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COMPARATIVE HISTOPATHOLOGY AND IMMUNE CELL RESPONSE OF SOME LYMPHOID TISSUES IN BROILERS ADMINISTERED *VERNONIA AMYGDALINA* AND ANTIBIOTICS

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ABSTRACT

The present study was carried out to explore the use of *Vernonia amygdalina* phytogenics for health promotion to reduce therapeutic antibiotics use in poultry production. One-hundred and fifty (150) day-old Cobb 2000 broiler chicks were randomly allotted to three treatment groups replicated five times with ten birds each in a completely randomized design. Treatment 1 served as control group (T1); birds administered antibiotics (Gentaryl-D) were assigned to treatment 2 (T2). Birds in treatment 3 (T3) were administered aqueous *V. amygdalina* (1 ml / bird) on days 8, 15 and 22; respectively. Feed and water were supplied *ad-libitum*, antibiotics was administered to birds in T2 for five days on day 15 of the experiment. The duration of the experiment was 28 days. One bird per replicate was sacrificed on day 14 and 28 to collect the spleen and proventriculus. This was fixed in 10% neutral buffered formalin for routine histological processing prior to histomorphology analysis of lymphocyte counts in the follicles and peri-arteriolar lymphoid sheet cells. Data were generated for spleen and proventriculus. Histological result on the spleen showed difference ($P< 0.05$) between *Vernonia amygdalina* and control on day 14 and day 28; respectively. Lymphocyte counts on day 28 in *Vernonia amygdalina* (140.54) was lower ($P<0.05$) than control (187.83). A value of 182.59 was recorded for antibiotics which was not different *(*$P>0.05$) from values recorded for the control and *Vernonia amygdalina*. Histopathology of the proventriculus showed that *Vernonia amygdalina* birds had the least abnormalities (lesions, hyperplasia and atrophy) than birds in the control and antibiotics. The results proved that the birds in *Vernonia amygdalina* were healthier than the control and antibiotics birds. It can thus be deduced that phytogenics such as *V. amygdalina* could be used as a suitable alternative to antibiotics to enhance immune response in broilers chickens.

Key words: Antibiotics, Phytogenics, Lymphoid tissues, Spleen, Proventriculus.

INTRODUCTION

The discovery of antibiotics as a growth promoting agent has led to its use as a feed additive in livestock production. Its use over the years have resulted in improved growth performance and has protected animals from pathogenic organisms. Their continuous and non-judicious use has posed a problem, with scientific evidence suggesting their large-scale use has led to antibiotic resistance (Munk *et al*., 2018) and residues in the food and environment, particularly aquatic ecosystems which has led to public health problems. To curb negative effects of antibiotic use in animal production, several alternatives have been suggested and they include; enzymes, prebiotics, probiotics, synbiotics, antimicrobial

peptides, phytogenics amongst others (Ohimain and Ofongo, 2012; Zhai, *et al.*, 2018). Phytogenics are plant-derived products which are natural growth promoters used as feed additives in livestock industry. Phytogenics also known as phytobiotics or botanicals include spices, plant extracts, herbs, essential oils and other plant materials. Phytogenics contain bioactive molecules such as carvacrol, thymol, capsaicin, cineole, etc. (Mountzouris *et al*., 2010). These properties of phytogenics prove its superiority to antibiotics as it has antimicrobial, antiviral, fungicidal, anticoccidial, and/or antioxidant properties (Applegate, *et al.,* 2010). Phytogenics have been shown to elicit antimicrobial property (Panghal *et al.,* 2011, Giannenas *et al.,* 2013), improve food intake and digestion (Basmacioglu *et al.,* 2010), immune-stimulating effect (Siani *et al*., 2013), and antioxidant properties (Zhang *et al.,* 2013).

The peripheral lymphoid tissue is also known as the secondary lymphoid tissue and they are the spleen, the MALT (Mucosa Associated Lymphoid Tissue) which involves the digestive, respiratory and reproductive tissue. The gut associated lymphoid tissue (GALT) which are the pharyngeal tonsil, diffuse lymphoid tissue and lymphoid follicles in the cervical and thoracic part of the oesophagus, and oesophageal tonsil, diffuse lymphoid tissue in the proventriculus, pyloric tonsil, peyer's patches, meckel's diverticulum, two caecal tonsils, diffuse lymphoid tissue in the rectum and the lymphoid tissues in the wall of the proctodeum form the integral part of the MALT (Pabst, 2007; Gino, 2010).

Phytogenics have been shown to exhibit immune stimulating ability on both cellular and humoral immunity. Kroismayr *et al.* (2008) reported a significant decrease in size of payer's patches in the intestine of weaned piglets, as well as mesenteric lymph nodes and spleen using essential oil and avilamycin. Also, a research conducted with oregano essential oil supplementation at higher doses (300 - 1000ppm) showed positive immune responses in new castle disease virus and avian influenza vaccinated broiler birds (Gala *et al.*,2016), lowered excreta oocyst counts of coccidia (Alp *et al.,* 2012), and increased hypersensitivity response, total antibody titer and heterophil/ lymphocyte ratio (Hashemipour *et al.,* 2013). Since several studies have shown that phytogenic plants have immuno-stimulating ability, this study was therefore, aimed at ascertaining the effect of *Vernonia amygdalina* on peripheral lymphoid tissues as an alternative to antibiotics considering the lymphoid tissues in the spleen and proventriculus.

MATERIALS AND METHODS

Experiment Location

The experiment was carried out in Niger Delta University Teaching and Research farm, Wilberforce Island, Bayelsa State which is geographically located within latitude $4^{\circ}15^{'}$N, $5^{\circ}23^{'}$S and longitude $5^{\circ}22^{'}$W and $6^{\circ}45^{'}$E. The experimental area is located in the rainforest vegetation.

Preparation of *Vernonia amygdalina* Extract

The plant was harvested first thing in the morning, washed to remove dirt and debris and excess water was allowed to drain from the leaves. The leaves were finely chopped to between 3 to 5 cm size thereafter 1000 g of the chopped leaves was weighed into an electric milling machine and milled with 1000 cl of clean drinkable water. The filtrate was obtained by passing through a clean cheese cloth. The obtained filtrate was administered to birds in treatment 3 at a dosage of 1 ml per bird in each replicate.

Experimental Design and Management of Experimental Animals

Before the experiment commenced, the pens were thoroughly washed and disinfected. One-hundred and fifty (150) day – old Cobb 2000 broiler chicks were distributed into three treatment groups replicated five times with ten birds each. Completely randomized design

(CRD) was used in which the birds were randomly assigned to the three (3) treatment groups. Treatment 1 served as control group (T1); birds administered antibiotics (Gentaryl-D: a combination of antibiotic and vitamins for growth stimulation/vitamin supplement during periods of disease, convalescence and stress) were assigned to treatment 2 (T2). Antibiotics was administered on day 15 for five days (1 gm / 1.5 liters of drinking water). Birds in treatment 3 (T3) were administered 1ml of aqueous *V. amygdalina* on days 8, 15 and 22. Each bird in the *V. amygdalina* treatment group was orally administered 1 ml of aqueous *V. amygdalina* extract on the respective days stipulated for administration (day 8, 15 and 22).

Hybrid feed broiler starter and finisher diets were used in feeding the birds for the period of the experiment. Feed and water were supplied *ad-libitum*. One bird per replicate per treatment was sacrificed on day 8 and day 28; respectively to collect the spleen and proventriculus. Respective samples were fixed in 10% neutral buffered formalin for routine histological processing (Winsor,1994) prior to histomorphology analysis of lymphocyte counts in the follicles and peri-arteriolar lymphoid sheet cells (Chu *et al*., 2014). Animal experimentation/data collection were according to ethics approved by Niger Delta University research ethics committee. Data collected were subjected to one-way analysis of variance using IBM SPSS version 25 and significant means separated with Duncans multiple range test.

Sample Collection

One (1) bird per replicate across each treatment was selected randomly on day 8 and day 28; respectively. The birds were euthanized through cervical dislocation, placed dorsally and the ventral region was cut open and the visceral were brought out in which the spleen and the proventriculus were then separated from other organs and placed in 10% neutral buffered formalin. Samples were collected on day 8 prior to *V. amygdalina* administration and day 28 post *V. amygdalina* administration, the experiment was terminated on day 28.

Histological Analysis

The labeled samples were taken to the laboratory and subjected to the following procedure: dissected, and appropriately labeled samples were fixed in 10% neutral buffered formalin for further fixing before being processed in an automatic tissue processor. The fixed samples were embedded in paraffin wax and sectioned at 5 microns on a rotary microtome mounted on glass slides. The stepwise protocol for the automatic tissue processor for histological examination slide was as described by Winsor (1994) and Hopwood (1996). Normal tissues were identified and pathological changes observed under light microscope. Photomicrographs were taken with the aid of computerized digital camera (Amscope MU900), and lymphoid cell quantification was done using Image.

Statistical Analysis

Data collected were subjected to one-way analysis of variance using IBM SPSS version 25 and significant means separated with Duncans multiple range test. The lymphoid cell counts were presented as Mean ± SD.

RESULTS

Histological changes in lymphoid tissues

There existed few observed changes in lymphoid tissues examined including hyperplasia, atrophy of lymphoid cells and inflammation when the treatment groups were compared as indicated in Table 1. Figures 1, 2 and 3 show the photomicrograph of lymphoid cells in spleen and proventriculus of 28 days old broiler chickens administered antibiotics and *V. amygdalina*

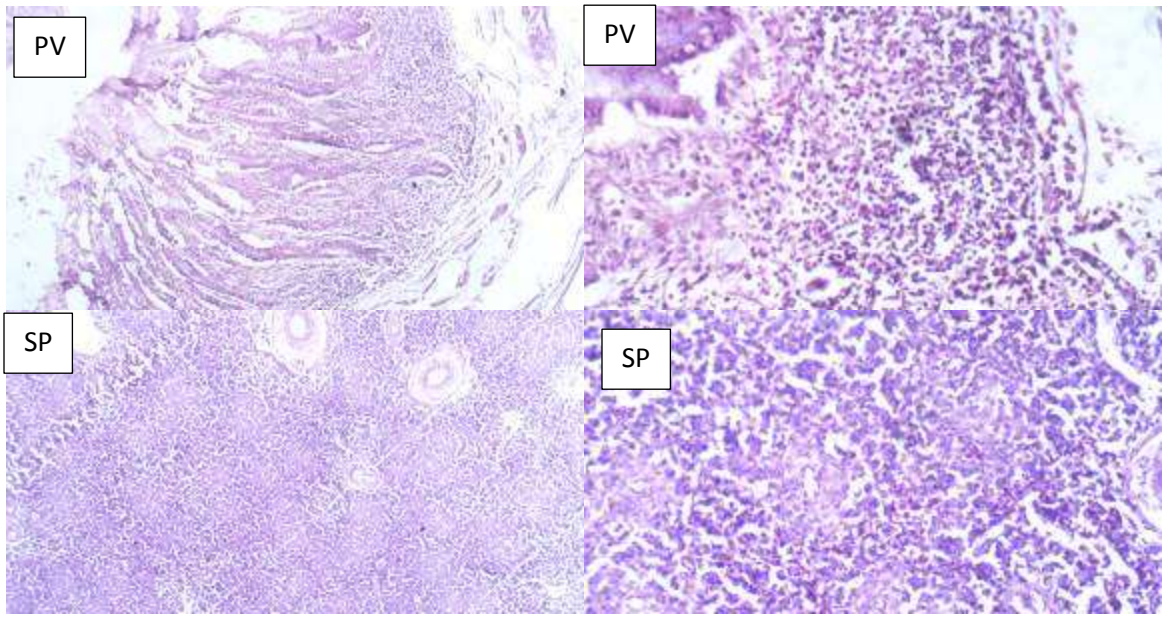


Figure 1: *Photomicrograph of spleen and Proventriculus (day 28) of T1 broiler chickens. PV: proventriculus; SP: spleen*

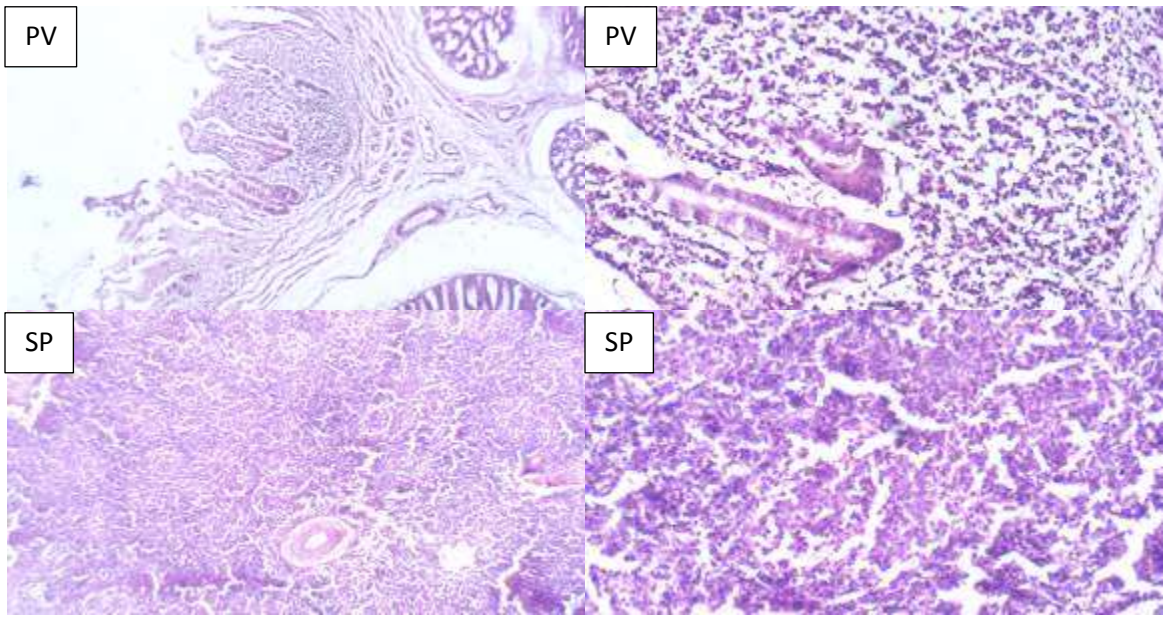


Figure 2: *Photomicrograph of spleen and proventriculus (day 28) of broiler chickens administered antibiotics. SP: Spleen; PV: Proventriculus*

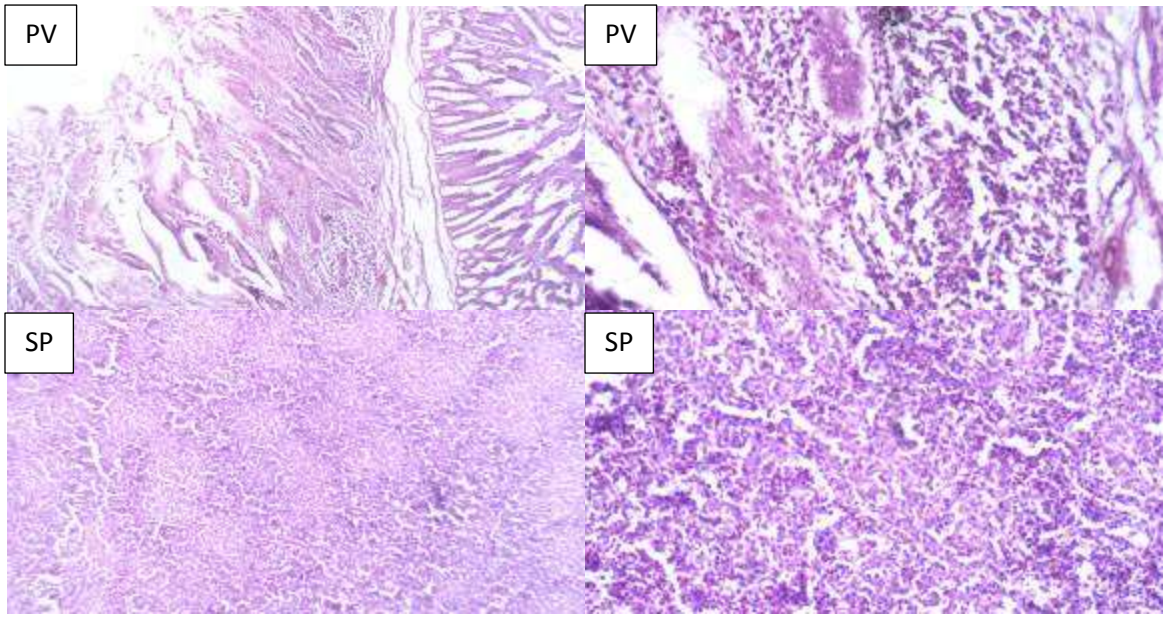


Figure 3: *Photomicrograph of Proventriculus and spleen (day 28) of broiler chickens administered V. amygdalina. SP: spleen; PV: Proventriculus*

Table 1: Histopathology of the proventriculus

Control	**Gentaryl-D (antibiotics)**	*Vernonia amygdalina*
There was no observable lesion.	Lymphoid hyperplasia, degeneration of gastric part cell.	Goblet hyperplasia.
SP Atrophy of mucous cells and gastric folds	Mucous cell hyperplasia	Nil
Atrophy of mucous cells and gastritis	Cellular infiltration in lamina propria of gastric folds.	Moderate lymphoid hyperplasia
Moderate lymphoid hyperplasia	Atrophy, inflammation	Nil
Nil	Lymphoid hyperplasia	Atrophy
Atrophy, inflammation	Lymphoid hyperplasia	Nil
Nil	Atrophy	Goblet hyperplasia, inflammation
No record was provided	Lymphoid hyperplasia	Nil
Atrophy	Goblet hyperplasia, inflammation	Nil

Lymphoid cell counts in spleen and proventriculus

Lymphoid cell counts in spleen and proventriculus of broilers administered antibiotics and *V. amygdalina* is presented in Table 2. The results showed significant difference (*P<0.05*) in lymphoid cell counts across the treatment groups with *Vernonia amygdalina* being the least on day 8 and 28; respectively. However, the highest value of lymphoid cell counts was seen in control (230.67 ± 26.57) on day 8 and 187.83 ± 7.96 on day 28. There was a gradual reduction in lymphoid cell counts from day 8 to day 28 across the treatment groups but values recorded in *Vernonia amygdalina* were the least in the experimental period. Spleen lymphoid cell counts in Gentaryl-D group was not different (*P>0.05)* from values recorded in control and *Vernonia amygdalina* but were numerically lower than that recorded in *Vernonia amygdalina* . Lymphoid cells number in proventriculus of broilers administered antibiotics and *V. amygdalina* were not different (*P>0.05)* but the least values were recorded in broiler chickens administered *V. amygdalina***.** The result however, showed that the highest value of lymphoid cell counts was recorded for Gentaryl-D (255.92 ± 53.73) on day 8. The least value of lymphoid cell counts in the proventriculus was recorded in *Vernonia amygdalina* on day 8 (196.31 ± 62.77) and day 28 (141.39 ± 5.64); respectively.

Table 2: Lymphoid cells number in the spleen and proventriculus of broiler chickens administered *V. amygdalina* as alternative to antibiotics.

Organ / time of sampling	Control (No antibiotics)	Gentaryl-D (Antibiotics)	*Vernonia amygdalina* (Phytogenic)
Spleen			
Day 8	230.67 ± 26.57[a]	204.29 ± 11.73[ab]	166.91 ± 6.66[b]
Day 28	187.83 ± 7.96[a]	182.59 ± 18.91[ab]	140.59 ± 6.65[b]
Proventriculus			
Day 8	241.69 ± 42.53	255.92 ± 53.73	196.31 ± 62.77
Day 28	168.28 ± 28	217.45 ± 53.11	141.39 ± 5.64

[ab] means along the same row with different superscripts are significantly different (P<0.05); T: control group; T2: Antibiotics group; T3: *V. amygdalina* group

DISCUSSION

The presence of lymphoid tissue in the spleen and proventriculus across the treatments proved that these organs could invoke immune response. The avian spleen played important roles in both innate and specific immune responses (Scanes, 2020). Stimulator of interferon genes (STING) is expressed in the spleen (Ran *et al*., 2018) and increased expression of pro – Inflammatory cytokines in the spleen such as interleukin (IL) 8 and IL 10 (Khare *et al*., 2019) under disease pathogenesis occured in chickens. Any metabolite or treatment that can induce immune response in the spleen will interplay in cases of disease pathogenesis. Fatty acids have been reported to

be involved in the regulation of intestinal immunity and immunological diseases, revealing that dietary fatty acids and their metabolites play an important role in the regulation of allergy, inflammation, and immunosurveillance in the intestine (Hosomi *et al*., 2020).

The constituent of *V. amygdalina* has been reported to consist of 9,12,15-Octadecatrienoic acid, methyl ester, (Z, Z, Z), hexadecenoic acid and phytol; respectively (Adeoye *et al*., 2018; Alara *et al*., 2019). The fatty acids 9,12,15-Octadecatrienoic acid, methyl ester, (Z, Z, Z) and Hexadecenoic acid have immune modulatory effect as reported earlier by Hosomi *et al*. (2020). These findings further corroborated the results obtained from this study. There is scarcely or limited report on the effect of phytogenics on the lymphoid tissue of the spleen and proventriculus (a component of the gut).

The present research carried out showed a significant effect of phytogenics on lymphoid tissue for *Vernonia amygdalina* broilers in the spleen when compared to other treatments while that of the proventriculus had no significant effect, but its mean values were lower than those found in both the control and antibiotic groups. Thus, indicating that the birds in the phytogenic group were healthier than the other treatments. Hall (2016) reported an increase in number of heterophil cells in the blood few hours after infection which causes inflammation.

The report by Hall (2016) was observed in this research when the pathology study showed that the birds on *Vernonia amygdalina* had the least lymphoid tissue count from which fewer lesions and hyperplasia regions were seen than for the control and antibiotic groups indicating that they had healthier tissues.

Ahmed *et al.*(2018) reported that use of herbal mixtures had positive effect on humoral immunity in broilers. This is in line with the findings from this study in which *V. amygdalina* was observed to have a positive effect on cellular immunity.This effect of *V. amygdalina* in invoking an immune response thereby resulting in healthier spleen and proventriculus, may be due to its bioactive ingredient such as flavonoids (Surai, 2014; Myburgh, 2014) which it has for its antimicrobial (Etxeberria *et al.*, 2013), anti-inflammatory, antioxidant and immune stimulating effect.

The study has proven that phytogenics such as *Vernonia amygdalina* is effective in improving immune response, thus, having a positive effect on the spleen and proventriculus as peripheral lymphoid tissue. It can be concluded that phytogenics such as *V. amygdalina* could be used as a suitable alternative to antibiotics.

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