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Augmenting avian immunity naturally: A comprehensive evaluation of the immunomodulatory efficacy of *Tephrosia purpurea* extract in broiler chickens

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Abstract

The present investigation was undertaken to evaluate the immunomodulatory potential of methanolic extract of *Tephrosia purpurea* in broiler chickens. Day-old broiler chicks of Vencobb strain ($n = 36$) were randomly allocated into three experimental groups with three replicates per group and four birds per replicate (Two each for cell-mediated and humoral immune response evaluation). The treatments consisted of: basal diet (Control), basal diet supplemented with Lincomycin @ 0.05% w/w (Standard), and basal diet with methanolic extract of *Tephrosia purpurea* @ 0.04% w/v administered through drinking water (Test). Cell-mediated immune response was assessed using the 2, 4-Dinitrofluorobenzene (DNFB) skin sensitization test, while humoral immune response was evaluated through the micro-haemagglutination assay against sheep red blood cells (SRBC). Haematological indices, including total and differential leukocyte counts, along with immune organ weight factors, were correlated with immune responsiveness. The findings demonstrated that supplementation with *Tephrosia purpurea* extract significantly enhanced both cellular and humoral immune responses in broiler birds, suggesting its promising potential as a phytogetic immunomodulator in poultry production systems.

Keywords: *Tephrosia purpurea*, immunomodulation, cell-mediated immunity, humoral immunity, broiler chicken, phytogetic feed additive

Introduction

The global poultry industry is undergoing a paradigm shift toward sustainable and antibiotic-free production systems due to growing concerns regarding antimicrobial resistance, drug residues in meat, and regulatory restrictions on antibiotic growth promoters. According to the World Health Organization (2023), antimicrobial resistance remains one of the top ten global public health threats, prompting urgent calls for alternative strategies to maintain animal health and productivity^[1]. In this context, phytogetic feed additives and plant-derived immunomodulators have emerged as viable and eco-friendly alternatives to conventional chemotherapeutic agents.

Immunomodulation refers to the alteration of immune responses through stimulation or suppression of specific and non-specific mechanisms. Herbal immunomodulators often exert their effects by enhancing macrophage activity, lymphocyte proliferation, cytokine production, and antibody synthesis^[2, 3]. Unlike synthetic immunostimulants, medicinal plants typically exhibit multi-targeted actions due to their diverse phytochemical constituents, including flavonoids, alkaloids, terpenoids, saponins, and phenolic compounds. Recent studies in poultry science have demonstrated that botanical extracts can improve vaccine responsiveness, enhance resistance to infectious diseases, and optimize growth performance under stress conditions^[4, 5].

Several medicinal plants such as *Curcuma longa*, *Withania somnifera*, *Ocimum sanctum*, *Panax ginseng*, and *Zingiber officinale* have been extensively studied for their immunomodulatory and adaptogenic properties in both laboratory animals and poultry species. These botanicals have demonstrated enhancement of antibody titers, stimulation of delayed-type hypersensitivity reactions, and improvement in lymphoid organ development.

Tephrosia purpurea (Family: Fabaceae), commonly known as Wild Indigo, is a perennial herb widely distributed across the Indian subcontinent. Traditionally used in Ayurvedic and Unani systems of medicine, the plant is reputed for its hepatoprotective, anti-inflammatory, antioxidant, antidiabetic, and anticancer properties [6]. Phytochemical investigations reveal that *T. purpurea* is rich in flavonoids, rotenoids, glycosides, sterols, and phenolic compounds, which are known to modulate immune signalling pathways and oxidative stress mechanisms [7].

Oxidative stress and immunosuppression are common challenges in intensive broiler production systems. Heat stress, vaccination, high stocking density, and pathogen exposure compromise immune efficiency and predispose birds to disease outbreaks. Emerging evidence suggests that plant-derived antioxidants can attenuate oxidative damage, thereby enhancing immune competence and overall performance [8]. Despite the documented pharmacological benefits of *Tephrosia purpurea*, limited scientific data are available regarding its immunomodulatory efficacy in poultry.

Therefore, the present study was designed to systematically evaluate the immunomodulatory potential of methanolic extract of *Tephrosia purpurea* in broiler chickens by assessing both cell-mediated and humoral immune responses, haematological indices, and immune organ development under controlled experimental conditions.

Materials and Methods

Plant Material Collection, Authentication and Extraction

The whole plant of *Tephrosia purpurea* (Family: Fabaceae) was collected from a local agro-climatic region and authenticated by a qualified taxonomist. The plant material was shade-dried at ambient temperature (25-30 °C) to preserve thermo-labile bioactive compounds and subsequently pulverized into coarse powder using a mechanical grinder.

Methanolic extraction was performed using Soxhlet apparatus following continuous hot percolation technique, a standardized method widely employed for efficient recovery of flavonoids and rotenoids [7]. Methanol was selected as the solvent due to its high polarity index and proven efficiency in extracting phenolic and immunoactive phytoconstituents [8]. The extract was concentrated under reduced pressure using a rotary evaporator and stored at 4 °C in airtight containers until further use to prevent oxidative degradation.

Experimental Birds, Housing and Ethical Compliance

All experimental procedures were carried out in accordance with guidelines prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India [9]. Institutional Animal Ethics Committee (IAEC) approval was obtained prior to the commencement of the study.

A total of 36 day-old Vencobb broiler chicks were procured from a commercial hatchery and randomly allocated into three treatment groups with three replicates per group (Four birds per replicate). Randomization was performed to minimize selection bias [10]. Birds were individually identified using numbered leg bands.

The birds were reared under standard deep litter system with controlled temperature (32 °C during first week with gradual

reduction), relative humidity (60-70%), and 23-hour light schedule during starter phase as per commercial broiler management guidelines [11]. Feed and water were provided ad libitum throughout the experimental period of six weeks.

Experimental Design and Treatment Regimen

The study followed a completely randomized design (CRD). The treatment groups were as follows:

- **Control Group (T₁):** Basal diet
- **Standard Group (T₂):** Basal diet supplemented with Lincomycin @ 0.05% w/w
- **Test Group (T₃):** Basal diet + *T. purpurea* extract @ 0.04% w/v in drinking water

The basal diet was formulated to meet or exceed nutrient requirements for broiler chickens as recommended by the National Research Council [12, 13]. Lincomycin was used as a standard antibiotic growth promoter owing to its established antimicrobial and growth-enhancing properties in poultry [14]. The dosage of plant extract was selected based on preliminary pilot trials and previously reported safe phyto-genic inclusion levels in poultry feed [15].

Assessment of Cell-Mediated Immune Response (CMI)

Cell-mediated immunity was evaluated using the 2,4-Dinitrofluorobenzene (DNFB)-induced delayed-type hypersensitivity (DTH) assay, a widely accepted *in vivo* method for assessing T-cell-mediated immune response in poultry [15].

A featherless area was demarcated on both sides of the abdomen and cleaned with acetone. The right side was sensitized with 2000 µg DNFB dissolved in acetone:olive oil (4:1). The left side served as vehicle control. Fourteen days post-sensitization, birds were challenged with 50 µg DNFB at the same site.

Skin thickness was measured at 0, 24, and 48 hours post-challenge using an engineer's micrometer. The mean increase in skin thickness represented DTH response and was interpreted as an indicator of T-lymphocyte activation and macrophage infiltration [16].

Assessment of Humoral Immune Response

Humoral immunity was assessed by micro-haemagglutination assay against sheep red blood cells (SRBC), a classical T-dependent antigen model extensively used in avian immunological studies [3]. Fresh sheep blood was collected aseptically in Alsever's solution and stored at 4 °C for stabilization. The erythrocytes were washed thrice with sterile normal saline by centrifugation at 3000 rpm for 10 minutes. A 7% SRBC suspension was prepared in sterile saline.

Six birds from each group were immunized intravenously with 1 ml SRBC suspension. Blood samples were collected on day 10 post-immunization. Serum was separated and heat-inactivated at 56 °C for 30 minutes to inactivate complement proteins. Serial two-fold dilutions were prepared in microtiter plates and incubated with SRBC suspension.

The highest serum dilution exhibiting complete haemagglutination was recorded as antibody titre and expressed in log₂ values, reflecting B-cell mediated antibody production [17].

Hematological Analysis

Heparinized blood samples were collected from the wing vein under aseptic conditions. Total leukocyte count (TLC) was determined using Natt and Herrick's diluting fluid and Neubauer hemocytometer following standard hematological protocols^[18].

Differential leukocyte count (DLC) was performed on Leishman-stained peripheral blood smears under oil immersion microscopy. Percentage distribution of lymphocytes, heterophils, monocytes, eosinophils, and basophils was recorded. The heterophil: lymphocyte (H: L) ratio was calculated as an indicator of physiological stress and immune competence^[19].

Immune Organ Weight Index

At the end of the experimental period, birds were humanely sacrificed. Primary and secondary lymphoid organs including spleen, thymus, and bursa of Fabricius were carefully excised and weighed using a precision digital balance.

Organ weight index was calculated as:

$$\text{Organ Weight Index} = \frac{\text{Organ Weight (g)}}{\text{Live Body Weight (g)}} \times 100$$

Relative organ weight is considered a reliable indicator of immune organ development and functional status in poultry^[20].

Statistical analysis

All data were expressed as Mean $\pm$ Standard Error (SE) for six birds per treatment group. Statistical analysis was performed using one-way Analysis of Variance (ANOVA) under completely randomized design. When significant differences were observed, Duncan's Multiple Range Test was applied for post-hoc comparison at $p < 0.05$ significance level^[21]. Statistical computations were performed using SPSS software version 26.0.

Results

Cell-mediated immune response (CMI)

Cell-mediated immunity was assessed through measurement of cutaneous hypersensitivity reactions following 2,4-dinitrofluorobenzene (DNFB) challenge, quantified as skin thickness at 24 and 48 hours post-exposure. Birds administered methanolic extract of *Tephrosia purpurea* (TPE) demonstrated a markedly enhanced delayed-type hypersensitivity (DTH) response compared to untreated controls.

At 24 hours post-challenge, the mean skin thickness in the TPE-treated group (3.59 ± 0.21 mm) was 54.74% significantly greater ($p < 0.05$) than that observed in the control group (2.32 ± 0.33 mm). This pronounced increase indicates robust T-cell-mediated immune activation and heightened local inflammatory responsiveness.

At 48 hours, the augmented response persisted, with the test group exhibiting a mean skin thickness of 3.52 ± 0.21 mm, surpassing both the standard group (2.92 ± 0.17 mm) and the control group (2.20 ± 0.23 mm). The sustained elevation in dermal thickness suggests that TPE potentiates both the initiation and maintenance phases of DTH reactions.

These findings corroborate earlier observations in murine models, where *Tephrosia purpurea* extract significantly enhanced phagocytic activity as measured by carbon

clearance assays and macrophage migration indices. Additionally, ethanolic extracts of the plant have been reported to modulate late-phase allergic responses in rodents. Comparable immunostimulatory patterns have been documented in avian species treated with botanicals from the same Fabaceae lineage, such as *Cajanus indicus*, wherein macrophage activation, polymorphonuclear leukocyte infiltration, increased vascular permeability, and localized oedema were associated with amplified T-cell-mediated immunity.

Collectively, the present findings indicate that TPE exerts a potent stimulatory influence on cellular immune mechanisms in broiler birds, outperforming both standard and untreated groups in DTH responsiveness.

Humoral Immune Response

The humoral immune response was evaluated by determining haemagglutination (HA) antibody titers against sheep red blood cells (SRBC) on day 42. Birds receiving TPE exhibited significantly elevated HA titers (4.83 ± 0.16) compared to the standard group (3.50 ± 0.22) and control group (2.17 ± 0.17) ($p < 0.05$).

The marked augmentation in antibody titers suggests enhanced B-cell activation and plasma cell differentiation, reflecting improved adaptive humoral immunity. The magnitude of increase in the test group indicates that TPE may act as an immunological adjuvant, promoting antigen-specific antibody production.

These results are consistent with previous reports in albino mice, where administration of *T. purpurea* extract resulted in significantly higher HA titers compared to untreated controls. Moreover, flavonoidal fractions isolated from the plant have demonstrated dual modulation of cellular and humoral immunity, suggesting the involvement of bioactive phytoconstituents capable of influencing lymphocyte proliferation and cytokine signalling pathways.

Total and differential leukocyte count

Hematological profiling revealed that birds treated with TPE had a significantly elevated total leukocyte count (TLC) of $33 \pm 0.96 \times 10^3/\text{mm}^3$, representing a 26.15% increase ($p < 0.05$) over the control group ($26.16 \pm 0.98 \times 10^3/\text{mm}^3$). This elevation indicates systemic immunological activation and enhanced leukopoiesis.

Differential leukocyte analysis showed a non-significant rise in heterophil percentage in the test group ($26.00 \pm 0.45\%$) compared to controls ($24.66 \pm 0.61\%$). Given that heterophils represent the avian equivalent of neutrophils and play a critical role in innate immunity, their elevation may reflect enhanced early-phase inflammatory responsiveness.

Notably, TPE-treated birds exhibited eosinopenia ($0.33 \pm 0.21\%$) relative to untreated birds ($1.00 \pm 0.63\%$). This reduction aligns with prior findings in rodent studies where ethanolic extracts of *T. purpurea* significantly reduced eosinophil counts, potentially indicating modulation of hypersensitivity or parasitic response pathways.

Overall, the haematological alterations observed in the test group suggest coordinated stimulation of both innate and adaptive immune compartments.

Organ weight factor

Post-sacrifice assessment of immune organ indices revealed no significant variation in spleen weight factor among control (1.53 ± 0.11), standard (1.52 ± 0.06), and test groups (1.48 ± 0.08).

However, non-significant increases were observed in the organ weight factors of the bursa of Fabricius (0.77 ± 0.03) and thymus (1.50 ± 0.57) in TPE-treated birds compared to controls (0.63 ± 0.04 and 1.48 ± 0.24 , respectively). The standard group exhibited intermediate values (bursa: 0.74 ± 0.06 ; thymus: 1.59 ± 0.56).

Although statistically non-significant, the upward trend in primary lymphoid organ indices may indicate subtle trophic effects on lymphoid development. Similar findings have been reported in birds treated with *Cajanus indicus*, wherein enhanced spleen, bursa, and thymus weights were observed relative to controls.

Discussion

The present investigation demonstrates that methanolic extract of *Tephrosia purpurea* exerts a comprehensive immunostimulatory effect in broiler birds, encompassing both cell-mediated and humoral arms of the immune system. The amplified delayed-type hypersensitivity response observed in TPE-treated birds signifies enhanced T-lymphocyte activation and macrophage recruitment hallmarks of a potentiated Th1-mediated immune response. DTH reactions are widely recognized as reliable *in vivo* correlates of cellular immunity, reflecting antigen-specific T-cell sensitization and cytokine-mediated inflammatory cascades.

The persistence of elevated dermal thickness at both 24 and 48 hours post DNFB challenge suggests that TPE not only accelerates immune activation but may also sustain effector mechanisms. Such sustained responses are indicative of improved immunological vigilance, particularly relevant in poultry production systems where rapid pathogen exposure is common. Contemporary immunopharmacological research has increasingly emphasized plant-derived immunomodulators as alternatives to antibiotic growth promoters in poultry, especially following global regulatory restrictions on antimicrobial use [22, 23]. Botanical extracts rich in flavonoids, alkaloids, and phenolic compounds have been shown to enhance macrophage function, nitric oxide production, and cytokine secretion, thereby fortifying host resistance.

The elevated haemagglutination titers observed in TPE-treated birds further reinforce the dual modulatory capacity of the extract. Enhanced antibody production implies effective antigen presentation, T-helper cell cooperation, and B-cell maturation. In poultry immunology, HA titers against SRBC serve as classical indicators of humoral competence and vaccine responsiveness. The pronounced increase in titers in the test group suggests that TPE may function as a natural immunological adjuvant, potentially improving vaccine efficacy and long-term protective immunity. Recent studies have highlighted the importance of phytochemical feed additives in optimizing antibody responses and modulating cytokine profiles in broilers [24, 25]. Haematological findings provide additional mechanistic insight. The significant elevation in total leukocyte count implies activation of hematopoietic pathways and systemic immune readiness. Increased heterophil proportions,

although not statistically significant, align with enhanced innate defense capacity. In avian species, heterophils are critical first-line effector cells, mediating phagocytosis and oxidative burst activity. The observed eosinopenia may reflect modulation of hypersensitivity-related pathways or redistribution of eosinophils during immune activation. Similar immuno-hematological trends have been documented following administration of phytochemical immunostimulants in poultry [26].

Interestingly, the absence of significant changes in spleen weight alongside modest increases in thymus and bursa indices suggests that TPE's immunomodulatory action may operate more at the functional and cellular signalling level rather than inducing gross anatomical hypertrophy. Primary lymphoid organs in birds are highly sensitive to nutritional and pharmacological influences, and even subtle increases may reflect improved lymphopoiesis and immune organ maturation.

Phytochemical analyses of *Tephrosia purpurea* have identified bioactive constituents such as flavonoids, rotenoids, and polyphenols, compounds known to influence NF- κ B signaling, cytokine transcription, and oxidative stress pathways. Contemporary immunological frameworks recognize that modulation of redox balance is intimately linked with immune competence. By potentially enhancing antioxidant defense systems, TPE may create a cellular microenvironment conducive to efficient lymphocyte proliferation and macrophage activity.

From a translational perspective, the implications of these findings are significant. With increasing emphasis on sustainable poultry production and reduction of synthetic immunomodulators, plant-derived agents such as *T. purpurea* offer promising alternatives. Their multimodal mechanism simultaneously enhancing cellular immunity, antibody production, and leukocyte proliferation positions them as viable candidates for integrated disease management strategies. However, advanced molecular studies are warranted to delineate the precise signalling pathways involved, quantify cytokine expression profiles, and isolate the principal active compounds responsible for immuno-potential.

Table 1: Effect of TPE Supplementation on Cell-Mediated Immune Response in Broiler Birds

Duration of DNFB application	Abdominal side	Skin thickness (mm) of group		
		Control (n = 6)	Standard (n = 6)	Test $\pm$ (n = 6)
Pre-challenge	Left	0.57 ± 0.02	0.57 ± 0.02	0.56 ± 0.01
	Right	0.56 ± 0.02	0.55 ± 0.002	0.55 ± 0.02
24 hrs post-challenge	Left	0.57 ± 0.03	0.57 ± 0.02	0.56 ± 0.01
	Right	2.32 ± 0.33^c	3.17 ± 0.22^b	3.59 ± 0.21^a
48 hrs post-challenge	Left	0.57 ± 0.02	0.56 ± 0.03	0.55 ± 0.02
	Right	2.20 ± 0.23^c	2.92 ± 0.17^b	3.52 ± 0.21^a

(Left side served as vehicle control; right side treated with DNFB)
Values are mean $\pm$ SE. Values in a row with different superscripts differ significantly ($p < 0.05$).

Table 2: Effect of TPE Supplementation on Humoral Immune Response Against SRBC in Broiler Birds

Particular	Group		
	Control (n = 6)	Standard (n = 6)	Test (n = 6)
HA titre Log ₂ value	2.17 ± 0.17^c	3.5 ± 0.22^b	4.83 ± 0.16^a

Values are mean $\pm$ SE. Values in a row with different superscripts differ significantly ($p < 0.05$).

Table 3: Effect of TPE Supplementation on TLC and DLC in Broiler Birds on Day 42

Leukocyte parameter	Group		
	Control (n = 6)	Standard (n = 6)	Test (n = 6)
TLC (thousand/mm ³)	26.16 ± 0.98 ^b	28 ± 1.52 ^b	33 ± 0.96 ^a
Heterophil (%)	24.66 ± 0.61	25.50 ± 0.22	26.00 ± 0.45
Lymphocyte (%)	72 ± 0.80	73.00 ± 0.37	72.83 ± 0.48
Monocyte (%)	1.83 ± 0.40	0.83 ± 0.40	0.67 ± 0.42
Eosinophil (%)	1.00 ± 0.63	0.50 ± 0.22	0.33 ± 0.21
Basophil (%)	0.33 ± 0.21	0.17 ± 0.17	0.17 ± 0.17

Values are mean ± SE. Values in a row with different superscripts differ significantly ($p < 0.05$).

Conclusion

The present study provides compelling evidence that methanolic extract of *Tephrosia purpurea* functions as a potent immuno-stimulatory agent in broiler birds. By significantly enhancing delayed-type hypersensitivity responses, elevating hemagglutination antibody titers, and increasing total leukocyte counts, the extract demonstrates a comprehensive capacity to strengthen both cellular and humoral immunity.

While changes in lymphoid organ indices were modest, the functional immunological enhancements observed underscore the therapeutic potential of this botanical extract in poultry health management. These findings support the strategic integration of *Tephrosia purpurea* as a phyto-genic immuno-modulator capable of improving disease resistance and reducing dependency on conventional antimicrobial agents.

Future investigations employing molecular immunological tools, cytokine profiling, and bioactive compound isolation are essential to fully elucidate its mechanistic pathways and optimize its application in commercial poultry production systems.

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