

Original Research Article

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

ABSTRACT

Aims: To investigate the *in-vitro* and *in-vivo* antityphoid activities of the crude ethanolic extract of unripe *Annona muricata* fruits in albino rats infected with *Salmonella typhi*.

Place and Duration of Study: Department of Microbiology Laboratory, Federal University of Technology, Akure, Ondo State, Nigeria, between June 2014 and December 2014.

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 (400mg/kg) accordingly for 7 days. During the treatment period, the fecal samples were collected to monitor the 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: The result of the *in-vitro* activity of the extract against the typed and clinical isolates did not show any zone of inhibition (ZI) at concentrations of 50-200mg/ml for clinical isolates but at higher concentration, a significant increase ($P<0.05$) was recorded (4.93-11.37mm). On the other hand, there was no inhibition up to 100mg/ml for typed isolates. However, a progressive increase (3.03-14.03mm) was observed at higher concentrations. The MIC values for typed and clinical isolates were 150 and 250mg/ml respectively while MBC values were 350 and 400mg/ml respectively. In the comparative sensitivity testing, ciprofloxacin (10µg) had the highest inhibitory activity against the typed (11mm) and clinical isolates (16mm) while both clinical and typed *S. typhi* were resistant to amoxicillin (30µg). For *in-vivo* examination, the infectivity dose (ID_{50}) of the test organism on experimental rats was 6.8×10^6 cfu/ml. Faecal shedding of *S. typhi* by rats treated with extract significantly decreased with increasing days of treatment while faecal shed in group given prophylactic treatment significantly increased ($P<0.05$). There was no significant change ($P<0.05$) in body weight in all groups when compared to weights before treatment but in group infected without treatment, a significant decrease ($P<0.05$) was recorded.

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* provide a scientific basis for its use in the treatment of typhoid infection. The extract might not possess prophylactic activity.

Keywords: *In-vitro*, *In-vivo*, *Annona muricata*, Anti-typhoid, 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 ft (7.5-9 m) in height. The fruit is covered with a leather-like, reticulated but inedible, 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 [8]. 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 therapy 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. MATERIAL AND METHODS

2.1 Plant Collection and Identification

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

65 **2.2 Source and Preservation of Bacteria**

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

70 **2.3 Extraction of the Bioactive Constituents of Unripe *A. muricata* Fruit**

71 The methods of [12] and [13] were employed. Unripe *A. muricata* fruits were washed with
72 sterile water. The peels and seeds were separated from the pulp and then cut into smaller
73 pieces. The fruits were oven dried at 50°C for 4days. The dried fruits were then pulverized
74 into fine powder by blending in a high-speed Philips Model blender. They were separately
75 kept in an airtight container to avoid the absorption of moisture. Three hundred grams (300g)
76 of the powdered sample was soaked in two thousand, five hundred milliliters (2000ml) of
77 70% ethanol as solvents to extract the bioactive compounds. The container was labeled
78 appropriately and left for 72hours (3days). After which it was sieved using muslin cloth and
79 then filtered using 0.45µm micropore filter. The filtrates were vaporized to dryness using
80 rotary evaporator and subsequently lyophilized to remove the extracting solvent. Then, the
81 ethanol extract was reconstituted with 30% dimethylsulphoxide (DMSO) to obtain varying
82 concentrations [14].

83 **2.4 Preparation of Standard Inoculums of *Salmonella typhi* for *In vitro* Assay**

84 Three isolated overnight colonies were transferred to a tube of sterile saline. The bacterial
85 suspension was compared to the 0.5McFarland standards against a sheet of white paper on
86 which sharp black lines were drawn. The bacterial suspension was adjusted to be the proper
87 density as the 0.5 McFarland by adding sterile saline or more bacterial growth. Then
88 bacterial suspension was diluted to obtain 10⁶ cfu/ml [11,15].

89 **2.6 Antibacterial Sensitivity Testing of the *A. muricata* Extract**

90 Using a sterile pipette, 0.1ml of the bacterial suspension was drawn out and was aseptically
91 introduced into sterile Petri dishes. Sterilized Mueller Hinton Agar (MHA, Difco, USA) that
92 had been cooled to about 45°C was aseptically poured into the Petri dishes containing 0.1ml
93 of *S. typhi*. Each Petri dish was gently swirled in a clockwise direction in order to ensure that
94 the bacterium is homogeneously distributed with MHA. The plates were allowed to stand for
95 40minutes for the inoculated bacteria to be established in the medium. After which three
96 wells were aseptically bored on each agar plate using a sterile cork borer (7mm) at
97 allowance of 30mm between opposite wells and the edges of the petri dishes. A 0.1ml of the
98 different concentrations of the extracts was then introduced into each well in the plates using
99 sterile pipette. A control well was in the centre with 0.1ml of the reconstituting reagent (30%
100 DMSO). The plates were incubated at 37°C for 24hours. The resulting zones of inhibition
101 were measured using a caliper. The experiment was done in triplicates and the average
102 reading was taken to be the zone of inhibition of the bacterial isolate at different
103 concentration of the extract [16].

104 **2.7 Antibiotic Assay**

Standard antibiotics such as gentamycin -30µg, chloramphenicol - 30µg, ciprofloxacin - 10µg, tetracycline - 30µg, amoxicillin - 30µg and nalixidic acid - 30µg were compared against the clinical and typed *S. typhi* isolates. With the aid of a sterile pipette, 0.1ml 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°C was aseptically poured into the Petri dishes containing 0.1ml 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 40minutes 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°C for 24hours. 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].

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

About 5ml of freshly prepared Mueller Hinton Broth (MHB) was drawn out with sterile pipette into test tubes, then 0.1ml of inoculum of *Salmonella typhi* (1×10^6 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°C for 24hrs. 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°C for 24hours. The least concentration at which no growth was observed was noted as the MBC [15].

2.9 Experimental Animals

Forty-two healthy Swiss albino rats (170-220g) between 5-7weeks 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 7days before the treatment started. The experimental procedures involving animals were conducted in conformity with international, national and institutional guidelines as reported by [16].

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

A 0.238g of sodium hydrogen phosphate was dissolved in 0.019g of potassium dihydrogen phosphate and sodium chloride respectively. The mixture was made up to 100mL with distilled water and pH was adjusted to neutral (7). Then standard inoculum of *S. typhi* was inoculated into 1000mL of nutrient broth and incubated for 24hrs. After incubation, the cells were centrifuged at 2000rpm 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].

2.9.2 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).

2.9.3 *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 400mg/ml ethanolic extract of unripe *A. muricata* fruit after the infection had set in. Group III was given prophylactic dose 400mg/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].

2.9.4 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 (1g) of the sample was serially diluted in physiological saline, plated in triplicates on *Salmonella Shigella* Agar (Difco, USA), and then incubated at 37°C 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].

2.9.5 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.

2.9.6 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 20ml 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.

2.9.7 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

The sensitivity pattern of clinical and typed isolates of *S. typhi* to ethanolic extract of unripe *A. muricata* fruits is shown in Table 1. 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 50-200mg/ml but at higher concentrations of 250-500mg/ml used, a significant increase in zones of inhibition which ranged from 4.93-11.37mm were observed. Also, there was no inhibition up to concentration of 100mg/ml for typed isolates. However, zones of inhibition which ranged from 3.03-14.03mm were observed at concentrations of 150-500mg/ml. The result of the *in-vitro* susceptibility test 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 250mg/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 200mg/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 50-200mg/ml for clinical *S. typhi* and 50-100mg/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 flavonoides 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.

Table 1: Zone of Inhibition (mm) of the Extract against Clinical and Typed *S. typhi*

Extract	<i>S. typhi</i> Clinical	<i>S. typhi</i> Typed
---------	--------------------------	-----------------------

	(mg/ml)	
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

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

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

251 The result of the sensitivity of clinical and typed *S. typhi* to conventional antibiotics is shown
252 in Table 2. Of all the antibiotics used in this study, ciprofloxacin (10µg) had the highest
253 antimicrobial activity against the clinical (11.00mm) and typed (16.00mm) isolates. This was
254 followed by Gentamycin (10.33-12.67mm), Tetracycline (8.67-12.33mm) and Nalixidic acid
255 (4.67-12.00mm). Clinical *S. typhi* was resistant to chloramphenicol (30µg) while the typed
256 was susceptible (3.50mm). However, both clinical and typed *S. typhi* were resistant to

amoxicillin (30µg). The sensitivity of both clinical and typed *S. typhi* to ciprofloxacin 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.

Table 2: Antibiotic Sensitivity Pattern of the *S. typhi* to extract of *A. muricata* Fruit

Standard Antibiotics	Clinical <i>S. typhi</i> (mm)	Typed <i>S. typhi</i> (mm)
GEN (30µg)	10.33 ^d ±0.88	12.67 ^c ±0.88
NAL (30µg)	4.67 ^b ±0.33	12.00 ^c ±2.52
COT (30µg)	0.00 ^a ±0.00	3.50 ^b ±0.50
AMO (30µg)	0.00 ^a ±0.00	0.00 ^a ±0.00
TET (30µg)	8.67 ^c ±0.88	12.33 ^c ±1.20
CIP (10µg)	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

The result of comparative zones of inhibition of standard antibiotics with crude fruit extracts using Duncan Multiple Range Test is shown in Table 3. The result showed that for both isolates, the activity of the extract (11.37-14.03mm) compared favourably with ciprofloxacin (11.00-16.00mm) widely used in the treatment of *S. typhi* infection at concentration of 500mg/ml while others such as gentamycin (10.33-12.67mm) and Tetracycline (10.33-12.67mm) compared favourably at concentration of 400-450mg/ml (10.03-12.37mm) of the extract. Also, the effect of the extract of unripe *A. muricata* fruit compared with the activity of

chloramphenicol drug on typed *S. typhi* at 150mg/ml. However, the activity of chloramphenicol was significantly lower ($p<0.05$) when compared to the activity of the extract on *S. typhi* at 300-350mg/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.

Table 3: Comparative Zones of Inhibition (mm) of Standard Antibiotics with Crude Fruit Extract

Antimicrobial Agents	<i>S. typhi</i> (Clinical)	<i>S. typhi</i> (Typed)
GEN (30µg)	10.33 ^f ±0.88	12.67 ^g ±0.88
NAL (30µg)	4.67 ^b ±0.33	12.00 ^g ±2.52
COT (30µg)	0.00 ^a ±0.00	3.50 ^b ±0.50
AMO (30µg)	0.00 ^a ±0.00	0.00 ^a ±0.00
TET (30µg)	8.67 ^e ±0.88	12.33 ^g ±1.20
CIP (10µg)	11.00 ^g ±0.58	16.00 ⁱ ±1.00
50mg/ml	0.00 ^a ±0.00	0.00 ^a ±0.00
100mg/ml	0.00 ^a ±0.00	0.00 ^a ±0.00
150mg/ml	0.00 ^a ±0.00	3.03 ^b ±0.09
200mg/ml	0.00 ^a ±0.00	5.13 ^{cd} ±0.03
250mg/ml	4.93 ^b ±0.07	4.93 ^c ±0.09
300mg/ml	5.53 ^c ±0.09	7.20 ^e ±0.15
350mg/ml	7.03 ^d ±0.15	8.10 ^f ±0.10
400mg/ml	10.03 ^f ±0.03	11.97 ^g ±0.88
450mg/ml	10.93 ^f ±0.07	12.37 ^g ±0.88
500mg/ml	11.37 ^g ±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 ($P<0.05$).

Key: GEN – Gentamycin, NAL – Nalixidic acid, CHL – Chloramphenicol, AMO – Amoxicillin, TET – Tetracycline, CIP – Ciprofloxacin

The dose of *Salmonella 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 is shown on Table 4. 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.

The decrease in *S. typhi* shed 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.

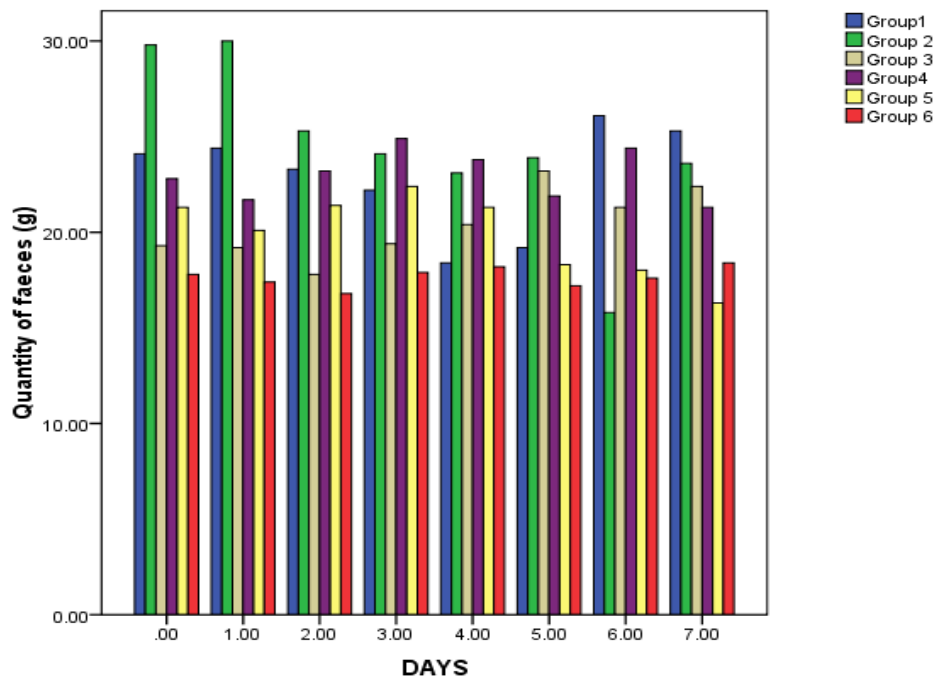
Table 4: Effect of Treatment on Faecal Shedding of *S. typhi*

<i>Salmonella typhi</i> Count ($\times 10^3$) (cfu/ml)						
Days	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

333 extract only, Group 5= Infected & treated with Ciprofloxacin, Group 6 = Basal diet and water
334 (Control), 0 day – Before treatment, 1-7 days = Treatment.

335 The result of the effect of treatment on the quantity of stool passed out by experimental rats
336 is shown in Figure 1. A decrease was observed in the quantity of stool passed in group I till
337 the fourth day. However, from the 4th day, a significant increase was observed. Also,
338 quantity of stool passed by group treated with extract (II) decreased. Stool passed in group
339 III decreased till 2nd day and increased accordingly from day 3. However, no significant
340 change was observed in group 6 (control). The significant decrease ($p<0.05$) observed till
341 the fourth day in group I might be that infection has not been established due to fighting
342 activity of body soldier. However, the sudden increase in stool passage observed from the
343 fourth day could be the result of the established infection after *S. typhi* had overcome the
344 body soldiers. Also, the decrease observed in the quantity of stool passed by rats infected
345 but treated with extract confirmed the use of *A. muricata* fruit as an anti-diarrheal agent [4].
346 The decrease observed however compared favourably with that reported for group V
347 administered with ciprofloxacin as positive control in this study.



348

349 **Figure 1: Effect of Treatment on the Quantity of Stool Passed out by Experimental**
350 **Rats**

351 00 day – Before treatment, 1-7 days = Treatment

352 In this study, the weights (g) of rats in their respective treatment groups were monitored as
353 shown in Table 5. There was no significant change in weight of rats in all the groups before
354 and after treatment except group I (only infected) where there was significant decrease in
355 weight till the fifth day. However, significant increase was observed on the sixth and seventh
356 day. The non-significant change in weights of rats observed in this study is in agreement
357 with the findings of [36] who reported that groups administered extract of *Coriandrum*
358 *sativum* up to 2000mg/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 400mg/kg and a corresponding decrease in weights of those treated with 200mg/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 non-significant 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.

Table 5: Effect of Treatment on Weight of Rats (g) Before and After Infection

Weight of Rats (g)						
Days	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

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

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

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

395 396 ETHICAL APPROVAL (WHEREEVER APPLICABLE) 397

398 All authors hereby declare that "Principles of laboratory animal care" (NIH publication No.
399 85-23, revised 1985) were followed, as well as specific national laws where applicable. All
400 experiments have been examined and approved by the appropriate ethics committee.

401 402 REFERENCES 403

- 404 1. Nau, R. and Tauber, S. C. Immunodulatory properties of antibiotics. Current Molecular
405 Pharmacology. 2008; 1:68-79.
- 406 2. Rajeswari, D. V., Gajalakshmi, S., Vijayalakshmi, S. Phytochemical and
407 pharmacological properties of *Annona muricata*: A review. International Journal of
408 Pharmacy and Pharmaceutical Sciences. 2012; 2(4):3-6.
- 409 3. Kumar, U. A., Manjunath, C., Thaminzhmani, T., Kran, Y. R. and Brahmaiah, Y. A
410 review on immunomodulatory activity of plants. Indian Journal Novel Drug Delivery.
411 2012; 4(2):93-103.
- 412 4. Taylor, L. Technical data report for graviola (*A. muricata*) In: The healing power of
413 rainforest herbs. Sage Press Inc. Austin, 2005.
- 414 5. Pathak, P., Saraswathy, D.R., Vora, A. and Savai, J. *In vitro* antimicrobial activity and
415 phytochemical analysis of the leaves of *Annona muricata*. International Journal of
416 Pharma Research and Development. 2010; 2(5): 1205-1210.
- 417 6. Mishra, S., Ahmad, S., Kumar, N. and Sharma, B. K. 2013. *Annona muricata* (The
418 Cancer Killer): A review. The Global Journal of Pharmaceutical Research. 2013; 2(1):
419 1613-1618.
- 420 7. World Health Organization (2003). *Background document: The diagnosis, treatment*
421 *and prevention of typhoid fever*. Department of Vaccines and Biologicals, CH-1211
422 Geneva 27, Switzerland. Pp. 1-30.
- 423 8. Khanam, F., Sayeed, M. A., Choudhury, F. K., Sheikh, A., Ahmed, D., Goswami D, M.
424 L. Hossain, A. Brooks, S. B. Calderwood, R. C. Charles, A. Cravioto, E. T. Ryan and F.
425 Qadri1. (2015). Typhoid Fever in Young Children in Bangladesh: Clinical Findings,
426 Antibiotic Susceptibility Pattern and Immune Responses. PLoS Negl Trop Dis. 2015;
427 9(4): e0003619. doi:10.1371/journal.pntd.0003619.
- 428 9. Gustavo, H. F. V., Jozeanne, A. M., Angela, M. A., Renata, A. C. and R. H. S. Dos
429 Fernandes Viera. Antibacterial effect (*in vitro*) of *Moringa oleifera* and *Annona muricata*

- 430 against gram positive and gram negative bacteria. *Revista do Instituto de Medicina*
431 *Tropical de Sao Paulo*. 2010; 52(3): 129.
- 432 10. Chukwuka, K. S., Ikheola, J. O., Okonko, L. O., Moody, J. O. and Makinde, T. A. The
433 antimicrobial activities of some medicinal plants on *Escherichia coli* as an agent of
434 diarrhea in livestock. *Adv.Appli. Sci. Res.*. 2011; 2(4):37-48.
- 435 11. Jothy, S. L., Zakaria, Z., Chen, Y., Lau, Y. L., Latha, L.Y., Sasidharan, S. Acute oral
436 toxicity of methanolic seed extract of *Cassia fistula* in Mice. *Molecules*. 2011; 16: 5268-
437 5282.
- 438 12. Nweke, C. N. and Ibiam, O. F. A. Pre and post harvest fungi associated with the soft rot
439 of the fruit of *Annona muricata* and their effects on the nutrient content of the pulp.
440 *American Journal of Food and Nutrition*. 2012; 2(4):78-85.
- 441 13. Solomon-Wisdom, G. O., Ugoh, S. C., Mohammed, B. Phytochemical screening and
442 antimicrobial activities of *A. muricata* (L) leaf extract. *American Journal Biological.*
443 *Chemical and Pharmaceutical Sciences*. 2014; 2(1): 1-7.
- 444 14. Cheesbrough, M. *District laboratory practice in Tropical countries*. 2nd edition,
445 Cambridge University Press, UK. 2006. Pp. 130-331.
- 446 15. Clinical Laboratory Standard Institute (CLSI). *Performance Standards for Antimicrobial*
447 *Susceptibility Testing*; Twenty-Fourth Informational Supplement. CLSI document M100-
448 S24. Wayne, PA: Clinical and Laboratory Standards Institute, 2014.
- 449 16. Adewole, S. O. and Caxton-Martins, E. A. Morphological changes and hypoglycemic
450 effects of *A. muricata* Linn. (Annonaceae) leaf aqueous extract on Pancreatic B-cells of
451 Streptozotocin-Treated diabetic rats. *African Journal of Biomedical Research*. 2006;
452 9:173-187.
- 453 17. Dada, E.O. and Komolafe, O. I. *In vivo* evaluation of the inhibitory effects of *Ocimum*
454 *gratissimum* on *Salmonella typhi*. *International Journal of Pharmaceutical and*
455 *Biological Research*. 2013; 4(4):185-191.
- 456 18. Reddy, Y. R. R., O. Lokanatha¹, K. S. V. P. Ratnam, C. S. Reddy, I. Naga Raju and C.
457 D. Reddy. Acute and sub acute toxicity of *Moringa oleifera* stem bark extract in Swiss
458 albino mice. *International Journal of Life Sciences Biotechnology and Pharma*
459 *Research*. 2013; 2(4):73-84.
- 460 19. Jang-Gi, C., Ok-Hwa, K., Young-Seob, L., Hee-Sung, C., You-Chang, O., Obiang-
461 Obounou, B., Min-San, K., Dong-Hwan, S., Hun-Soo, K., Hyun, P., Dong-Won, S.,
462 Jung-Rae, R. and Dong-Yeul, K. *In vitro* and *In vivo* Antibacterial Activity of *Punica*
463 *granatum* Peel Ethanol Extract against Salmonella. *Evidence-Based Complementary*
464 *and Alternative Medicine*. 2011; 1-8.
- 465 20. Ugoh, S. C. and Haruna, I. M. Phytochemical Screening and Antibacterial Activity of the
466 Fruit and Leaf Extracts of *Tamarindus Indica* (Linn.). *Report and Opinion*. 2013;
467 5(8):18-27.
- 468 21. Joshi, B., Sah, G. P., Basnet, B. B., Bhatt, M. R., Sharma, D., Subedi, K., Pandey, J.
469 and Malla, R. Phytochemical extraction and antimicrobial properties of different
470 medicinal plants: *Ocimum sanctum* (Tulsi), *Eugenia caryophyllata* (Clove), *Achranthes*

- 471 *bidentata* (Datiwan) and *Azadirachta indica* (Neem), Journal of Microbiology and
472 Antimicrobials. 2011; 3(1): 1-7.
- 473 22. Bahir, A., Saeed, B., Talat, Y.M. and Nayar J. Comparative study of antimicrobial
474 activities of *Aloe vera* extracts and antibiotics against isolates from skin infections.
475 African Journal of Biotechnology. 2011; 10(19): 3835-3840.
- 476 23. Faleye, O. S. (2015). The anti-typhoid activities of the ethanolic pulp extract of unripe
477 *Annona muricata* fruit in Swiss albino rats infected with *Salmonella typhi*. M.Tech Final
478 Thesis, Department of Microbiology, Federal University of Technology, Akure, Ondo
479 State, 2015, Pp.
- 480 24. Eze, S. O. and Obinwa, E. (2014). Phytochemical and nutrient evaluation of the leaves
481 and fruits of *Nauclea latifolia*. Communication in Applied Sciences. 2014; 2(1): 8-24.
- 482 25. Agyare, C., A. S. Dwobeng, N. Agyepong, Y. D. Boakye, K. B. Mensah, P. G. Ayande,
483 Adarkwa-Yiadom and M. Antimicrobial, antioxidant, and wound healing properties of
484 *Kigelia africana* (Lam.) Beneth. and *Strophanthus hispidus* DC. Advances in
485 Pharmacological Sciences. 2013; 1-10.
- 486 26. Nagham, M. A. Study effect of medical plant extracts in comparison with antibiotic
487 against bacteria. Journal of Scientific and Innovative Research, 2013; 2(5): 843-845.
- 488 27. Zeidan, R., S. Oran, K. Khleifat and S. Matar. Antimicrobial activity of leaf and fruit
489 extracts of Jordanian *Rubus sanguineus* Friv. (Rosaceae). African Journal of
490 Microbiology Research. 2013; 7(44):5114-5118.
- 491 28. Oskay, M., Oskay, D. and Kalyoncu. Activity of some plant extracts against multi-drug
492 resistant human pathogens. Iranian Journal of Pharmaceutical Research. 2009; 8(4):
493 293-300.
- 494 29. Alabi, O. A., M. T. Haruna, C. P. Anokwuru, Jegede, T., Abia, H.,V. U. Okegbe and
495 Esan, B. E. Comparative Studies on antimicrobial properties of extract of fresh and
496 dried leaves of *Carica papaya* (L) on clinical bacterial and fungal isolates. Advances in
497 Applied Science Research. 2012; 3(5): 3107-3114.
- 498 30. Adnan, S. J. Antimicrobial resistance pattern and plasmid profile of some *Salmonella*
499 spp. Isolated from clinical samples in Riyadh area. European Scientific Journal. 2015;
500 11(6):136-143.
- 501 31. Al-Bahry, S. N. Plasmid profiling of antibiotic resistant *Salmonella* species isolated in
502 Muscat, Oman. Pakistan Journal of Biological Sciences. 2000; 3: 215-218.
- 503 32. Akhtar, A., A. A. Deshmukh, A.V. Bhonsle, P. M. Kshirsagar and M. A. Kolekar. In vitro
504 Antibacterial activity of *Pimpinella anisum* fruit extracts against some pathogenic
505 bacteria. Veterinary World. 2008; 1(9): 272-274.
- 506 33. Lunga, P. K., Jean de Dieu Tamokou, S. P.C. Fodouop, Jules-Roger Kuate, J.
507 Tchoumboue and D. Gatsing. Antityphoid and radical scavenging properties of the
508 methanol extracts and compounds from the aerial part of *Paullinia pinnata*.
509 SpringerPlus. 2014; 3:302-311.

- 510 34. Adeyi, A. O., Jinadu, A. M., Arojoye, O. A., Alao, O. O., Ighodaro, O. M. and Adeyi, O.
511 E. In vivo and in vitro antibacterial activities of *Momordica charantia* on *Salmonella*
512 typhi and its effect on liver function in typhoid-infected rats. *Journal of Pharmacognosy*
513 and *Phytotherapy*. 2013; 5(11):183-188.
- 514 35. Patel, D., Desai, S., R. Devkar and A.V. Ramachandran. Acute and sub-chronic
515 toxicological evaluation of hydro-methanolic extract of *Coriandrum sativum* L. Seeds.
516 *EXCLI Journal*. 2012; 11:566-575.
- 517 36. Centres for Disease Control and Prevention. Questions and Answers: Sickness caused
518 by *E. coli* O157:H7. [https://www.cdc.gov/ncidod/dbmd/diseaseinfo/Escherichia_coli-](https://www.cdc.gov/ncidod/dbmd/diseaseinfo/Escherichia_coli-g.htm)
519 [g.htm](https://www.cdc.gov/ncidod/dbmd/diseaseinfo/Escherichia_coli-g.htm). 2006.