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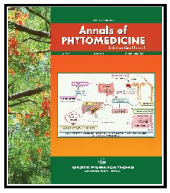

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Evaluation of immunomodulatory and antioxidant activity of *Curcuma longa* L., *Ocimum sanctum* L. and *Piper nigrum* L. alone and in combination in broiler

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Abstract

The emergence of AMR and the potential transfer of antibiotic-resistant genes to human microbiota have led to regulatory restrictions on antibiotic use in poultry. In this context, alternative strategies are imperative. Phytogenic feed additives, such as *Curcuma longa* L. (turmeric), *Ocimum sanctum* L. (tulsi) and *Piper nigrum* L. (black pepper), have emerged as promising candidates. *C. longa* has curcuminoids, renowned for their antioxidant capabilities, has demonstrated anti-inflammatory and immune-modulating effects. Similarly, *O. sanctum* has pharmacological actions ranging from antimicrobial to cardioprotective effects, offers a compelling option. *P. nigrum* containing piperine exhibits a broad spectrum of pharmacological activities, including antioxidant and anti-inflammatory properties. A total of 120 chicks were allocated randomly to 10 groups each of 12 chicks. A total of 120 chicks were allocated randomly to 10 groups each of 12 chicks to evaluate immunomodulatory and antioxidant properties of *C. longa*, *O. sanctum* and *P. nigrum* powder and compare the result with control and standard control treatment. Results indicate significant enhancements in cutaneous basophil hypersensitivity response and humoral immune response, akin to conventional vitamin E and selenium supplementation. Moreover, supplementation with these additives led to improvements in serum protein levels and antioxidant enzyme activity, suggesting their potential as viable alternatives to antibiotic growth promoters. These findings underscore the potential of phytogenic feed additives as sustainable alternatives to mitigate the challenges posed by AMR in poultry production.

1. Introduction

Over the last fifty years, global chicken production has experienced the most rapid growth among agricultural sub-sectors, with projections indicating a sustained rapid expansion in the upcoming decades (Bruinsma, 2003). In 2019, India's poultry population saw a significant increase of 16.8 per cent (851.81 million) compared to 2012 figures, with backyard poultry contributing up to 317.07 million and commercial poultry reaching 534.74 million, according to the 20th Livestock Census-2019 by the Government of India. India has 5th rank globally in poultry production (DADF, 2018). The expansive chicken industry, comprising over 21 billion individuals, equating to about three per person on the planet, significantly contributes to food security and nutrition. It provides essential energy, protein, and micronutrients to humans (Muir *et al*., 2008; FAO, 2016). Over the time, low-dose antibiotics have been utilized in poultry production as immunomodulators to uphold health and productivity (Aarestrup, 2000; 2012). However, apprehensions regarding the emergence of antimicrobial resistance (AMR) and the potential transfer of antibiotic-resistant genes from animals to the human microbiota have resulted in the prohibition of immunomodulatory antibiotics in poultry birds (Sweeney *et al*., 2018). With the rapid development of antimicrobial resistance (AMR), the global consumption of antimicrobials in food animal production is projected to increase by 67% from 2010 to 2030 (Van Boeckel *et al*., 2015; Liu *et al*., 2016). The World Health Organization (WHO) forecasted that without additional interventions, the annual death toll attributable to AMR could reach 10 million by 2050, surpassing other causes such as cancer (WHO, 2014).Therefore, it is a matter of great global concern to take effective strategies to reduce antibiotic use in chicken production to limit the spread of antimicrobial-resistant bacteria (Liu *et al*., 2016; Williams Nguyen *et al*., 2016).

Phytogenic feed additives, including herbs and spices in powder or extract forms, are employed in poultry to enhance flavor, palatability, and overall productive performance. Studies on phytogenic herbs have uncovered numerous beneficial characteristics, such as antioxidative, immunomodulatory, and antimicrobial effects. The phytochemicals present in the *Nigella sativa* extracts may be responsible for the antioxidant, antidiabetic, and anti-inflammatory activities (Balyan and Ahmad, 2022). Extracts of *Bauhinia variegata, Peltophorum pterocarpum* and *Syzygium cumini* leaves can be served as a vital source of phenoilcs and flavonoids for having antioxidant potential (Bhatt *et al*., 2019). Additionally, these additives have been found to improve diet palatability, regulate gut function, and promote growth (Windisch *et al*., 2008). The roots and leaves of *Glycyrrhiza glabra* were extracted to yield saponins and tannins,

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which demonstrated comparable or superior antibacterial and antioxidant properties (Soulef *et al*., 2021). Herbs and spices have garnered significant attention as a phytogenic or phytobiotic alternative to antibiotic growth promoters due to their antibacterial, antioxidant, anti-inflammatory, digestive stimulant, immuno-stimulant, and growth-promoting properties (Eevuri and Putturu, 2013). Clove oil showed dose-dependent anti-inflammatory activity @ 100, 250 and 500 mg/kg body weight in female Wistar rats (Humbal *et al*., 2019). Ginger may be used as a natural substitute for prescription antibiotics when treating *Streptococcus mutans* caused oral diseases (Mohammad *et al*., 2023). Trikatu pretreatment in goats significantly increased the bioavailability of levofloxacin (Patel *et al*., 2019). The active components of *Passiflora foetida* exhibit anthelmintic action in both the hydroalcoholic and ethyl acetate extracts (Kommu *et al*., 2023).

C. longa, commonly known as turmeric, is a perennial herb belonging to the *Zingiberaceae* family and is extensively cultivated in Asia, particularly in India and China. The rhizome, which is the medicinally used part of the plant, yields a yellow powder. The active constituents of turmeric are flavonoids known as curcuminoids, including curcumin (diferuloylmethane), monodexmethoxy curcumin, and bisdesmethoxy -curcumin, with curcumin comprising approximately 90% of the curcuminoid content in turmeric. Turmeric and curcumin have been reported to possess antioxidant, immunomodulatory and anti-inflammatory properties (Reddy and Lokesh, 1994; Joe and Lokesh, 1997; South *et al*., 1997; Wei and Shibamoto, 2007). *O. sanctum*, commonly known as Tulsi, belongs to the family *Lamiaceae*. *O. sanctum* contains numerous chemical constituents such as oleanolic acid, rosmarinic acid, ursolic acid, eugenol, linalool, carvacrol, β-elemene, β-caryophyllene, and germacrene (Falagas and Bliziotis, 2007). It exhibits various pharmacological actions, including antimicrobial, antifungal, anticancer, antiarthritic, antifertility, hepatoprotective, antispasmodic, analgesic, antiemetic, and cardioprotective (Rao *et al*., 2013). *P. nigrum*, commonly known as black pepper, is a member of the family *Piperaceae*. It contains the major pungent alkaloid piperine (1-peperoyl piperidine). Piperine exhibits a wide range of pharmacological activities like antihypertensive, antiplatelet, antioxidant, antitumor, antipyretic, analgesic, anti-inflammatory, antidiarrheal antispasmodic, hepatoprotective, antibacterial, antifungal and insecticidal (Taqvi *et al*., 2008; Matsuda *et al*., 2008; Manoharan *et al*., 2009). *C. longa, O. sanctum* and *P. nigrum* ethanolic extracts evinced antibacterial activity against *Staphylococcus aureus, Bacillus cereus* and *Listeria monocytogenes,* which stated that it may support immune system by its antibacterial nature (Bhavsar *et al*., 2022).

2. Materials and Methods

2.1 Experimental materials

Powder of *C. longa* and fruit of *P. nigrum* (certified by FSSAI, Ministry of Health and Family Welfare, Government of India) were purchased from the local market of Anand, Gujarat. Leaves of *O. sanctum* were procured from the Medicinal and Aromatic Plant Research Station, Anand Agricultural University, Anand. Fruits of *P. nigrum* and dried leaves of *O. sanctum* were made to powder using grinder. Iodonitrotetrazolium chloride (INT) dye, 96 well cell culture plates (flat bottom with lid) and phytohemagglutinin PHA-P lyophilized powder were purchased from Sigma-Aldrich Chemicals Private Limited, Bengaluru. Avilamycin antibiotic powder was received as a gift from Poshak Poultry Medicine, Anand, Gujarat. Newcastle disease virus and Newcastle disease-positive serum were received as a gift from Hester Biosciences Ltd., Mehsana, Gujarat. Commercially available Newcastle disease vaccine and vitamin E and selenium-containing products were used during the experiment. Reagents used for estimation of serum total protein and albumin were purchased from Coral Clinical System (A Division of Tulip Diagnostics (P) Limited, Goa, India). Kits used for estimation of antioxidant enzymes Superoxide dismutase, Catalase and Malondialdehyde were purchased from Cayman Chemicals, Michigan, USA.

2.2 Experimental birds and their management

The experiment was conducted at the Department of Veterinary Pharmacology and Toxicology in collaboration with the Poultry Research Station (PRS, Anand Agricultural University, Anand, Gujarat), and the Department of Veterinary Physiology and Biochemistry, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Anand, Gujarat. Day-old Ven-cobb broiler chicks were procured from Shakti Hatcheries Pvt. Ltd., Anand, Gujarat, and were maintained under standard managemental conditions. 4 Animals will be kept in an environmentally controlled room with 25 ± 40°C temperature and 30-70% humidity. Light/dark cycle of 12 /12 h will be provided throughout the study period. Each day floor will be swept and mopped with a disinfectant solution.The experimental design (Project No.: 340/VPT/2021) was approved by the Institutional Animal Ethics Committee (IAEC). Standard medication programme along with strict biosecurity measures were implemented. All birds were vaccinated to protect them from various diseases according to standard vaccination protocols.

Broiler starter and finisher feed were prepared at the feed manufacturing unit of PRS, Anand. The powder of *C. longa, O. sanctum* and *P. nigrum* powder alone and in combination at different doses were mixed with basal feed to make different treatment feeds. Different treatment feeds were offered treatment-wise twice a day during the whole experimental period. Mixing and stirring of feed in the feeder was carried out two times a day.

2.3 Experimental design

Evaluation of immunomodulatory activity of *C. longa, O. sanctum* and *P. nigrum* powder was conducted using chicks. A total of 120 chicks were randomly divided in to 10 groups (each of 12 chicks) as shown in Table 1. The treatment given to different groups of birds for 35 days are mentioned in Table 1.

2.4 Parameters for immunomodulatory activity

2.4.1 Cutaneous basophil hypersensitivity (CBH)

Cell-mediated immunity was estimated by conducting cutaneous basophil hypersensitivity response on the 14th day by using classical toe web assay (Corrier and Deloach, 1990). Two different doses (100 µg and 200 µg) were used to perform the test in different birds of the same group. Six birds for 100 µg and the other six birds for 200 µg dose were selected randomly on the 14th day. Phytohemagglutinin-P (PHA-P) in 0.10 mL of sterile physiological saline solution (PSS) was injected intradermally in the skin between the 3rd and 4th digits of the right foot. The CBH response to PHA-P was determined by measuring the thickness of the interdigital skin before injection and at 12 and 24 h after injection with the help of the vernier caliper. The CBH response was calculated by subtracting pre-injection skin thickness from the post-injection skin thickness of the right foot.

Table 1: Different type of treatments given to broiler birds

Groups	Treatment details	Total number of birds/treatments
I	Control group (Basal diet only)	12
II	Basal diet + (vitamin E and selenium combination)(@1.5 g/100 birds for first two weeks and 5 g/100 birds for next 3 weeks)	12
III	Basal diet + *C. longa* powder (@ 2.5 g/kg feed)	12
IV	Basal diet + *C. longa* powder (@ 5.0 g/kg feed)	12
V	Basal diet + *O. sanctum* powder (@ 2.5 g/kg feed)	12
VI	Basal diet + *O. sanctum* powder (@ 5.0 g/kg feed)	12
VII	Basal diet + *P. nigrum* powder (@ 5.0 g/kg feed)	12
VIII	Basal diet + *P. nigrum* powder (@ 10.0 g/kg feed)	12
IX	Basal diet + *C. longa, O. sanctum* and *P. nigrum* powder (@ 2.5, 2.5 and 5.0 g/kg feed, respectively)	12
X	Basal diet + *C. longa, O. sanctum* and *P. nigrum* powder (@ 5.0, 5.0 and 10.0 g/kg feed, respectively)	12
		Total number of birds = 120

2.4.2 Determination of antibody titer

Blood was collected from the wing veins of birds with a 2 ml syringe equipped with a 26 G needle in plain vials from each group on the 7th, 21st and 35th day. Samples were centrifuged at 3000 rpm (5-6 min). The serum was separated, transferred to 2 ml centrifuge tubes, and stored at – 55ºC temperature until analysis. Antibody titers against the ND vaccine were determined by the haemagglutination inhibition (HI) test as described by Buxton and Frazer (1977) with slight modifications.

2.4.2.1 Haemagglutination (HA) test

The blood was collected from chicken, and an equal quantity of Elsevier's solution was added and then centrifuged at 1500 RPM for 10 min. Then the supernatant was discarded and again added PBS, centrifuged and the supernatant was discarded. Likewise, three washes were given to obtain pure chicken RBCs. The packed red blood cells were re-suspended in PBS to make 1% v/v suspension. A clean microtitre plate having 96 'V' shaped wells was taken. This was dispensed (25 µl) into 10 wells of the first row of the plate with the help of micropipette. The virus from stock solution (25 µl) was added to the first well of the row and mixed well using a micropipette and then serial dilution was made in 1:1 ratio the ix well. In the ix well it was mixed again and 25 µl fluid was discarded while the x well received only PBS and served as control. One percent v/v RBC suspension prepared in PBS and 25 µl was added in all the ten wells. The plate was then shaken to mix the ingredients properly and incubated at room temperature for 45 min, followed by observations taken. The endpoint of HA activity of the virus was the highest dilution of the virus that produced complete agglutination (Positive pattern of the RBCs).

2.4.2.2 Haemagglutination inhibition (HI) test

Preparation of 4HA unit

The dilution factor for the stock virus was determined by dividing HA titer by 4 to make 4 HA units. A clean microtitre plate having 96 'V' shaped wells was taken. This dilution in PBS was dispensed (25 µl) into 9 wells of the first row of the plate with the help of a micropipette. The serum (25 µl) was added to the first well of the row and mixed well using a micropipette and then then serial dilution was made in 1:1 ratio up to the viii well. Virus containing 4 HA units was added (25 µl) in wells i to vii and the virus was not added in the viii and ix wells and kept as serum control RBCs control, respectively. One percent v/v RBC suspension prepared in PBS and 25 µl was added in all the ten wells. The plate was then incubated at room temperature for 45 min without disturbing. The HI titer was expressed as the reciprocal of the highest dilution of serum, inhibiting agglutination of the RBC. The data of antibody titer was converted into $\log_2$ value and these converted values were subjected to statistical analysis.

2.4.3 Estimation of biochemical parameters

Serum was separated from blood samples collected in plain vials on the 7th, 21st and 35th day and stored at -55ºC till further biochemical estimations. Total protein (g/dl) and albumin (g/dl) were analyzed using standard assay kits (total protein kit and albumin kit) with the help of an auto serum chemistry analyzer (Mindray BS-120, Mumbai, India). Globulin (g/dl) was calculated by subtracting values of albumin (g/dl) from total protein (g/dl). The albumin to globulin ratio (A/G ratio) was calculated by dividing the values of albumin (g/dl) by globulin (g/dl).

2.4.4 Haematological investigation

On the 35th day, blood samples were collected from birds from each group thin blood smears were prepared on grease-free clean slides, dried at room temperature and fixed in alcohol. Later on, blood smears were then stained in the field's stain, allowed to dry and examined microscopically for differential leucocyte counts under the oil immersion objective (100X). The heterophil-to-lymphocyte ratio was calculated manually from DLC obtained data.

2.4.5 Histopathological examination of organs

At the end of the experimental period, birds of all groups were euthanized with cervical dislocation. The birds were given *ad libitum* water for 24 h and kept off feed 6 h before to euthanasia. Tissues like

the thymus, spleen and bursa of fabricius were collected in tissue collection bottles containing 10% formalin solution and processed by paraffin embedding technique for histopathological examination. Sections of tissues were cut at 4-5 micron thickness with the help of an automatic section-cutting machine. Tissues were stained using hematoxylin and eosin (H and E) stains and slides were observed under the light microscope.

2.5 Determination of oxidative stress markers

Antioxidant enzymes such as superoxide dismutase (SOD), catalase and malondialdehyde (MDA) were measured from serum samples of various groups. Serum was separated from blood samples collected in plain vials on the 35th day and above-mentioned enzymes were estimated using standard assay kits and protocol provided by Cayman Chemical, USA.

2.5.1 Superoxide dismutase

Cayman's superoxide dismutase assay kit utilizes a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. The principle of the method is to formation of formazan dye from tetrazolium salt upon reduction by superoxide anions that are formed during the oxidation by xanthine oxidase. The formed nitro blue tetrazolium (NBT) is based on the spectrophotometric measure of the absorbance of the color at 440-460 nm using a plate reader (Infinite M Nano - TECON). One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

2.5.2 Catalase

Cayman's catalase assay kit utilizes the peroxidation function of CAT for the determination of enzyme activity. Catalase reacts with methanol in the presence of hydrogen peroxide (H_2O_2) which leads to the formation of formaldehyde and $2H_2O$. The formaldehyde produced is measured colorimetrically (540 nm) with 4-amino-3-hydrazino-5-mercapto-1, 2, 4-triazole (Purpald) as the chromogen, which upon oxidation changes from colorless to a purple color.

2.5.3 Malondialdehyde

Malondialdehyde (MDA) is a naturally occurring product of lipid peroxidation. The MDA-TBA adduct formed by the reaction of malondialdehyde (MDA) and thiobarbituric acid (TBA) under high temperature (90-100°C) and acidic conditions is measured colorimetrically at 530-540 nm by using a plate reader (Infinite M Nano - TECON).

2.6 Statistical analysis

Completely randomized design and one-way-analysis of variance (ANOVA) were used to compare the means of various parameters of immunomodulatory and growth-promoting effect by using SPSS statistics software (version 20.0). Significant differences (p<0.05) between different experimental groups were analyzed by Duncan's multiple range test (Duncan, 1995). All the data have been presented as Mean ± S. E.

Table 2: Effect of dietary supplementation of *C. longa*, *O. sanctum* and *P. nigrum* powder alone and in combination on cutaneous basophil hypersensitivity response against phytohaemagglutinin-P in broiler (n=6)

Groups	Mean toe web skin thickness (mm)					
	Pre-injection (PHA-P @ 100 µg)	Post injection (PHA-P @ 100 µg)		Pre-injection (PHA-P @ 200 µg)	Post injection (PHA-P @200 µg)	
		12 h	24 h		12 h	24 h
I	1.08 ± 0.04	1.45 ± 0.04^{a}	1.48 ± 0.07^{a}	1.13 ± 0.05	1.48 ± 0.08^{a}	1.53 ± 0.08^{a}
II	1.06 ± 0.01	1.88 ± 0.13^{d}	1.90 ± 0.06^{d}	1.19 ± 0.04	1.91 ± 0.07^{c}	1.95 ± 0.06^{d}
III	1.07 ± 0.01	1.74 ± 0.02^{bcd}	1.76 ± 0.01^{bc}	1.19 ± 0.01	1.73 ± 0.02^{b}	1.84 ± 0.02^{bcd}
IV	1.08 ± 0.01	1.80 ± 0.02^{cd}	1.83 ± 0.04^{cd}	1.16 ± 0.02	1.88 ± 0.03^{c}	1.92 ± 0.04^{cd}
V	1.04 ± 0.01	1.74 ± 0.02^{bcd}	1.79 ± 0.03b^{cd}	1.12 ± 0.01	1.69 ± 0.03^{b}	1.81 ± 0.03^{bcd}
VI	1.08 ± 0.02	1.77 ± 0.03^{bcd}	1.89 ± 0.03^{d}	1.12 ± 0.02	1.73 ± 0.02^{b}	1.84 ± 0.03^{bcd}
VII	1.04 ± 0.01	1.63 ± 0.02^{b}	1.71 ± 0.04^{bc}	1.12 ± 0.01	1.64 ± 0.03^{b}	1.71 ± 0.04^{b}
VIII	1.05 ± 0.02	1.67 ± 0.03^{bc}	1.83 ± 0.02^{cd}	1.12 ± 0.01	1.65 ± 0.02^{b}	1.80 ± 0.04^{bc}
IX	1.06 ± 0.01	1.64 ± 0.02^{b}	1.68 ± 0.03^{b}	1.13 ± 0.02	1.63 ± 0.02^{b}	1.82 ± 0.03^{bcd}
X	1.07 ± 0.01	1.69 ± 0.03^{bc}	1.78 ± 0.02^{bcd}	1.13 ± 0.02	1.63 ± 0.02^{b}	1.84 ± 0.03^{bcd}

Values (Mean ± S.E.) bearing different superscripts (a, b, c, d) in a column differ significantly (p<0.05).

I - Control group; II- Positive control group; III- *C. longa* powder @ 2.5 g/kg feed, IV- *C. longa* powder @ 5.0 g/kg feed, V- *O. sanctum* powder @ 2.5 g/kg feed, VI- *O. sanctum* powder @ 5.0 g/kg, VII- *P. nigrum* powder @ 5.0 g/kg feed, VIII- *P. nigrum* powder @ 10.0 g/kg feed, IX- *C. longa, O. sanctum* and *P. nigrum* powder mixture @ 2.5, 2.5 and 5.0 g/kg feed, X- *C. longa, O. sanctum* and *P. nigrum* powder mixture powder @ 5.0, 5.0 and 10.0 g/kg feed.

3. Results

3.1 Assessment of immunomodulatory activity

3.1.1 Cutaneous basophil hypersensitivity (CBH) response

The cutaneous basophil hypersensitivity (CBH) response, as indicated by the thickness of the skin between the toes, demonstrated significant improvement in chicks when supplemented with varying doses of *C. longa* powder (2.5 and 5.0 g/kg feed), *O. sanctum* powder (2.5 and 5.0 g/kg feed), *P. nigrum* powder (5.0 and 10.0 g/kg feed), a mixture of *C. longa*, *O. sanctum*, and *P. nigrum* powder at lower doses (2.5, 2.5, and 5.0 g/kg feed), and a mixture of *C. longa*, *O. sanctum*, and *P. nigrum* powder at higher doses (5.0, 5.0, and 10.0 g/kg feed). This enhancement was observed at both doses (100 and 200 µg) of PHA-P, evaluated at 12 and 24 h post-injection, in comparison to chicks from the control group. After 12 h of PHA-P injection (100 µg), the toe web skin thickness in chicks supplemented with *C. longa* (2.5 and 5.0 g/kg feed) and *O. sanctum* (2.5 and 5.0 g/kg feed) powder resembled that of chicks supplemented with vitamin E and selenium (Group II). Similarly, after 24 h of PHA-P injection (100 µg), the toe web skin thickness in chicks supplemented with *C. longa* (5.0 g/kg feed), *O. sanctum* (2.5 and 5.0 g/kg feed), *P. nigrum* (10.0 g/kg feed), and their respective mixtures (2.5, 2.5, and 5.0 g/kg feed and 5.0, 5.0, and 10.0 g/kg feed) was akin to the toe web skin thickness observed in chicks supplemented with vitamin E and selenium (group II). Following 12 h of PHA-P injection (200 µg), the toe web skin thickness in chicks supplemented with *C. longa* (5.0 g/kg feed) was similar to that of chicks supplemented with vitamin E and selenium (group II). After 24 h of PHA-P injection (200 µg), the toe web skin thickness of chicks supplemented with *C. longa* (2.5 and 5.0 g/kg feed), *O. sanctum* (2.5 and 5.0 g/kg feed), and a mixture of *C. longa*, *O. sanctum*, and *P. nigrum* (at doses of 2.5, 2.5, and 5.0 g/kg and 5.0, 5.0, and 10.0 g/kg feed) was comparable to the toe web skin thickness observed in chicks supplemented with vitamin E and selenium (group II). The details of CBH response in different groups have been presented in Table 2.

3.1.2 Antibody titer against ND vaccine

Antibodies are produced by B lymphocytes and plasma cells, playing a crucial role in the humoral immune response, with key immunoglobulins being IgG and IgM. They contribute to opsonization, complement activation, and toxin neutralization (Miller, 1991). The specifics of the haemagglutination inhibition (HI) antibody titer against Newcastle disease (ND) vaccine in different groups are outlined in Table 3.

During the first week of age, no significant changes in antibody titer were observed among the different groups. However, by the third week of age, there was a significant increase in antibody titer in birds supplemented with *C. longa* (5.0 g/kg feed), *O. sanctum* (2.5 and 5.0 g/kg feed), and *P. nigrum* (5.0 and 10.0 g/kg feed) powder individually and in combination (at doses of 2.5, 2.5 and 5.0 g/kg and 5.0, 5.0 and 10.0 g/kg feed) compared to birds in the control group. By the fifth week of age, a significant increase in antibody titer was observed in chicks supplemented with *C. longa* (2.5 and 5.0 g/kg feed), *O. sanctum* (2.5 and 5.0 g/kg feed), and *P. nigrum* (5.0 and 10.0 g/kg feed) powder individually and in combination (at doses of 2.5, 2.5 and 5.0 g/kg and 5.0, 5.0 and 10.0 g/kg feed) compared to birds in the control group. Moreover, at the third and fifth weeks of age, the log2 values of HI antibody titers in chicks supplemented with *C. longa* (2.5 and 5.0 g/kg feed), *O. sanctum* (2.5 and 5.0 g/kg feed), and *P. nigrum* (5.0 and 10.0 g/kg feed) alone and in combinations (at doses of 2.5, 2.5 and 5.0 g/kg and 5.0, 5.0 and 10.0 g/kg feed) (groups III, IV, V, VI, VII, VIII, IX, and X) were comparable to the log2 values of HI antibody titers of chicks supplemented with vitamin E and selenium (group II). This suggests that *C. longa*, *O. sanctum*, and *P. nigrum*, along with their mixtures, have a similar effect to that of the standard supplement. Before vaccination at the age of day 7 (1st week), birds from all groups exhibited mean HI titers ranging from 4.67 to 5.33. This observation indicated a significant presence of maternal antibody titers in the chicks. This phenomenon can be attributed to the vertical transfer of maternal antibodies, wherein immunoglobulins are transferred from immunized hens to egg yolks, or the transmission of natural passive immunity from hens to chicks.

Table 3: Effect of dietary supplementation of *C. longa, O. sanctum* and *P. nigrum* powder alone and in combination on HI antibody titer against ND vaccine in broiler (n=12)

Groups	HI Antibody titer		
	1st week (Day 7)	3rd week (Day 21)	5th week (Day 35)
I	4.67 ± 0.21	5.00 ± 0.37^{a}	5.00 ± 0.37^{a}
II	4.50 ± 0.43	6.50 ± 0.43^{b}	6.67 ± 0.33^{bc}
III	5.33 ± 0.33	6.00 ± 0.37^{ab}	6.50 ± 0.22^{b}
IV	5.33 ± 0.33	7.17 ± 0.31^{b}	7.50± 0.22^{c}
V	5.33 ± 0.21	6.67 ± 0.33^{b}	6.67 ± 0.33^{bc}
VI	4.67 ± 0.21	7.17 ± 0.31^{b}	7.50 ± 0.22^{c}
VII	4.50 ± 0.43	6.00 ± 0.37^{ab}	6.50 ± 0.22^{b}
VIII	5.33 ± 0.33	6.50 ± 0.43^{b}	6.67 ± 0.33^{bc}
IX	5.33 ± 0.33	7.17 ± 0.31^{b}	7.50 ± 0.22^{c}
X	5.33 ± 0.21	7.17 ± 0.31^{b}	7.50 ± 0.22^{c}

Values (Mean ± S.E.) bearing different superscripts (a, b, c) in a column differ significantly (p<0.05).

3.1.3 Biochemical investigations

3.1.3.1 Total proteins

Total protein levels were significantly elevated in birds supplemented with *C. longa* (2.5 and 5.0 g/kg feed), *O. sanctum* (2.5 and 5.0 g/kg feed), and *P. nigrum* (5.0 and 10.0 g/kg feed) powder individually and in combinations (at doses of 2.5, 2.5 and 5.0 g/kg and 5.0, 5.0 and 10.0 g/kg feed) (groups III, IV, V, VI, VII, VIII, IX, and X) compared to chicks in the control groups. At the 3rd week of age, the total protein levels in birds supplemented with *C. longa* (2.5 and 5.0 g/kg feed), *O. sanctum* (5.0 g/kg feed), and *P. nigrum* (10.0 g/kg feed) powder individually and in their respective mixtures (at doses of 2.5, 2.5 and 5.0 g/kg and 5.0, 5.0 and 10.0 g/kg feed) exhibited a similar effect to birds supplemented with vitamin E and selenium (group II). The total protein level at the 5th week of age in birds supplemented with *C. longa* (2.5 and 5.0 g/kg feed), *O. sanctum* (2.5 and 5.0 g/kg feed), and *P. nigrum* (5.0 and 10.0 g/kg feed) powder individually exhibited a similar effect to birds supplemented with vitamin E and selenium (group II). Additionally, at the 5th week of age, the total protein level of birds supplemented with a mixture of *C. longa*, *O. sanctum*, and *P. nigrum* powder (at doses of 2.5, 2.5 and 5.0 g/kg and 5.0, 5.0 and 10.0 g/kg feed) (groups IX and X) was significantly higher than that of birds supplemented with vitamin E and selenium (group II). Details of serum total protein in different groups have been presented in Table 4.

Table 4: Effect of dietary supplementation of *C. longa*, *O. sanctum* and *P. nigrum* powder alone and in combination on serum total protein in broiler (n=12)

Groups	Serum total protein (g/dl)		
	1st week (Day 7)	3rd week (Day 21)	5th week (Day 35)
I	3.05 ± 0.17	2.55 ± 0.11^{a}	2.94 ± 0.25^{a}
II	2.88 ± 0.21	3.48 ± 0.22^{de}	4.00 ± 0.30^{b}
III	2.86 ± 0.06	3.17 ± 0.07^{bcd}	3.69 ± 0.09^{b}
IV	3.09 ± 0.08	3.48 ± 0.09^{de}	3.97 ± 0.13^{b}
V	2.79 ± 0.06	3.15 ± 0.08^{bc}	3.68 ± 0.08^{b}
VI	2.93 ± 0.17	3.52 ± 0.09^{e}	3.94 ± 0.09^{b}
VII	2.74 ± 0.06	2.94 ± 0.08^{b}	3.59 ± 0.07^{b}
VIII	2.92 ± 0.05	3.45 ± 0.06^{cde}	3.95 ± 0.04^{b}
IX	2.86 ± 0.06	3.17 ± 0.08^{bcd}	4.45 ± 0.05^{c}
X	3.09 ± 0.06	3.54 ± 0.04^{e}	4.51 ± 0.12^{c}

Values (Mean ± S.E.) bearing different superscripts (a, b, c, d, e) in a column differ significantly (p<0.05).

Table 5: Effect of dietary supplementation of *C. longa*, *O. sanctum* and *P. nigrum* powder alone and in combination on albumin to globulin ratio (A/G ratio) in broiler (n=12)

Groups	Albumin/globulin ratio		
	1st week (Day 7)	3rd week (Day 21)	5th week (Day 35)
I	0.61 ± 0.04^{a}	0.87 ± 0.04^{c}	0.76 ± 0.07^{d}
II	0.73 ± 0.06^{abc}	0.61 ± 0.07^{ab}	0.52 ± 0.06^{bc}
III	0.71 ± 0.4^{ab}	0.59 ± 0.04^{a}	0.45 ± 0.02^{ab}
IV	0.69 ± 0.09^{ab}	0.54 ± 0.02^{a}	0.44 ± 0.05^{ab}
V	0.76 ± 0.04^{abc}	0.64 ± 0.04^{ab}	0.49 ± 0.02^{bc}
VI	0.69 ± 0.09^{ab}	0.54 ± 0.02^{a}	0.45 ± 0.03^{ab}
VII	0.79 ± 0.03^{bc}	0.85 ± 0.03^{c}	0.58 ± 0.02^{c}
VIII	0.88 ± 0.03^{c}	0.62 ± 0.02^{ab}	0.55 ± 0.02^{bc}
IX	0.64 ± 0.01^{ab}	0.72 ± 0.04^{b}	0.36 ± 0.01^{a}
X	0.61 ± 0.03^{a}	0.55 ± 0.02^{a}	0.36 ± 0.02^{a}

Values (Mean ± S.E.) bearing different superscripts (a, b, c, d) in a column differ significantly (p<0.05).

3.1.3.2 Albumin to globulin ratio (A/G ratio)

During the 1st week, no significant difference was observed in the albumin to globulin (A/G) ratio between birds supplemented with *C. longa*, *O. sanctum*, and *P. nigrum* powder individually and in combination, compared to birds supplemented with vitamin E and selenium (group II). However, by the 3rd week, there was a significant decrease in the A/G ratio in birds supplemented with *C. longa* powder (2.5 and 5.0 g/kg feed), *O. sanctum* powder (2.5 and 5.0 g/kg feed), *P.*

nigrum powder (5.0 g/kg feed), and mixtures of *C. longa*, *O. sanctum*, and *P. nigrum* powder at lower and higher doses (2.5, 2.5, and 5.0 g/kg and 5.0, 5.0, and 10.0 g/kg feed) compared to birds in the control group. At the 3rd week, the albumin to globulin (A/G) ratio in birds supplemented with *C. longa* (2.5 and 5.0 g/kg feed), *O. sanctum* (2.5 and 5.0 g/kg feed), *P. nigrum* (10.0 g/kg feed), and mixtures of *C. longa*, *O. sanctum*, and *P. nigrum* powder at lower and higher doses (2.5, 2.5, and 5.0 g/kg feed and 5.0, 5.0, and 10.0 g/kg feed) (groups III, IV, V, VI, VIII, IX, and X) were similar to all groups supplemented with vitamin E and selenium (group II), except for the birds supplemented with *P. nigrum* powder at the dose rate of 5.0 g/kg (group VII). At the 5th week, the A/G ratio was significantly decreased in birds supplemented with *C. longa*, *O. sanctum*, and *P. nigrum* powder alone or in combination compared to birds in the control group. In the 5th week, the albumin to globulin (A/G) ratio in birds supplemented with *C. longa*, *O. sanctum*, and *P. nigrum* alone was similar to birds given the standard supplement (group II). However, there was a significant decrease in the A/G ratio in birds supplemented with a combination of *C. longa*, *O. sanctum*, and *P. nigrum* at lower and higher doses (2.5, 2.5, and 5.0 g/kg feed and 5.0, 5.0, and 10.0 g/kg feed) (groups IX and X) compared to birds supplemented with vitamin E and selenium (group II). The details of albumin to globulin ratio in different groups have been presented in Table 5.

3.1.4 Haematological investigation

3.1.4.1 Heterophil to lymphocyte ratio (H/L ratio)

The details of mean values of DLC and heterophil to lymphocyte ratio in different groups have been presented in Table 6. The counts of heterophils and lymphocytes, as well as the H/L ratio, showed improvement in birds supplemented with *C. longa*, *O. sanctum*, and *P. nigrum* powder. Specifically, heterophil counts decreased significantly in birds supplemented with *O. sanctum* (5.0 g/kg feed) and a mixture of *C. longa*, *O. sanctum*, and *P. nigrum* at a higher dose (5.0, 5.0, and 10.0 g/kg feed) compared to birds supplemented with vitamin E and selenium. The lymphocyte count increased significantly in birds supplemented with *O. sanctum* powder (5.0 g/kg feed) and a mixture of *C. longa*, *O. sanctum*, and *P. nigrum* at a higher dose (5.0, 5.0, and 10.0 g/kg feed) compared to birds supplemented with vitamin E and selenium. Additionally, the heterophil-to-lymphocyte ratio decreased significantly in birds supplemented with *O. sanctum* powder (5.0 g/kg feed) and the mixture of *C. longa*, *O. sanctum*, and *P. nigrum* given at a higher dose (5.0, 5.0, and 10.0 g/kg feed) compared to birds supplemented with vitamin E and selenium.

Table 6: Effect of dietary supplementation of *C. longa, O. sanctum* and *P. nigrum* powder alone and in combination on differential leukocyte counts and H/L ratio in broiler (n=12)

Groups	Differential leukocyte counts (Per cent)				
	Heterophils	Lymphocytes	Monocytes	Eosinophils	H/L Ratio
I	38.50 ± 1.06^{e}	53.50 ± 1.31^{a}	4.67 ± 0.42	3.33 ± 0.42^{ab}	0.72 ± 0.04^{e}
II	29.17 ± 1.74^{bcd}	62.50 ± 2.05^{bc}	4.83 ± 0.31	3.50 ± 0.34^{b}	0.47 ± 0.04^{cd}
III	30.33 ± 1.45^{cd}	62.83 ± 1.74^{abc}	4.16 ± 0.31	2.66 ± 0.21^{ab}	0.49 ± 0.04^{cd}
IV	25.50 ± 1.26^{ab}	66.33 ± 1.02^{cde}	4.67 ± 0.33	3.50 ± 0.22^{b}	0.39 ± 0.02^{abc}
V	29.33 ± 1.76^{bcd}	63.83 ± 1.87^{bcde}	4.33 ± 0.21	2.50 ± 0.22^{a}	0.47 ± 0.04^{bcd}
VI	24.00 ± 1.06^{a}	67.83 ± 1.17^{de}	4.83 ± 0.40	3.33 ± 0.21^{ab}	0.36 ± 0.02^{ab}
VII	32.67 ± 1.28^{d}	59.50 ± 1.84^{b}	4.83 ± 0.31	3.00 ± 0.37^{ab}	0.55 ± 0.04^{d}
VIII	27.83 ± 1.74^{abc}	64.33 ± 1.73^{bcde}	4.66 ± 0.33	3.16 ± 0.17^{ab}	0.44 ± 0.04^{abc}
IX	28.83 ± 1.51^{bcd}	64.50 ± 1.71^{bcde}	4.16 ± 0.31	2.50 ± 0.22^{a}	0.45 ± 0.04^{abcd}
X	23.50 ± 1.26^{a}	68.67 ± 1.41^{e}	4.66 ± 0.33	3.16 ± 0.17^{ab}	0.34 ± 0.03^{a}

Values (Mean ± S.E.) bearing different superscripts (a, b, c, d, e) in a column differ significantly ($p<0.05$).

3.1.5 Gross and histopathological examination

During necropsy, no appreciable gross changes were observed in the bursa of fabricius, thymus and spleen of any experimental birds. On histopathological examination, normal architecture was observed in the sections of the bursa of fabricius, thymus and spleen of birds given *C. longa, O. sanctum* and *P. nigrum* powder either alone or in a mixture and birds of control and standard control group and are depicted in Figures 1 to 6.

3.2 Assessment of antioxidant enzymes

Antioxidant enzymes play a crucial role in cellular defence mechanisms against oxidative stress-induced damage. These enzymes are proteins responsible for catalyzing the transformation of reactive species, commonly known as free radicals, into stable and non-toxic molecules. By facilitating this process, antioxidant enzymes help to mitigate the harmful effects of oxidative stress, which can lead to cellular damage and various health issues. In essence, they serve as the frontline defence mechanism in combating oxidative stress and maintaining cellular homeostasis.

The serum antioxidant enzyme activity was assessed at 5th week of age in different birds of the same group. The antioxidant enzyme values (Mean ± S.E.) of different experimental groups have been presented in Table 7.

3.2.1 Superoxide dismutase (SOD)

Superoxide dismutase enzyme activity were significantly elevated in chicks supplemented with *C. longa* powder, *O. sanctum* powder, and *P. nigrum* powder, as well as *C. longa*, *O. sanctum*, and *P. nigrum* powder mixtures at both lower and higher doses compared to birds in the control group. Additionally, there was a significant increase in SOD enzyme levels in chicks supplemented with vitamin E and selenium (group II) compared to the control group (group I).

Furthermore, the levels of superoxide dismutase enzyme in birds supplemented with *C. longa*, *O. sanctum*, and *P. nigrum* alone and in mixtures (Groups III, IV, V, VI, VII, VIII, IX, and X) were similar to those observed in birds supplemented with vitamin E and selenium (group II).

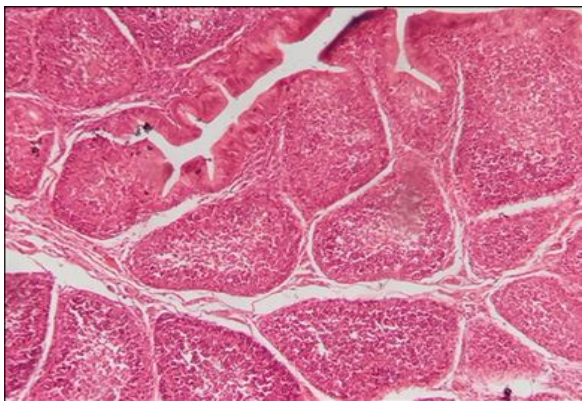

Figure 1: Section of the bursa of Fabricius from the bird of control group showing normal architecture on 35th day of the experiment (H and E, 120 X).

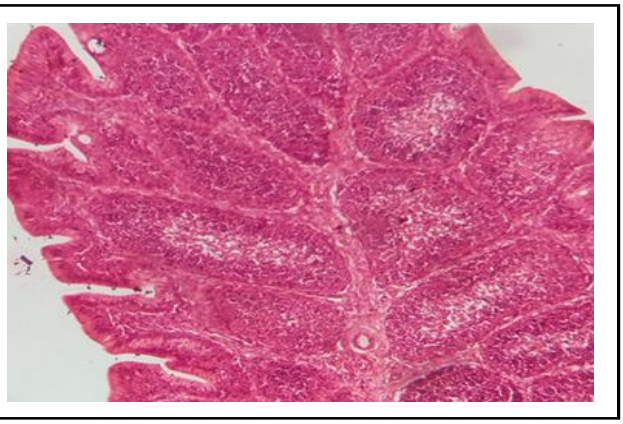

Figure 2: Section of the bursa of Fabricius from bird of *C. longa* powder, *O. sanctum* powder, and *P. nigrum* powder mixture at higher dose supplemented group showing normal architecture at 35th day of the experiment (H and E).

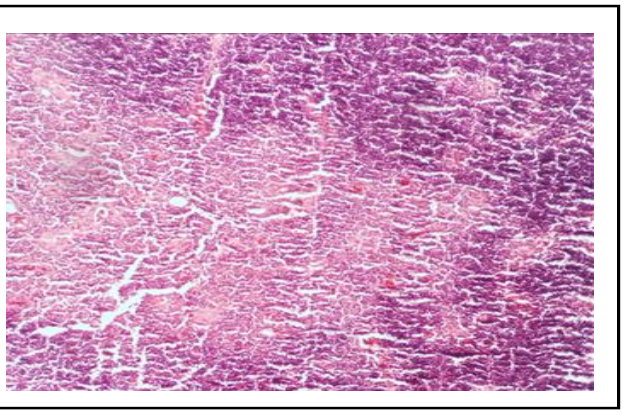

Figure 3: Section of thymus from bird of control group showing normal architecture at the 35th day of the experiment (H and E, 240 X).

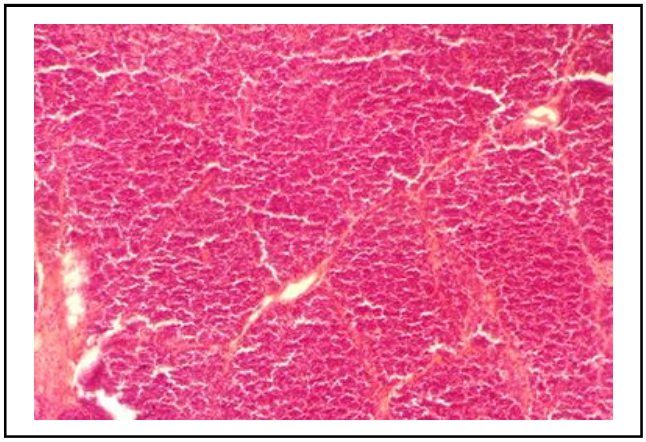

Figure 4: Section of thymus from bird of *C. longa* powder, *O. sanctum* powder, and *P. nigrum* powder mixture at higher dose supplemented group showing normal architecture at 35th day of the experiment (H and E, 240 X).

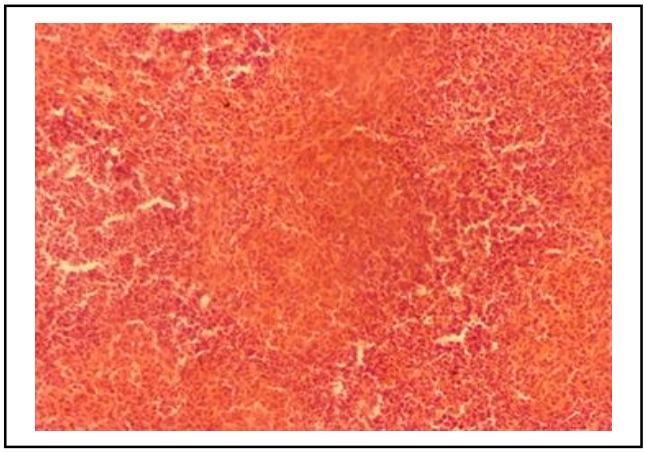

Figure 5: Section of spleen from bird of control group showing normal architecture on the 35th day of the experiment (H and E, 240 X).

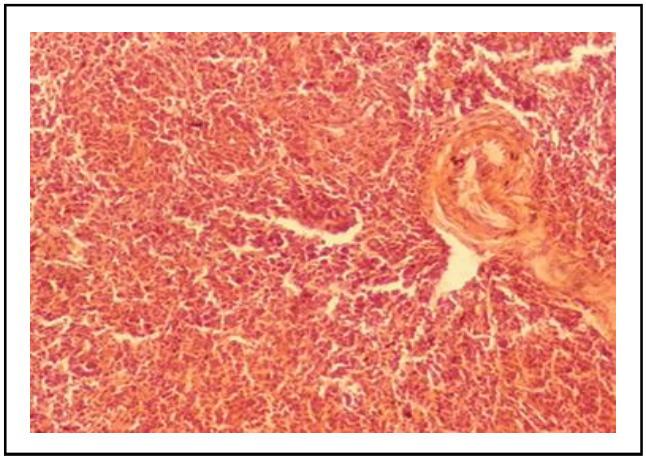

Figure 6: Section of spleen from bird of *C. longa* powder, *O. sanctum* powder, and *P. nigrum* powder mixture at higher dose supplemented group showing normal architecture at 35th day of the experiment (H and E, 240 X).

3.2.2 Catalase

Catalase enzyme activity were significantly increased in chicks supplemented with *C. longa* powder, *O. sanctum* powder, and *P. nigrum* powder, as well as *C. longa*, *O. sanctum*, and *P. nigrum* powder mixtures at both lower and higher doses compared to birds in the control group. Additionally, catalase enzyme levels in birds supplemented with *C. longa* powder (2.5 and 5.0 g/kg feed), *O. sanctum* powder (2.5 g/kg feed), *P. nigrum* powder (5.0 and 10.0 g/kg feed), *C. longa*, *O. sanctum*, and *P. nigrum* powder mixtures at lower dose (2.5, 2.5, and 5.0 g/kg feed), and *C. longa*, *O. sanctum*, and *P. nigrum* powder mixtures at higher dose (5.0, 5.0, and 10.0 g/kg feed) were similar to birds supplemented with vitamin E and selenium. However, catalase levels in chicks supplemented with *O. sanctum* powder at the dose rate of 5.0 g/kg feed (group VI) were significantly higher than those in birds in the control, standard control, and other treatment groups.

3.2.3 Malondialdehyde (MDA)

Malondialdehyde levels were significantly reduced in chicks supplemented with *C. longa* powder, *O. sanctum* powder, and *P. nigrum* powder, as well as *C. longa*, *O. sanctum*, and *P. nigrum* powder mixtures at both lower and higher doses compared to birds in the control group. Furthermore, there was a significant decrease in malondialdehyde levels in chicks supplemented with vitamin E and selenium (group II) compared to the control group (group I). Additionally, malondialdehyde levels in birds supplemented with *C. longa*, *O. sanctum*, and *P. nigrum* (groups III, IV, V, VI, VII, VIII, IX, and X) were similar to birds supplemented with vitamin E and selenium (group II). This suggests that *C. longa*, *O. sanctum*, and *P. nigrum* powder have similar antioxidant effects as the standard supplement.

Table 7: Effect of dietary supplementation of *C. longa, O. sanctum,* and *P. nigrum* powder alone and in combination on antioxidant enzymes of broiler (n=12)

Groups	Serum antioxidant enzymes (5th Week)		
	SOD enzyme (U/ml)	Catalase enzyme (nmol/min/ml)	MDA (µM)
i	0.34 ± 0.03^{a}	57.96 ± 6.53^{a}	14.94 ± 1.53^{d}
ii	0.57 ± 0.02^{bcd}	82.12 ± 6.25^{b}	8.44 ± 1.40^{abc}
iii	0.58 ± 0.02^{bcd}	83.99 ± 9.05^{b}	6.81 ± 0.89^{ab}
iv	0.62 ± 0.00^{cd}	100.84 ± 15.80^{bc}	6.40 ± 0.92^{ab}
v	0.60 ± 0.05^{cd}	91.70 ± 5.03^{b}	6.57 ± 1.56^{ab}
vi	0.69 ± 0.04^{d}	117.49 ± 7.28^{c}	5.91 ± 0.79^{a}
vii	0.46 ± 0.03^{b}	80.68 ± 3.73^{b}	6.60 ± 0.46^{ab}
viii	0.51 ± 0.03^{bc}	82.87 ± 3.82^{b}	6.55 ± 0.50^{ab}
ix	0.54 ± 0.06^{bc}	87.10 ± 3.87^{b}	10.83 ± 1.40^{c}
x	0.57 ± 0.04^{bc}	97.22 ± 7.64^{bc}	9.66 ± 1.15^{bc}

Values (Mean ± S.E.) bearing different superscripts (a, b, c, d) in a column differ significantly ($p<0.05$).

4. Discussion

For the cutaneous basophilic hypersensitivity (CBH) response, similar results were reported by Sethy *et al.* (2017), who observed a significantly higher CBH response in chicks supplemented with turmeric at concentrations of 0.5% and 1.0%. Additionally, Sahoo *et al.* (2019) reported a significantly higher CBH response in chicks supplemented with turmeric at a concentration of 1%. Singh and Doley (2012) and Swathi *et al.* (2013) reported a significantly increased cell-mediated immune response following PHA-P inoculation in chicks supplemented with 1% and 0.25 % tulsi leaf powder, respectively.

During the first week, no significant changes in antibody titers were observed, likely due to maternal transfer. However, by the third and fifth weeks, birds supplemented with *C. longa*, *O. sanctum*, and *P. nigrum* powder, along with vitamin E and selenium, showed significantly increased antibody levels compared to the control group. Moreover, the HI antibody titers in the supplemented groups closely matched those of the vitamin E and selenium groups, indicating similar effectiveness between the supplements.

The findings regarding antibody titers against the Newcastle disease virus vaccine were consistent with Kumari *et al.* (2007), who noted a significant increase in HI antibody titer against Newcastle disease virus in chicks supplemented with *C. longa* (1 g/kg feed). Similarly, Reddy *et al.* (2012) observed a significant increase in antibody titers against Newcastle disease in chicks fed with turmeric (0.25% and 0.50%) and tulsi (0.25% and 0.50%) compared to those in the control group. Fallah and Mirzaei (2016) also reported a significant increase in antibody titers against Newcastle disease in chicks supplemented with 0.50% turmeric powder compared to the control group at the 18th and 28th days of age. Additionally, Rubaee (2020) investigated the immunomodulatory effect of turmeric powder at 0.2% and 0.4% in feed and observed a significant increase in antibody titers against Newcastle disease vaccine in broiler birds supplemented with turmeric powder. Khodadadi *et al.* (2021) also observed markedly higher antibody titers against Newcastle disease during the second vaccination on the 19th day in broiler chicks supplemented with 250 and 500 mg/kg of turmeric powder. Swathi *et al.* (2013) documented notably increased antibody titers against the ND vaccine in chicks supplemented with tulsi powder (0.25% and 0.50%). Singh and Doley (2012) identified a pronouncedly heightened humoral immune response against the ND vaccine in chicks supplemented with 1% tulsi leaf powder. Prasad *et al.* (2012) reported a noteworthy potential

increase in the mean haemagglutination inhibition (HI) antibody titer against the ND vaccine in chicks treated with tulsi powder (2 g/kg). Nayak *et al*. (2016) observed a marked improvement in haemagglutination inhibition titers against the ND vaccine in a group of chicks fed with 4 g/kg of *O. sanctum* dry leaf powder. Mohammed *et al.* (2017) documented a significantly elevated antibody titer against the ND vaccine in chicks supplemented with 0.6% *Ocimum basilicum* seeds. Jahejo *et al.* (2019) observed an enhancement in Newcastle disease humoral immunity in chicks treated with *O. basilicum* powder at a dosage of 5 g/kg feed. Mohamed and Abd Elaziz (2020) noted a significant improvement in the hemagglutination inhibition (HI) antibody titer against ND vaccine in chick groups fed with 0.5% and 1% *O. basilicum* leaves powder. Valiollahi *et al.* (2014) observed a significant increase in ND vaccine antibody titer at 21, 35, and 42 days in broilers fed with 2% black pepper (T2) and 1% ginger + 1% black pepper powders. Abou-Elkhair *et al*. (2014) reported an enhanced antibody titer against ND vaccine at 35th day of age in broilers fed with a mixture of 0.5% black pepper and 2% coriander seed, as well as a mixture of 0.5% black pepper, 0.5% turmeric powder, and 2% coriander seeds in chicks.

The findings regarding serum biochemical investigations were consistent with Al-Jaleel (2012), who noted significantly increased globulin levels in broilers supplemented with turmeric powder at concentrations of 0.25%, 0.50%, 1%, and 1.5% in feed. Similarly, Prasad *et al*. (2012) reported significantly increased serum total protein and globulin levels in groups supplemented with turmeric powder compared to control birds. Moreover, they observed a significant decrease in the albumin-to-globulin ratio in the turmeric powder-supplemented groups compared to the control. ELnaggar *et al.* (2021) also reported a significantly decreased albumin-to-globulin ratio in Japanese quails supplemented with black pepper powder (0.5% in feed) compared to the control birds.

The findings regarding haematological investigations were consistent with Al-Jaleel (2012), who noted a significantly decreased heterophil-to-lymphocyte ratio in chicks supplemented with turmeric powder (0.25%, 0.50%, 1%, and 1.5%) compared to the control. Similarly, Widhowati *et al.* (2017) found significantly reduced heterophils and H/L ratio in chicks receiving 40%, 50%, and 60% ethanolic extract of *C. longa* in drinking water compared to the control group. Al-Kassie *et al.* (2011) reported reduced heterophil count, decreased H/L ratio, and increased lymphocyte count in birds supplemented with *P. nigrum* (0.50%, 0.75%, and 1% feed) compared to the control. Additionally, Al-Kassie *et al*. (2012) observed a reduced H/L ratio in birds supplemented with black pepper powder (0.75% and 1%) compared to the control birds. Aikpitanyi and Egweh (2020) reported a reduced H/L ratio in birds supplemented with black pepper powder (1%) compared to the control birds.

The findings regarding histopathological investigation were consistent with Gupta *et al.* (2007), who observed no histopathological changes in the spleen and bursa sections of birds supplemented with 600 mg of dried leaves powder of *O. sanctum* per chicken daily for 15 days. Similarly, Nath *et al.* (2012) reported no histopathological changes in the thymus, spleen, and bursa supplemented with Tulsi-Black pepper-Clove (TBC) extract at a dose of 1ml/liter in drinking water. Additionally, Biswas *et al.* (2017) reported no significant histopathological changes in any internal organs of broilers supplemented with tulsi leaf extract at a dose of 1ml/liter in drinking water.

The findings concerning serum superoxide dismutase (SOD) investigation were consistent with Hosseini-Vashan *et al.* (2012), who reported an increase in SOD activity in chicks fed 8 g/kg turmeric powder compared to the control diet. Similarly, Wang *et al.* (2015) noted a significant increase in SOD levels in the group of birds supplemented with turmeric rhizome extract (300 mg/kg feed) compared to the control group at 42, 56, and 84 days of age. Additionally, Osman *et al.* (2017) observed a significant increase in SOD activities in the breast and thigh muscles of birds supplemented with turmeric powder (0.5% and 1.0% in feed) compared to the control birds. Sethy *et al.* (2017) also reported a significant increase in SOD levels in the group of birds supplemented with turmeric powder (0.5% and 1.0% in feed) compared to the control group at 42 days of age. Additionally, Badran (2020) documented a notable increase in SOD levels in broiler groups treated with curcumin (100 mg/kg) compared to the control birds. Daramola *et al.* (2020) reported significantly increased SOD levels in the groups of birds supplemented with turmeric powder (0.5% and 1.0% in feed) compared to the control birds. Moreover, Reddy *et al.* (2014) observed a significant increase in SOD levels (50% pyrogallol auto-oxidation/min/mg) in the group of birds supplemented with *O. sanctum* (0.5%) compared to the control birds. Al-Shammari *et al.* (2019) reported a significant increase in SOD levels in the group of birds fed with 1% black pepper powder. Additionally, ELnaggar *et al.* (2021) reported an improvement in SOD levels in Japanese quails supplemented with a mixture of 2% turmeric powder + 0.5% black pepper powder/kg diet.

The findings concerning serum catalase investigation were consistent with those of Sethy *et al.* (2017), who reported a significant increase in catalase levels in the group of birds supplemented with turmeric powder (0.5% and 1.0% in feed) compared to the control birds at 42 days of age. Similarly, Daramola *et al.* (2020) reported significantly increased catalase levels in the group of birds supplemented with turmeric powder (0.5% and 1.0% in feed) compared to the control birds. Additionally, Reddy *et al.* (2014) observed a significant increase in catalase levels in the group of birds supplemented with *O. sanctum* (0.5%) compared to the control birds. Furthermore, Al-Shammari *et al.* (2019) reported a significant increase in catalase levels in the group of birds fed with 1% black pepper powder.

The findings regarding serum malondialdehyde (MDA) investigation were consistent with those of Hosseini-Vashan *et al.* (2012), who reported a significantly reduced MDA plasma concentration in chicks fed with 4 and 8 g/kg turmeric powder compared to control birds under pre and post heat stress conditions. Similarly, Daneshyar *et al.* (2012) reported a significantly decreased serum MDA level in birds supplemented with turmeric powder at 5 mg/kg compared to control birds. Additionally, Wang *et al.* (2015) found that the serum MDA concentration reduced in birds supplemented with dietary turmeric rhizome extract (100, 200, and 300 mg/kg feed) compared to control birds at 5, 8, and 12 weeks of age, respectively. Kanani *et al.* (2017) reported a significant reduction in MDA levels in birds supplemented with cinnamon and turmeric (0.25% turmeric + 0.25% cinnamon) compared to control birds. Furthermore, Sethy *et al.* (2017) reported a significant decrease in MDA levels in the group of birds supplemented with turmeric powder (0.5% and 1.0% in feed) compared to control birds at 42 days of age. Osman *et al*. (2017) noted a significant decrease in MDA activities in breast and thigh muscles of birds supplemented with turmeric powder (0.5% in feed) compared to control birds. Sahoo *et al.* (2019) also observed a

significant decrease in MDA levels in birds supplemented with turmeric powder (0.5% and 1.0% in feed) compared to control birds. Similarly, Badran (2020) reported significantly reduced MDA levels in broilers treated with curcumin (50 and 100 mg/kg) compared to control birds. Additionally, Reddy *et al.* (2014) found significantly decreased MDA levels in birds supplemented with *O. sanctum* (0.5%) compared to control birds. Puvaca *et al.* (2015) reported that black pepper significantly reduced lipid oxidation in breast tissue. Furthermore, Al-Shammari *et al.* (2019) observed a significant decrease in MDA levels in birds fed with 1% black pepper powder compared to control birds.

5. Conclusion

C. longa, *O. sanctum*, and *P. nigrum* powders, individually and in combination, significantly enhanced the cutaneous basophil hypersensitivity response in broilers, akin to the effect seen with vitamin E and selenium supplementation. This suggests that including these powders in the diet stimulates cell-mediated immune response in broilers. Moreover, broiler birds supplemented with these powders showed significantly higher haemagglutination inhibition (HI) antibody titers, comparable to those observed with vitamin E and selenium supplementation. These findings indicate that incorporating *C. longa*, *O. sanctum*, and *P. nigrum* powders, either alone or in combination, stimulates humoral immune response in broilers. Incorporating *C. longa*, *O. sanctum*, and *P. nigrum* powders, individually and in combination, significantly enhanced serum total protein and globulin levels while decreasing the albumin-to-globulin ratio ($p<0.05$). The combined supplementation of these powders exhibited superior effects compared to individual supplementation and even to standard supplements like Vit. E and selenium. Additionally, dietary inclusion of these powders, alone and in combination, led to a reduction in the H/L ratio. Notably, the combination of *O. sanctum* (5.0 g/kg feed) and *C. longa*, *O. sanctum*, and *P. nigrum* powders at a higher dose (5, 5, 10 g/kg feed) demonstrated a lower H/L ratio compared to the standard supplements of Vit. E and selenium. The significant increase in superoxide dismutase and serum catalase levels, along with the notable reduction in malondialdehyde observed in birds supplemented with *C. longa*, *O. sanctum*, and *P. nigrum* powders, both individually and in combination, demonstrates antioxidant activity comparable to the standard supplement of Vit. E and selenium. These findings suggest that *C. longa*, *O. sanctum*, and *P. nigrum* powders, alone or in combination showed immunostimulant and antioxidant effects in broiler.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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