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# Effect of Different Combinations of Methanolic Extract of *Moringa oleifera* and *Thymus vulgaris* on Production Performance, Gut Morphology, Hematology and Nutrient Digestibility in Broilers

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**Key words**
***Moringa oleifera, Thymus vulgaris,* Gut health, Digestibility, Production performance**

## ABSTRACT

The present study was designed to find the effect of methanolic extract of *Moringa oleifera* and *Thymus vulgaris* on the performance, hematology, digestibility and gut health in broiler chickens. A day-old 270 broiler chicks (Ross-308) were randomly assigned to 6 treatment groups with 3 replicates and 15 birds per replicate. CON (Control) group; M100 (*M. oleifera* 200mg/L); T100 (*T. vulgaris* 300mg/L); M50T50 (*M. oleifera* 100mg/L and *T. vulgaris* 150mg/L); M75T25 (*M. oleifera* 150mg/L and *T. vulgaris* 75mg/L); and M25T75 group (*M. oleifera* 50mg/L and *T. vulgaris* 225mg/L). Methanolic extract of both *M. oleifera* and *T. vulgaris* were supplemented in drinking water. The duration of the experiment was 42 days. The results showed significantly higher feed intake, body weight gain, feed conversion ratio, broiler performance efficiency factor, and broiler farm economy index in the M50T50 diet group. Digestibility of ash, dry matter, ether extract, crude fiber, and nitrogen-free extract were not affected except for crude protein which was significantly higher in the M50T50 diet group. Similarly, hematological parameters were not affected, while villus height, width, and crypt depth were significantly improved in the M50T50 diet group. These findings demonstrate that the supplementation of methanolic extract of *M. oleifera* and *T. vulgaris* in drinking water either alone or in combination improves the production performance, nutrient digestibility and gut health in broilers.



## INTRODUCTION

The poultry industry is the second largest sector of Pakistan and for the last several decades performed an emerging role in the country's economic sector contributing 5.77% to the agriculture sector, 26.7% to total meat in the form of superior protein, and 1.3% to national GDP (PPA, 2018-2019). More than 1.5 million people in the country are involved in the poultry industry in the form of employment. In the last few decades, different synthetic and chemical antibiotics were used as growth promoters that harm the natural physiology of the bird microflora and also induced drug resistance in humans and bacteria. There is an intense need to decrease the application of synthetic drugs such as antibiotics in poultry production because of antimicrobial resistance (Borazjanizadeh *et al*., 2011). This is possible through the inclusion and use of phytogenic plants which replace antibiotics and work as growth promoters in poultry production. Therefore, poultry scientists are involved to investigate and use unconventional plants and plant by-products to decline the heavy economic damage in the form of different microbial diseases and improve poultry health and production. To date incorporation of phytogenic extract generated an essential role in the improvement of the poultry industry in the form of health and performance. *Moringa oleifera* is a drought, a resistant

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and fast-growing tree grown in sub-Himalayan zones of northern Pakistan, Afghanistan, India, and Bangladesh. It is growing worldwide now in the subtropics and tropics zone (Fahey *et al*., 2001). Siddhuraju and Becker (2003) reported that *M. oleifera* contains active ingredients such as crude protein 27.51%, ether extract 22.3%, crude fiber 19.25%, ash 7.13%, and dry matter 76.53%. It has also been reported to contain caffeic acid and chlorogenic acid also which are known for antioxidant effects. *M. oleifera* reduces and inhibits the degradation of amino acids and positively improves the growth performance of birds (Alagawany *et al*., 2017). Ebenebe et al. (2012) have reported that *M. oleifera* supplementation improves the feed conversion ratio of meat-type birds. Supplementation of *M. oleifera* in the feed of broiler and layer birds suppresses the gut microbial count such as *C. perfringens*, coliforms, and *E. coli*, and also has a gut integrity role (Arif *et al*., 2019). *Thymus vulgaris* known for cosmetic, medical, and culinary purposes. It has strong growth stimulant properties mostly used as an alternative for synthetic and chemical materials in the poultry industry. *T. vulgaris* have some important functions such as antioxidant, antiseptic, antispasmodic, and expectorant (Hertrampf, 2001; Adu-Darwish *et al*., 2009). *T. vulgaris* contains thymol which is the fundamental active ingredient and also contains 21-56% volatile oils, carvacrol (5-isopropyl-2-methyl phenol), and phenolic compound (5-methyl-1-2-isopropyl phenol) with well-known antimicrobial activity (Rahimi *et al*., 2011). Thyme contains intrinsic bioactivities and also has appetite and digestion-stimulating properties (Herendez et al., 2004). This appetizing property increases feed utilization and growth performance (Bolukbasi et al., 2006; Al-Mashadani et al., 2011). Plant extract protects the absorptive surface and villi of the gut and intestine against toxins produced by pathogens mostly viruses and bacteria (Rolfe *et al*., 2000). Thyme in combination with other phytogenic significantly improves gut integrity by enhancing the villus height and width (Demir *et al*., 2003). To date, very less studies reported on the extract of *M. oleifera* and *T. vulgaris* but no such study reported on the combined use of *M. oleifera* and *T. vulgaris* extract on poultry health and performance. Therefore; the current study was designed to determine the effect of different combinations of methanolic extract of *M. oleifera* and *T. vulgaris* on overall performance, gut morphology, hematology, and nutrient digestibility in broilers.

## MATERIALS AND METHODS

*Preparation of methanolic extract of* Moringa oleifera *and* Thymus vulgaris

*M. oleifera* and *T. vulgaris* fresh leaves were harvested and collected from local areas of Peshawar, Khyber Pakhtunkhwa Pakistan, and were separately air and shade dried because directly expose to extreme sun light effect the active ingredient of the plant leaves. For inhibiting the fungal growth, leaves were turned over at constant intervals for at least one week. After complete drying, the leaves were ground and sieved with a 0.15-mm sieve and made a fine powder. About 100 ml of absolute methanol and 200 gm powder of both *M. oleifera* and *T. vulgaris* leaves dissolved separately in soxhlet apparatus overnight. After 24 h about 50ml extract was dissolved in dimethyl sulfoxide and kept for 72 h. After 72 h a cohesive mass was obtained which contained 10 mg/ml of methanolic extract of *M. oleifera* and *T. vulgeris* approximately. Experimental diets are presented in Table I.

**Table I. Composition of feed during the starter and finisher phase of experimental birds.**

| Ingredients (%) | Starter phase | Finisher phase |
|---|---|---|
| Corn gluten meal | 1.98 | 7.14 |
| Corn | 54.1 | 60.2 |
| Corn oil | 2.18 | 2.70 |
| Soyabeen meal | 37.6 | 24.0 |
| Limestone | 0.81 | 0.67 |
| Dicalcium phosphate | 2.35 | 2.10 |
| VM mix[a] | 0.50 | 0.50 |
| Salt | 0.50 | 0.55 |
| Lysine HCL | 0.21 | 0.40 |
| DL-Methionine | 0.22 | 0.12 |
| Cholin chloride | 0.06 | 0.06 |
| Threonine | 0.12 | 0.10 |
| **Chemical composition** | | |
| Crude protein (%) | 23.0 | 21.0 |
| ME (Kcal/kg) | 3000 | 3150 |
| Methionine (%) | 0.54 | 0.44 |
| Sulphur amino acid (%) | 0.94 | 0.78 |
| Lysine (%) | 1.40 | 1.22 |
| Calcium (%) | 1.06 | 0.90 |
| Phosphorus (%) | 0.50 | 0.46 |
| Threonine (%) | 0.95 | 0.88 |

[a]Vitamin-minral premix contains the following ingredient per kg of feed: vitamen A, (2,400,000 IU); vitamen E, (16,000 IU); vitamin K, 800 mg; vitamin D, 1,000,000 IU; vitamin B12, 6 mg; vitamin B6, 1000 mg; vitamin B2, 1600 mg; vitamin B1, 600 mg; folic acid, 400 mg; biotin 40 mg; niacin, 8000 mg; pantothenic acid, 3000 mg; antioxidant, 3000 mg; copper, 2000 mg; selenium, 60 mg; cobalt, manganese, 18,000 mg; 80 mg; iron, 1200 mg; iodine, 400; zinc and14,000 mg.

*Experimental design and bird's husbandry*

A total of 270 days old chicks (Ross-308) were

randomly assigned to six different treatment groups in a completely randomized design (CRD). Each treatment group was further divided into 3 replicates with 15 birds per replicate. The same basal diet was fed to birds of all experimental groups. Control group with (no supplementation); M100 (Supplemented with *Moringa* 200mg/L); T100 (supplemented with *Thyme* 300mg/L); M50T50 (supplemented with *M. oleifera* 100mg/L and *Thymus vulgaris* 150mg/L); M75T25 (supplemented with *Moringa oleifera* 150mg/L and *T. vulgaris* 75mg/L); M25T75 (supplemented with *M. oleifera* 50mg/L and *T. vulgaris* 225mg/L). All the supplementation was given in the drinking water. The optimum number of the drinker, feeder, and other requirements were provided to the birds in such a way that the bird's genetics show the best potential results of all the experimental parameters. The experiment was extended for 42 days including 7 days of adaptation.

*Performance parameters*

Feed was offered to the broiler chicks *ad libitum* and daily feed intake was measured by subtracting the refused feed from the feed offered while weight gain was calculated by subtracting the initial body weight from the final body weight. The feed conversion ratio was calculated by dividing feed intake by weight gain x 100. Mortality was recorded throughout the experiment. The percent livability was calculated at the end of the experiment by the formula:

Livability (%) = Birds sold ÷ number of birds present at the beginning x 100

The broiler performance efficiency factor (BPEF) was computed by the formula;

BPEF= Bird live body weight in Kg ÷ feed conversion ratio x 100

while the broiler performance efficiency index was calculated by the formula:

BPEI= live weight in kg x % livability ÷ feed conversion ratio x growing period in days

*Hematology and gut morphometry*

Blood parameters were determined on the last day of the experiment and 3 to 5 ml of blood was collected from the wing vein of 03 birds of each replicate. Hemocytometer was used for the manual calculation of total white blood cells (WBCs) and total red blood cells (RBCs) (Campbell, 1995). The packed cell volume (PCV) was measured by a standard manual technique using microhematocrit capillary tubes and blood was centrifuged at 2500 rpm for 5 min. The cyanmethemoglobin methodology was implemented for the estimation of hemoglobin (HB) concentration in the blood. For gut morphometry, the procedure used by Shuaib *et al*. (2022) was followed.

*Nutrient digestibility*

Nutrient digestibility was calculated by using the indigestible and insoluble marker $Cr_2O_3$ on day 35. From each replicate, 5 birds were randomly shifted to metabolic cages on day 35 of the experiment for total excreta collection. The birds remained for 4 days in these metabolic cages provided with a ration containing 0.2% $Cr_2O_3$ as an indigestible marker. Fresh fecal samples were collected twice a day in a plastic bag and stored at -20°C. Excreta samples, ileal digesta, and ground feed were properly dried, ground, and sieved through a 0.5-01 mm sieve and kept at -20°C. Proximate analyses of feed and excreta samples were done as defined by (Adejumo *et al*., 2005). The concentrations of chromium were determined with the help of a UV absorption spectrophotometer (Shimadzu, Kyoto, Japan Shimadzu, UV-1201) as described by Williams *et al*. (1962). The nutrient digestibility was determined according to the procedure used by (Stefanello *et al*., 2020).

*Statistical analysis*

Complete randomized design (CRD) was used for the analysis and computing of the collected data. For significance, the difference means were reviewed by least significant difference (LSD) (Guide, 2010).

## RESULTS

Results regarding feed intake, weight gain, feed conversion ratio, production traits, and gut health are shown in Table II. During the starter phase higher ($P$<0.05) feed intake was recorded in the M50T50 diet group but at the finisher phase in the M100, M50T50, and M75T25 diet groups. Overall, significantly higher feed intake was recorded in the M50T50 and M75T25 diet groups. Weight gain during the starter and finisher phases as well as overall was calculated higher ($P$<0.05) in the M50T50 diet group. At the starter, and finisher phases and an overall significantly better FCR was recorded in the M50T50 and M75T25 diet groups. The highest significant BPEF was noted for M50T50, M25T75, and M75T25 diet groups, and the highest broiler farm economy index was noted for the M50T50 group. Mortality was recorded as non-significant among the groups. The villus height and villus width were recorded higher ($P$<0.05) in the M50T50 diet group while the crypt depth had a higher ($P$<0.05) value in the control group. Results regarding nutrient digestibility and hematology are presented in Table III. Digestibility of dry matter, ash, crude fiber, ether extract, and the nitrogen-free extract was not affected but crude protein had a significantly higher digestibility in the M100, M50T50, and M75T25 diet groups than the all-other groups. Blood parameters were not affected ($P$<0.05) among the groups.

**Table II. Effect of methonolic extract of *Moringa oleifera* and *Thymus vulgaris* alone or in different combinations on feed intake, weight gain, FCR, production traits and gut health of broiler birds.**

| Parameters | Groups | | | | | | P value |
|---|---|---|---|---|---|---|---|
| | Control | M100 | T100 | M50T50 | M75T25 | M25T75 | |
| **Feed intake (g)** | | | | | | | |
| Starter phase | 982.3±7.35 $^{c}$ | 1009±7.26 $^{b}$ | 976.6±2.33 $^{c}$ | 1048.3±3.28 $^{a}$ | 1014.2±5.02 $^{b}$ | 982±9.50 $^{c}$ | 0.001 |
| Finisher phase | 2442.7±5.81 $^{c}$ | 2745.3±11.7 $^{a}$ | 2641.7±1.45 $^{b}$ | 2780.7±3.93 $^{a}$ | 2781.3±3.92 $^{a}$ | 2668±23.3 $^{b}$ | 0.003 |
| Overall (day1-42) | 3425±3.51 $^{d}$ | 3754.7±5.36 $^{b}$ | 3618.3±1.45 $^{c}$ | 3829±38.6 $^{a}$ | 3795.5±8.65$^{ab}$ | 3650±15.8 $^{c}$ | 0.002 |
| **Weight gain (g)** | | | | | | | |
| Starter phase | 601.8±4.37 $^{d}$ | 633.5±8.25 $^{c}$ | 602.2±3.05 $^{d}$ | 678.5±5.81 $^{a}$ | 649.8±1.33 $^{b}$ | 608.8±3.45 $^{d}$ | 0.001 |
| Finisher phase | 1017±29.1 $^{d}$ | 1399.3±12.3 $^{c}$ | 1355.3±37.9 $^{c}$ | 1650.7±10.7 $^{a}$ | 1564.7±10.4 $^{b}$ | 1342.3±8.25 $^{c}$ | 0.002 |
| Overall (day1-42) | 1618.9±26.9 $^{e}$ | 2032.9±18.6 $^{c}$ | 1957.5±38.5 $^{d}$ | 2329.2±9.07 $^{a}$ | 2214.5±11.4 $^{b}$ | 1951.2±11.7 $^{d}$ | 0.003 |
| **FCR** | | | | | | | |
| Starter phase | 1.63±0.01 $^{a}$ | 1.59±0.01 $^{b}$ | 1.62±4.60 $^{ab}$ | 1.54±9.30 $^{c}$ | 1.56±7.30 $^{c}$ | 1.61±8.50 $^{ab}$ | 0.004 |
| Finisher phase | 2.40±0.06 $^{a}$ | 1.96±0.02 $^{b}$ | 1.94±0.05 $^{b}$ | 1.68±0.02 $^{c}$ | 1.77±0.01 $^{c}$ | 1.98±0.02 $^{b}$ | 0.001 |
| Overall (day1-42) | 2.11±0.04 $^{a}$ | 1.84±0.01 $^{b}$ | 1.84±0.03 $^{b}$ | 1.64±0.01 $^{c}$ | 1.71±0.01 $^{c}$ | 1.87±0.01 $^{b}$ | 0.002 |
| Livability (%) | 93.7±2.22 | 100.0±0.00 | 100.0±0.00 | 100.0±0.00 | 100.0±0.00 | 97.7±2.22 | 0.366 |
| BPEF (%) | 82.3± 4.08 $^{d}$ | 109.3± 1.21 $^{b}$ | 95.2± 2.66 $^{c}$ | 121.4±1.31 $^{a}$ | 116.4±0.41 $^{a}$ | 118.3±1.50 $^{a}$ | 0.003 |
| BFEI | 1.95± 0.30 $^{c}$ | 2.63± 0.32 $^{b}$ | 1.96 ±0.17 $^{c}$ | 3.32± 0.11 $^{a}$ | 2.67± 0.22 $^{b}$ | 2.72± 0.33 $^{b}$ | 0.000 |
| Mortality (%) | 1.00±0.57 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.33±0.33 | 0.366 |
| **Gut health** | | | | | | | |
| Villus height(µm) | 610± 4.08 $^{c}$ | 824± 1.21 $^{b}$ | 755± 2.66 $^{bc}$ | 862±1.31 $^{a}$ | 803±0.41 $^{b}$ | 788±1.50 $^{bc}$ | 0.003 |
| Villus width (µm) | 54± 0.30 $^{c}$ | 68± 0.32 $^{b}$ | 57±0.17 $^{bc}$ | 74± 0.11 $^{a}$ | 64± 0.22 $^{b}$ | 59± 0.33 $^{b}$ | 0.045 |
| Crypt depth (µm) | 68±1.23 $^{a}$ | 59±0.53 $^{b}$ | 56±2.78 $^{b}$ | 47±2.22 $^{d}$ | 56±2.78 $^{b}$ | 53±2.22 $^{c}$ | 0.003 |

Means with different superscripts in the same row to each treatment are significantly different at α= 0.05. Control, untreated; M100, supplemented with 100% *Moringa oleifera*; T100, supplemented with 100% *Thymus vulgaris*; M50T50, supplemented with 50% *M. oleifera* and 50% *T. vulgaris*; M75T25, supplemented with 75% *M. oleifera* and 25% *T. vulgaris*; M25T75, supplemented with 25% *M. oleifera* and 75% *T. vulgaris;* BFEI, broiler farm economy index; BPEF, broiler performance efficiency factor (%).

**Table III. Effect of methonolic extract of *Moringa oleifera* and *Thymus vulgaris* alone or in different combinations on nutrient digestibility and hematology of broilers.**

| Parameters | Groups | | | | | | P. value |
|---|---|---|---|---|---|---|---|
| | Control | M100 | T100 | M50T50 | M75T25 | M25T75 | |
| DM (%) | 66.3±0.76 | 70.2±0.47 | 68.5±0.38 | 71.7±0.37 | 70.6±0.34 | 69.1±0.60 | 0.140 |
| Ash (%) | 41.5±3.53 | 45.6±2.44 | 43.8±0.11 | 47.7±0.53 | 44.9±1.00 | 44.7±0.40 | 0.348 |
| CP (%) | 70.3±0.25$^{c}$ | 72.2±0.13$^{ab}$ | 70.7±0.22$^{c}$ | 73.0±0.16$^{a}$ | 71.9±0.04$^{ab}$ | 71.2±0.80$^{bc}$ | 0.002 |
| EE (%) | 77.9±2.83 | 81.3±1.23 | 78.6±2.29 | 85.0±1.21 | 81.7±1.26 | 80.1±0.68 | 0.128 |
| CF (%) | 79.8±1.33 | 83.5±0.92 | 81.6±0.74 | 83.9±0.01 | 83.6±0.33 | 81.2±0.28 | 0.299 |
| NFE (%) | 80.4±0.95 | 83.5±1.39 | 80.8±1.15 | 85.5±0.93 | 84.7±0.46 | 80.4±2.31 | 0.053 |
| RBC ($10^{12}$/L) | 1.98±0.03 | 2.30±0.06 | 2.14±0.05 | 2.50±0.07 | 2.38±0.30 | 2.08±5.35 | 0.122 |
| WBC ($10^{9}$/L) | 10.5±0.85 | 7.97±1.19 | 8.85±2.38 | 6.98±0.03 | 6.32±0.57 | 6.97±0.05 | 0.188 |
| PCV (%) | 28.2±2.43 | 30.4±1.00 | 30.6±1.00 | 32.5±1.70 | 31.0±0.86 | 30.0±2.08 | 0.632 |
| HB (g/dl) | 10.2±1.00 | 10.9±0.60 | 10.8±0.20 | 12.6±0.40 | 12.0±0.87 | 11.2±1.08 | 0.357 |

Means with different superscripts in the same row to each treatment are significantly different at α=0.05. DM, Dry matter; CP, Crude protein; EE, Ether extract; CF, Crude fiber; NFE, Nitrogen free extract; RBC, Red blood cells; WBC, White blood cells; PCV, Packed cell volume; HB, Hemoglobin.

## DISCUSSION

In the current study, the M50T50 diet group exhibit the highest performance in the form of feed intake, body weight, and improved FCR while all the performance parameter were observed better in the phytogenic (*M. oleifera* and *T. vulgaris*) combination group than in the control group. Our results are similar to the findings of Feizi *et al*. (2013) who reported that thyme extracts contain thymol which activates broiler's digestive system by secreting endogen a digestive enzyme that speeds up the absorption rate of the intestine and subsequently improved the utilization and absorption of feed. Our results are also in line with the results of Mikhail *et al*. (2020) who stated that kaempferol and quercetin flavoring glycosides are abundant in the *M. oleifera* leaf. Provision of M50T50 significantly increased the bird's final body weight gain which might be due to synergetic effect of both *M. oleifera* and *T. vulgeris*. *M. oleifera* contains flavonoids having antimicrobial activity and the thymus contains thymol and carvacrol principle active molecules which increased the palatability of feed and also help to boost the nutrient absorption of broilers which ultimately leads to increased weight gain. Similarly, Alabi *et al*. (2017) reported that the phenolic compounds containing flavonoid content present in *M. oleifera* seeds had an encouraging effect on broiler growth rate. Alagawany *et al*. (2017) reported that *M. oleifera* could decrease and inhibit the degradation of amino acids and hence positively improve growth performance. Similarly, Feizi *et al*. (2013) supplemented the birds with *T. vulgaris* (200cc/1000 liter) and reported higher body weight gain, and Saki et al. (2014) mixed 0.2 ml/liter extract of *T. vulgaris* in drinking water and reported higher weight gain. Similarly, the best FCR was recorded in the M50T50 diet group which might be due to the synergetic effect of both *M. oleifera* and *T. vulgaris*. Similar results were also reported by Kout *et al*. (2015), who fed MOLM to birds with 0.2% concentration in the feed. Ebenebe et al. (2012) also found better FCR by supplementation of *M. oleifera*. Our results are also in line with Ocak *et al*. (2008) by the inclusion of 2% thyme. Cross *et al*. (2003), Dahal and Farran (2011) also favor our results. Supplementation of the methanolic extract significantly improved the broiler performance and economical parameters which are similar to the findings of Zanu *et al*. (2012) who reported improved feed cost in meat-type birds by supplementation of *M. oleifera* mix with fish meal. Abbas et al. (2012) also concluded that 10% *Moringa* leaf meal is cost-effective in broilers. Supplementation of methanolic extract of *M. oleifera* and *T. vulgaris* significantly ($P<0.05$) affected the crude protein which is in line with the results of El-Badawi *et al*. (2014) who fed 0.15 or 0.30% *M. oleifera* dry leaves powder to the rabbits in the ration. Supplementation of methanolic extract of *M. oleifera* and *T. vulgaris* significantly ($P<0.05$) increased the height and width of the ilium portion of villi in all supplemented groups which is due to the synergetic effect of both phytogenic methanolic extract and this synergetic effect increases the surface area which directly increases the absorptive capacity and ultimately leads to increased digestibility and nutrient utilization. Generally, crypt depth decreased by increasing villus height and width and similarly, our findings also exhibited a higher decrease in crypt depth in group M50T50. Similarly, Saeed *et al*. (2018) found that broilers fed with herbs significantly stimulate the jejunum histology which enhanced the absorption of nutrients and growth performance.

## CONCLUSION

The administration of methanolic extract of *M. oleifea* and *T. vulgaris* enhanced the utilization of feed efficiency, and body performance and also potentially modulate and improve gut health which beneficially improves the crude protein digestibility. Hence supplementation of methanolic extract of *M. oleifera* and *T. vulgaris* alone and in combination form enhanced the broilers' performance but the most effective improvement was exhibited by the M50T50 group in drinking water on the production performance, nutrient digestibility, and gut health in broilers.



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*IRB approval*

The experimental work was approved by the Advanced Studies and Research Board (ASRB) (No.712/ASRB-56/UAP), The University of Agriculture, Peshawar, KP, Pakistan.

*Ethical statement*

This study was approved by the animal welfare and care committee of the Faculty of Animal Husbandry and Veterinary Sciences, The University of Agriculture, Peshawar, Pakistan, and all the measures and tools was considered to minimize the pain and discomfort of birds during the conduction of this experiment.

*Statement of conflict of interest*

The authors have declared no conflict of interest.

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