including tannins present in the extract. Also, [27] established that the miswak plant had high antibacterial activity against *S. aureus* and *E. coli* because of the presence of flavonides and saponins in high state. In addition, the non-inhibition observed at such dose levels could also be that the ethanol used as solvent in this study did not sufficiently extract the bioactive components capable of causing effective inhibition. It is well known that different solvents have diverse solubility capacities for different phytoconstituents [28].

It was also noted in this study that the clinical *S. typhi* was more resistant to the extract of unripe *A. muricata* fruit when compared to the typed isolate. The resistance might be due to the previous exposure of patients from which the isolate was obtained to some commonly used antibiotics. This opinion however differed from the reports of [29] who stated that antibiotic resistance does not interfere with antibacterial action of plant extracts and that the extracts might have different modes of action on test organism. Therefore, the demonstration of antibacterial activity of unripe fruit extract against both clinical and typed isolates used in this study provides a scientific basis for its use in the treatment of typhoid infection.

The MIC values for typed and clinical *S. typhi* were 150 and 250 mg/ml respectively while the MBCs were 350 and 400 mg/ml respectively. The MIC is the lowest concentration of the extract needed to inhibit the growth of test organism while MBC is that lowest concentration that kills the test organism (*S. typhi*). It was revealed in this study that the plant extracts can be both bacteriostatic and bactericidal. The MIC values obtained in this study is lower compared to the findings of [21] who reported that the aqueous and methanolic leaf extract of *Tamarindus indica* were not able to inhibit the growth of *S. aureus* and *P. aeruginosa* at the highest experimented concentration (500 mg/ml). However, lower MIC values were seen with the ethanolic *T. indica* fruit extracts where 50mg/ml of the extract inhibited the growth of *E. coli* [21]. Similarly, [30] reported the MIC value for fresh leaf extract of *Carica papaya* to be 75mg/ml and 100mg/ml against *E. coli* and *Enterococcus faecalis* respectively. MIC values indicated the definite nature of the antimicrobial activities of plant and the inhibition values showed the extent of effectiveness of the extract with increasing concentration [30]. Therefore, the high MIC and MBC values for the extract of unripe *A. muricata* fruit against clinical and typed *S. typhi* is an indication of its impaired efficacy against the test bacteria and/or the possibility that the bacteria may possess the capacity to develop resistance against the plant extracts [21].

The sensitivity of both clinical and typed *S. typhi* to ciprofloxacin (Table 2) in this study is similar to the findings of [31] who reported that the *Salmonella* strains obtained from patients of different age categories were susceptible to ciprofloxacin. However, the observed sensitivity of the isolates to gentamicin, nalixidic acid and tetracycline is in contrast to a report that clinical isolate of *S. typhi* were resistant to nalixidic acid, gentamicin and tetracycline administered [31]. The susceptibility of the clinical isolate to tetracycline in this study was unexpected because of the high use of tetracycline as an antimicrobial agent in animal husbandry and human medicine. The sensitivity however might be due to the fact that the *Salmonella* strain involved in this study had not been discriminately exposed to antibiotics and thus has not developed any resistance. Also, the resistance of both clinical and typed *S. typhi* to amoxicillin is in contrast to the works of [31] who reported clinical *S. typhi* to be susceptible to amoxicillin. In addition, the resistance of the clinical isolate to chloramphenicol was expected since it is one of the most discriminately used antibiotics and the resistance might be plasmid encoded. This corroborated a previous reports by [32] and [31] who in their plasmid profiling of antibiotic resistant *Salmonella* strains revealed that the isolates contain various sizes of R-plasmid. They added that the number of plasmids found per cell is proportional to the number of antibiotics the isolated strains are resistant to.

In addition, when the results of the zones of inhibition observed for crude fruit extract (Table 1) and standard antibiotics (Table 2) against both isolates of *S. typhi* were compared using Duncan Multiple Range Test, it was revealed that the activity of the extract (11.37-14.03 mm) compared favourably with ciprofloxacin (11.00-16.00 mm) widely used in the treatment of *S. typhi* infection at concentration of 500 mg/ml. Meanwhile, others such as gentamycin (10.33-12.67 mm) and Tetracycline (10.33-12.67 mm) compared favourably at concentration of 400-450 mg/ml (10.03-12.37 mm) of the extract. The effect of the extract of unripe *A. muricata* fruit compared with the activity of chloramphenicol on typed *S. typhi* at 150 mg/ml. However, the activity of chloramphenicol was significantly

lower ($p<0.05$) when compared to the activity of the extract on *S. typhi* at 300-350 mg/ml. When compared to the antimicrobial activity of *A. muricata* fruits extract at various concentrations in this study, ciprofloxacin was more effective. This is in agreement with findings of [33] who reported that the reference drug (ciprofloxacin) was more effective than the *Pimpinella anisum* fruits extract against all the pathogenic test bacteria used. The differences observed between the activities of the extract and the standard antibiotics might be due to the proportion of bioactive constituents present in the crude extract compared to the pure compound contained in the standard drugs.

The decrease in *S. typhi* shed (Table 3) observed in groups administered extract and ciprofloxacin is similar to the findings of [34] who reported significant ($p<0.05$) drop in the *S. typhi* load with time in groups of experimental rat administered the methanolic leave extract of *Paullinia pinnata* and conventional antibiotics (ciprofloxacin and oxytetracycline). Similarly, [35] reported that the treatment of infected rats with the leaf extract of *Momordica charantia* and chloramphenicol antibiotics significantly reduced ($p<0.05$) the colony counts of *S. typhi*. However, the continuous increase in *Salmonella typhi* shed in the infected but untreated group is similar to the works of [34] who observed that the *Salmonella* discharged in the stool remained relatively high in the untreated group. In this study, the group treated with the ciprofloxacin (V) had lesser faecal counts of *S. typhi* when compared to the group treated with extract of unripe *A. muricata* fruit. This differed from a previous report that group of experimental rats treated with the leaf extract of *Momordica charantia* recorded significantly lower ($p<0.05$) colony counts than rats treated with chloramphenicol [35].

The decrease observed in *S. typhi* shed in group treated with the extract throughout the experiment suggests that a steady administration of the ethanolic extract of unripe *A. muricata* fruit will be effective in combating Salmonella infection and might be useful when treating such infection. However, the increasing *S. typhi* shed reported in group given extract before infection is an indication that the extract might not possess prophylactic activity. The significant decrease ($p<0.05$) observed till the fourth day in group I might be that infection has not been established due to fighting activity of body soldier. However, the sudden increase in stool passage observed from the fourth day could be the result of the established infection after *S. typhi* had overcome the body soldiers. Also, the decrease observed in the quantity of stool passed by rats infected but treated with extract confirmed the use of *A. muricata* fruit as an anti-diarrheal agent [4]. The decrease observed however compared favourably with that reported for group V administered with ciprofloxacin as positive control in this study.

In this study, the insignificant change in weights of rats reported (Table 3) is in agreement with the findings of [36] who reported that groups administered extract of *Coriandrum sativum* up to 2000 mg/g did not show any significant alteration in body weights of the rats. However, this result is in contrast to the works of [18] who reported an increase in the average body weights of the rats treated with 400 mg/kg and a corresponding decrease in weights of those treated with 200 mg/kg of the extract of *O. gratissimum* but they attributed the observed changes to dose-dependent nature of the antimicrobial activity of *O. gratissimum* extract. Similarly, [35] found out that all groups of experimental rats treated with leaf extract of *M. charantia* recorded steady increase in body weight. The expected significant decrease ($p<0.05$) observed in group 1 infected but untreated might be due to the establishment of infection in the rats leading to loss of weight. However, a sudden increase in body weight observed from the sixth day might be due to the recovery of the rats as a result of the fighting activity of the increased White Blood Cell differentials. This observation is similar to that of a human case in which some victims of *E. coli* O157:H7 infection recovered without treatment within 5-10 days [37].

The body weight change serves as a sensitive indicator of the general health status of animals and it will be significant if the body weight loss that occurred is more than 10% from the initial weight [12]. Hence, the insignificant difference observed in body weight of rats given extract in this study indicates that the extract of *A. muricata* fruits does not interfere with the metabolism of the rats.

4. CONCLUSION

The demonstration of antibacterial activity of unripe fruit extract against both clinical and typed isolates as well as the reduction observed in the faecal shedding of *S. typhi* in group of rats treated with fruit extract of *A. muricata* provides a

scientific basis for its use in the treatment of typhoid infection. The increasing *S. typhi* shed reported in group given the extract before infection is an indication that the extract might not possess prophylactic activity. However, the high MIC and MBC observed in the *in-vitro* studies indicates the possibility that the bacteria may possess the capacity to develop resistance against the plant extracts.

It is recommended that a comparative study of the antibacterial activity of the extract should be carried out using other solvents apart from ethanol used in this study in order to determine which solvent will best isolate active component of the fruit extract.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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