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MODULATING THE ACTIVITIES OF T-LYMPHOCYTES IN BROILER CHICKS USING PLANT SUPPLEMENTS

Iheukwumere, I. H.[1], Iheukwumere, C. M.[2], Nwakoby, N.E[1] and Nnadozie, H. C. [1]

1. Department of Microbiology, Faculty of Natural Science, Chukwuemeka Odumegwu Ojukwu University, Anambra State

2. Department of Applied Microbiology and Brewing, Faculty of Biosciences, Nnamdi Azikiwe University, Anambra State

Email: ik.iheukwumere@coou.edu.ng / ikpower2007@yahoo.com

ABSTRACT

Botanical products supplemented with vitamin C plays vital roles in augmenting the functioning the immune cells and organs through the synergistic activities of the phytochemicals and vitamin C. This study was carried out to evaluate the tendency of modulating the activities of T-lymphocytes in broiler chicks using plant supplements. Equal concentration (100 mg/mL) of extracts from *Azadirachta indica* leaves (Neem plant), *Curcuma longa* roots (turmeric) and *Allium sativum* rhizomes (garlic) were mixed with equal concentration (100 mg/mL) of vitamin C to prepare NeemViC (NE), TumeriViC (TU), and GarliViC (GA), respectively. A 0.5 mL/100 g of *Baphia nitida* leaf extract (BN), GA, NE, and TU each was orally administered to 3 weeks old broiler chicks, and assessed the delayed type hypersensitivity and population of T cells of the blood samples drawn from the chicks after 11 days. There was significant ($P<0.05$) increase in delayed type hypersensitivity (DTH) responses among the experimented chicks as compared to the control group, of which BN showed the highest activity. The maximum DTH responses were elicited after 24 h, but retarded after 48 h and 72 h. There increase in the population of absolute lymphocytes of the experimented chicks as compared to the control groups, of which TU recorded the maximum and significant ($P<0.05$) counts. Therefore, the plant supplements showed pronounced increase in DTH responses and population of lymphocytes, of which TU was most effective.

Keywords: *Curcuma,Allium, Baphia, Azadirachta*

INTRODUCTION

Background of study

The immune system protects our body from environmental invaders and maintains the balance in health. The immunostimulants, including medicines, chemicals, and natural products might improve immune responses through non-specific as well as specific defense mechanisms (Abid *et al.,* 2012). The major side effects caused by the frequent use of chemotherapeutic agents for immunomodulation include cytotoxicity to normal cells and immunosuppressive actions. Cyclophosphamide (CTX) is one of the most widely used anticancer agent and a potent immunosuppression drug that can inhibit both humoral and cell mediated immunity. CTX is also used to induceimmunosuppression in animal models. The administration of CTX induces immunosuppression and myelosuppression, which are occasionally life-threatening. Alternatively, there has been a large increase in the number of studies focused on medicinal plants used in various traditional systems. Herbal medicines and dietary therapies are customarily used as alternative medicine for treatment in conjunction with conventional medicine or after the conventional treatment (Rasheed *et al.,* 2016). Natural products demonstrate an alternative potential to chemotherapies for many illnesses, particularly when the host defense system needs to be activated in immune response. *Houttuynia cordata* is a medicinal plant widely distributed in Asia and South-east Asia. In Thailand, *H. cordata* is mostly found in the Northern and Northeastern regions, and customarily used as a vegetable side dish with local food. *H. cordata* is commonly known as Plu-khao or Khao-tong in Thailand due to its fishy smell. *H. cordata* has been reported to have several biological activities such as anti-virus, anaphylaxis inhibition, anti-cancer, anti-allergic, and anti-inflammation. Further, it has also been revealed that *H. cordata* stimulates the immune response. *H. cordata* water extract has been shown to stimulate the proliferation of mouse splenic lymphocytes and T cells in vitro as well as possess anti-SARS activities (Abid *et al.,* 2012). *H. cordata* fractions showed valuable therapeutic effects on Th2-mediated (IL-4 and IL-5) or allergic skin disorders. *H. cordata* has a potential role in modulation of innate immune mediators in oral health. Essential oils from *H. cordata* show a potential for growth as well as replacing antibiotics in fish immune responses.

Presently, it is believed that fermentation of medicinal plants can

promote good health as well as cure diseases. In fact, the fermentation process has been shown to increase flavonoid content as well as the fermented *H. cordata* extract containing identified *Bacillus* strains from the fermentation process. As mentioned above, fresh *H. cordata* plants have several pharmaceutical activities; however, little is known regarding the pharmaceutical activity of *H. cordata* fermentation products (HCFPs). More specifically, the immunomodulatory activity of the commercial HCFPs available throughout Thailand has not yet been investigated. The oral toxicityof this product in male and female rats was also established (Rasheed *et al*., 2016).

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.

Delayed type hypersensitivity: This was carried out using the modified method described and published by Anartlhe *et al*., (2014), Ejike *et al*. (2017), Nwobodo *et al*. (2018) and Ekesiobi *et al*. (2025). On the 7th day prior to injection, right hand footpad thicknesses of the broiler chicks were measured with micrometer screw gauge (Mitutoyo Digimatic). Then the chicks were challenged by injecting 20 ml of 1% SRBC into the right hind foot pad. On the 9th, 10th and 11th days, the

footpad thickness of the experimented rats were again measured and the mean differences of the pretreated and post treated rats were calculated and recorded in millimeters

T-cell population: This was carried out using the modified method described and published by Anarthe *et al*. (2014), Iheukwumere *et al*. (2022a) and Iheukwumere and Iheukwumere (2022a). On the 11th day, blood samples were collected from tails and ears of the broiler chicks and these were mixed with Alsever's solution in test tubes. These were kept, in sloping position (45^{o}) and incubated at 37^{o}C for 1h. The RBCs were allowed to settle at the bottom of the test tubes, and supernatant was collected from each test tube using micropipette and this contains the Lymphocytes

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

Delayed type hypersensitivity (DTH) responses for the plant supplements are shown in Table1. The study revealed that the maximum responses were seen after 24 h, and then progressive decrease followed after 48 h and 72 h, respectively. It was also observed that *Baphia nitida* leaf extract recorded the highest response after 24 h, 48 h, and 72 h, respectively, followed by TumeriViC whereas GarliViC recorded the least response only after 24 h, but 48 h and 72 h, it alternates with vitamin C and NeemViC.

The study revealed pronounced activities of the plant supplements on the population of lymphocytes in the experimented broiler chicks (Table 2). The study revealed that there was significant ($P<0.05$) increase in the population of lymphocytes in every 3 days intervals, and these increase was mostly pronounced among the broiler chicks administered tumeriViC. It was also observed that the population of lymphocytes from those broiler chicks administered plant supplements were higher than that of statistically significant ($P<0.05$) among those broiler chicks administered *Baphia nitida* extract, turmeric and garliViC.

Table 1: Delayed type hypersensitivity (DTH) responses for the plant supplement

Sample	24 h	48 h	72 h
Baphia nitida	9.50±0.13	8.13±0.47	6.50±0.19
TumeriViC	9.00±0.33	8..00±0.00	6.13±0.21
GarliViC	7.50±0.21	6.50±0.19	5.67±0.33
NeemViC	8.13±0.41	7.00±0.00	5.67±0.33
Vitamin C	7.77±0.19	6.50±0.19	5.50±0.19
Control	4.00±0.00	3.00±0.00	1.00±0.00

Table2: The population of lymphocytes in the experimented broiler chicks

Sample	Day 0	Day 3	Day 6	Day 9
Baphia nitida	157.11±1.08	188.23±1.08	227.14±1.24	281.62±1.27
Tumeric	159.01±1.12	182.17±1.41	242.18±1.52	311.17±1.31
GarliViC	156.26±1.22	198.16±1.51	231.03±1.42	289.15±1.33
NeemViC	158.08±1.31	181.03±1.27	219.62±1.21	251.31±1.16
VitaminC	156.31±1.21	178.01±1.22	210.13±1.12	226.91±1.42
Control	159.17±1.22	172.06±1.41	182.10±1.13	192.16±1.22

DISCUSSION

The significant increase in the delayed type hyper sensibility (DTH) and population of T cells from the blood samples drawn the experimented chicks indicate immunostimulatry activities of the plant supplements and these agree with the findings of Dashputre and Naikwode (2010), Yapo *et al*., (2011), Sumalatha *et al*., (2012), Tripathi *et al*., (2012), Anarthe *et al*., (2014), Ramesh *et al*., (2016) and Obi *et al*., (2019) and disagree with the findings of Ahirwal *et al*., (2013) and John *et al*., (2017).

Anarthe *et al*., (2014) reported that DTH involved initial sensitization phase and effecter phase. In initial sensitization phase TH^1 cells are activated and clonally expand by antigen presenting cell APC) with class II MHC molecule. In effecter phase subsequent exposure to the SRBC antigen induces DTH response, where TH^1 cells secrete a variety of cytokines and other non-specific inflammatory mediators. The above explanation was also made by Daslpute and Naikwode (2010) and Tripathi *et al*., (2012).

The increased in the Lymphocyte population and rosette formation in the present study supported the findings of Yapo *et al*., (2011), Anarthe *et al*., (2014) and Obi *et al*., (2019) but disagrees with the findings of Johnson *et al*., (2017). This increase is an indication of cell mediated immunity. Also the plant supplements have the capability to activate CD 4 and CD8 cells and this influences the population of T cells. Similar report was presented by Anarth *et al*., (2014). Momoh *et al*. (2010) observed a significant increase in the total T cell population among the investigated broiler chicks. Several researchers documented similar enhancement in the T cell population (Yashraj *et al*., 2011; Singh *et al*., 2011; Youcef *et al.,* 2013; Badria and Attia, 2007). The increase in the total T cell population could be ascribed to the presence of phytochemicals such as flavonoids, phenolics, tannins and alkaloids. The immuomodulatory potential of the phytochemical components of the plant extract was responsible for the increase in the total T cell count as reported by Yashraj *et al.* (2011).

CONCLUSION: The study has shown that the plant supplements exhibited pronounced increase in DTH responses and population of lymphocytes, of which TumeriVic was most effective, and these proved that the plant supplements had immune support potential.

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