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Antibacterial and Immuno-Modulatory Roles of *Ocimum Gratissimum* in the Control of *E. coli* O157:H7 Chicken Colibacillosis

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ABSTRACT

O. gratissimum is one of the leafy vegetables used by Nigerians as a food additive in preparing some local dishes. It has also been documented by some researchers to have some medicinal values. This research aimed at monitoring the immunomodulatory and antibacterial roles of *Ocimum gratissimum* ethanolic extract in the control of colibacillosis in broilers; as a natural alternative to antibiotic therapy.1.5 ml 10^7 cfu/ml *E. coli* O157:H7 isolated from nono (sour milk) and identified with 16s rDNA sequencing, was used to elicit colibacillosis infection in three weeks old broilers. Oral administration of the plant extract (40 g/l) to the infected birds over a 1 month period resulted in regulated haemoglobin, blood electrolytes, urea, creatinine, C-reactive protein and liver enzyme values. Microbial counts in the intestine revealed a decrease in total *E. coli* count. Histopathology examination of the intestinal tissues revealed the activity of gut associated lymphoid tissue in immune response. *O. gratissimum* ethanolic extract can serve as a natural alternative to antibiotic therapy in the control of colibacillosis in broilers.

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INTRODUCTION

O. gratissimum is used by Nigerians for the preparation of native foods. It grows usually as a small shrub with many branches and simple oval leaves. The leaves are used as food additives, where it serves medicinal and nutritive values, as well as add aroma or flavor to the food (Okoye and Madumelu, 2013). According to Edeoga and Eriata (2011), it is mainly used as spice. However, it also serves a lot of medicinal purposes which include antibacterial, antifungal, and antihelminthic.

Colibacillosis in chicken is caused by invasive strains of *E. coli* commonly called Avian pathogenic *E. coli* (APEC). It is a threat to the intestinal health of chicken and other poultry birds, predominantly found occurring in all age group of chickens, with high prevalence rate in adult layer birds and 1-5 weeks chicks. This adversely affects expected economic returns on the poultry by farmers. Colibacillosis in poultry is frequently associated with *E. coli* strains of serotypes 078, K80, 01: K1 and 02:K1 (Kabir *et al*., 2005). This infection has been controlled over the years with conventional antibiotics by most Nigerian farmers but has major draw-backs which are high antibiotic float in the human ecosystem and emergence of multiple drug resistant forms of *E. coli*. Hence, there is need to explore natural treatment alternatives such as the use of medicinal plants. Thus, this work is directed at observing the antibacterial and immunomodulatory roles of *O. gratissimum* in the control of *E. coli* O157:H7 chicken colibacillosis.

METHODS

Isolation of Organism

Escherichia coli isolates were obtained from *Nono* using one in ten fold serial dilutions in sterile peptone

water, and culturing on Eosin methylene blue agar. Cultures were incubated at 35^0C for 24 h aerobically according to the methods of Makut *et al*., (2014).

Identification of Organism

Escherichia coli isolates were presumptively identified using routine biochemical tests. Confirmation of isolates was by 16s rDNA molecular typing, carried out at Macrogen Incorporate, South Korea.

Screening of *E. coli* Isolates for Avian Pathogenicity

Twenty, three-week old broiler chicks were orally infected with 10^7 cfu/ml of *E. coli* in phosphate buffered saline, with the aid of a sterile Pasteur pipette. The chicks were then monitored for thirty days for pathological signs such as malaise and occurrence of watery and bloody stools (Ezema, 2013).

***In-vitro* Antibacterial Assessment of *O. gratissimum* Ethanolic Extract**

The tube dilution assay was employed to first determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the ethanolic plant extract. Two-fold dilution of 500 mg of the plant ethanolic extract was made serially in 10% Dimethylsulfoxide, to get 250 mg/ml, 125 mg/ml, 62.5 mg/ml, 31.25 mg/ml, 15.625 mg/ml, 7.813 mg/ml, 3.906 mg/ml and 1.953 mg/ml. Thereafter, 1 ml aliquot of each diluted extract was transferred into test tubes containing 1 ml peptone water with 0.1 ml 24 h *Escherichia coli* culture. The set-up was incubated for 24 h at room temperature and turbidity was checked for in each tube. The agar well diffusion method was employed to determine the zones of inhibition of the MIC with Ciprofloxacin as the standard. A 100 µl aliquot of each concentration of plant extract was placed in a well cut in sterile Eosin methylene Blue Agar plate that was already seeded with *E. coli*. The plates were incubated in an anaerobic chamber at 35^0C for 24 h and the diameters of zone of inhibition were measured (Kabir *et al*., 2005).

Preparation of Experimental Birds

Broiler chicks (*Gallus domesticus*) were obtained from Aroma Farms, Awka, Anambra State Nigeria, as day-old chicks and were raised till they got to three weeks old before they were used for experiment. They were provided with feeds starting with top starter for the first seven days, and top finishers for the remaining weeks.

Five groups of ten, three-week old broiler chicks were used as stated below:

- Group A: Healthy control.
- Group B: Infected with APEC without treatment.
- Group C: Infected with APEC and treated with 15 g/L Norfloxacin (Antibiotic control).
- Group D: Infected with APEC and controlled with 40 g/L *O. gratissimum* ethanolic extract.

Test chicken samples were known to be diseased by the discharge of watery and bloody stool.

Infection of Chicks with APEC and Administration of the Extract

Groups B, C, and D were orally dosed initially with 1.5 ml of 1.3 x 10^7 cfu/ml of *Escherichia coli* mixed with 0.5 ml phosphate buffer saline (pH 6) with the aid of a sterile pipette and left for a period of two days to give room for proper pathogen incubation and disease establishment. Afterwards, Group C was controlled with 15 g/L Norfloxacin, Group D was dosed with 40 g/L of *O. gratissimum* ethanolic extract in order to control the infection; while group B was left without treatment as stated already in the group arrangements (Pascual *et al*., 2009; Emmanuel and Obiezue, 2014).

Effect of Oral Administration of *O. gratissimum* on Haematological Profile of Chicks

Haemoglobin, was determined using an automated haemoglobin reader which displays haemoglobin results in a digital pattern. Total white blood cell count was determined by collecting the Blood samples from the chickens via the under-side of their wings and transferred to an EDTA bottle. 0.02ml of the blood samples were mixed with 0.038 of Tursk diluent in a test tube. A little aliquot was used to fill the counting chamber of the already charged Neubauer chamber. This set-up was charged again for 5-10minutes by placing the counting chamber on a damp towel. Thereafter, the under-side of the chamber was cleaned and placed under the microscope where it was viewed using x10 objective lens. Differential white blood cell counts were monitored by making a thin blood film on a slide. Four drops of Leishmann stain and eight drops of dilution buffer was added and mixed, then allowed to stand for 8-10 minutes. The stain was washed off and slide was allowed to dry before it was viewed under x40 magnification with the microscope Cheesbrough (2006).

Effect of Oral Administration of *O. gratissimum* on Blood Chemistry parameters of Chicks

Blood electrolytes (Sodium, Potassium, Chlorine and Bicarbonate), Urea, Creatinine and C-reactive protein were monitored according to the methods described by Reddy *et al*., (2011).

Effect of Oral Administration of *O. gratissimum* on Liver Enzyme Parameters of Chicks

Serum aspartate amino transferase, alanine amino transferase and acid phosphatase enzymes were

monitored according to the methods of Reitman & Frankel, (1957); Babson & Read, (1959).

Effect of Oral Administration of *O. gratissimum* on Intestinal Microflora of Chicks

Intestinal lavage was performed on dead chickens from each group with 1 ml of phosphate buffer saline; lavage fluid was serially diluted and plated on Eosine Methylene Blue (EMB) agar. Colony forming units from lavage cultures after 24 h were used to determine the intestinal *E. coli* burden (Pascual *et al*., 2009).

Gross Morphology and Histopathological Examination of Intestinal Tissues

Gross morphological examination was performed on the birds by exsanguination, followed by opening up of the chicks from the lateral view, with the help of dissecting tools. Histopathological examination was done according to the method described by Ikele *et al*., (2014).

RESULTS

Table 1: Antibacterial Activity of *O. gratissimum* Ethanolic Extract against Avian pathogenic *E. coli* *In-vitro*

Concentrations (mg/ml)	Results	Diameter Zone of Inhibition (mm)
250	+	15
125	+	14
62.5	+	13.6
31.25	+	13
16.625	-	0
7.812	-	0
3.906	-	0
1.953	-	0
Ciprofloxacin	+	12

+ = Zones of Inhibition Present.
- = Zones of Inhibition Absent.

Table 2: Effects of Oral Administration of *O. gratissimum* on some Haematological Profile of Broilers

Parameters	Baseline (Values at Zero Time)	Treatment Groups	Values after 21 days Treatment	Values after 28 days Treatment
Haemoglobin (g/dl)	5.05 ± 1.05	A	6.50 ± 1.15	6.05 ± 1.15
		B	3.60 ± 0.55[a]	2.45 ± 0.35[a]
		C	5.05 ± 1.15	8.35 ± 1.25
		D	6.45±1.25[b]	11.25±1.35[b]
Total white blood Cell count(x10^9)	9.05 ± 1.2	A	10.80 ± 1.45	12.79± 1.45
		B	12.90 ± 1.15[b]	14.10± 1.20[b]
		C	13.20 ± 0.85	12.60 ± 1.20
		D	12.10 ± 1.15[a]	13.90± 1.00[a]
Neutrophil	26.20 ± 1.10	A	28.20± 1.15	30.30 ± 1.20
		B	22.60 ± 1.45	26.00±1.15[b]
		C	29.65 ± 1.75	28.65± 0.82
		D	22.30 ± 1.45	18.30±1.45[b]
Lymphocyte	60.06 ± 1.75	A	65.60 ± 1.20	65.60± 6.60
		B	86.60 ± 1.20[b]	86.40±1.40[b]
		C	62.65 ± 1.70	68.00± 1.10
		D	74.00 ± 1.15[a]	79.00±1.15[a]
Eosinophil	0.00 ± 0.00	A	0.65 ± 0.65	0.00 ± 0.00
		B	1.00 ± 1.00	1.00 ± 1.00
		C	1.00 ± 1.00	0.67 ± 0.67
		D	0.67 ± 0.67	0.70 ± 0.27

Basophil	0.00 ± 0.00	A	0.00 ± 0.00	0.33 ± 0.33
		B	0.00 ± 0.00	0.33 ± 0.33
		C	0.33 ± 0.33	0.67 ± 0.33
		D	0.67 ± 0.03	0.00 ± 0.00
Monocytes	1.00 ± 0.37	A	1.33 ± 0.05	2.00 ± 1.05
		B	0.66 ± 0.66	0.55 ± 0.16
		C	2.00 ± 0.48	2.48 ± 0.67
		D	1.88 ± 0.62	2.00 ± 0.58

a= significant decrease at p< 0.05

b= significant increase at p< 0.05

A = Healthy control, B= Infected and untreated chicks, C= Infected and treated with Norfloxacin, D= Infected and treated with *Ocimum gratissimum*.

Table 3: Effects of Oral Administration of *O. gratissimum* on some Blood Chemistry Parameters of Broilers

Parameters	Baseline (Values at Zero Time)	Treatment Groups	Values after 21 days Treatment	Values after 28 days Treatment
Sodium (MEq/L)	124.00 ± 1.15	A	145.00 ± 1.15	146.00 ± 3.06
		B	132.32 ± 1.20	130.00 ± 1.15[a]
		C	140.32 ± 1.45	135.32 ± 1.40
		D	135.00 ± 1.10	140.00 ± 1.15[b]
Potassium (MEq/L)	2.15 ± 1.00	A	3.37 ± 1.0	2.15 ± 1.80
		B	2.35 ± 1.15	2.25 ± 0.80
		C	2.00 ± 1.05	2.20 ± 1.10
		D	2.75 ± 1.00	2.55 ± .60
Chlorine (MEq/L)	94.06 ± 1.15	A	138.00 ± 1.15	145.00 ± 1.15
		B	115.60 ± 1.40	105.00 ± 1.15[a]
		C	120.65 ± 1.75	135.00 ± 1.15
		D	125.00 ± 1.15	140.00 ± 1.15[b]
Bicarbonate (MEq/L)	14.00 ± 1.15	A	25.30 ± 1.45	27.00 ± 1.15
		B	20.00 ± 1.5[b]	15.00 ± 1.15[a]
		C	18.00 ± 1.15	24.00 ± 1.10
		D	17.60 ± 1.80[a]	25.30 ± 1.45[b]
Urea (mg/dl)	4.19 ± 2.81	A	6.06 ± 1.52	7.02 ± 0.78
		B	11.96 ± 1.34[b]	17.41 ± 3.22[b]
		C	8.87 ± 3.02	9.40 ± 2.33
		D	9.33 ± 1.28[a]	10.34 ± 2.97[a]
Creatinine (mg/dl)	0.13 ± 0.03	A	0.83 ± 0.60	0.51 ± 0.31
		B	2.55 ± 1.96[b]	1.19 ± 0.16[b]
		C	0.52 ± 0.19	0.57 ± 0.25
		D	0.45 ± 0.32[a]	0.22 ± 0.11[a]
C-reactive Protein	4.00 ± 1.15	A	8.00 ± 3.46	10.00 ± 3.46
		B	20.00 ± 3.42[b]	18.00 ± 6.00[b]
		C	8.67 ± 2.31	6.00 ± 0.00
		D	12.00 ± 6.00[a]	10.00 ± 3.46[a]

a= significant decrease at p< 0.05
b= significant increase at p< 0.05
A = Healthy control, B= Infected and untreated chicks, C= Infected and treated with Norfloxacin, D= Infected and treated with *Ocimum gratissimum*.

Table 4: Effects of Oral Administration of *O. gratissimum* on some Liver Enzyme Parameters of Broilers

Parameters	Baseline Values (Values at Zero Time)	Treatment Groups	Values after 21 days Treatment	Values after 28 days Treatment
AST	24.12 ± 3.33	A	33.33 ± 7.64	35.00 ± 7.55
		B	74.33 ± 7.02[b]	88.00 ± 6.64[b]
		C	52.67 ± 6.86	74.00 ± 6.56
		D	66.67 ± 7.62[a]	75.00 ± 8.36[a]
ALT	28.22 ± 5.35	A	34.00 ± 8.19	36.00 ± 5.29
		B	63.67 ± 6.66[b]	48.67 ± 6.66[b]
		C	47.67 ± 4.93	27.00 ± 7.55
		D	48.00 ± 2.65[a]	29.33 ± 7.37[a]
ACP	15.15 ± 3.33	A	18.10 ± 5.87	28.87 ± 0.03
		B	44.94 ± 7.27[b]	47.19 ± 6.26[b]
		C	23.70 ± 4.77	24.56 ± 5.40
		D	32.31 ± 13.83 [a]	32.07 ± 8.48[a]

a= significant decrease at p< 0.05
b= significant increase at p< 0.05
A = Healthy control, B= Infected and untreated chicks, C= Infected and treated with Norfloxacin, D= Infected and treated with *Ocimum gratissimum*.

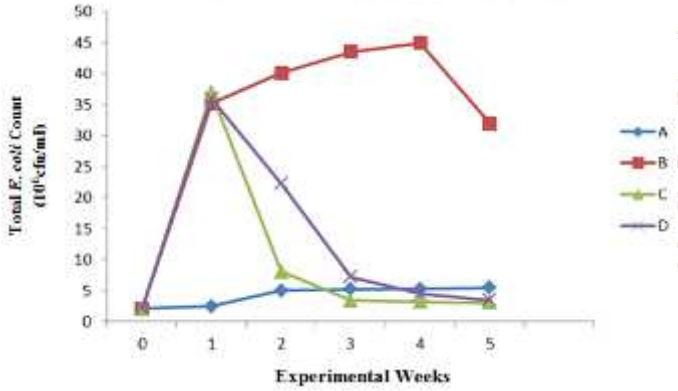

Figure 1: Mean Intestinal Total *E. coli* Count of the Test Chicken.

A: Healthy control.

B: Diseased without Treatment.

C: Antibiotic control using Norfloxacin (15g/L).

D: Prebiotic control using *O. gratissimum* (40 g/L).

Fig 2: Dissection of Broiler Chick in Group B (infected not treated) showing Severe Intestinal Ulceration.

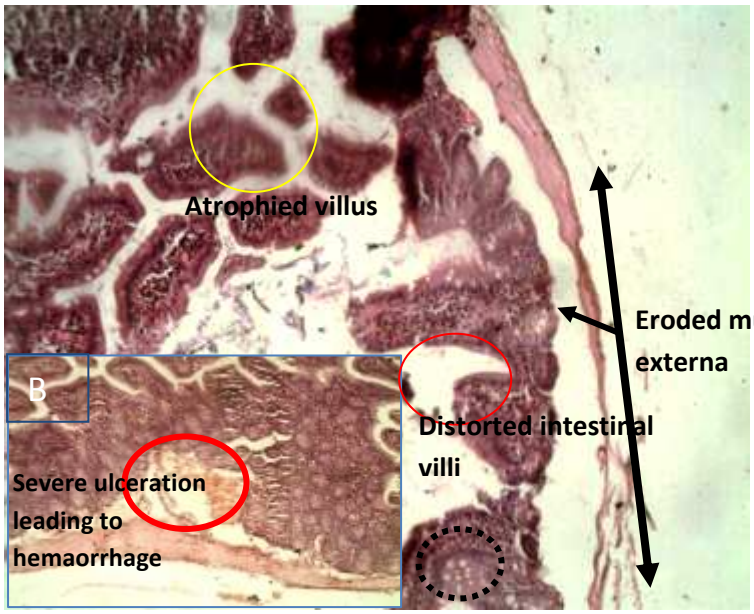

Fig 3: Photomicrograph of duodenum of Group B chicken (infected not treated) showing: (1). Severe erosion of the muscularis externa down to the submucosa (black arrow) with loss of mucosal epithelial cells (2). Distortion/or complete breaking of the intestinal villi and almost complete loss of the villi (red circles) and also villous atrophy was equally observed (yellow circle). (3). There is severe reduction/ or degeneration of goblet cells (dotted circle). (4). Severe observable intestinal hemorrhage due to ulceration is seen (red circle). H&E. mag. 100X.

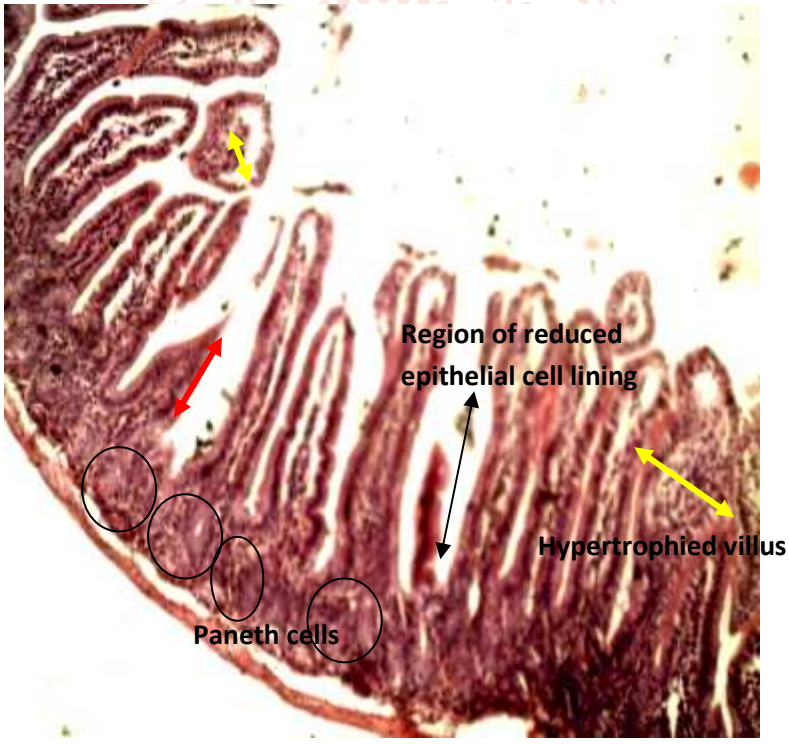

Fig 4: Photomicrograph of duodenum of Group C chicken (antibiotic control) showing:(1) Minor reduction of epithelial cells lining(black arrow head) in the villi although nuclei of the columnar epithelium are evident and clear. (2) Minor reduction of villous length (red arrow head). (3) Increased size of the villi which is usually one of the characteristics pathological changes observed in cases of mucosal hypertrophy (yellow arrow head). (4) That the paneth cells (circles) are observed and increased in number and they are usually the key effectors of innate mucosal defense. H&E. mag. 100X.

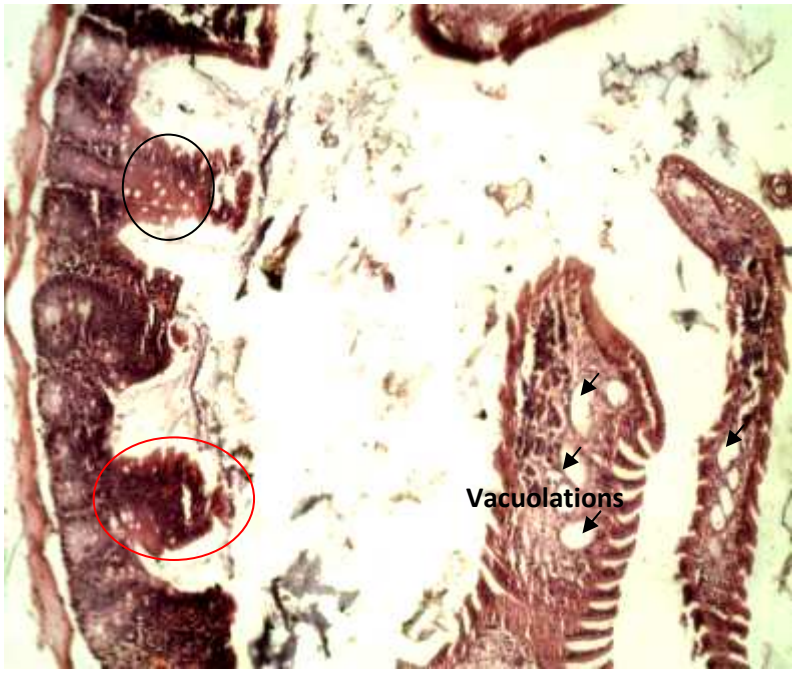

Fig 5: Photomicrograph of small intestine of Group F (diseased and treated with *O. gratissimum*) showing: (1) Gradual villous regeneration (red circle).(2) Regeneration of goblet cells which are usually the frontline of innate host defense (black circle). (3) Vacuolation of epithelial cells which is seen at the apical portion of epithelial cells of the upper third of the villus keeping for mucosal lipidosis(black arrow). H&E. mag. 100X.

DISCUSSION

Table 1 shows that *O. gratissimum* had a Minimum Inhibitory Concentration (MIC) of 31.25 mg/ml against *E. coli*. However, the work of Ladipo *et al.* (2010), recorded 50mg/ml against *E. coli*.

Oral administration of *O. gratissimum* produced some significant effects on the haematological profile of Broiler chicks. There was marked reduction in haemoglobin count in the infected and untreated chicks (B) compared to the infected chicks treated with *O. gratissimum* (D). There was also an observable difference in Total white blood cell count, Neutrophil and Lymphocyte counts between the two groups. No significant ($P \geq 0.05$) differences were recorded in Eosinophil, Basophil and Monocyte counts (Table 2). The action of *O. gratissimum* on leucocytes agrees with the works of Arhoghro *et al.* (2009).

O. gratissimum produced some significant effects ($p \leq 0.05$) on some blood chemistry profile of Broiler chicks, with marked reduction in Sodium, Chlorine and Bicarnonate values which indicate electrolyte loss through diarrhea, in the infected and untreated chicks (B); while such conditions were not noticed in the infected chicks treated with *O.gratissimum* (D). There was also an observable difference in urea and creatinine, with Group B having high urea and creatinine values and indicates improper urea and creatinine clearance, which points out a possibility of kidney function impairment in the birds. However, such conditions were not observed in the Group D chicks. C-reactive protein values between the two groups show that Group B had high C-reactive protein values which indicate high levels of inflammation while such was not also observed in Group D. No significant ($P \geq 0.05$) differences were recorded in Potassium values (Table 3).

There was also significant ($p \leq 0.05$) effects on some liver enzyme values of Broiler chicks. There was marked increase in Serum Aspartate aminotransferase, Alanine aminotransferase and Acid phosphatase values in the infected and untreated chicks (B) compared to the infected chicks treated with *O. gratissimum* (D) (Table 4). Surana and Jain (2010) stated that *O. gratissimum* ethanolic extract has hepato-protective effect in rats. There was steady increase in *E. coli* count of infected and untreated chicks (B), from the first week to the third week of monitoring, compared with other groups. However, there was a slight decrease in the count on the fourth week, which is still also highest in value when compared to other groups. The antibiotic treated chicks (C) showed a sharp decline in *E. coli* count after the first week of infection, and same was observed in the *O. gratissimum* treated group (D) (Figure 1). Kabir et *al.* (2005) reported that *O. gratissimum* has the potency to control intestinal infections.

Figures 2-5 show the levels of damage *E. coli* O157:H7 infection caused in the intestine of test broilers. Histopathology examination shows that there was eliciting of the actions of gut associated

lymphoid tissues as an innate immune response by *O. gratissimum* administration (D). However, there was gross disorganization of intestinal wall architecture for group B.

CONCLUSION

O. gratissimum is a common Nigerian vegetable rich in antibacterial phytochemicals and has good therapeutic use against Avian pathogenic *E.coli* (APEC). It equally acts as a boost to chicken immune system.

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