



## ***In vitro* and *in vivo* Activities of *Psidium guajava* and *Azadirachta indica* Leaf Extracts and solvent Fractions against *Salmonella Typhi***

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### **Authors' contributions**

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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### **ABSTRACT**

Typhoidal salmonella infections remain a challenge in the health care system in sub-Saharan Africa. Carrier status and advent of multi-drug resistant *S. Typhi* strains have necessitated the search for new drug leads. Hence, this study aims at investigating *P. guajava* and *A. indica* leaves for anti-salmonella activities. Guava and neem leaves were extracted by maceration in methanol and fractionated by solvent partitioning. *In vitro* activities were assessed by agar well diffusion and broth micro-dilution methods. Sixty male rats were randomized to 10 groups of 6 animals each for the *in vivo* experiments. Groups of rats except, normal control, were induced with 0.5McFarland of *S. Typhi* suspension orally. Treatment groups received 200 mg/kg body weight of extracts and fractions, and the control groups were treated with 14.29mg/kg body weight of ciprofloxacin and 1%v/v DMSO for 7 days post-infection. Biochemical parameters were determined

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spectrophotometrically. Hematological parameters were analyzed with automated hematology diagnostic machine. All fractions of *P. guajava* and three of *A. indica* inhibited *S. Typhi* growth with Zone of Inhibition (ZI) ranging from 11-15 mm. Active fractions inhibited 48.60-62.45% of *S. Typhi* biofilm formation at 25 mg/mL with Minimum Bactericidal Inhibitory Concentration (MBIC) of 0.39-12.5 mg/mL. All fractions improved body weight of treated rats and inhibited bacteremia at 44.75 and 95.94%. Hematological parameters improved in all fractions-treated rats. MDA was not significantly ( $p < 0.05$ ) altered in all groups. One fraction of *P. guajava* (ePg) lowered the elevated level in concentration of Nitric oxide (NO) while all fractions enhanced the lowered activity of SOD. Elevated (lactate dehydrogenase (LDH), aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and bilirubin (BIL) were lowered by all fractions to various extents in treated rats. Fractions of *P. guajava*, and *A. indica* could be further considered for identification of active anti-salmonella principle(s).

**Keywords:** Typhoid; *P. guajava*; *A. indica*; *S. Typhi*; biofilm; bacteremia.

## 1. INTRODUCTION

Typhoid fever is an acute, systemic and febrile illness caused by the infection of the reticulo-endothelia system, intestinal lymphoid tissue and gall bladder by a bacterium, *Salmonella enterica* serovars Typhi (*S. Typhi*). It is endemic in the tropics and sub-tropics especially in the developing countries of the world, with estimated annual global incidence of between 11- 21million incidences and 128,000-161,000 deaths [1,2], salmonella infections are among major prevalent diseases in Nigeria [3]. *S. Typhi* is an aerobic, gram-negative, non-sporing and rod-shaped bacterium transmitted through ingestion of food and water contaminated by faeces and urine of ill persons or chronic, asymptomatic and healthy carriers [4]. Although there are two vaccines for typhoid enteric fever, their effectiveness are limited and treatment has been by use of antibiotics. Expanding antibiotic resistance to current drugs being used for treatment of typhoid fever coupled with the persistence of carrier state in human population has necessitated urgent search for new drug leads and natural products for preventive potential [5].

*Psidium guajava* L is a fruit-bearing tree of the family Myrtaceae and commonly known as guava, with nativity in the tropics and it has reported for traditional treatment of typhoid (Kamath *et al.*, 2008). Many scientific evidences abound for medicinal values of *P. guajava*. *P. guajava* was previously reported for anti-mutagenic [6], anti-fungal [7], antidiarrheal and anti-diabetic [8] as well as antibacterial [9] activities. In addition, *Azadirachta indica* (common name: neem, family: Meliaceae) widely spread in tropical and semitropical regions has been equally known for age-long use for the treatment of fever like typhoid and malaria. Anti-

nociceptive and antioxidant [10], anticancer [11], anti-inflammatory, pro-apoptotic, and anti-proliferative [12], anti-diabetic [13], antimicrobial [14] activities, among several others have been reported for various parts of the neem plant.

*P. guajava* and *A. indica* are two plants that have been used by local people in management of typhoid and have not been scientifically proven to have therapeutic values. This study aims at investigating *P. guajava* and *A. indica* leave extracts for anti-salmonella activities.

## 2. MATERIALS AND METHODS

### 2.1 Materials

#### 2.1.1 Plant samples and *Salmonella enterica* serovar Typhi (*S. Typhi*)

Fresh leaves of *Psidium guajava* and *Azadirachta indica* were harvested in the premises of Ladoke Akintola University of Technology, Ogbomoso, Nigeria and authenticated at the Pure and Applied Biology Department of same institution. Leaves were washed under running water to remove sand and debris, air dried and pulverized with the aid of an electric blender. Clinical isolates of *S. Typhi* were obtained from the Medical Microbiology unit of University of Ilorin Teaching Hospital and maintained on Salmonella-Shigella agar throughout the study period.

#### 2.1.2 Reagents and chemicals

Solvents (methanol, ethanol, n-hexane, chloroform and ethyl acetate) were purchased from British Drug House, Poole, United Kingdom while agars and broth were products of HiMedia

laboratories, India. Assay kits (ALT, AST, Total protein, Albumin, Globulin, LDH, ALP, Bilirubin, and SOD) were manufactured by Fortress Diagnostic limited, United Kingdom. NO and MDA were assayed for using Oxford Biomedical research kit (Oxford, USA). Other reagents used were of analytical grade.

## 2.2 Methods

### 2.2.1 Extraction and Fractionation of plant samples

Powdered plant samples were macerated in methanol (1:10 w/v) at room temperature for 72 hours with intermittent shaking. The suspension was thereafter filtered, first through a Whatman No1 filter paper and concentrated using a rotary evaporator (40°C at reduced pressure).

A modified method of Kupchan and Tosu [15] was employed for solvent partitioning of the plant extracts. Extracts were re-suspended in 60% aqueous methanol and poured into a 1000 mL separating funnel, equal volume of n-hexane was added, mixed and allowed to separate, n-hexane fraction was removed and fresh n-hexane was added. This was repeated until a clear n-hexane layer was seen. The whole process was repeated first, with chloroform and then ethyl acetate. The fractions were concentrated at 40°C. Four fractions viz n-hexane-, Chloroform-, ethyl acetate- and residual aqueous were derived for each of the two plants and designated as follows: n-hexane fraction of *P. guajava* – hPg; chloroform fraction of *P. guajava* – cPg; ethyl acetate fraction of *P. guajava* – ePg; residual aqueous fraction of *P. guajava* – aPg; n-hexane fraction of *A. indica* – hAi; chloroform fraction of *A. indica* – cAi; ethyl acetate fraction of *A. indica* – eAi; residual aqueous fraction of *A. indica* – aAi.

### 2.2.2 Assessment of *in vitro* sensitivity of *S. Typhi* to extracts and fractions

#### 2.2.2.1 Zone of inhibition

Agar well diffusion method, as described by Balouiri *et al.* [16], was employed for determination of zones of inhibition of bacterial growth. Wells of 6mm in diameter were aseptically bored into freshly prepared Mueller Hinton agar. *S. Typhi* suspension (0.5 McFarland) was used for inoculation of the agar and 50 mg/mL of extracts or fractions in dimethyl sulphoxide (10% v/v DMSO) was added to each well. DMSO (10%) and 10mg/mL ciprofloxacin served as negative and positive controls respectively. Plates were incubated at 37°C for

24 hours. Clear zones of inhibition were measured with a millimeter ruler and recorded for each extract/fraction. Tests were performed in triplicate for reproducibility.

#### 2.2.2.2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of fractions with antisalmonella activities were evaluated using standard broth micro-dilution method. Fifty microliter (50 µL) of active fractions (fractions to which *S. Typhi* was sensitive) in freshly prepared Muller Hinton Broth (MHB), concentration range of 25-0.39 mg/mL, were added to each well of 96-well microtitre plate. Fifty microliter (50 µL) of *S. Typhi* suspension (0.5 McFarland) was added to each well except the sterility control well. A well with only 50 µL of 10% DMSO in MHB and 50 µL of bacterial suspension served as negative control while ciprofloxacin (1.28-0.02 mg/mL) served as the standard drug control. The OD<sub>630</sub> was recorded immediately before incubation and after 24 hours of incubation at 37°C. Wells with difference in pre-and post-incubation OD<sub>630</sub> that are lesser than 0.01 are adjudged to have no growth and the lowest concentration at which no growth was recorded taken as the MIC. Fifty microliter (50 µL) of content of wells with no bacterial growth were plated on freshly prepared MHA plates and incubated at 37°C for 24 hours. The concentration at which no growth was observed on the agar plate was taken as MBC for each fraction.

#### 2.2.2.3 Minimum biofilm inhibition concentration

The ability of the active extracts and fractions of *P. guajava* and *A. indica* to prevent the formation of *S. Typhi* biofilm was evaluated using a modified crystal violet assay described by Rakhmawatie *et al.* [17]. In order to simulate growth conditions of *S. Typhi* on cholesterol gall stone, microtitre plates were pre-coated with cholesterol (by dispensing 5 mg/mL of cholesterol in 1:1 ethanol: isopropanol in each well of 96-well polystyrene micro-titre plate) and allowed to dry at room temperature overnight. The plates were then prepared as described for MIC assay and incubated at 37 °C for 48 hours without agitation. Contents of each well were gently removed. Each well was then washed thrice with 200 µL of sterile phosphate buffered saline (PBS) to remove free-floating bacteria.

Biofilms formed by adherent cells in plate were stained with 1% crystal violet and incubated at the room temperature for 30 minutes. Excess stains were removed by washing thrice with sterile PBS. Stained biofilm was solubilized by adding 100  $\mu$ L of 33% acetic acid. Optical densities (OD) of stained adherent bacteria were measured at 570 nm using an ELISA microtitre plate reader.

The percentage biofilm inhibition was calculated using the following formula:

$$\text{Percentage (\%)} \text{inhibition} = \frac{OD_{\text{growth control}} - OD_{\text{sample}}}{OD_{\text{growth control}}} \times 100$$

Where  $OD_{\text{growth control}}$  = optical density of the growth control

$OD_{\text{sample}}$  = optical density of sample

The lowest concentration of extract that showed inhibition on the biofilm formation was taken as the minimum biofilm inhibition concentration (MBIC).

### 2.2.3 In vivo assessment of selected fractions of *P. guajava* and *A. indica* leaf extract against *S. Typhi*

#### 2.2.3.1 Experimental animals

Sixty (60) male Wister rats were randomly assigned to ten (10) groups of 6 animals each. The animals were housed in plastic cages under standard laboratory conditions ( $25 \pm 2^\circ\text{C}$ , 30-70% humidity, 12hr light/12hr dark cycle) and allowed to acclimatize for 7 days before the commencement of the experiment. They were allowed free access to food and water throughout the experiment. Animals in groups 1-9 were infected, while those of group 10 were not infected and served as normal control group.

#### 2.2.3.2 Inoculum preparation

A few colony of *S. Typhi* were picked, with sterile wire loop, from an overnight SSA culture into sterile MHB and incubated at  $37^\circ\text{C}$  for 18 hours. The suspension was centrifuged at 4000rpm for 10 minutes to remove the broth (the supernatant) and washed twice with normal saline. The bacterium (pellet) was re-suspended in normal saline and adjusted to 0.5 McFarland standard containing about  $1.5 \times 10^8$  CFU/mL. Animals in groups 1-9 were given 1 mL of the adjusted suspension with the aid of an oral cannula [18].

Establishment of infection was confirmed by determining the load of *S. Typhi* in the blood of the animals 48 hours after infection. About 50  $\mu$ L of blood were drawn from the caudal vein of infected animals into 1 mL of normal saline and plated on freshly prepared SSA. The inoculum was allowed to be assimilated into the agar for about 5 minutes and thereafter incubated at  $37^\circ\text{C}$  for 18 hours. Clear growth with black center indicated the presence of *S. Typhi* [19]. Only animals with established infection were used in the experiment. Experimental Animal were grouped accordingly for preliminary *in vivo* anti-salmonella screening of *P. guajava* and *A. indica*. Since the LD50 of both *P. guajava* and *A. indica* are over 5000mg/kg body weight [20,21], animals were treated with 200mg/kg b.w. extract or fraction. Group 1 received extract of *P. guajava*, group 2-4 received hPg, cPg and ePg respectively. Group 5 received extract of *A. indica*, groups 6 and 7 received cAi and aAi respectively. Animals in group 8 were administered 14.29 mg/kg b.w. of Ciprofloxacin while those of group 9 were given 10% DMSO. Treatment was administered for seven (7) days at 24 hours intervals. Weights of animals were taken every 48hours. Caudal blood was plated of freshly and aseptically prepared SSA for enumeration of bacterial load every 48 hours.

#### 2.2.3.3 Collection and preparation of samples

After the administration of last dose of treatment, animals were fasted overnight and sacrificed by cervical dislocation. Blood was collected via cardiac puncture into EDTA-containing and plain sample bottles for hematological assay and serum preparation respectively. Blood in plain bottles was allowed to clot and thereafter centrifuged at 4000xg for 5 minutes. The serum (supernatant) was removed with a Pasteur pipette and used for biochemical assays while the pellet was discarded. The liver was quickly excised from the animal, rinsed in PBS, cut into small pieces and homogenized in ice-cold phosphate buffered saline using a Teflon homogenizer to achieve a 10% liver homogenate used for assessment of some antioxidant assays.

#### 2.2.3.4 Biochemical and hematology assays

Serum biochemical parameters (lactate dehydrogenase (LDH), aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and bilirubin BIL) were

assayed for using spectrophotometric methods as outlined the manufacturers' manuals for each assay kit.

Hematological parameters (WBC, RBC, HGB, HCT, PLT & LYM) were analyzed with the aid of Sysmex Automated Hematology Diagnostic Machine (XP-300), Mundelein, USA.

#### 2.2.3.5 Liver oxidative markers

Concentration of nitric oxide (NO) and malondialdehyde (MDA) as well as activity of superoxide dismutase (SOD) were evaluated in the liver homogenate were determined spectrophotometrically using standard procedures as outlined in the kit's manufacturer's manual.

#### 2.2.3.5 Histological examination

Cross sections of livers and small intestines were prepared and analyzed using standard procedures as described by Di Fiore [20]. After sacrificing the animals, small pieces of liver were allowed to fix in 10% formalin for 48 hours, serially passed through ascending concentration of alcohol for dehydration and cleared in xylene. Embedding was done in paraffin wax. The tissue was sectioned at 4 microns using rotary microtome (LEICA RT2115) and mounted on microscopic slides the sections to pre-labeled slides. The sections were dried on hot plate and ready for stained with Hematoxylin and Eosin. The sections were examined microscopically at X40 and X100 with light microscope.

#### 2.2.4 Statistical analysis

Data were expressed as mean  $\pm$  SEM (n=3 for in vitro and n=4 for animal samples analysis) and analyzed by one-way analysis of variance (ANOVA) test using GraphPad Prism5. The difference between mean were analyzed by Duncan's multiple range test (DMRT) at level  $p < 0.05$ .

### 3. RESULTS AND DISCUSSION

#### 3.1 *In vitro* Anti-salmonella Activities of *Azadirachta indica* and *Psidium guajava* Leave Extracts and Fractions

All four fractions of *P. guajava* (hPg, cPg, ePg and aPg) inhibited *S. Typhi* growth on MHA plates with diameters of inhibition zones ranging from 12 to 15.5mm while the chloroform (cAi), ethyl acetate (eAi) and aqueous (aAi) fractions of *A. indica* both had *in vitro* anti-salmonella activity with diameters of between 11 and 13mm (Table 1). *n*-hexane fraction of *A. indica* (hAi) did not inhibit *S. Typhi* on agar plate. cPg and ePg has the lowest and highest MIC values respectively. cPg has the highest MBC to MIC ratio. cAi had MBC of 6.25mg/ml whereas other fractions has MBC of  $\geq 25$ mg/ml (Table 1). hPg, cPg, ePg, cAi and aA inhibited 62.45, 51.67, 48.60, 56.15 and 50.48% of biofilm formation in *S. Typhi* at 25mg/mL respectively (Fig. 1). The minimum biofilm inhibiting concentration (MBIC) ranges from 0.39-12.5mg/mL (Table 1).

**Table 1. *In vitro* activities of *A. indica* and *P. guajava* leave extracts and fractions against *S. Typhi***

Plants	Fractions	ZI (mm) at 50mg/ml	MIC (mg/ml)	MBC (mg/ml)	MBC:M IC	MBIC (mg/ml)
<i>P. guajava</i>	methanol extract (mPg)	15.07 $\pm$ 0.07	3.13-6.25	6.25	1-2	-
	n-Hexane (hPg)	13.33 $\pm$ 0.36	12.5	25	2	12.5
	Chloroform (cPg)	14.33 $\pm$ 0.27	3.13	25	8	12.5
	Ethyl acetate (ePg)	12.00 $\pm$ 0.15	25	25	1	3.13
	Aqueous (aPg)	15.50 $\pm$ 0.26	>25	>25	-	-
<i>A. indica</i>	methanol extract (mAi)	11.00 $\pm$ 0.25	1.56	25	16	-
	n-Hexane (hAi)	-	-	-	-	-
	Chloroform (cAi)	13.00 $\pm$ 0.30	6.25	6.25	1	0.39
	Ethyl acetate (eAi)	11.00 $\pm$ 0.00	6.25	>25	-	-
	Aqueous (aAi)	13.00 $\pm$ 0.11	12.5	25	2	3.13
	Ciprofloxacin (10mg/ml)	28.00 $\pm$ 0.02	0.08	0.08	1	$\leq 0.02$

ZI-zone of inhibition; MIC-minimum inhibition concentration; MBC-minimum bactericidal concentration; MBIC-minimum biofilm inhibition concentration

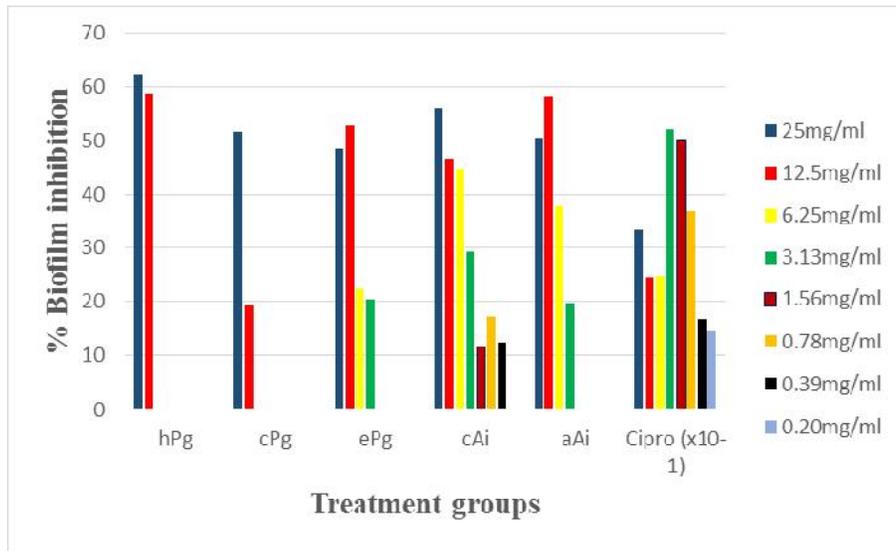


Fig. 1. *In vitro* inhibition of *S. Typhi* biofilm by *P. guajava* and *A. indica* leaves fractions

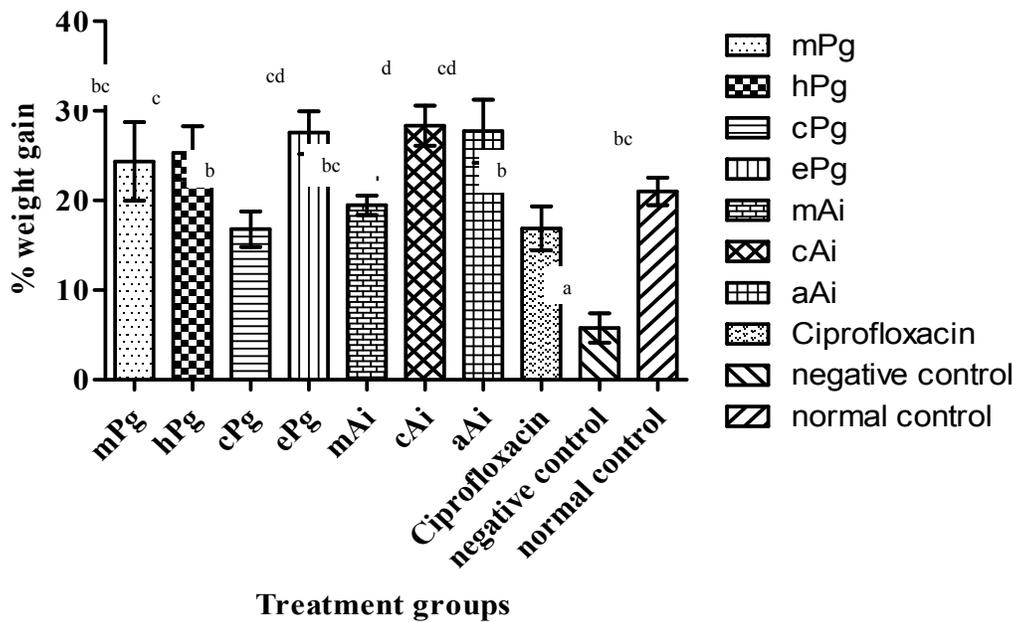


Fig. 2. Effect of administration of extract and fractions of *P. guajava* and *A. indica* on percentage weight gain of *S. Typhi*- infected rats

### 3.2 *In vivo* Experiment

#### 3.2.1 Effect of treatments on body weight of *S. Typhi*-infected rats

There was a significant ( $p < 0.05$ ) weight gain in all the experimental groups. The highest

percentage weight gain ( $28.35 \pm 2.22\%$ ) was observed in cAi-treated *S. Typhi*-infected rats with least in cPg- and ciprofloxacin-treated animals ( $16.80 \pm 1.97$  and  $16.89 \pm 2.45\%$  respectively) compared to a sparsely  $5.79 \pm 1.62\%$  gained by the negative control rats (Fig. 2).

### 3.2.2 Bacteremia progression and inhibition in rats induced with *S. Typhi* and treated with *P. guajava* and *A. indica* leave extracts

As shown in Fig. 3A, bacteremia was established in all infected animals 72 hours post infection and progressed in the negative control group until the end of the experiment. Administration of all extracts and fractions led to reduction in the blood bacterial load in all test groups. The percentage inhibition of bacterial growth in ePg- and hPg-treated groups were significantly ( $p < 0.05$ ) higher than in those treated with ciprofloxacin while those of cAi-, aAi- and mAi-treated groups were not significantly different from that of ciprofloxacin treated group. mPg- and cPg-treated groups recorded lower percentage inhibition of bacterial multiplication in comparison with ciprofloxacin-treated animals (Fig. 3B).

### 3.2.3 Hematological parameters of rats induced with *S. Typhi* and treated with *P. guajava* and *A. indica* leave extracts

Some hematological parameters of all treatment and control groups are presented in Table 3. There was a slight decrease in the white blood cell (WBC) count of negative control, this was significantly ( $P < 0.05$ ) reversed to values comparable to those of normal control in groups treated with mPg, ePg, mAi, aAi, and ciprofloxacin. WBC count in hPg- and cPg-treated *S. Typhi*-treated animals are not significantly ( $P < 0.05$ ) different from that of negative control while cAi-treated animals recorded values significantly higher than those of normal control.

A significant ( $P < 0.05$ ) drop in relative lymphocyte (LMP %) was observed in negative control, cPg- and ePg-treated groups while there was no significant difference ( $P < 0.05$ ) in same for infected animals administered ciprofloxacin, mAi and aAi when compared with normal control. LMP % in mPg- hPg-, cAi-treated groups were significantly ( $P < 0.05$ ) higher than in normal control (Table 2).

The platelet count (PLT) was significantly ( $P < 0.05$ ) raised in negative control animals compared to normal control. The PLT was not significantly different in groups fed hPg, cPg and ePg when compared to that of normal control group. cAi, aAi and ciprofloxacin-treated animals had PLT count significantly ( $P < 0.05$ ) higher than that of normal control (Table 2).

Although the lowering of the anemic indices (red blood cell count, hemoglobin concentration and hematocrit) occasioned by *S. Typhi* was not significant ( $p < 0.05$ ) when compared with the normal control group, there were observable amelioration across all fraction-treated groups.

No significant difference ( $p < 0.05$ ) was observed in the concentration of malondialdehyde (MDA) in liver homogenate of animals across all treatment and control groups. (Fig. 3A)

### 3.2.4 Effect of treatment with *P. guajava* and *A. indica* leave extracts on oxidative stress markers in liver of *S. Typhi*-infected rats

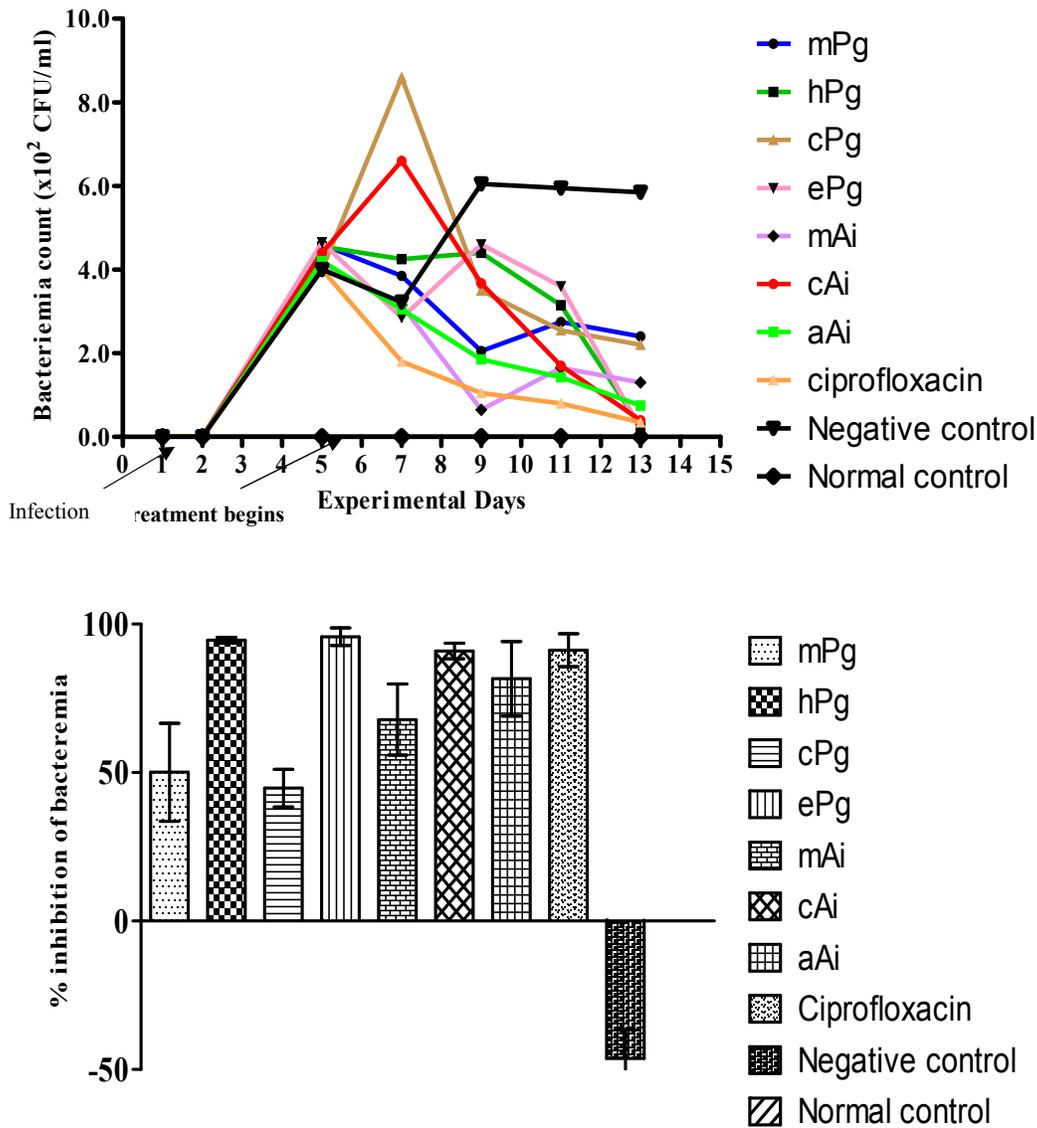
*S. Typhi* infection elicited a was significant ( $p < 0.05$ ) rise in concentration of nitric oxide (NO) in negative control, mPg-, hPg-, cPg-, cAi- and aAi-treated animals when compared to normal control while those of ePg-, mAi- and ciprofloxacin-treated groups were not significantly ( $p < 0.05$ ) different from that of normal control. (Fig. 4A)

As shown in Fig. 4B, activity of superoxide dismutase (SOD) was reduced significantly ( $p < 0.05$ ) in the negative control and mPg-treated groups when compared to normal control, however treatment with mAi and all fraction raises the activity significantly ( $p < 0.05$ ) in the aAi > cPg > ciprofloxacin > mAi > hPg > ePg > cAi.

### 3.2.5 Effect of extracts and fractions *P. guajava* and *A. indica* leave on some biochemical parameters in of *S. Typhi*-infected rats

Although there was no significant ( $p < 0.05$ ) alteration in activity of serum alkaline phosphatase (ALP) in the negative control group when compared to normal control group, administration of the extracts and fraction raised serum ALP activity significantly ( $p < 0.05$ ) (Table 3).

*S. Typhi* infection led to an increase in the activity of serum lactate dehydrogenase (LDH) in negative control significantly ( $p < 0.05$ ), however all extracts and fraction lowered same. LDH activity in mPg-, hPg-, cAi-, aAi- and ciprofloxacin-treated animals are not significantly ( $P < 0.05$ ) different from those of normal control group (Table 3).



**Fig. 3. Bacteriemia in *S. Typhi*-infected rats treated with *P. guajava* and *A. indica* extracts and fractions: (A.) bacteremia progression (B) percentage inhibition of bacteremia by extracts and fraction 7-days post-treatment**

Serum aspartate transaminase (AST) activity was significantly ( $p < 0.05$ ) higher in negative control and mPg-treated animals than in normal control animals. All other treatments lower the activity of serum AST raised by *S. Typhi* infection. Serum AST activity in mAi-, aAi- and ciprofloxacin-treated animals are not significantly ( $p < 0.05$ ) different from those of normal control while those of hPg-, cPg- ePg-treated animals are slightly lower those of normal control (Table 3).

Activity of serum alanine transaminase (ALT) was significantly ( $p < 0.05$ ) higher in negative control group followed by mAi- and cAi-treated groups when compared with normal control. cPg-, ePg-, aAi- and ciprofloxacin-treated animals had serum ALT activity significantly lower than those of negative control but significantly higher than that of normal control. Activity of serum ALT in mPg- and hPg-treated groups was not different from that of normal control (Table 3).

**Table 2. Hematological parameters of *S. Typhi*-infected rats administered extracts and fractions of *P. guajava* and *A. indica***

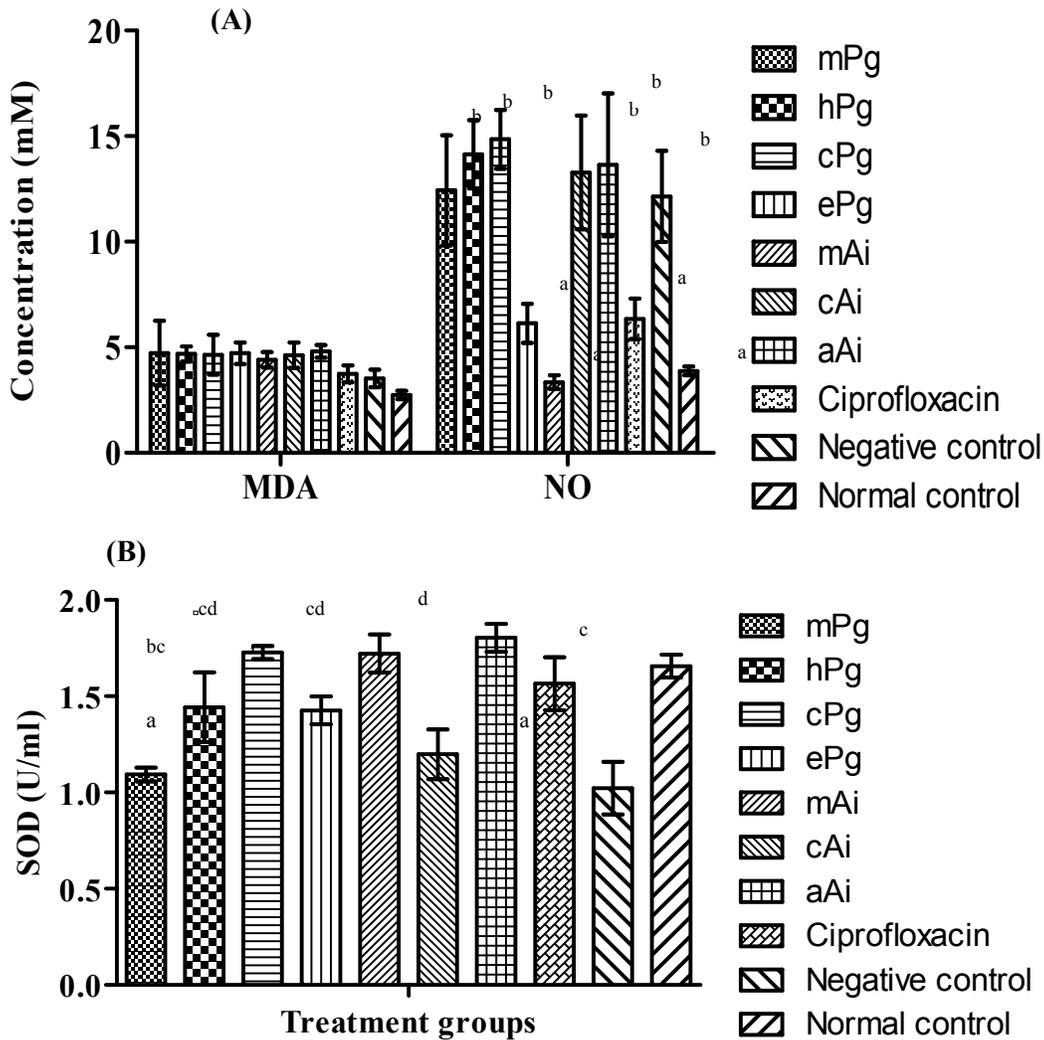
Treatment Group(s)	WBC (X10 <sup>3</sup> /μl)	LYM (%)	RBC (X10 <sup>6</sup> /μl)	HGB (g/dL)	HCT (%)	PLT (X10 <sup>3</sup> /μl)
mPg*	5.90±0.66 <sup>ab</sup>	73.67±5.33 <sup>d</sup>	4.90±1.65	8.93±1.68	26.67±9.84 <sup>d</sup>	416.67±105.93 <sup>a</sup>
hPg*	4.00±0.49 <sup>a</sup>	67.33±2.31 <sup>bcd</sup>	5.40±0.55	10.45±0.29	32.67±2.19 <sup>c</sup>	500.33±6.15 <sup>ab</sup>
cPg*	3.77±0.67 <sup>a</sup>	52.67±5.24 <sup>ab</sup>	3.04±1.38	8.33±0.73	18.00±7.09 <sup>a</sup>	342.67±48.31 <sup>a</sup>
ePg*	4.90±0.72 <sup>ab</sup>	50.00±3.06 <sup>a</sup>	2.86±0.33	8.00±1.35	16.00±1.53 <sup>a</sup>	531.67±40.07 <sup>ab</sup>
mAi*	4.27±0.13 <sup>ab</sup>	58.00±1.15 <sup>c</sup>	5.92±0.28	10.00±0.36	33.33±1.20 <sup>c</sup>	1014.67±24.67 <sup>d</sup>
cAi*	7.10±1.50 <sup>b</sup>	69.33±3.76 <sup>cd</sup>	4.99±0.49	9.50±0.35	30.67±3.48 <sup>c</sup>	753.33±188.97 <sup>c</sup>
aAi*	5.73±1.24 <sup>ab</sup>	61.67±7.22 <sup>c</sup>	5.25±0.71	9.63±0.47	32.00±4.04 <sup>c</sup>	654.00±51.29 <sup>d</sup>
Ciprofloxacin#	6.17±0.72 <sup>ab</sup>	63.67±1.45 <sup>c</sup>	4.76±1.13	10.67±0.03	29.00±6.08 <sup>bc</sup>	602.67±41.16 <sup>d</sup>
Negative control	4.07±1.19 <sup>a</sup>	55.00±7.51 <sup>bc</sup>	3.02±1.86	8.07±2.58	18.33±1.46 <sup>a</sup>	854.00±115.82 <sup>d</sup>
Normal control	4.43±0.29 <sup>ab</sup>	64.00±6.43 <sup>c</sup>	3.98±0.36	9.17±1.35	24.67±2.03 <sup>b</sup>	420.00±21.28 <sup>a</sup>

\*administered at 200mg/kg body weight; # administered at 14.29mg/kg body weight. Values were expressed a mean ± SEM (n=4) and considered significant at P value <0.05. Different alphabet superscript represent significant difference between groups

**Table 3. Some Biochemical parameters of *S. Typhi*-infected rats administered extracts and fractions of *P. guajava* and *A. indica***

Treatment groups	LDH (U/L)	ALP (U/L)	ALT (U/L)	AST (U/L)	TBIL
mPg*	74.23±5.59 <sup>ab</sup>	310.96±83.13 <sup>a</sup>	92.97±6.39 <sup>a</sup>	1127.49±184.11 <sup>b</sup>	174.15±9.41 <sup>a</sup>
hPg*	64.55±3.73 <sup>ab</sup>	702.88±94.13 <sup>cd</sup>	109.36±8.81 <sup>a</sup>	578.27±40.00 <sup>a</sup>	198.57±24.36 <sup>ab</sup>
cPg*	90.37±0.00 <sup>b</sup>	590.02±122.27 <sup>c</sup>	142.64±6.62 <sup>bc</sup>	534.08±143.04 <sup>a</sup>	171.19±3.98 <sup>a</sup>
ePg*	118.34±2.15 <sup>b</sup>	420.75±28.75 <sup>ab</sup>	144.40±8.69 <sup>c</sup>	686.68±94.02 <sup>a</sup>	206.52±4.13 <sup>ab</sup>
mAi*	137.70±17.61 <sup>bc</sup>	390.69±54.42 <sup>ab</sup>	164.79±10.50 <sup>cd</sup>	919.15±70.09 <sup>ab</sup>	240.09±15.61 <sup>bc</sup>
cAi*	68.85±2.15 <sup>ab</sup>	440.37±16.26 <sup>bc</sup>	178.06±10.07 <sup>d</sup>	597.30±22.97 <sup>a</sup>	337.61±7.29 <sup>e</sup>
aAi*	77.46±3.73 <sup>ab</sup>	838.43±33.07 <sup>d</sup>	146.90±3.50 <sup>c</sup>	837.92±271.68 <sup>ab</sup>	268.53±3.21 <sup>cd</sup>
Ciprofloxacin#	60.24±7.76 <sup>a</sup>	544.03±71.50 <sup>bc</sup>	148.28±4.64 <sup>c</sup>	729.51±48.17 <sup>ab</sup>	288.14±27.48 <sup>d</sup>
Negative control	161.37±68 <sup>c</sup>	582.67±47.18 <sup>b</sup>	186.19±14.59 <sup>d</sup>	1097.92±81.44 <sup>b</sup>	292.74±18.40 <sup>d</sup>
Normal control	71.00±3.73 <sup>ab</sup>	579.60±154.64 <sup>b</sup>	116.74±9.06 <sup>a</sup>	733.58±10.01 <sup>ab</sup>	284.61±4.34 <sup>cd</sup>

\*administered at 200mg/kg body weight; # administered at 14.29mg/kg body weight. Values were expressed a mean ± SEM (n=4) and considered significant at P value <0.05. Different alphabet superscript represent significant difference between groups



**Fig. 4. Oxidative stress markers of *S. Typhi*-infected rats administered extracts and fractions of *P. guajava* and *A. indica*: (A) concentration of malondialdehyde (MDA) and nitric oxide (NO) (B) activity of superoxide dismutase (SOD)**

Total bilirubin level was elevated significantly ( $p < 0.05$ ) in negative control, cAi- and ciprofloxacin treated groups compared with normal control group. aAi-treated rats had their total bilirubin levels not significantly different from those of normal control while total bilirubin level in other groups was lower. Direct bilirubin level was significantly ( $p < 0.05$ ) higher in cAi- and aAi-treated animals and lower in all other groups when compared with normal control group. Concentration of indirect bilirubin was significantly elevated in negative control, mAi-, cAi- and ciprofloxacin treated animals in comparison with normal control while it was

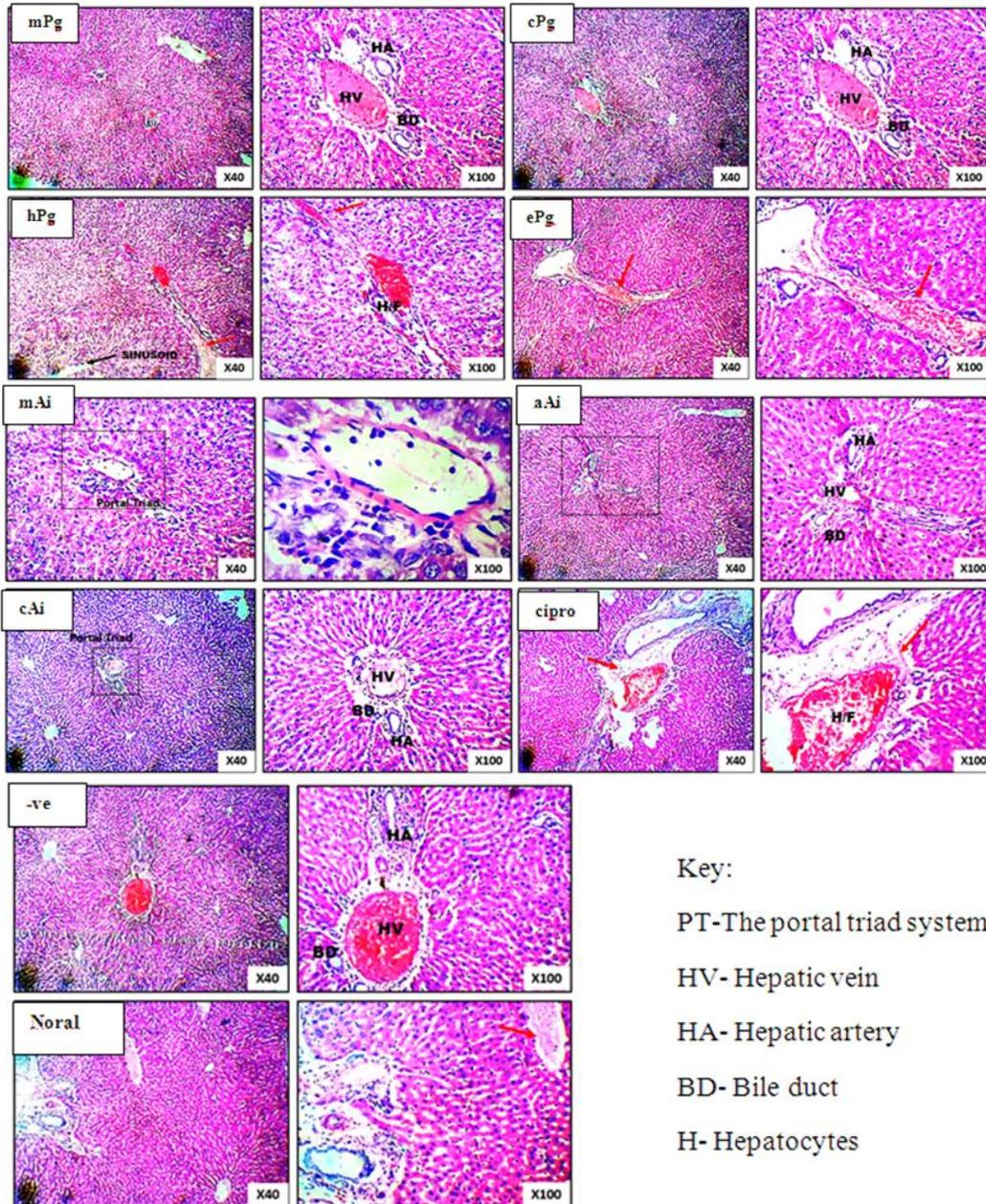
significantly lowered in other treatment groups (Table 3).

### 3.2.6 Liver histological study

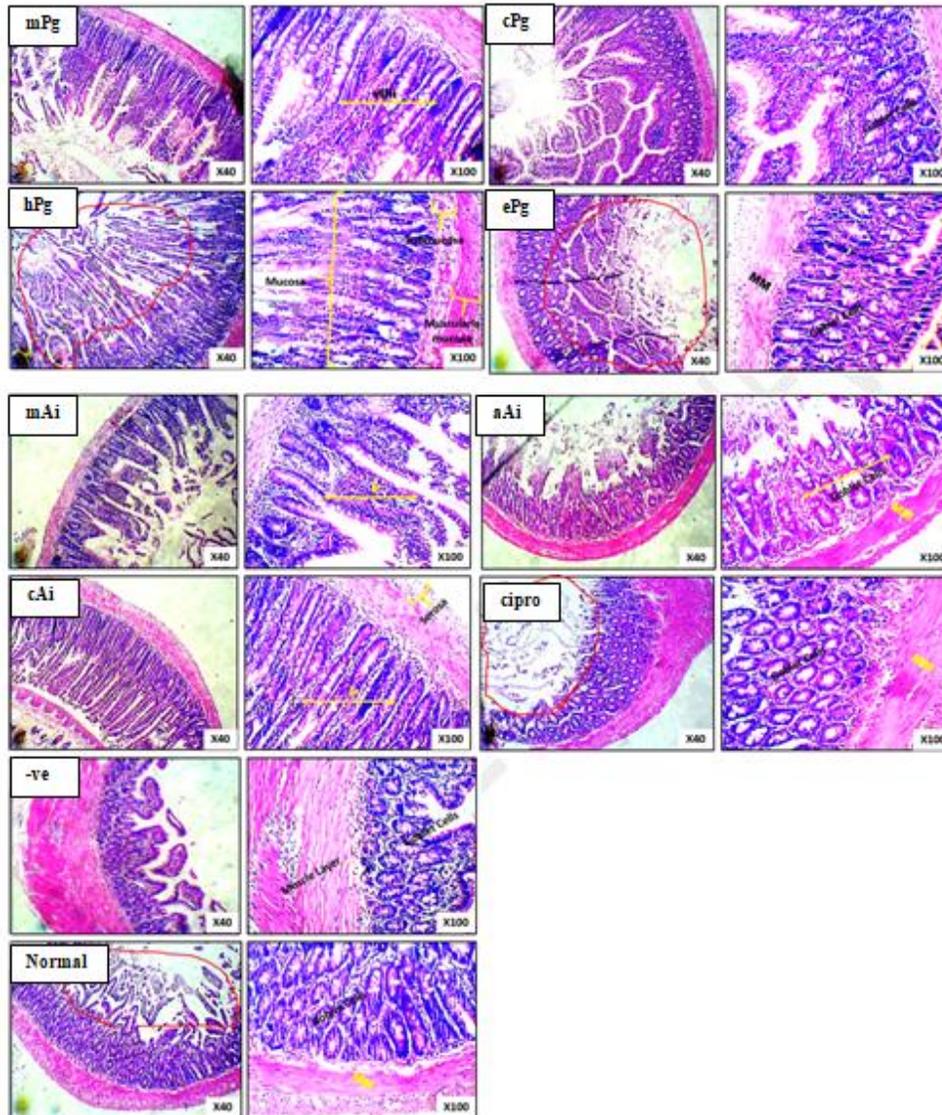
Presented in Plate 1 are photomicrographs of representative livers of rats from each experimental group. Comparative observation across the micrographs shows some well outlined arrays of hepatic cells and vessels, without any observable cytoarchitectural distortion seen in groups treated with mAi, cAi and aAi treatments. Mild structural alterations that is characterized with mild fibrosis,

disorganized portal triad system (mildly poor layering), some hemorrhage localized within the walls of the portal vessels is seen groups treated with mPg and cPg as well as negative control groups. Observable severe bleeding and fibrosis across the hepatic profile were seen in hPg-, ePg- and ciprofloxacin-treated groups. Also seen in this groups include; distorted blood vessel

walls and some degenerative hepatocytes, presence of inflammatory cells within and around the central vein with sinusoids. There were also variations in the sizes and shapes of the nuclei with foci sclerosis of the portal vessels. Some regenerating hepatocytes clustered around the portal triad were seen in normal control group.



**Plate 1. Representative light photomicrographs livers of *S. Typhi*-infected rats administered extracts and fractions of *P. guajava* and *A. indica* (x40); magnified view at (x100) (H & E)**



**Plate 2. Representative light photomicrographs ileum of *S. Typhi*-infected rats administered extracts and fractions of *P. guajava* and *A. indica* (x40); magnified view at (x100) (H & E)**

### 3.2.7 Ileum histological study

There were no appreciable observable significant structure alteration mAi-, cAi-, aAi- treatment and normal control groups. Ileums of hPg-, ePg-, ciprofloxacin-treated animals showed hypertrophy of the muscularis layer (red circle), considerably distorted mucosa layer, mild cellular fragmentations and vacuolation as well as condensation of Brunner's glands (BG). There was mild cellular fragmentations and vacuolation in groups treated with mPg, cPg and negative control (Plate 2).

### 4. DISCUSSION

Treatment of typhoid fever ever has become suboptimal due to advent of multi-drug resistant (MDR) strains of *S. Typhi*. Inability of current treatment to completely eradicate the carrier status of the disease has maintained the bacterial infection in human population till date. In recent decades, attention has shifted to herbal sources of curatives. Methanol extracts of both *P. guajava* and *A. indica* showed inhibitory activities against *S. Typhi in vitro*, thus their solvent fractions were investigated for various

activities against the bacterium. Hexane and ethyl acetate fractions were considered bactericidal since their MBC: MIC <4 while chloroform fraction was bacteriostatic. (MBC: MIC>4). Only the chloroform and aqueous fractions of *A. indica* (cAi and aAi) showed considerable *in vitro* activities with both of them being bactericidal in nature. Antibacterial agents with MBC: MIC ≤4 are termed bactericidal while those with values greater than 4 are bacteriostatic [21]. Ethyl acetate fraction of *A. indica* was not investigated further owing to a MIC value of >25mg/ml. The ability of these fractions to inhibit *S. Typhi* on agar plate could be attributed to the presence of phytochemicals with ability to interrupt vital bacterial processes. These findings are in tandem with those of [22] who reported a satisfactory result for hot water extract of *P. guajava* leaves against *Salmonella sp.* and *E. coli*. Since being bacteriostatic or bactericidal does not impact on clinical outcomes of antibacterial agents [23] as well as their anti-biofilm activities, all five active fractions were considered for anti-biofilm testing. Formation of *S. Typhi* biofilm on gall stones as well as epithelial tissue of gall bladder has been implicated in the development of typhoid carrier status, which is in turn responsible for the persistence of the disease in human population. It is, therefore, pertinent to source antibacterial agents with inherent anti-biofilm activity [24].

All five active fractions (hPg, cPg, ePg, cAi and aAi) inhibited biofilm formation in *S. Typhi* at various concentration and to different extent. Although, *A. indica* had activities in only two fractions, the anti-biofilm activity was more pronounced than that of *P. guajava* fractions, with low MIBC values. Potentials of *A. indica* in inhibiting bacterial biofilm in gram-negative bacteria have been reported. Possible mechanisms of actions include disruption of cell wall and inhibition of quorum sensing as well as reducing the level of biofilm components in as in *Pseudomonas aeruginosa* [25].

The *in vivo* study accented to the *in vitro* anti salmonella effects of the active fractions of *P. guajava* (hPg, cPg and ePg) and *A. indica* (cAi and aAi). Two fractions from each plant achieved inhibition rate not significantly different from what was achieved by ciprofloxacin. Anti-infective agents with ability to relieve the host of pathogen load are capable of improving the overall treatment outcome. Antibacterial activities have been associated with many plant secondary metabolites, both polar (tannins, glycosides etc.)

and non-polar (e.g sterols) which either act directly on the bacterial and/or induce the immune system to fight off the infection. Antibacterial activities of both *P. guajava* and *A. indica* against gram negative bacteria have been reported [26]. There is a correlation between percentage inhibition of bacterial multiplication and weight gain. The weight gain could be attributed to improved appetite and absorption of nutrient which salmonella infection normally impaired during salmonella infection. Protection of the ileum against perforation as seen in the histological examination of the ileum could have contributed to improved absorption of nutrients in the groups treated with fractions of *A. indica*.

One of the ways salmonella exerts virulence is suppression of the bone marrow and haemophagocytosis [27], thereby hampering the response of the immune system to the bacterial infection. Anemia and thrombocytopenia may also result. Relieving the bone marrow suppression and subsequent induction of the host innate immunity could also be a mechanism of actions the fractions as depicted by significant rise in total white blood cell count (WBC) and relative lymphocyte (%LMP) in the test groups. Nwankpa *et al.* [28] reported a similar outcome with ethanol leave extract of *Phyllanthus amarus* in *Salmonella Typhi*-infected rats. The slightly altered anemic indices were also ameliorated by treatment with the fractions.

Nitric oxide (NO) and superoxide dismutase (SOD) are known oxidative stress markers associated with typhoid fever. NO, and other reactive nitrogen species are effectors of the innate immune system that responds to salmonella invasion. This rise in NO was reversed only in ePg-treated animals despite the lowering of bacteria load by all fractions. Reasons for this might be due to the fact that the bacteremia was not zeroed as at the time the experiment was terminated. SOD, an important and first line defense against ROS catalyses the disintegration of superoxide anion ( $O_2^-$ ) to oxygen ( $O_2$ ) and hydrogen peroxide ( $H_2O_2$ ) and thus protects cells against damaging effects of superoxide anion, the later will further brake down to oxygen and water by catalase. Both salmonella and the host produces enormous amount of  $O_2^-$  and thus the enzyme SOD becomes overwhelmed during active infection. This result in depression of SOD, this depression of activity was relieved in all fraction-treated animals. This indicated antioxidant values of these fractions and consequently, protection of

the liver against the damaging effects of free radicals occasioned by of salmonella invasion of the vital organ.

Alanine transaminase (ALT) and aspartate transaminase (AST) are commonly used biomarkers of liver necrosis. Levels of these intracellular enzymes rise in the serum during tissue damages that disrupt the integrity of cell membrane. Although AST can be found in extra hepatic tissues including the heart and kidney, concomitant rise in serum AST activity alongside that of ALT can point to hepatic damage since both of them are cytosolic enzymes. Invasion of the liver by *S. Typhi* and its attendant consequences (hypoxia, endotoxin production and stimulation of production of cytokines) give rise to elevated serum activities of ALT and AST as seen in the untreated *S. Typhi*-infected animals. Increase in serum activity of lactate dehydrogenase (LDH) has also been attributed to hepatic damage during typhoid fever [29]. Fractions of *P. guajava* performed better in lowering the elevated serum activity of ALT than those of *A. indica*. These definitely owes to the difference in their phytochemical components, however all fractions ameliorated the elevated serum activity of AST and LDH. Serum total bilirubin level is another biomarker of derangement of hepatic functions in typhoid fever as instances of jaundice has been reported in typhoid patients [30-32]. All fractions showed ameliorative potentials in lowering the elevated level of serum total bilirubin as seen in the negative control animals. This efficacy could be due to the protective effects of the fractions on the biliary system. Histological findings however, suggest that the fractions of *A. indica* are more hepatoprotective than those of *P. guajava*. This was corroborated by elevated levels of alkaline phosphatase (ALP). At dose administered, fractions of *P. guajava* could be damaging to the liver contrast to the crude methanol extract.

## 5. CONCLUSION

The study revealed that the anti-salmonella potentials of *P. guajava* leaves are more in n-hexane and ethyl acetate soluble fractions while that of *A. indica* reside more in aqueous and chloroform soluble fractions. Overall, the active fractions of *A. indica* are more tolerated than those of *P. guajava* as seen in the histological examination. These fraction are currently under further investigation with a view to identifying the active anti-salmonella principles.

## SIGNIFICANT STATEMENT

*P. guajava*, *A. indica* extracts have been used since time immemorial for the treatment of typhoid without recourse to ascertain its efficacies by scientific means. This study evaluated the use of *P. guajava* and *A. indica* for the treatment of typhoid.

## ETHICAL APPROVAL

The standard guideline and protocol on animal care, handling and treatment of animals were duly followed as prescribed by the ethical approval given by FBMS LAUTECH, Ogbomoso

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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