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Effects of *Moringa oleifera* Leaf Powder Supplements on Histomorphometry of Lymphoid Organs and Immune Responses of Broilers Under Dexamethasone-Induced Stress

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Key words
Broiler, Bursa of Fabricius, Dexamethasone, Broilers immunity, Phytobiotics, Stress, *Moringa oleifera*, Lymphoid organs

ABSTRACT

In this study, dexamethasone (De)-induced stress was studied on immune organs and immune responses of broilers to *Moringa oleifera* leaf powder (MOLP) supplements. Day-old 100 chicks were divided into 5 groups having 4 replicates, (5 birds/replica). Negative control/Group A was given basal diet (BD), positive control/B with De (15mg/kg of diet after day 21st) + BD, C with De + 0.8% MOLP, D with De + 1.2% MOLP, and E with De + 1.6% MOLP. On the 35th day, 2 birds/replica were slaughtered for sampling bursa of Fabricius (BF), cecal tonsils and intestine. Lymphatic follicular width and area of BF were significant ($P \leq 0.05$) in the MOLP group 1.6%, in all groups, lymphatic follicular length and number were non-significant. Lymphatic nodules length, width, area, and number of cecal tonsils were non-significant in all groups. Antibody titers against Newcastle disease virus were unaffected and antibody titers against sheep red blood cells were significant ($P \leq 0.05$) in 1.6% MOLP-treated birds on the 21st and 35th day, cell-mediated immunity was non-significant. Also, after 72 h, cell-mediated immunity was significant ($P \leq 0.05$) in MOLP groups (1.2% and 1.6%), and goblet cell counts were significant ($P \geq 0.05$) in MOLP-treated groups in all intestinal portions except acidic goblet cells in the duodenum. Intra-epithelial lymphocyte number was non-significant among MOLP groups except for ileum ($P \leq 0.05$). It was observed that 1.6% MOLP in broiler feed had a moderate effect on the broiler's immunity.

INTRODUCTION

Poultry production mainly focuses on the commercial keeping and breeding of birds including chickens, ducks, turkeys, and quails. Chicken production accounts for more than 90% of all poultry produced in most of the world's countries (Biswal *et al*., 2022). Several factors including short lifespans, low collagen levels, inexpensive protein sources, enrichment of minerals (copper, iron, zinc, etc.), significant sources of pantothenic acid, vitamin B6, thiamin, and egg production make poultry production a healthier choice. Also, significant progress has been made in the fields of poultry genetics, fat reduction, enhancement in breast muscles, and food conversion ratio (Rehman *et al*., 2018; Mishra and Jha, 2019; Ademu *et al*.,2021).

The production performance of poultry birds is mostly affected by stressors; among them, excessive heat, stocking density, unavailability of proper nutrients, and pathogenic infections are obvious which in severe conditions can lead to oxidative stress. Prolonged

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exposure to oxidative stress causes a hormonal cascade in stressed animals, which raises glucocorticoid levels, when glucocorticoids are secreted in excessive concentrations, they suppress birds immune systems, inhibit growth, hurt animal performance, and ultimately result in diseases in birds (Yang *et al*., 2015). Such harmful effects of glucocorticoids are apparent in birds, and stress responses have been documented worldwide in response to synthetic glucocorticoids, such as dexamethasone, which are primarily administered to simulate the effects of excessive glucocorticoids on livestock (Chang *et al*.,2015; El-Senousey *et al*., 2018).

Birds' immunity mainly comprises primary lymphoid organs like the thymus and Bursa of Fabricius (BF) and secondary lymphoid organs like the spleen and gut-associated lymphoid tissues (GALT). B lymphocyte production and humoral immunity are concerned with BF, while along with cellular immunity, cecal tonsils produce T and B cells. Oxidative stress modifies the normal morphological and physiological functions of BF and thymus, and high levels of free radicals are obvious in such stressed birds (Chen *et al*., 2014; Balami *et al*., 2018; Wang *et al*., 2019). Various stressors activate the hypothalamus pituitary adrenal axis to evoke the hypothalamus which in turn secretes corticotrophin-releasing factors, these factors stimulate the pituitary gland to secrete adrenocorticotropic hormone (ACTH), and secretions of ACTH release glucocorticoids from adrenal cortex just after the broiler are exposed to oxidative stress (Ademu *et al*., 2018). Glucocorticoids are crucial for an animal's body to function normally, and the assessed amounts of these hormones hurt an animal's body and result in hypertension, gluconeogenesis, immunosuppression, and cardiovascular diseases (Kandeil *et al*., 2019). The levels of glucocorticoids depend upon exposure time, intensity of stressors, animal type, genetic makeup, experiences, and the concentrations of glucocorticoids are also different in the blood of various species and even different in the same species after exposure to stress, it mostly depends upon the body status, sex, and breeding conditions (Cockrem, 2013).

The ban on the usage of antibiotics in poultry feed by European Unions in 2006 has led scientists to think about alternatives such as probiotics, prebiotics, trace minerals, and phytobiotics to overcome antimicrobial resistance in bacterial strains and antibiotics residues in human food (Osho and Adeola, 2020). Nutritional supplements play a significant part in the development of different immunological organs. Probiotics, prebiotics, phytobiotics, and trace minerals are some of the nutritional supplements with the potential to alter the immune system has been thoroughly studied (Nabi *et al*., 2020). However, phytobiotics are always on the menu when it comes to alternatives to antibiotics and due to their beneficial aspects, plant derivates are widely administrated in birds' feed to boost immunity against diseases and enhance growth performance. Phytobiotics have a wide range of functions, including increasing gut digestibility, improving gut secretions, detoxifying microbial toxins, and enhancing gut histology. Hence, inflammation is reduced, and the energy is used for growth and development rather than immunity. *Moringa oleifera*, a fast-growing, drought-resistant tree that mostly grows to the height of 5-12 meters and is commonly used in poultry feed and medicine, is one such promising antibiotic substitute (Ullah *et al*., 2022; Diaz-Sanchez *et al*., 2015). *M. oleifera* (Family Moringaceae), is used in medicines mainly, seeds, leaves, roots, flowers, and pods have a variety of vitamins (such as folic acid, E, A, B2, B5, and B6), and minerals (such as calcium, iron, potassium, magnesium, phenols). Its roots, and flowers contain pterygospermin , and are known for antibacterial and antifungal activities. *M. oleifera* leaf powder (MOLP) supplements in feed, has shown growth-enhancing, anti-inflammatory, antioxidant, anti-ulcerous, anti-cancerous, and immune modulatory activities. Moreover, *M. oleifera* does not pose a significant risk to an animal's life (Khan *et al*., 2017; Mousa *et al*., 2017; Sogut and Mohammad, 2018). Therefore, in a healthy immune system, the body needs energy, various proteins for antibody production, and a wide range of minerals and enzymes like vitamins E and A to communicate information to various parts of the animal body about infections. Surprisingly, *M. oleifera* contains most of the carbohydrates, proteins, vitamins, and minerals that improve immunity against any stressful conditions. Thus, immune-modulatory effects of *M. oleifera* have gained the attention of researchers and it is in high demand for its medicinal uses (Balami *et al*., 2018). However, very little is known about the effect of *M. oleifera* on poultry's immunity. The goal of this study was to highlight the potential benefits of MOLP supplements in broiler feed under dexamethasone-induced stress.

MATERIALS AND METHODS

Birds grouping and feeding plan

Day-old 100 broiler chicks were divided into 5 groups, A, B, C, D, and E each of 20 at a poultry farm in Abdul Wali Khan University, Mardan, Pakistan. Each group was various percentages of MOLP were administrated from day first to chicks in BD and chicks were vaccinated on days 7th, 14th, and 21st against Newcastle disease virus. Group A (Negative control received only basal diet (BD), while other groups were given 15 mg/Kg of

the diet Dexamethasone (De) after day 21st of the trial along with (MOLP). Group B (positive control) received dexamethasone (De) supplemented BD, group C received 0.8% MOLP, group D received 1.2% MOLP, and group E received 1.6% MOLP in the de-supplemented BD throughout the trail. The various percentages of MOLP were administered from day one to chicks in BD and chicks were vaccinated on days 7, 14 and 21 against Newcastle disease virus.

Sampling of tissues and hisological studies

From each replicate two birds were randomly chosen for sampling on day 35th. Lymphoid organs, bursa of Fabricus (BF), and cecal tonsils (CT) were removed for tissue processing. Intestinal samples (duodenum, jejunum, and ileum) were taken for intra-epithelial lymphocytes (IEL) and goblet cell counts and were fixed in formaline. Bancroft et al. (2018) protocol was used for tissue processing, paraffin embedding and hematoxylin and eosin (H & E) staining.

Progress capture Pro 2.7.7. Labomed USA was used to examine the stained slides and under a bright field microscope, the number, width, length, and lymphatic nodules (LN) area in CT were examined with 4X objective lens, and measued. Lymphatic nodule areas (LNA) were calculated by LNA = NW x NL, where NW represents nodular width and NL represents nodular length.

The number, width, length, and lymph follicles area were studied in the bursa of Fabricus. Areas of lymphatic follicles (LFA) were measured by LFA = FW x FL, in which FW represented follicular width while FL represented the length of the follicle (Khan *et al*., 2017).

Immune responses of broilers

Antibody titers were assessed against NDV and sheep red blood cells (SRBCs) to determine humoral immune response. Two chickens were randomly marked from each replicate on day 14th and injected 5% SRBCs. Blood samples were obtained on 21st and 35th day from chosen birds. Blood was centrifuged at 2500 x g for 15 min to obtain serum and kept at -45°C for further examination. Micro-titer assays for hemagglutination inhibition (HI) and hemagglutination (HA) were employed to determine the immune response to NDV and SRBCs (Darabighane et al., 2012).

Phytohemagglutinin (PHA) was injected to examine cell-mediated immune response in broilers (Corrier, 1990). Two broilers per replicate were randomly chosen on day 17th, and PHA (0.1g/0.1ml/chicken) was injected intra-dermally (between 3rd and 4th digit) in right foot. The control group (left foot) was administered 0.1 ml of sterile phosphate buffer solution. Swelling of the injection site was measured with the help of the vernier caliper at the 24th, 48th, and 72nd h after inoculation. Cell-mediated immune response was calculated by subtracting the thickness of the right foot's digits from the thickness of the left foot's.

A bright field microscope was used for slides (H & E stained), five well-intact villi were examined to count intra-epithelial lymphocytes (IEL) and were identified as spherical cells with pale cytoplasmic borders and richly stained spherical basophilic nuclei. Alcian blue periodic acid Schif staining procedures were used and goblet cells were identified as cells having wide apical portions and narrow bases (Khan *et al*., 2017).

Statistical analysis

Data was statistically analyzed as standard error mean (SEM) using SPSS (Version 22.0). Using one-way ANOVA, group means were examined, post-hoc Tukey's test was used for differences in groups, and a significant level was observed at ($P \leq 0.05$).

RESULTS

Morphometry of Bursa of Fabricius and cecal tonsils

Table I shows effect of MOLP on morphometry of bursa of Fabricius and cecal tonsils. The lymphatic follicular area and width in the 1.6% MOLP treated group was significantly higher ($P \leq 0.05$), in comparison with the positive control group.

The histomorphology of cecal tonsils of broiler chickens in response to MOLP supplements under dexamethasone-induced stress showed that in experimental groups the number, length, width, and area of lymphatic nodules were not affected throughout the experimental period.

Immune responses under induced stressed

The humoral immunological response was determined by measuring antibody titers against sheep red blood cells (SRBCs) and NDV by hemagglutination inhibition (HI) and hemagglutination (HA) assays The antibody titer against NDV was not higher in all the treated groups in comparison with the positive control group, while in 1.6 % MOLP administrated bird's antibody titer against SRBCs were higher ($P \leq 0.05$) on day 21st and 35th as shown in Table II.

Table II also does not show significant changes in cell-mediated immunity after 24 and 48 h of phytohemagglutinin injection. However, after 72 h of phytohemagglutinin injection, there was an increase in cell-mediated immunity ($P \leq 0.05$) in 1.2% and 1.6% of MOLP.

Table I. Bursa of Fabricius and Cecal tonsils morphometry in birds administered *Moringa oleifera* leaves powder (MOLP) during dexamethasone-induced stress.

Parameters	NC	PC	0.8% MOLP	1.2% MOLP	1.6% MOLP	P value
Bursa of Fabricius morphometry						
LFN	28.0±1.0	25.5±5.5	26.0±4.0	32.0±1.0	23.5±1.5	0.47
LFL (µm)	1521.6±2.8	1054.4±198.0	1310.2±102.0	1091.2±79.4	1489.3±130.3	0.11
LFW (µm)	637.7±76.6^{ab}	481.8±170.9^{b}	729.7±38.8^{ab}	669.7±164.5^{ab}	1300.0±97.9^{a}	0.03
LFA (µm)^2	970213.8±114875.5^{ab}	541973.8±275723.7^{b}	960119.7±125336.1^{ab}	717844.4±126372.3^{b}	1948994.1±315398.7^{a}	0.03
Cecal tonsils morphometry						
LNN	4.5±0.5	3.0±0.0	4.5±0.5	5.5±0.5	5.5±0.5	0.49
LNL (µm)	377.5±2.6	287.7±16.7	454.5±5.3	377.4±127.3	492.4±131.5	0.51
LNW (µm)	392.2±7.3	337.3±60.3	462.7±97.6	432.7±197.6	538.1±139.1	0.80
LNA (µm)^2	148101.0±3813.7	98073.2±23040.0	210872.8±46854	188517.5±129720.5	283356±139328.4	0.66

LFN, lymphatic follicles number; LFL, lymphatic follicles length; LFW, lymphatic follicles width; LFA, lymphatic follicles area. LNN, number of lymphatic nodules; LNL, length of lymphatic nodules; LNW, width of lymphatic nodules; LNA; area of lymphatic nodules. $^{a-b}$ Means in the same respective row with various superscripts differ significantly ($P \leq 0.05$). NC, negative control; PC, positive control; MOLP, *Moringa oleifera* leaf powder.

Table II. Effect of *Moringa oleifera* leaf powder (MOLP) supplementation on humoral and cell mediated immunity of broilers.

Antibody titer	NC	PC	0.8% MOLP	1.2% MOLP	1.6% MOLP	P-Value
Humoral immunity						
NDV: 21-day	2.06±0.30	1.85±0.20	2.09±0.40	2.18±0.40	2.00±0.20	0.62
NDV: 35-day	2.18±0.30	1.92±0.25	2.01±0.20	2.24±0.30	1.99±0.25	0.20
SRBC: 21-day	5.21^{c}±0.25	5.33^{bc}±0.2	5.41^{b}±0.25	5.59^{ab}±0.40	5.68^{a}±0.40	0.05
SRBC: 35-day	7.25^{ab}±0.60	6.09^{c}±0.40	6.74^{b}±0.40	7.38^{a}±0.50	7.58^{a}±0.60	0.02
Cell mediated immunity						
24-h	0.62±0.02	0.60±0.02	0.65±0.03	0.66±0.02	0.71±0.04	0.65
48-h	0.51±0.04	0.53±0.04	0.51±0.03	0.63±0.05	0.57±0.04	0.18
72-h	0.39^{bc}±0.02	0.36^{c}±0.02	0.43±0.02	0.59^{a}±0.02	0.49^{b}±0.02	0.04

$^{a-c}$ Means in the same respective row with various superscripts differ significantly ($P \leq 0.05$). NDV, Newcastle disease virus; SRBC, sheep red blood cells. For other abbreviations, see Table I.

Table III. Effect of *Moringa oleifera* leaves supplementation on goblet cells counting of various intestine portions of broiler chickens.

Intestinal portions	Goblet cells	NC	PC	0.8% MOLP	1.2% MOLP	1.6% MOLP	P Value
Duodenum	AGC	53.0±9.0	40.0±6.0	55.0±0.0	59.0±0.0	69.0±2.5	0.06
	MGC	29.5±6.5^{b}	18.0±1.0^{b}	35.5±11.5^{ab}	54.5±2.5^{ab}	56.5±6.5^{a}	0.03
	TGC	82.5±2.5^{c}	58.0±7.0^{c}	90.5±11.5^{c}	113.5±2.5^{ab}	126.0±9.0^{a}	0.00
Jejunum	AGC	64.0±3.0^{ab}	57.0±1.0^{b}	71.0±5.0^{ab}	75.0±0.0^{a}	78.0±2.0^{a}	0.01
	MGC	31.0±2.0^{ab}	22.0±3.0^{b}	39.5±7.5^{ab}	49.5±3.5^{a}	52.5±5.5^{a}	0.02
	TGC	95.0±1.0^{ab}	79.0±4.0^{b}	110.5±12.5^{ab}	124.5±3.5^{a}	130.5±7.5^{a}	0.01
Ileum	AGC	35.5±6.5^{ab}	39.5±1.5^{b}	53.5±3.5^{ab}	73.0±3.0^{a}	67.5±1.5^{a}	0.00
	MGC	24.0±4.0^{c}	19.0±0.0^{c}	43.0±1.0^{b}	50.0±2.0^{ab}	58.5±0.5^{a}	0.00
	TGC	77.5±10.5^{bc}	58.5±1.5^{c}	96.5±2.5^{ab}	123.0±5.0^{a}	126.0±1.0^{a}	0.00

$^{a-c}$ Means in the same respective row with various superscripts differ significantly ($P \leq 0.05$). AGC, Acidic goblet cells; MGC, mixed goblet cell0; TGC, total goblet cell. For other abbreviations, see Table I.

Table IV. Influences of *Moringa oleifera* leaves supplementation on intra epithelial lymphocytes of various intestine segments of broilers.

IEL	NC	PC	0.8% MOLP	1.2% MOLP	1.6% MOLP	P Value
Duodenum	25±1.0	16.0±1.0	20.5±1.5	22.5±4.5	21.5±1.5	0.22
Jejunum	23.5±1.5	15.0±1.0	20.0±2.0	22.0±3.0	21.0±2.0	0.16
Ileum	24.0±1.0[a]	15.0±0.5[b]	19.0±1.0[ab]	21.0±1.0[ab]	19.5±1.5[ab]	0.01

[a-c] Means in the same respective row with various superscripts differ significantly ($P \leq 0.05$). For other abbreviations, see Table I.

Goblet cell counting

Table III shows effect of different concentrations of MOLP on goblet cells in various intestinal portions of broiler chicken. Acidic goblet cells in the duodenum were not significantly affected by the MOLP supplementation while mixed and total goblet cells in the 1.6% MOLP treated group were significant higher ($P \leq 0.05$). Acidic, mixed, and total goblet cells of the 1.2 % and 1.6 % MOLP administrated groups in the jejunum and ileum of broiler chickens were significant higher ($P \leq 0.05$).

Intra-epithelial lymphocytes (IEL) count

Table IV shows the effect of different concentrations of MOLP on IEL count of broiler chicken. Only in the ileum of MOLP-supplemented birds, the IEL counts were significantly ($P \leq 0.05$) higher in comparison with the positive control group. The IEL count in the duodenum and jejunum of MOLP-administrated birds were not significantly affected.

DISCUSSION

The medicinal properties of *M. oleifera* and its products in poultry feeds have created a substantial demand as it contains all the essential nutrients, and vitamins that boost immunity. It was observed that supplementing broiler chickens with MOLP in feed strengthens their immune systems and improves their digestive health (Sogut and Mohammad., 2018; Khan *et al*., 2017). Therefore, the current study was designed to investigate the immunomodulatory effects of MOLP supplements on broiler's immune responses and histomorphometry of lymphoid organs under dexamethasone-induced stress.

The current research trial suggested that MOLP improves the immunity of broilers and morphological parameters of the bursa of Fabricius indicated that the lymphatic follicular area and width were significant ($P \leq 0.05$) in 1.6% MOLP treated birds as compared with positive control group birds. Such results are comparable with the those of Khan *et al*. (2017). The comparative analysis of the lymphatic follicular length and number across all the experimental groups was not higher in all the MOLP-treated groups. The Bursa of Fabricius is a vital immunological organ that is responsible for the growth and development of B lymphocytes and the increase in the lymphatic follicular area can be linked to the proliferation of lymphocytes or possibly due to the release of lymphokines, further investigations are required to explore the precise mechanisms (Saleh, 2014; Cai *et al*., 2012).

Cecal tonsils are known to maintain cellular immunity and provide protection against infectious agents. The comparative analysis of all the MOLP administrated groups revealed that the morphological parameters of the cecal tonsils did not change throughout the trial. The current study showed that the length, width, area, and number of the lymphatic nodules were non-significant among all treated birds throughout the research trial. In contrast, positive results ($P \leq 0.05$) were reported by Khan *et al*. (2017) and the mechanism, is however, not yet known.

The humoral immunological response of broiler chickens under the influence of MOLP administration in all the experimental groups were similar to those reported by Wahab *et al*. (2020) against NDV in their study. Such immunomodulation might be due to the presence of phytochemicals in the MOLP supplementation such as saponins and alkaloids and possibly it can be also linked to the presence of trace minerals like iron, zinc, selenium, manganese, and magnesium (Faluyi and Agbede, 2018).

Cellular immunity is concerned with the removal of infectious agents that have invaded the cells or formed inside the cell due to viral protein and the current investigations have reported that the cell-mediated immune responses of broiler chickens after the 24th and 48th h were non-significant throughout the experimental bird's groups. However, the 1.2% and the 1.6% MOLP administrated groups showed significant results ($P \leq 0.05$) regarding cell-mediated immunity after 72nd h as compared to the positive control group while in contrast to the current research work, non-significant results were reported by Eladia and Ampode (2021).

The innate gut immune system relies mainly on goblet cells to produce a mucus layer, that in turn forms

mucin glycoproteins, that act as a transport medium, barrier, and solvent between the intestine luminal contents and the epithelial lining. This study showed significant increase in goblet cell counts in all intestinal parts, except for acidic goblet cells in the duodenum. These findings are comparable with those of Khan *et al*. (2017). Additionally, the presence of vitamin A in *M. oleifera* may be related to the rise in goblet cell count, as vitamin A is essential for goblet cell maturation and regulates mucin differentiation.

The gut-associated IELs are known for being the intestinal immune system's first line of defense because they directly interact with luminal antigens. The IEL count largely changes in circumstances like the development of illnesses conditions and dietary modifications. In this study experimental groups showed significant ($P < 0.05$) alterations in ileum. While Khan *et al*. (2017) reported unfavorable outcomes in comparison to the current study. All other MOLP-administered groups exhibited non-significant effects when compared with the positive control group Khan *et al*. (2021) had reported that the increase in the IEL counts can be associated with the presence of selenium, which suppresses the overproduction of oxidants in T cells and increases T cell proliferation.

CONCLUSION

Moringa oleifera leaf powder supplements up to 1.6% in poultry feed have shown potential health benefits regarding broiler immunity and immune responses. It has improved the morphological and physiological aspects of lymphoid organs, immune responses, Intra-epithelial lymphocytes, and goblet cell counts under dexamethasone-induced stress. However further investigations are needed to gain insight and knowledge about the use of various components of *Moringa oleifera* in a comparative approach to the overall health and performance of poultry.

DECLARATIONS

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IRB approval

This research project was duly approved by the committee in the 7th meeting of Sub-ASRB (Advanced Studies & Research Board), Faculty of Chemical & Life Sciences AWKUM, held on April 26, 2022, vide notification No. Dir/A&R/AWKUM/2022/9396 dated June 28, 2022.

Ethical approval

The current study was duly approved by the Ethical Review Committee of the Department of Zoology, Abdul Wali Khan University Mardan, Pakistan.

Statement of conflict of interest

The authors have declared no conflict of interest.

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