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COROLLARY OF PLANT SUPPLEMENTS ON HEMATOLOGICAL INDICES AND ABSOLUTE LYMPHOCYTES OF BROILER CHICKS

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ABSTRACT

Consumption of supplements from botanical origin in order to amplify the activities of immune cells and organs is a huge success in immune modulatory potential of botanical products. This study investigated the effects of plant supplements on hematological indices and absolute lymphocytes in broiler chicks. The supplements, prepared by mixing *Azadirachta indica* (Neem), *Curcuma longa* (turmeric), and *Allium sativum* (garlic) with vitamin C, were orally administered to 3-week-old broiler chicks. The results showed a non-significant ($P>0.05$) increase in lymphocytes and neutrophils, with the highest values observed in the *Baphia nitida* leaf extract (BN) group (62.14% lymphocytes and 35.56% neutrophils) compared to the control group (56.80% lymphocytes and 30.50% neutrophils). However, there was a significant ($P<0.05$) increase in absolute lymphocytes, with the BN group recording the highest value (3244 Lymphs/mcL) compared to the control group (1920 Lymphs/mcL). The plant supplements exhibited a pronounced increase in blood lymphocytes, neutrophils, and absolute lymphocytes, with BN being the most effective. These findings suggest that these plant supplements, particularly BN, may have immunomodulatory effects in broiler chicks.

Keywords: *Azadirachta, Curcuma, Allium, Baphia*

INTRODUCTION

Medicinal plants play a vital role in the treatment of human and animal diseases. The application of these herbal medicines has contributed significantly to the search for a new drug for prevention and treatment of infectious agents (Adeshina *et al*., 2017). Recently, much interest has been directed to the use of natural compounds to enhance host immunity. Plant extracts play a significant role in the prevention and curing of infections by modulating the immune system. As a result, their application and use has increased dramatically (Anisuzzama 2018).

Herbal medicines act on the immune system by either suppressing or stimulating innate or adaptive immune cells/molecules. Immune regulation is important in maintaining normal immunity, and the search for herbal immunomodulatory compounds to treat various infections by enhancing the body's natural resistance is of growing interest. *Dendropanax morbifera* Léveille, also knowns as *Dendropanax trifidus,* is an economically and medicinally important subtropical broad-leaved tree that is endemic to Korea. The tree has been used in the treatment of different human infections and reported to have anti-thrombotic, anti-diabetic and anti-atherogenic components (Arunkumar *et al*., 2016).

Polyacetylene from plant leaves has been shown to have an anti-complement effect. The plant is also known to increase the excretion of toxic elements, namely, cadmium from the kidney, and to reduce cadmium-induced oxidative stress in the hippocampus. Ayati *et al*. (2015) reported the anti-cancer and anti-oxidant activity of the methanolic leaf and debarked stem extracts. The bioflavonoid extract, rutin, prevents rotenone-induced cell injury through inhibition of the JNK and p38 MAPK signaling pathways in a Parkinson's disease model. Furthermore, the chloroform extract suppresses proinflammatory mediators and cytokines through inhibition of NF-κB.

Numerous studies have been conducted using plant extracts with different techniques and the extracts had shown to have an immunostimulatory activity (Balaji and Chempakkam, 2010). This research is aimed at evaluating the corollary of plant supplements on hematological indices and absolute lymphocytes of broiler chicks.

MATERIALS AND METHODS

Purification of the Isolates

The plate that showed discrete colonies were selected after 24 - 48 h and each colony was aseptically streaked using a sterile wire loop on a sterile poured plate (90mm x 15mm) containing nutrient agar (BIOTECH) prepared according to the manufacturers description. after which it was incubated at their required growth conditions (Iheukwumere and Iheukwumere, 2022c; Iheukwumere *et al*., 2022d; and Iheukwumere and Iheukwumere, 2022e).

Characterization of the Bacteria Pure Isolates

The pure isolates were characterized using the morphological, biochemical and molecular characteristics as described by Iheukwumere *et al.* (2018), Iheukwumere *et al*. (2022f), Iheukwumere *et al*. (2023a) and Iheukwumere *et al*. (2023b).

Morphological characteristics of the Bacteria isolates

The cultural descriptions (size, appearance, edge, elevation, colour) of the isolates were carried out as described in Iheukwumere *et al*. (2024) and Iheukwumere *et al.* (2022g). The Gram staining technique which revealed the Gram reaction, cell morphology and cell arrangement were also carried out using the procedure described by Obianom *et al*., (2024), Egbe *et al*. (2025a) and Manasseh *et al*. (2025). The presence or absence of capsule was also carried out as described by Ekechukwu *et al*. (2025a). The presence or absence of flagellum was determined by carrying out motility test as described by Ekechukwu *et al*. (2025b).

Gram staining technique

A thin smear was made in a cleaned grease free microscopic slide (75mm×25mm), air dried heat fixed. The smear was flooded with crystal violet solution (0.2%) for 60 seconds and rinsed with cleaned water. Gram iodine solution (0.01%) was then applied and allowed for 60 seconds. This was rinsed with cleaned water. This was followed by decolourizing the slide content with 95%w/v ethyl alcohol for 10seconds and then rinsed with cleaned water. The smear was then counter stained with safranin solution (0.025%) for 60 seconds, rinsed with cleaned water, blot drained and air dried. The stained smear was covered with a drop of immersion oil and observed under a binocular compound light microscope using × 100 objective lens as described

by Ekechukwu *et al*. (2025c), Egbe *et al*. (2025b) and Egbe *et al*. (2025c).

Motility test: This was done using the method described by Iheukwumere *et al*. (2025a),Iheukwumere *et al*. (2025b) and Iheukwumere *et al*. (2025c). A semi-solid medium prepared by mixing 5.0g of bacteriological agar (BIOTECH) with 2.0g of nutrient broth (BIOTECH) in 1 Litre of distilled water was used. The solution was dissolved and sterilized using autoclaving technique after dispensing 10 ml portion in different test tubes. The test tubes were allowed to set in vertical positions and then inoculate the test organisms by performing a single stab down the centre of the test tube to half the depth of the medium using sterile stabbing needle. The test tubes were kept in an incubator in vertical position at 35 ± 2^0C for 24h.T

Biochemical characteristics of the isolates

Indole test: Indole is a nitrogen containing compound formed when the amino acid tryptophan is hydrolyzed by bacteria that have the enzyme tryptophanase. This is detected by using KOVAC's reagent. This was done using the method described by Iheukwumere *et al*. (2025d), Iheukwumere *et al*. (2025e) and Iheukwumere *et al*. (2025f). The isolates were cultured in peptone water in 500.0 ml of deionized water. Ten millilitres of peptone water was dispensed into the test tubes and sterilized. The medium was then inoculated with the isolates and kept in an incubator at 37˚C for 48 hr. Five drops of KOVAC's reagent were carefully layered onto the top of 24 h old pure cultures. The presence of indole was revealed by the development of red layer colouration on the top of the broth cultures.

Sugar fermentation test: The capability of the isolates to metabolize some sugars (glucose, xylose, ducitol, maltose, arabinose, inositol, mucate and lactose) with the resulting formation of acid and gas or either were carried out using sugar fermentation test. One litre of 1% (w/v) peptone water was added to 3 mL of 0.2% (w/v) bromocresol purple and 9 ml was dispensed in the test tube that contained inverted Durham tubes. The medium was then sterilized by autoclaving. The sugar solution were prepared at 10% (w/v) and sterilized. One milliliter of the sugar was dispensed aseptically into the test tubes as described by Dim *et al*. (2025a) and Dim *et al*. (2025b). The medium was then inoculated with the appropriate isolates and the cultures incubated at

37˚C for 48 h and were examined for the formation of acid and gas. Change in colour from purple to yellow indicated acid formation while gas formation was assessed by the presence of bubbles in the inverted

Methyl red test: Using the method described by Dim *et al*. (2025c), Iheukwumere *et al*. (2025g). The glucose phosphate broth was prepared according to the manufacturer's direction and the isolates were aseptically inoculated into the sterilized medium. This was incubated at 37˚C for 48 hr. After incubation, five drops of 0.4 % solution of alcoholic methyl red solution was added and mixed thoroughly, and the result was read immediately. Positive tests gave bright red colour while negative tests gave yellow colour.

Voges-Proskauer test: Using the method described by Iheukwumere *et al*. (2025h), Ike *et al*. (2025a). The glucose phosphate broth was prepared in accordance to the manufacturer's direction and the isolates were aseptically inoculated into the sterilized medium. This was incubated at 37˚C for 48hr. After incubation, 1.0 mL of 40% potassium hydroxide (KOH) containing 0.3% Creatine and 3 ml of 5% solution of α-naphthol was add ed in the absolute alcohol. Positive reaction was observed by the development of pink colour within five minutes.

Citrate utilization test: The Simmon's Citrate Agar was prepare according to the manufacturer's direction and the isolates were inoculated by stabbing directly at the center of the medium in the test tubes and incubated at 37˚C for 48 hr. Positive test was shown by the appearance of growth with blue colour, while negative test showed no growth and the original green colour was retained as described by Ike *et al*. (2025b) and Ike *et al*. (2025c).

Catalase test: The test was carried out as described by Ike *et al*. (2025d) and Ike *et al*. (2025e). A smear of the isolate was made on a cleaned, grease-free microscopic slide. Then, a drop of 30% hydrogen peroxide (H_2O_2) was added on the smear. Prompt effervescence indicated catalase production.

Oxidase test: The test was carried out using the method described by Ugwu *et al*. (2025a). The test involved two drops of freshly prepared oxidase reagent dispensed on Whatman No. 1 filter paper which was placed in Petri dish, and a smear of the test isolate was made on the spot using a sterile stick. The

development of blue-black colouration was checked within 15 seconds.

Urease test: This was carried out as described by Ugwu *et al*. (2025b). The urea agar slant was prepared in accordance to the manufacturer's direction and the isolates were aseptically inoculated into sterilized medium. This was incubated at 37^0C for 48 h. After incubation, observation was made for the presence of purple-pink colouration.

Molecular characterization of the isolates

Extraction and purification of DNA: All strains were plated on Nutrient Agar (Biotech) and incubated at 37°C for 24 hr. Using the Zymo Research (ZR) DNA miniprepTM kit (Category No. D6005; Irvine, California, USA), bacterial genomic DNA was extracted and purified as described by Iheukwumere et al. (2018), Iheukwumere *et al*. (2020) with the procedures outlined in the kit.

Determination of the quality of extracted DNA: Using mass spectrophotometer (Nanodrop), One micro litre (1µL) was aseptically dropped into a fresh space in the chamber and the chamber was lightly closed which was then linked to a computer system which showed the window that discovered the value of the sample at 260/280nm as described by (Iheukwumere *et al.,* 2017a; Chude *et al*., 2020).

Amplification of DNA and gel electrophoresis of PCR product: This was analysed using Master cycler Nexus Gradient (Eppendorf). A mixture of primer (20 µL), template DNA (20µL), water (72 µL) and master mix (108 µL), which comprises taq polymerase, dimethylsulfoxide (DMSO), magnesium chloride ($MgCl_2$) and nucleotides triphosphates (NdTPs), was made in 1.5 mL tube and homogenized using vortex mixer (Eppendorf). This was then positioned in the block chamber of the master cycler and then programmed. The PCR program for conditions were as follows: initial incubation at 94°C for 5 mins, followed by 35 cycles of denaturation at 94°C for 15 secs, annealing at 55°C for 15 secs, elongation at 72°C for 21 secs and final extension period for 10 mins at 72°C. The amplified products were electrophorezed in 1.0% agarose gel and a1kb DNA ladder was used as a size reference. After staining with 3µL of nucleic acid stain (GR green), the gel was documented with gel documentation apparatus (Iheukwumere *et al.,* 2017b;

Iheukwumere *et al*., 2017c; and Iheukwumere *et al*., 2018b).

DNA sequencing of 16s rRNA fragment: The 16S rRNA amplified PCR products generated from universal primer (16S), was used for the sequencing using ABI DNA sequencer (Applied Biosystem Inc) at International Institute of Tropical Agriculture (IITA), Ibadan using the method of Iheukwumere *et al*. (2017d), and Iheukwumere *et al.* (2018c).

Computational Analysis: This was analysed making use of the modified method of Iheukwumere *et al.* (2025i) and Iheukwumere *et al*. (2025j). The chromatograms generated from the sequences were cleaned to obtain regions with normal sequences. The cleaned nucleotides were aligned using pair wise alignment tool. The consensus sequences formed by the alignment of the forward and reverse sequences were used to perform the Basic Local *Alignment* Search Tool (BLAST) using National Centre for Biotechnology Information BLAST over the internet. The sequences of the isolates with 95% and above similarities were accepted. Also the maximum scores, total scores and accession numbers of the isolates were assessed. The relatedness of the isolates was determined by tracing their phylogenetic tree using DNA distance neighbour phylogenetic tree tool.

Experimented Chicks: A total of twenty four (24) broiler chicks (3 weeks old) were purchased from poultry market located at Ihiala market, Ihiala L. G. A. in Anambra State were used for the study. The chicks were kept in separate, thoroughly cleaned and disinfected house and provided with feeds and water ad libitum. All the chicks were vaccinated against Newcastle disease using Lasota vaccine strains at 6 and 19 days of age, against infectious bronchitis using live H120 strain at 6 days old and also against avian influenza (A1) disease using inactivated H5N1virus vaccine strain at 7 days old. All the vaccines were given via eye drop instillation except (A1) vaccine which was given through subcutaneous route at the back of the neck from the folder report collected from the poultry farmer.

Preparations of Plant Materials: The leaves of *Azadirchta indica,*(Neem plant) leaves of *Baphia nitida*, rhizomes of *Allium sativum* (garlic) and roots of *Curcuma longa* were collected from Onitsha, Anambra State, Nigeria. The plant material was authenticated appropriately Dr B. Garuba, in Botany Department, Michael Okpara Federal

University of Agriculture, Umudike. The plant material was washed and dried under shade at room temperature for 14 days. The dried plant material was ground to powder form using sterile electric grinder. (Iheukwumere *et al.,* 2020; Ejike *et al*. 2017; Nwobodo *et al*., 2018; and Ekesiobi *et al*., 2025).

Extraction Procedure: A 2000 mL Soxhlet extractor that has three main sections: a percolator (boiler and reflux) which circulates the solvent, a thimble (usually made of thick filter paper) which retains the solid to be extracted, and a siphon mechanism, which periodically empties the thimble was used for process. Twenty grams (100 g) of the plant material to be extracted was placed inside the thimble. The thimble was then loaded into the main chamber of the Soxhlet extractor. Then 1000 mL of ethanol was placed in a 1000 mL distillation flask. The flask was placed on the heating mantle (2000 mL, 220 V, 500 W). The Soxhlet extractor was placed at the top of the flask. A reflux condenser was placed at the top of the extractor. When the ethanol was heated to reflux, the solvent vapour travelled up a distillation arm, and flooded into the chamber housing the thimble of solid. The condenser ensured that any solvent vapour cooled, and dripped back down into the chamber housing the solid material. The chamber containing the solid material slowly filled with warm solvent. When the Soxhlet chamber was almost full, the chamber was emptied by the siphon. The solvent then returned to the distillation flask. The thimble ensured that the rapid motion of the solvent did not transport any solid material to the still pot. This cycle was allowed to repeat many times for 12 h. After extraction, the solvent is removed, typically by means of a rotary evaporator to collect the extract.

Preparation of Extracts: The plant extracts were each reconstituted with phosphate buffer saline (PBS). One (1.0) g of the ethanolic plant extracts were each dissolved in 10 ml of PBS to make 0.10 ppm of the extracts using sterile conical flasks. This was evenly homogenized and stored in clean sterile containers for use (Iheukwumere *et al.,* 2020; Iheukwumere *et al*., 2025k; Iheukwumere *et al*., 2025l).

Preparation of Plant Supplements: A 50 mL portion of the prepared extract (100 mg/mL or 0.10 ppm) was carefully mixed 50 mL portion of vitamin C (100 mg/mL or 0.10 ppm) in order to form 100 mL portion of the respective solution of NeemVic (NE), TumeriVic (TU) and GarliVic (GA).

Antigen preparation: This was carried out using the method described and published by Nfambi et al. (2015). Fresh blood sample was collected from healthy sheep from Uli in Ihiala L. G. A., Anambra State, and this was mixed with sterile Alsever's solution (1:1). The sample was centrifuged at 2000 xg for 5 min to enable the red blood cells (RBCs) settled at the bottom of the test tube. Then the supernatant was discarded and the sediment was collected as the sheep red blood cells (SRBCS). The SRBC was then washed three times with pyrogen- free phosphate buffered saline (PH 7.2). This was then kept under refrigeration for the study.

Experimental Protocols for the *In vivo* Models: A total of 36 broiler chicks were used for this study. The broiler chicks were grouped into six groups, and each group comprises 6 chicks. . A 0.5 mL/100 g of *Baphia nitida* leaf extract (BN), GA, NE, and TU each was orally administered to each of group of broiler chicks, and the remaining group was giving only feed and water as control group. The body weights and blood absolute lymphocytes were assessed from the blood samples drawn from the chicks after 11 days.

Hematological Indices: The blood samples collected from the broiler chicks were examined using Automated Hematology Analyzer (MIN DRAY BC – 360), and the variations in the red blood cells (RBCs), lymphocytes, monocytes, neutrophils, eosinophils and basophils were assessed and recorded as described in the work published by Agiang *et al.* (2017), Iheukwumere *et al*. (2022a), Iheukwumere and Iheukwumere (2022a)

Absolute Lymphocytes: The blood samples collected from the broiler chicks were examined using Automated Hematology Analyzer (MIN DRAY BC – 360), and the differential white blood cell (WBC) counts were carried out and the percentage of lymphocytes were calculated. The absolute lymphocytes were calculated as stated below, assessed and recorded as described in the work published by Agiang *et al.* (2017)

Absolute Lymphocytes = WBC ($\times 10^3$ cells/mcL) × 1000 × % Lymphs

Statistical Analysis: The data obtained in this study were presented in tables and figures. Their percentages were also calculated. The sample means and standard deviations of some of the analytical data were also calculated. The significance of this study was determined at 95% using one way analysis of variance (ANOVA). Pairwise comparison was analyzed using student

"t" test as described by Okeke *et al*. (2017), Iheukwumere *et al*. (2022b), Iheukwumere *et al*. (2017e), Nwike *et al*. (2017), Amadi *et al*. (2017), and Iheukwumere *et al*. (2025l).

RESULTS

The study showed elevated in the value of white blood cells (WBC), Lymphocytes. Monocytes, Lymphocyte counts and neutrophil counts when compared to the control group. There was slight decrease in the PCV and RBC values. The values of Hb varied; there was an increase among chicks administered *Baphia nitida* extract while those administered tumeriViC, GarliViC, NeemViC and VitaminC showed slight decrease when compared to the control group.

The percentage of neutrophils of neutrophils also showed slight decrease. There were variations in the values of basophils and eosinophils, and these variations were statistically non-significant ($P>0.05$). Also, the increase in lymphocytes and WBC observed among the broiler chicks administered Baphia nitida extract, tumeriViC, GarliViC, NeemiViC and Vitamin C were statistically non-significant ($P>0.05$). The study showed pronounced increase in the absolute lymphocytes as shown in Table 2. The absolute lymphocyte values were significantly ($P<0.05$) higher among the broiler chicks administered *Baphia nitida* extract, TumeriVic, GarliViC and NeemViC, and those broiler chicks that received *Baphia nitida* extract recorded the highest absolute lymphocyte values, this was followed by TumeriViC and Vitamin C recorded the least value.

Table1: Hematological indices of the blood samples drawn from the experimented chicks

Parameter	BN	TUM	GAR	NEE	VC	CON
PCV(%)	28.67	27.22	25.46	27.29	25.62	28.77
Hb(g/dL)	8.86	8.64	8.14	8.68	8.08	8.81
RBC($X10^{12}$ cells/L)	5.69	5.14	4.82	5.21	4.91	5.71
WBC($X10^{9}$ cells/L)	5.22	5.10	4.27	4.02	3.71	3;38
Lym(%)	62.14	61.86	59.41	58.27	57.70	56.80
Mon(%)	3.90	4.20	4.60	4.36	3.91	3.90
Neu(%)	30.56	31.18	33.60	34.92	34.71	35.50
Eos(%)	1.10	0.80	1.30	1.40	1.10	1.20
Bas(%)	0.2	0.2	0.5	0.2	0.3	0.3
Lym($X10^{3}$/µL)	8.02	8.11	6.82	6.47	5.82	5.76
Mon($X10^{3}$/µL)	0.26	0.29	0.34	0.37	0.31	0.30
Neu($X10^{3}$/µL)	5.11	5.07	4.76	4.41	4.38	4.25
Eos($X10^{3}$/µL)	0.40	0.35	0.50	0.55	0.40	0.45

Table2 : Absolute lymphocytes of the blood samples drawn from the experimented broiler chicks

Samp[le	Lymphocytes (%)	WBC WBC($X10^3$cells/MCL)	Absolute Lymphocytes (Lymphs/mcL)
Baphia nitida	62.14	5.22	3244
TumeriViC	61.86	5.10	3154
GarliViC	59.41	4.27	2537
NeemViC	58.27	4.02	2342
Vitamin	57.70	3.71	2141
Control	56.80	3.38	1920

WBC = White blood cell counts

DISCUSSION

The increase in the lymphocytes and red blood cells (RBCs) associated with the present study supported the findings of Yapo *et al*., (2011), Sumalatha *et al*., (2012) Anarthe *et al*., (2014) and Obi *et al*., (2019) but disagree with the findings of Johnson *et al*., (2017). Variations in the values of immune c ells/blood cells observed in the present study could be attributed to the variation in the ability of the plant supplements to argument the hematopoietic processes in the cells of the experimented chicks. An increase in the hematological indices was observed in broiler chicks administered plant extracts as documented by Mwale *et al*. (2013). Several researchers had reported similar effect on broiler chicks (Bonsu *et al*., 2012; Ewuola and Egbunike, 2008; Ganesan and Bhatt, 2008; Huff *et al*., 2008; Mwale and Masika, 2009; Mwale and Masika, 2011; Mwale and Masika, 2012; Mwale *et al.,* 2013). The increase in the hematological indices could be attributed to high anti-oxidative potentials of the plant extracts as reported by Mwale *et al.* (2013). The increase observed in the hematological indices showed that the plant extracts are capable of promoting an d stimulating the activities of immune system as documented by Bonsu *et al.* (2012). The increase could also be attributed to the ability of the plant extract to inhibit process of oxidative break down of erythrocyte as reported by Ewuola and Egbunike (2008). The presence of phytochemicals such as flavonoids, tannins, and terpenes in the plant extracts could be responsible for the increase in hematological indices as documented by Mwale and Masika (2008). The ability of the plant extracts to stimulate stem cells in the bone marrow could be responsible for the increase in the hematological indices in the broiler chicks as reported by Mwale and Masika (2011). Mwale and Masika (2011) also attributed the increase to the action of erythropoietin, which is a glycoprotein hormone.

Jiwuba *et al*. (2017) documented an increase in absolute lymphocyte count on broiler chicks administered plant extracts. Similar observation was reported by several researchers (Oyeyemi *et al*., 2014; Tothova *et al*., 2016; Oh *et al*., 2013). The increase in the absolute lymphocyte count could be attributed to high anti-oxidative potentials of the extract (Oh *et al*., 2013). The increase could also be attributed to the ability of the extracts to enhance mechanisms that produce lymphocytes. Meanwhile, Jiwuba et al. (2017) also reported that viral, bacterial, and fungal infections could elevate lymphocytic counts. The increase in the total

lymphocytes count be attributed to stress on the chicks when they were restrained for blood collection as documented by Orsatti *et al*. (2010b)

CONCLUSION: The study has shown that the plant supplements exhibited pronounced increase in lymphocytes, neutrophils, WBC and absolute lymphocytes of which BN was most effective, and these proved that the plant supplements had immune support potential.

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