Evidence of *Milletia Aboensis* Ability to Reverse the Adverse Haematological Parameters and Gastro-Intestinal Histopathological Changes Caused by *Salmonella Typhi* in Wistar Rats

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Abstract
Salmonella typhi is a human-adapted bacteria and the cause of typhoid fever, a prominent source of global morbidity and mortality. This study investigated haematological and Gastro-intestinal histopathological changes connected with (2.0 x 10^8cfu/ml) Salmonella typhi infectivity and the potential of ethanol root extract of Millettia aboensis (EREMA) to reverse these changes. 51 Wistar rats were divided into six groups: group 1 was normal control without treatment but were given feed and water ad libitum, group 2 was infected without treatment (negative control), group 3, 4 and 5 were infected and treated with 100mg/kg, 200mg/kg and 400mg/kg of the extract respectively, while group 6 was infected and treated with 7.14mg/kg of ciprofloxacin. Treatments were done for fifteen days, following confirmation of infection. The rats were humanely sacrificed using diethyl ether anesthesia and blood samples taken for haematological indices including [Total White Blood Cell count (WBC), Neutrophil count, Lymphocyte count, Monocyte count, Platelet count, Red Blood Cell -(RBC) count, Hemoglobin concentration (Hgb), Hematocrit (HCT), Mean Corpuscular volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC)]. Also a portion of the intestine was harvested and processed for histological assessment. Inoculation with S. typhi caused a significant decrease in the haematological indices except for lymphocytes and monocytes and different extent of damages to the intestinal cells. These were all reversed on treatment with ethanol root extract of Millettia aboensis. Thus the extract demonstrated both anti-Salmonella typhi as well as Gastro-intestinal-curative potentials.

Keywords: *Chromolaena odorata; Haematological; histopathological; Salmonella typhi; Wistar Rats*

Introduction

Crump et al., (2010), explained that typhoid fever so called enteric fever is caused by the human-adapted (*S. typhi*). It has an infective dose range of < 10^8-10^9CFU/ml of the bacteria (Judy, 2010).Typhoid fever is an essential source of morbidity and mortality globally (Buckle *et al*., 2012). The Utah Department of Health (2015) stated that the initial indications of infection with typhoid fever typically include sustained fever, abdominal pain, anorexia, lethargy, malaise, dull continuous headache, and a non-productive cough and elevated frequency of constipation than diarrhoea in adults, adding that nausea and vomiting may also occur. The report continued that, diarrhoea is frequent in children, especially infants under one year and that following the first week, some cases develop a macular rash on the trunk and upper abdomen ("rose spots"), followed by a protracted fever and mental dullness in the second week. In their report they established that other symptoms include intestinal bleeding, slight deafness, parotitis, neurologic symptoms (acute psychosis, myelitis, meningitis, and encephalitis; rare focal central nervous infections) as well as common mild and atypical infections and relapses in up to 15-20% of patients. This causes such patients to remain chronically infected and become carriers and spreaders of the bacteria without having the symptoms anymore. In this stage the bacteria are primarily localized in the biliary tract and gall-bladder (Gonzalez-Escobedo *et al*., 2011). In a research to evaluate the extract and fermented liquors of beniseed in management of diarrhoea in *S. typhi* infected rats, Momoh et al., (2013), described that the organism produced a reduction in packed cell volume, Red blood cell and hemoglobin and an elevation in monocyte, neutrophil and WBC. According to him, the observed differences were not significant. Also in a study to evaluate the anti-*salmonella tphi* potentials of *Chromolaena odorata*, Isirima et al., (2018), reported that inoculation of Wistar rats with *Salmonella typhi* caused a decrease in total white blood cell count (WBC) and neutrophil levels, an increase in the lymphocytes and monocytes levels, decrease in platelets, red blood cell count (RBC), Hemoglobin concentration (HB), percentage hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). The view that typhoid fever causes severe hematological changes which include anemia, leucopenia, thrombocytopenia, eosi-nophilia and sub clinical distributed intra-vascular clotting was described by Abro *et al*., (2009). Two different studies by Khosla *et al*., (1995) and Synder & Hedli (1996) found that *S. enterica* infection decreased MCH, MCV, RBC, PCV, Hb, and MCHC significantly. These researchers associated hemo-phagocytosis to be responsible for hematological changes and added that the observed low levels of Hb, PCV and RBC were attributed to hematopoiesis prevention, causing anemia. In other studies by Dangana *et al*., (2010) and Kayode *et al*., (2011), it was also reported that *S typhi* produced diminished levels of PCV in typhoid and paratyphoid patients and they explained that the destruction of RBC and reduction in Hb were accountable for the diminished levels of MCHC, MCH and MCV. To estimate the Intestinal histopathology in *S. typhimurium* infected mice Zainab (2012), reported hyperplasia of goblet cells and infiltration of inflammatory cells in the lamina propria of atrophic villi. He also reported that electron microscope observation of the tissues showed that *S. typhimurium* lay close to the brush border of the villi of the ileum of infected mice, and observed a degeneration of the brush border and the apical cytoplasm with cavity formation occurring near a bacterium in another section of the tissue. The study also revealed in addition, swelling, budding and elongation of microvilli. Also, in a study to evaluate the effect of *Chromolaena odorata* on the gastro-intestinal tract in Wistar rats, Isirima et al., (2018) reported intensely disrupted tissues in animals inoculated with *S. typhi*. The use of plant extracts for the treatment of human diseases has sure advantages besides being readily available and cheap, they produce little or no adverse effects and are also biodegradable, hence produce no toxic effects to the environment (Ray et al., 2004). This has allowed plant extracts to serve as suitable alternatives to synthetic and chemical drugs, being

loaded with secondary metabolites such as essential oils, anti-bacterial, antifungal agents and other products (Bibalani and Mosazadeh-Sayadmahaleh 2011; and Joudi and Bibalani 2010). Herbal drugs are prescribed extensively because of their less adverse effects, effectiveness, and comparatively low cost (Odhav et al., 2010). One of such plants with medicinal potentials is *Milletia aboensis*. This plant is a specie in the plant kingdom, in the phylum of flowering plants called Magnoliophyta, classified as Magnoiiphisida, in the order of Fabales (milkworts, snakeroot and legumes) which belongs Fabaceae family (Pea family) and *Millettia* Genius (Blessing et al., 2013). The family is mostly shrubs with streaked-dark-reddish or chocolate-coloured wood with typical alternate and compound leaves, having seriated margins and stipule that are either inconspicuous, thorn-like (robinia) or leaf-like (pisum) (Blessing et al., 2013). Leaf margins are entire or occasionally, seriate. Both the leaves and the leaflets often have wrinkled pulvini to permit mastic movements. Most members of the fabaceae accommodate bacteria in their root nodules offering them the ability to 'take-up' nitrogen from the air for nitrogen fixation. The family name fabaceae was coined from Latin word 'Faba' which means 'bean'. Leguminosae is an older name still considered valid (Blessing et al., 2013).

Methods

Plant Collection

The plant was harvested from natural habitat in Ika community, Akwa-Ibom State, Nigeria in the month of September and Plant roots was identified and authenticated at herbarium unit, in the department of plant science and biotechnology, Faculty of Sciences, University of Port Harcourt, Rivers State, Nigeria with herbarium number UPH/P/104 by Mr. Ekeke Chimezie (Ph.D.)

Isolation of Test Organisms

The test organism, *S typhi* was isolated from patients with typhoid fever in University of Port Harcourt Teaching Hospital (UPTH), Rivers State. The enrichment media used in course of the isolation of the organism include; strep-tokinase broth (Watson, 1978) and Bile salt broth (Watson, 1954). The samples presenting perceptible turbidity were sub-cultured on the medium "Mac-Conkey agar". Subsequently, traditional biochemical tests and PCR were used to identify the isolates exhibiting specific colonies

Extraction Method

The bark of the root of the plant were shredded out using cutlass, washed with clean tap water and allowed to dry at room temperature between 32-35^{o}C, until they attained a constant weight. The extraction method used was adapted from Hanan *et al.*, (2013) cold maceration extraction protocol, with diminutive adjustment. The powdered *M aboensis* root bark of about 50g was soaked in 70% ethanol of about 1000ml in a 2 litre flask and mixed forcefully at 1hr intermission, for 12 hrs and allowed to settle over-night (35^{o}C) to allow for adequate extraction. Subsequently, the concoction was filtered by means of a filter paper with pore size of 0.45milli-pore. The concentration of the extract was increase using rotary evaporation process at 40^{o}C and 200 rpm. The final semi-solid extract was obtained by drying the content of the rotary evaporator over a steam bath at 40^{o}C. The resultant extract obtained 20% yield, was kept safe at room temperature in desiccators, until it was needed for the study.

Experimental design

Fifty one (51) animals were separated into 6 groups. Group 1 (normal) had three (3) animals, Group 2 (negative control) had twelve (12) animals, while groups 3-6 each had nine (9) animals. Group 1 animals were not treated throughout the experimental period but were given

free access to normal animal feed and water *ad labitum*. Group 2 contained *Salmonella typhi*-infected rats not treated after disease induction. Group 3 contained *Salmonella typhi*-infected rats treated with100mg/kg (low dose) of *Milletia aboensis* root extract. Group 4 contained *Salmonella typhi*-infected rats treated with 200mg/kg (medium dose) of ethanol root extract of *Milletia aboensis*. Group 5 contained *Salmonella typhi*-infected rats treated with 400mg/kg (high dose) of ethanol root extract of *Milletia aboensis*. Group 6 contained *Salmonella typhi*-infected rats treated with 500mg/70kg (7.14mg/kg) of a standard antibiotic drug (Ciprofloxacin). On day 0, (when the animals were confirmed infected, through observation of anorexia, weakness and diarrhea from the animals as well as isolation of the organism from the animal stool), and at six day intervals and on day sixteen, 3 animals from each group were humanely sacrificed and blood sample was collected for hematological evaluation as well as a portion of the intestine for histopathological examination, respectively.

Challenging apparently healthy animals with *Salmonella typhi*

Forty eight (48) animals (groups 2-6) were orogastrically challenged with an infective dose (2.0 x 10^8cfu/ml) of *Salmonella typhi*. After infection had set in (through observation of signs like weakness, anorexia, non-productive cough, watery stool, standing of the hairs as in cold condition and isolation of the organism from the animal stool) (day 0), three animals were sacrificed and blood samples and liver tissues collected for preliminary screening while the other 45 animals were treated with the ethanol extract of *Milletia aboensis* according to the different doses and the standard antibiotic (Ciprofloxacin), once daily, for fifteen days.

Preparation of the Extract Concentrations and Antibiotic

Stock solution for the extract was prepared by dissolving 500 mg in 1 ml of sterile distilled water. An antibiotic control was made by dissolving 500mg of ciprofloxacin in sterile distilled water.

Blood collection and dissection

Blood was collected from each animal by cardiac puncture method after the animals were anaesthetized with diethyl ether in a desiccator. The blood was immediately transferred into appropriately labelled sample bottles containing anticoagulant and the liver was removed aseptically and was weighed and a portion was kept for histological analysis.

Hematological analysis

Hematological analysis was carried out as described by Ghai, (2007) within 24 h of sample collection, to determine the levels of red blood cell (RBC) and white blood cell (WBC) counts, differential leucocyte count (DLC), platelet count, haemoglobin concentration (Hb), and red cell indices, including mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV) and mean cell hemoglobin concentration (MCHC).

Histopathology studies

The animals were anaesthetized with diethyl ether, dissected aseptically to remove the liver which was then transferred into 10% chloroform and later trimmed down to a size between 2mm to 4mm thickness, to allow the fixative to readily penetrate the tissue. The tissues were exposed to different stages of processing by standard methods as described by Baker (1945), including, fixation, dehydration, clearing, impregnation, embedding, sectioning and staining with hematoxylin and eosin (H&E) and finally mounting.

Statistical analysis

The results are presented as Mean ± Standard error of mean. Differences between means were assessed using Analysis of variance (ANOVA) and post-test using LSD multiple comparison test (Mead, & Curnow, 1982).

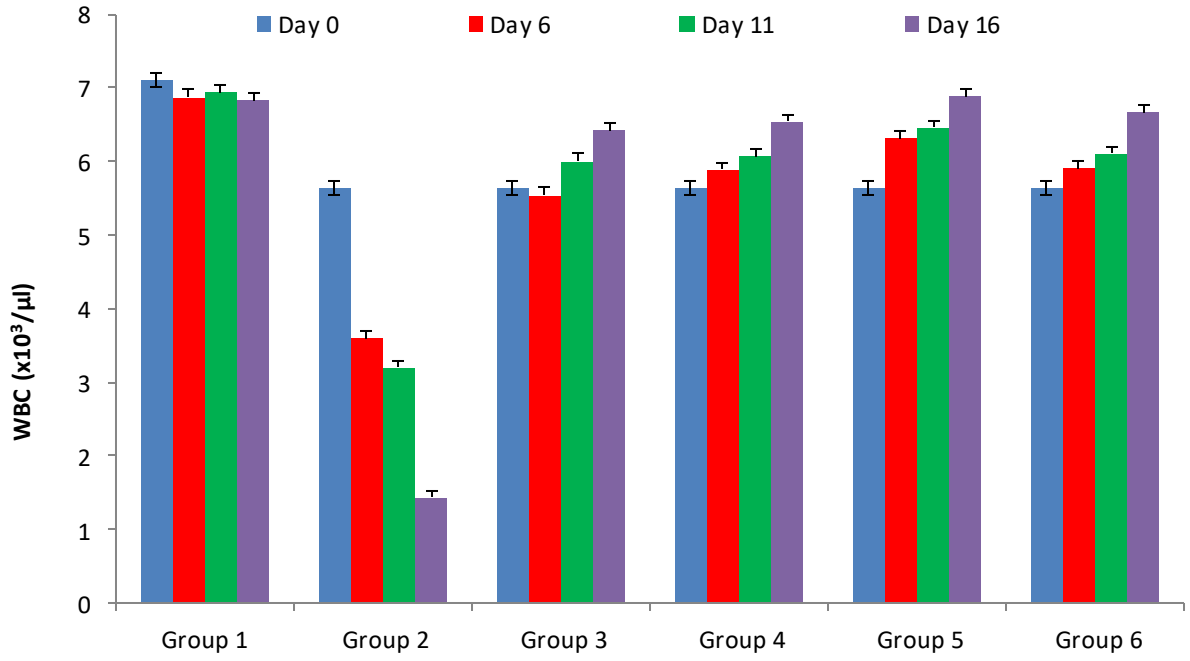

Figure 1: White Blood Cells of Albino Rats exposed to *Salmonella typhi* bacteria before treatment with *Milletia aboensis*

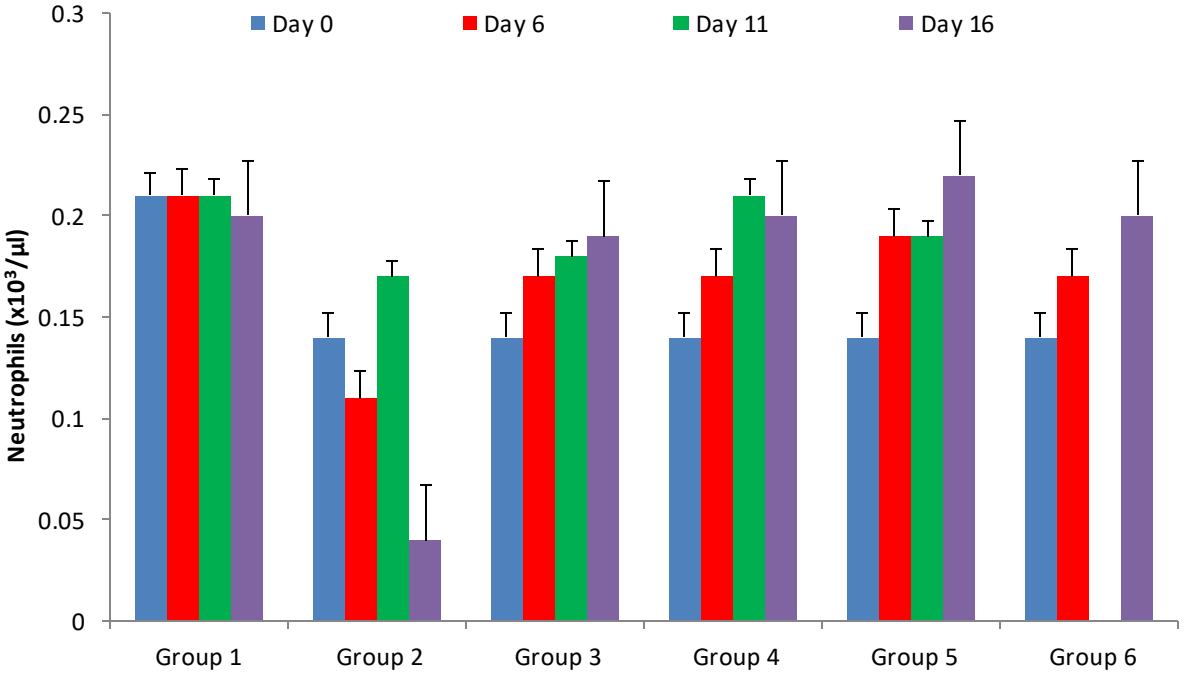

Figure 2: Neutrophils of Albino Rats exposed to *Salmonella typhi* bacteria before treatment with *Milletia aboensis*

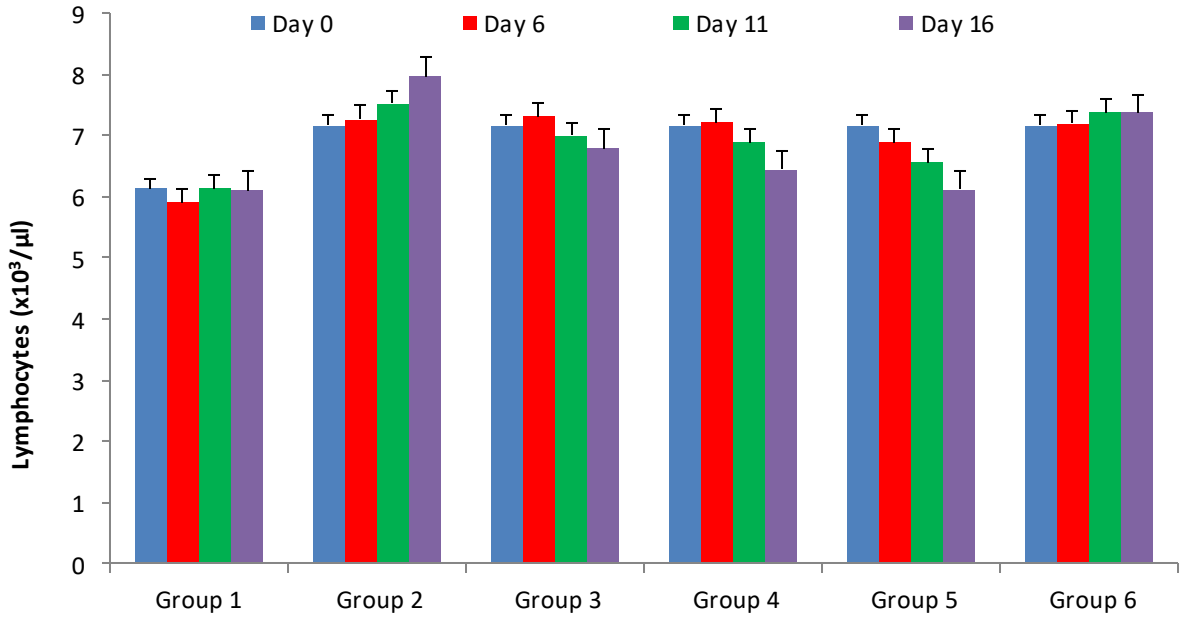

Figure 3: Lymphocytes of Albino Rats exposed to *Salmonella typhi* bacteria before treatment with *Milletia aboensis*

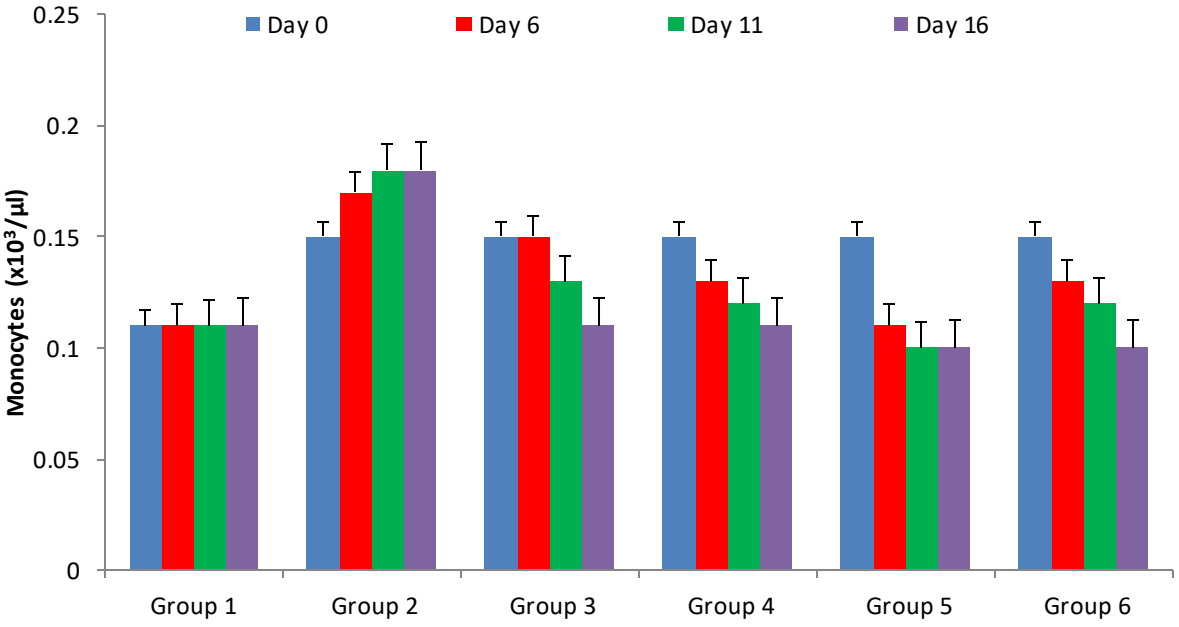

Figure 4: Monocytes of Albino Rats exposed to *Salmonella typhi* bacteria before treatment with *Milletia aboensis*

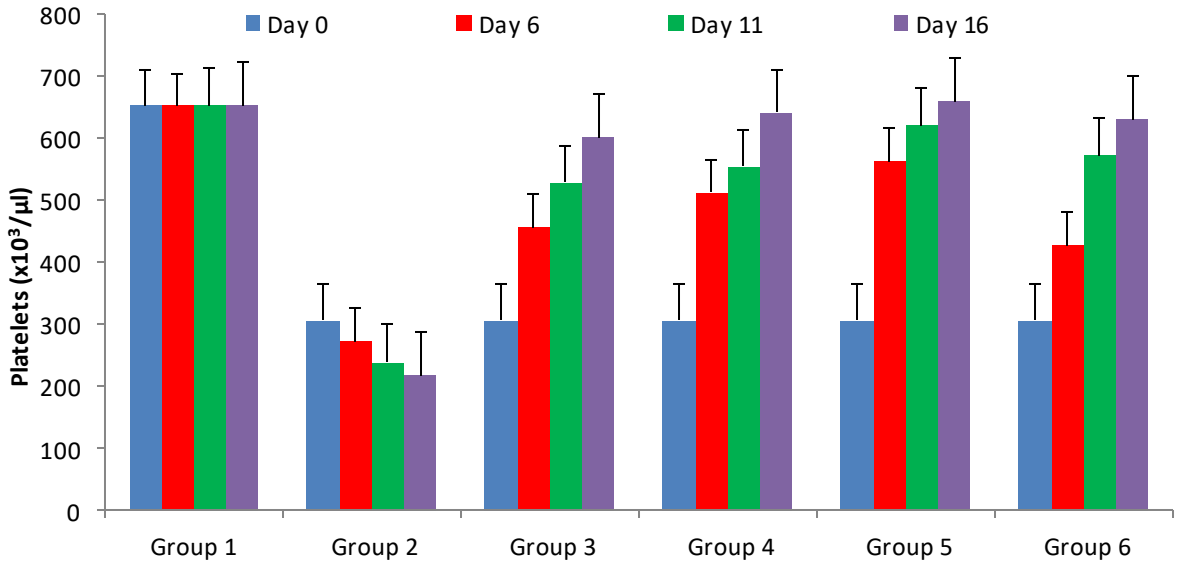

Figure 5: Platelets count of Albino Rats exposed to *Salmonella typhi* bacteria before treatment with *Milletia aboensis*

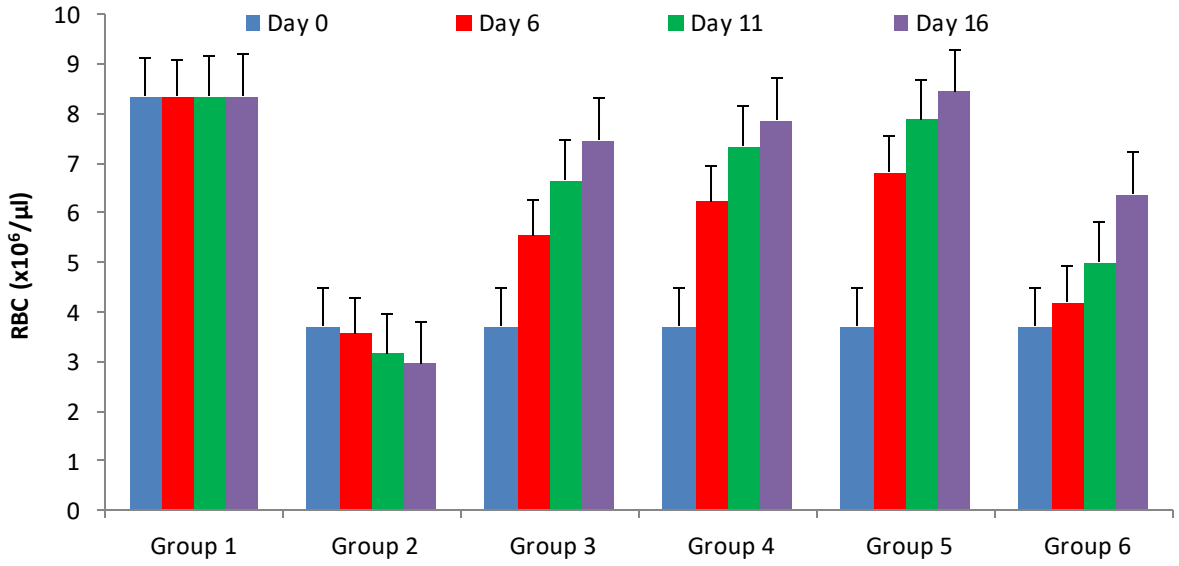

Figure 6: Red Blood Cell (RBC) count of Albino Rats exposed to *Salmonella typhi* bacteria before treatment with *Milletia aboensis*

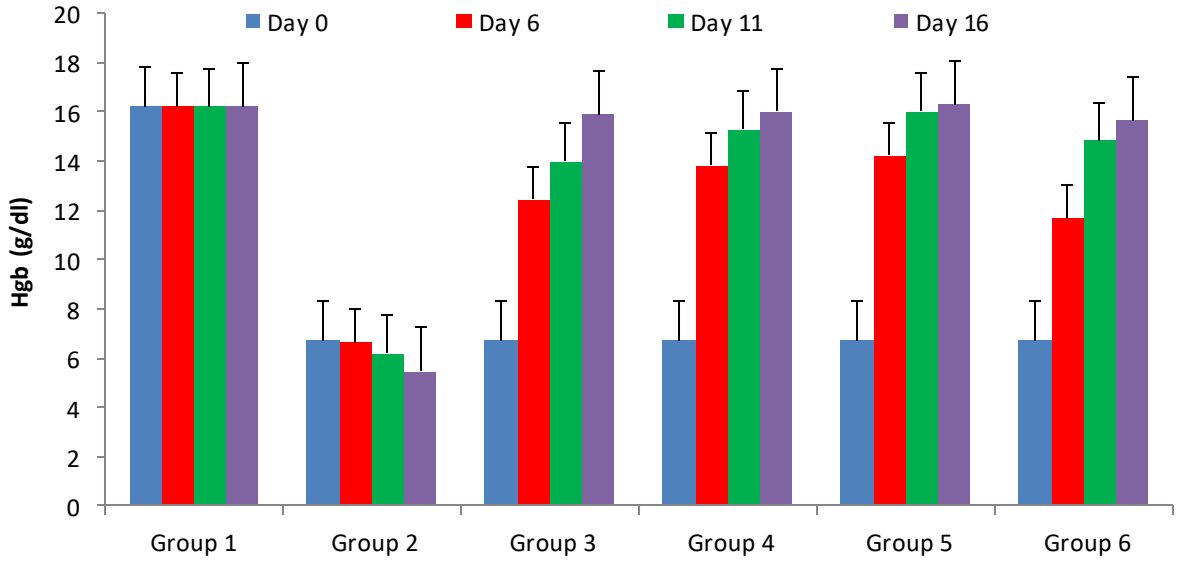

Figure 7: Hemoglobin Concentration (Hgb) of Albino Rats exposed to *Salmonella typhi* bacteria before treatment with *Milletia aboensis*

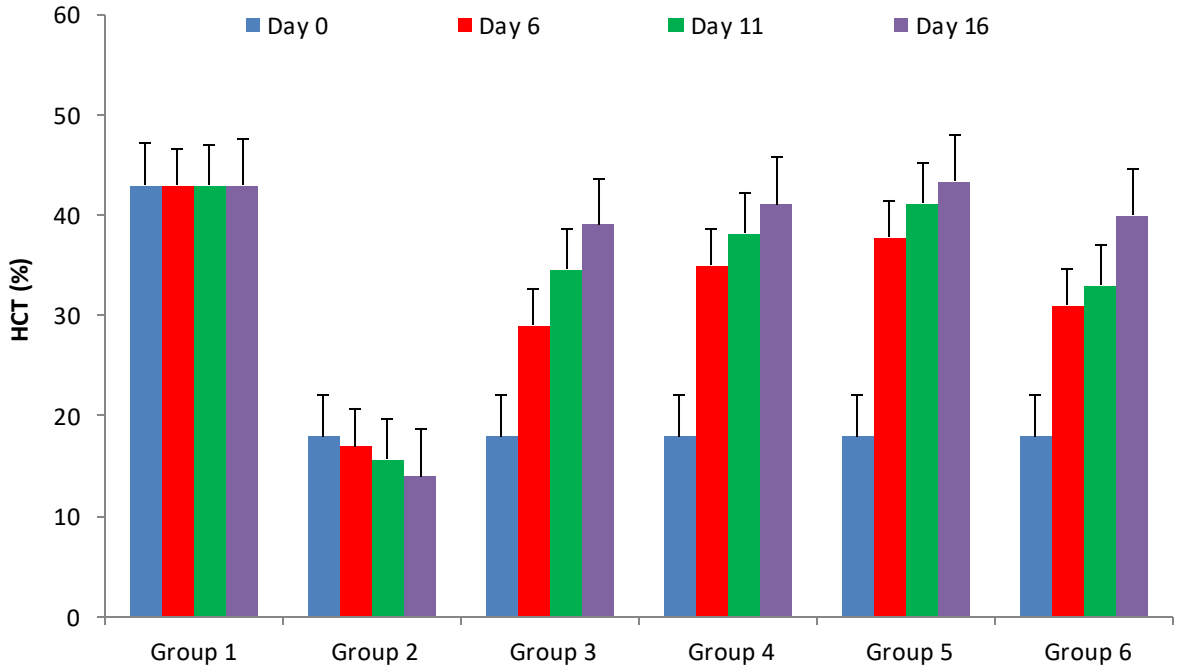

Figure 8: Hematocrit (HCT) of Albino Rats exposed to *Salmonella typhi* bacteria before treatment with *Milletia aboensis*

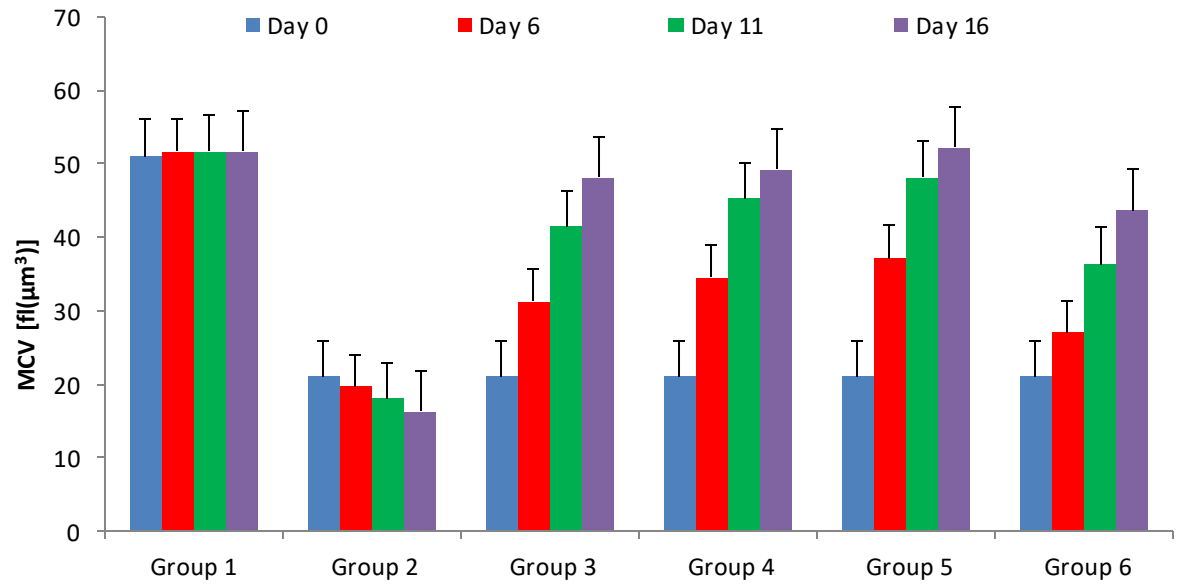

Figure 9: Mean Corpuscular Volume (MCV) of Albino Rats exposed to *Salmonella typhi* bacteria before treatment with *Milletia aboensis*

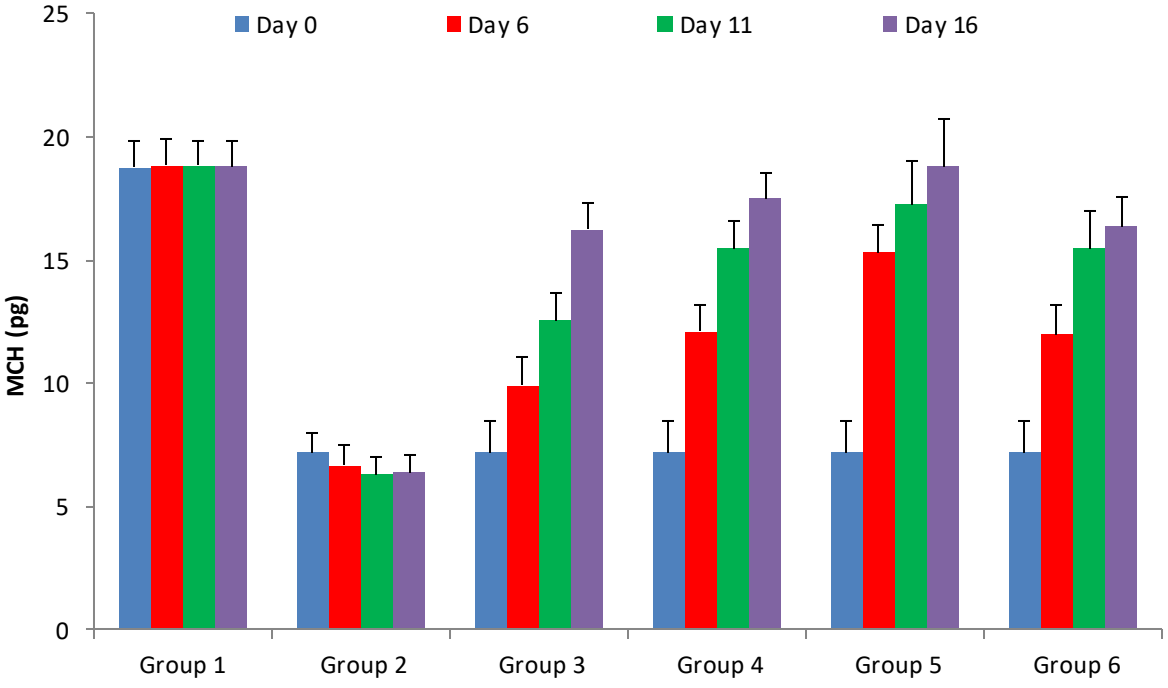

Figure 10: Mean Corpuscular Hemoglobin (MCH) of Albino Rats exposed to *Salmonella typhi* bacteria before treatment with *Milletia aboensis*

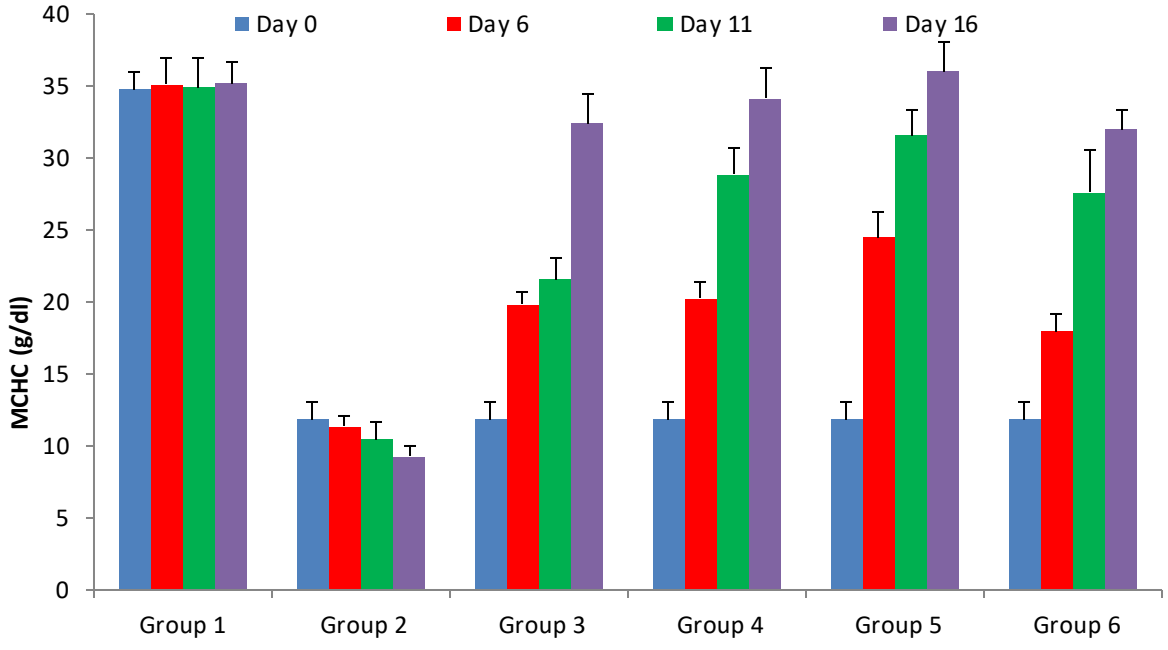

Figure 11: Mean Corpuscular Hemoglobin Concentration (MCHC) of Albino Rats exposed to *Salmonella typhi* bacteria before treatment with *Milletia aboensis*

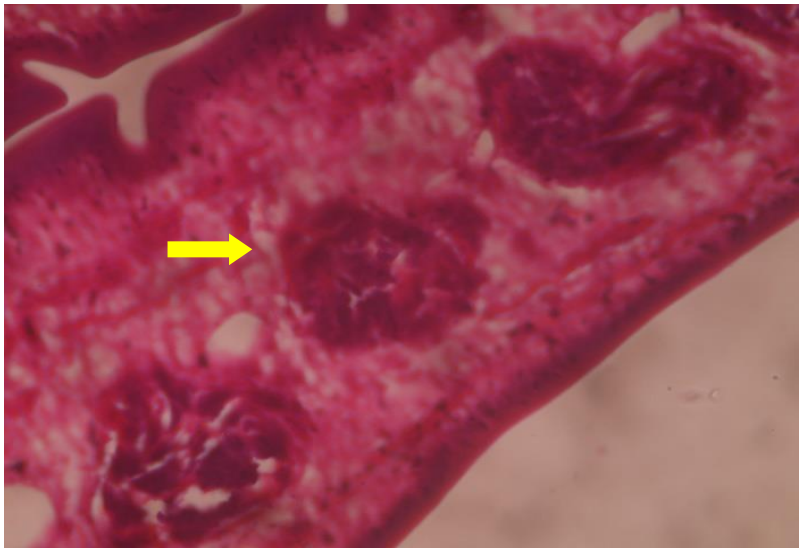

Plate 1: Photomicrograph of intestinal tissues of normal rats (group one) after five days of study, showing normal tissue with the epithelial lining, typically of the columnar variety, as shown by the arrow.

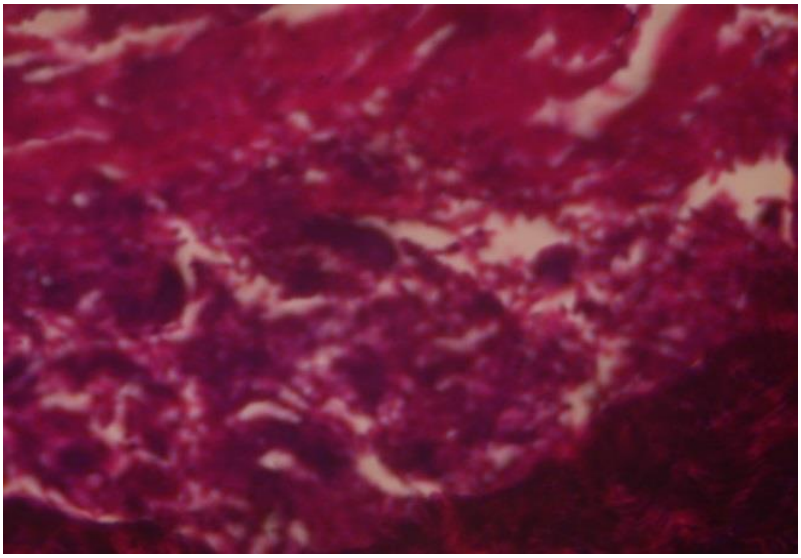

Plate 2: Photomicrograph of intestinal tissues of rats infected with *S. typhi*, without treatment (group two) for five days showing intensely tissue disruption.

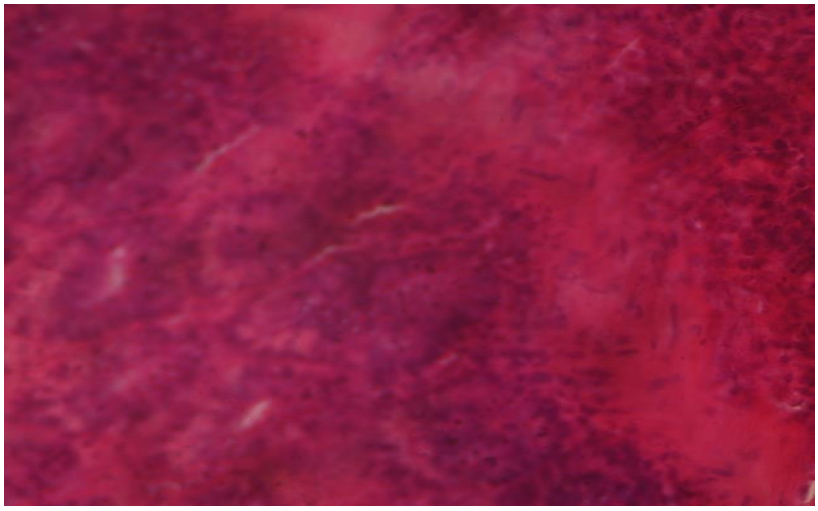

Plate 3: Photomicrograph of intestinal tissues of rats infected with *S. typhi* and treated with 100mg/kg of *M. aboensis* (group three) for five days, showing tissues replete with inflammatory cells (WBCs).

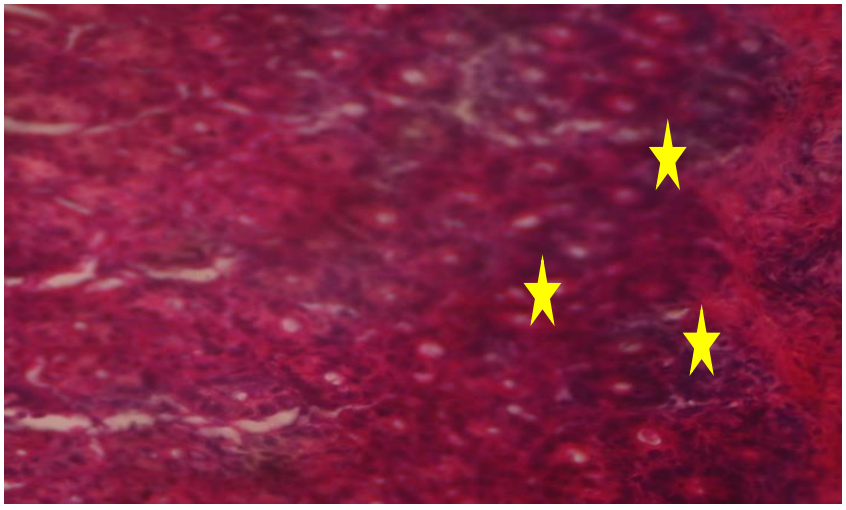

Plate 4: Photomicrograph of intestinal tissues of rats infected with *S. typhi* and treated with 200mg/kg of *M. aboensis* (group four) for five days, showing numerous duodenal glands with the muscularis mucosa seen at the edge (as indicated by the star shapes), without histological disruption.

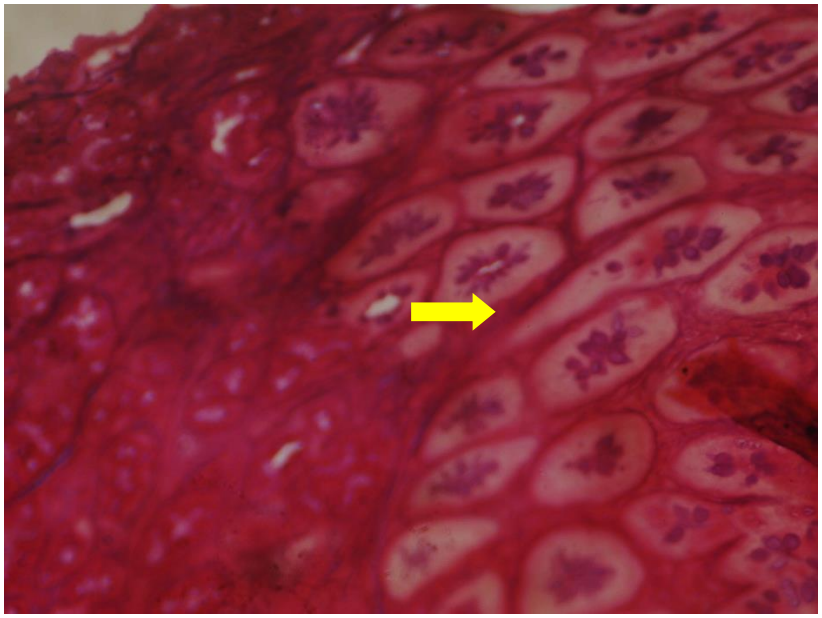

Plate 5: Photomicrograph of intestinal tissues of rats infected with *S. typhi* and treated with 400mg/kg of *M. aboensis* (group five) for five days, showing intact duodenal glands and normal tissue architecture (as indicated by the arrow).

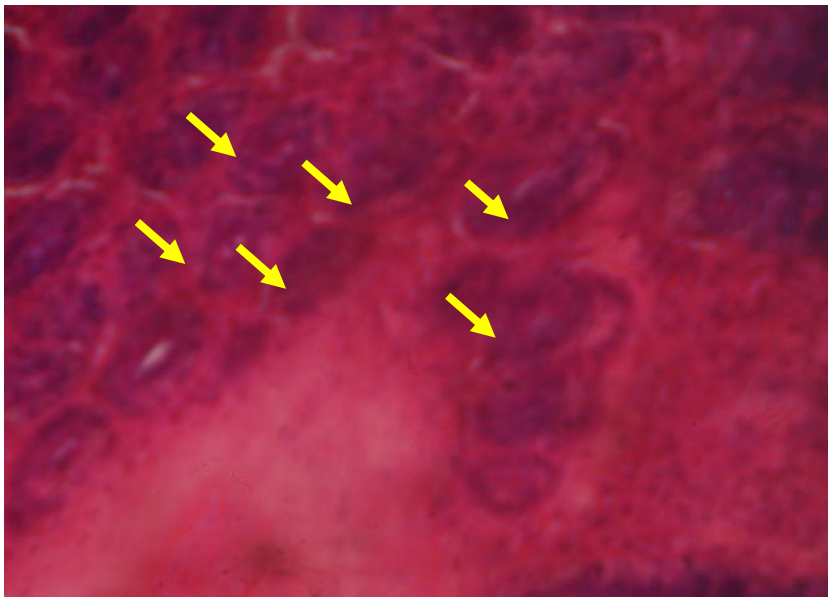

Plate 6: photomicrograph of intestinal tissues of *S. typhi* infected rats treated with 7.14mg/kg of *Ciprofloxacin* (group six) for 5 days, showing replete with transverse sections of the numerous microvilli that are highly basophilic.

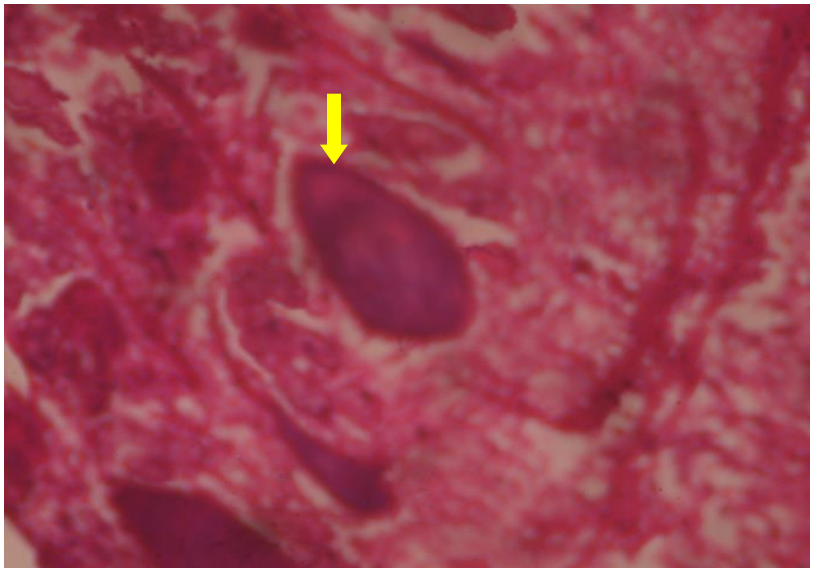

Plate 7: Photomicrograph of intestinal tissues of normal rats (group one) after ten days of study, showing intact duodenal glands and normal tissue architecture as indicated by the arrow.

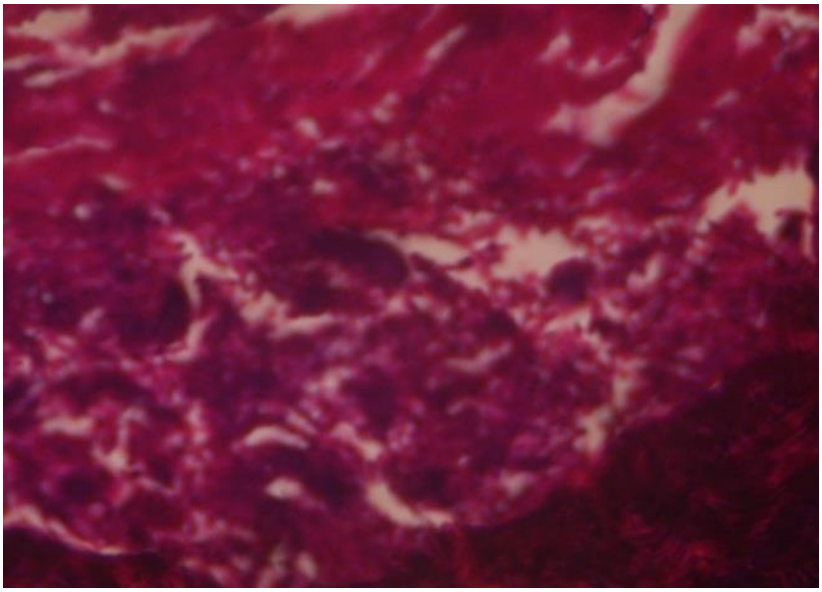

Plate 8: Photomicrograph of intestinal tissues of rats infected with *S. typhi*, without treatment (group two) for ten days showing intensely tissue disruption.

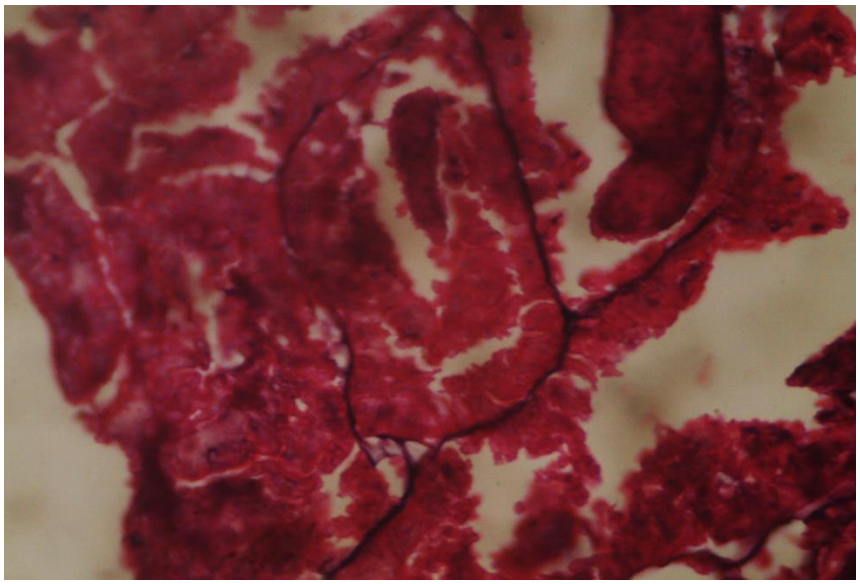

Plate 9: Photomicrograph of intestinal tissues of rats infected with *S. typhi* and treated with 100mg/kg of *M. aboensis* (group three) for ten days, showing disrupted duodenal glands of the small intestine.

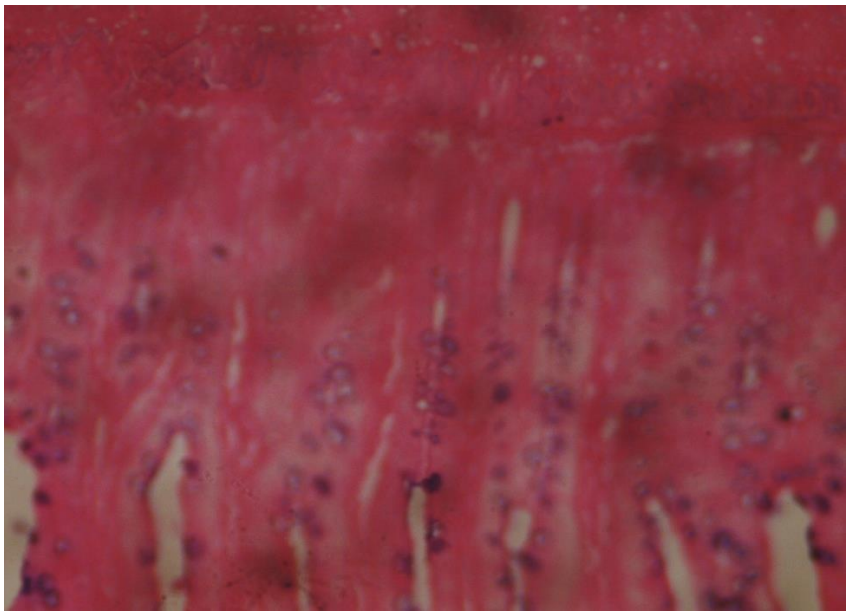

Plate 10: Photomicrograph of intestinal tissues of rats infected with *S. typhi* and treated with 200mg/kg of *M. aboensis* (group four) for ten days, showing disrupted duodenal glands.

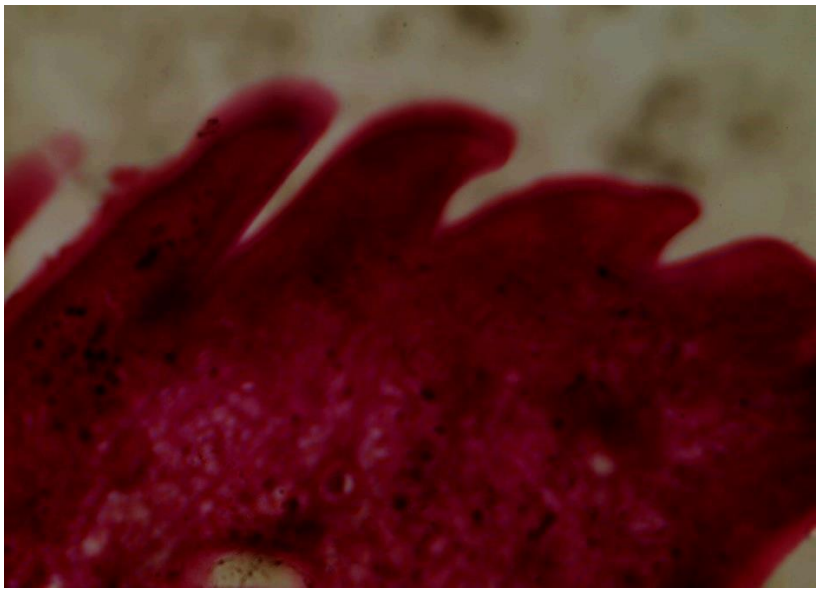

Plate 11: Photomicrograph of intestinal tissues of rats infected with *S. typhi* and treated with 400mg/kg of *M. aboensis* (group five) for ten days, showing villi and epithelial lining characterized by columnar epithelium, with no proliferation of gut associated lymphatic tissue cells (normal).

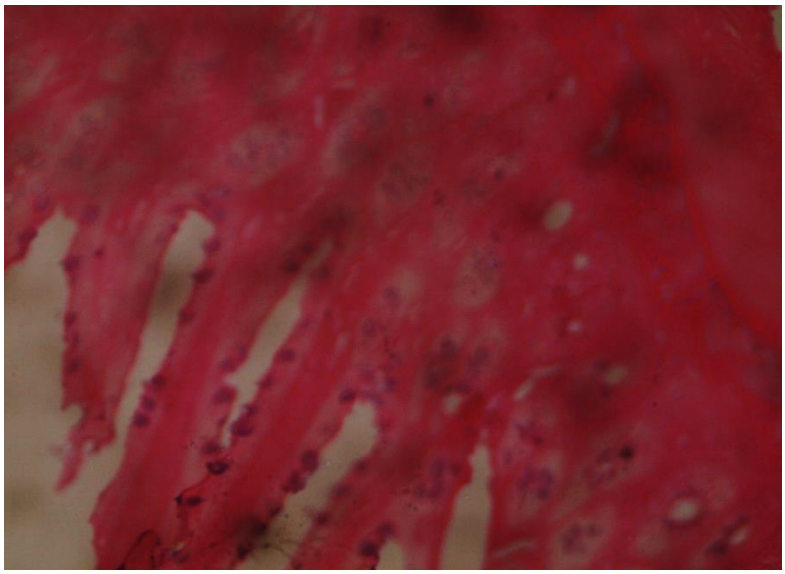

Plate 12: photomicrograph of intestinal tissues of *S. typhi* infected rats treated with 7.14mg/kg of *Ciprofloxacin* (group six) for 10 days, showing intact duodenal glands and normal tissue architecture.

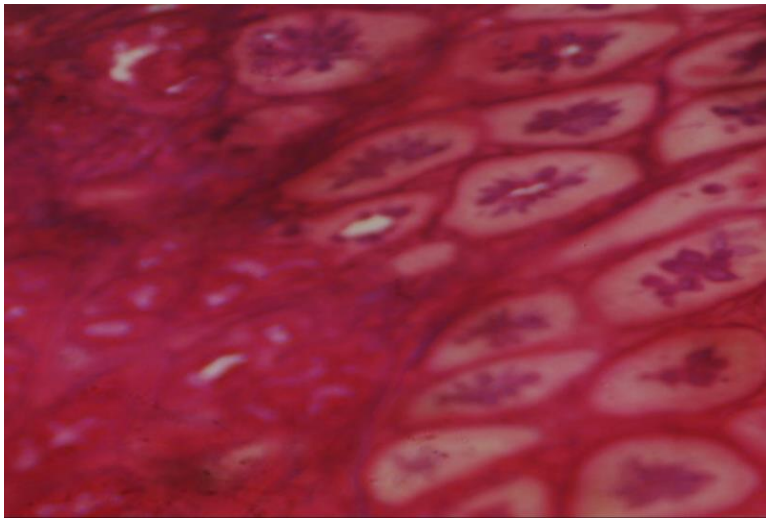

Plate 13: Photomicrograph of intestinal tissues of normal rats (group one) after 15 days of study, showing normal architecture and intact duodenal glands.

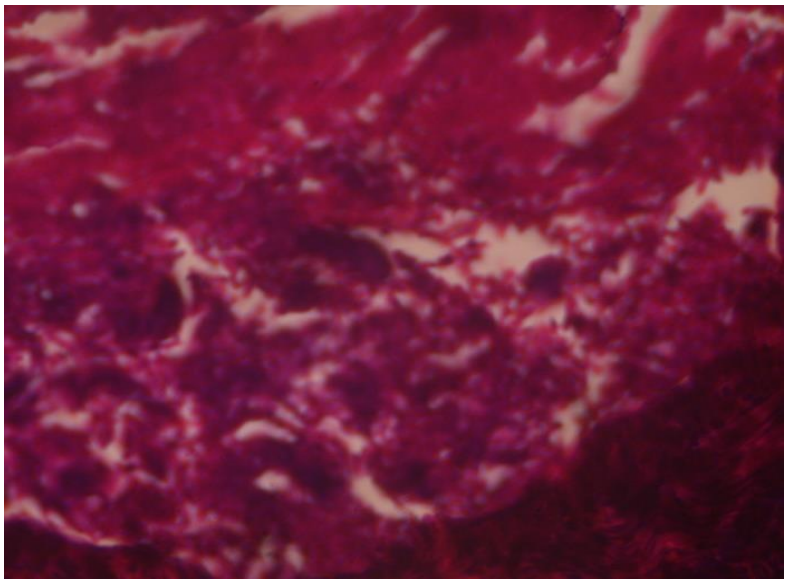

Plate 14: Photomicrograph of intestinal tissues of rats infected with *S. typhi*, without treatment (group two) for 15 days showing intensely tissue disruption.

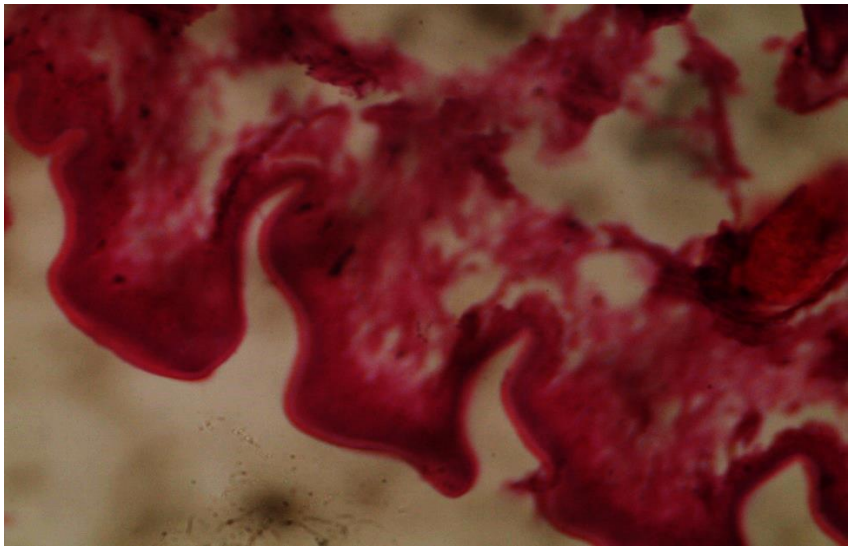

Plate 15: Photomicrograph of intestinal tissues of rats infected with *S. typhi* and treated with 100mg/kg of *M. aboensis* (group three) for 15 days, showing disrupted duodenal glands of the small intestine.

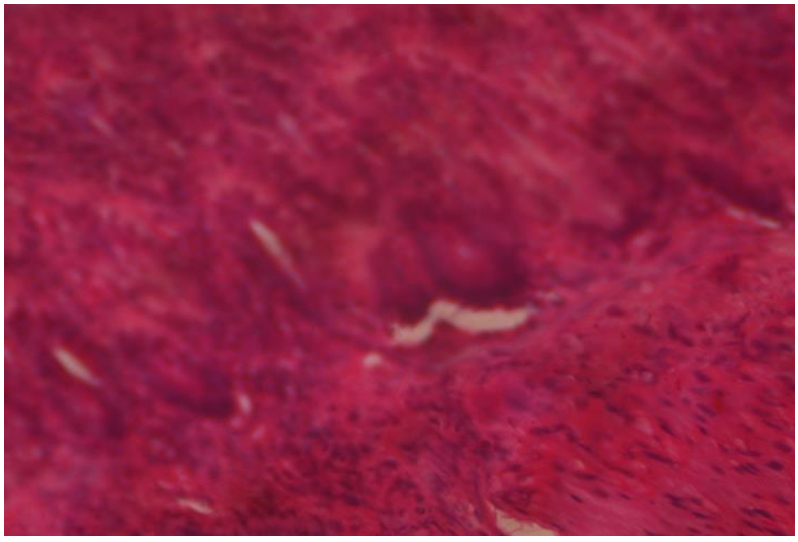

Plate 16: Photomicrograph of intestinal tissues of rats infected with *S. typhi* and treated with 200mg/kg of *M. aboensis* (group four) for 15 days, showing normal tissues of the junction between the inner circular and outer longitudinal muscle layer.

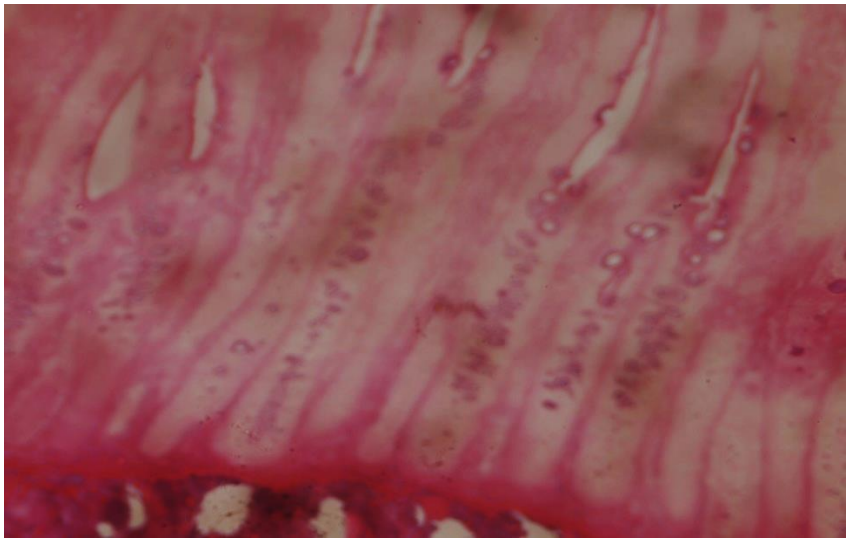

Plate 17: Photomicrograph of intestinal tissues of rats infected with *S. typhi* and treated with 400mg/kg of *M. aboensis* (group five) for 15 days, showing normal tissues with intact villi.

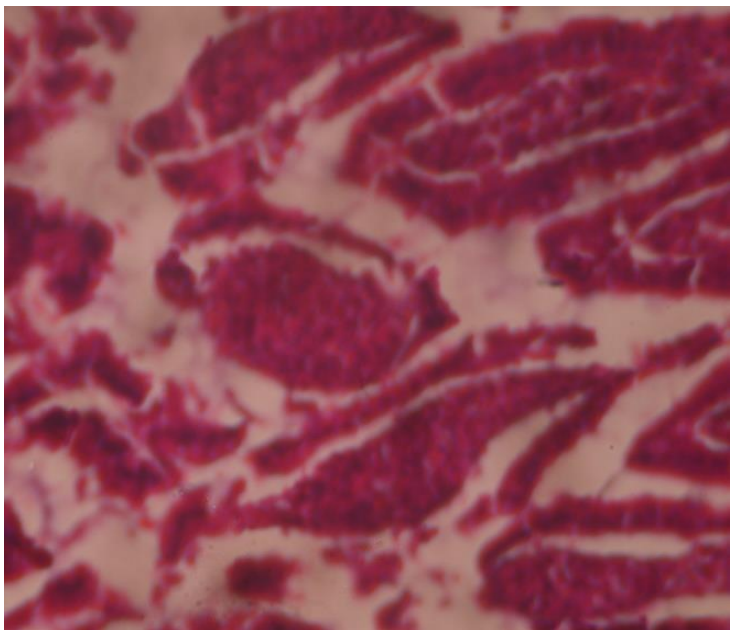

Plate 18: photomicrograph of intestinal tissues of *S. typhi* infected rats treated with 7.14mg/kg of *Ciprofloxacin* (group six) for 15 days, showing villi with columnar epithelium, without pathologies seen.

Results

Inoculation of rats with *S. typhi* showed reduction in neutrophil and WBC levels and a non-significant elevation in monocytes and lymphocytes levels compared to normal. These modifications were nullified on treatment with the extract and Ciprofloxacin, excluding lymphocytes. Treatment with ethanol root extract of *Milletia aboensis* and Ciprofloxacin demonstrated a rise in WBCs, neutrophils, lymphocytes and a trivial reduction in monocytes (figures 1, 2, 3 and 4 respectively). Contrarily, the negative control revealed a constant reduction in neutrophils and WBCs and relentless elevations in monocytes and lymphocytes counts. ANOVA comparison showed a significant difference ($p<0.05$) between normal and negative control and between treated groups and negative control on the 6^{th}, 11^{th} and 16^{th} day in WBC count, as well as monocytes and neutrophil counts on the 16^{th} day. Inoculation with

just a dose of *S. typhi* ($1.5x10^8$ CFU/ml) revealed a significant reduction ($p<0.05$) in platelets, RBC Hgb, HCT, MCV, MCH, and MCHC compared to control (normal) as shown in figures 5, 6, 7, 8, 9, 10 and 11 respectively. However, treatment with ethanol root extract of *Milletia aboensis* and Ciprofloxacin annulled these variations producing a gradual rise across the hematological parameters. ANOVA comparison between normal and negative control demonstrated significant difference in all the parameters. Correspondingly, a statistically significant variation was recorded between treated groups and negative control on the 11^{th} and 16^{th} day in RBCs and platelets and also between treatment groups and negative control on days (6, 11 & 16) in HCT and Hgb. Histological examination of the section of control rats showed normal epithelial lining of the typically columnar variety (plate 1), while those infected without treatment showed intensely disrupted or destroyed tissues (plate 2). The small intestines of animals infected and treated for 5 days with 100mg/kg of ethanol root extract of *Milletia aboensis* showed tissues replete with inflammatory cells (plate 3), those treated for 5 days with 200mg/kg showed numerous duodenal glands with the muscularis mucosa seen at the edge without histological disruption (plate 4); while those treated for 5 days with 400mg/kg, showed intact duodenal glands with normal architecture (plate 5). Equally, the small intestines of infected rats administered with (500mg/70gk) of Ciprofloxacin for 5 days showed small intestinal tissue replete with transverse sections of numerous microvilli that were highly basophilic (plate 6). The photo-micrographs of infected rats treated for 10 days with 100mg/kg showed disrupted duodenal glands of the small intestine (plate 9); while those treated with 200mg/kg and 400mg/kg of ethanol root extract of *Milletia aboensis* for 10 days showed disrupted duodenal glands and normal tissues respective (plates 10 and 11) while those treated with 500mg/70kg of ciprofloxacin for 10 days also showed normal tissues (plate 12). Examination of the histo-architecture of the small intestines of *S. typhi* infected animals treated for 15 days with 100mg/kg showed disrupted duodenal gland (plate 15); while those administered with 200mg/kg & 400mg/kg of ethanol root extract of *Milletia aboensis* and ciprofloxacin (500mg/kg) for 15 days, showed intact tissue (plates 16, 17 and 18) respectively.

Discussion

During typhoid fever infection, the common hematological changes include, lymphocytosis, anemia and monocytosis (Preeti *et al.*, 2016); thrombocytopenia, eosinophilia and leucopaenia (Abro *et al.*, 2009); a significant decrease ($p<0.05$) in mean levels of MCHC,MCH, HCT, pack cell volume (PCV), Hgb, MCV and RBC (Preeti *et al.*, 2016). Our study also agrees with these reports. The neutropenia and leucopenia are attributed to *S. typhi*'s invasion of the hemopoietic organs (bone marrowand spleen) which slows down leucopoiesis (Anusuya and Sumathi, 2015). The monocytosis and lymphocytosis are ascribed to elevated release of these cells from the myeloid/lymphoid tissues in response to the infection (Das and Mukherjee, 2003). The anaemia and decrease MCHC, MCV, Hgb, MCH and RBC are due to destruction of RBC (Dangana *et al.*, 2010), hemophagocytosis and bone marrow suppression (Khosla *et al.*, 1995). Treatment of the infected rats in this study revealed that the extract reversed the usual significant decrease ($p<0.05$) in WBC, neutrophil, Hgb, MCHC, MCV, HCT, MCH, RBC and significant increase ($p<0.05$) in monocytes and lymphocytes normally associated with *S. typhi* infection. This implies that the extract reversed *S. typhi;* invasion of the hemopoietic organs, invasion of the macrophages, destruction of RBC, hemophagocytosis and bone marrow suppression, these effects suggests curative potentials of ethanol root extract of *Milletia aboensis* against *S. typhi*. Histological examination of the section of small intestine of normal control group showed normal epithelial lining that is typically of the columnar variety, while those of the infected group showed intensely disrupted tissues which agree with the report of Zainab (2012) who

reported tissue hyperplasia and infiltration well as degeneration of the brush border and the apical cytoplasm, these disruptions were reversed by the ethanol root extract of *Milletia aboensis* indicating therapeutic potential of the extract against *S. typhi*.

Conclusion

Administration of ethanol root extract of *Milletia aboensis* in *S. typhi* infected rats reversed the adverse haematological and histopathological changes of the gastro-intestinal tract, indicating curative potentials of this extract.

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