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Effect of *Emblica officinalis* Supplementation on Expression Level of Toll-Like Receptors (TLRs) in Broilers

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ABSTRACT

The present study was aimed to evaluate the effect of supplementing different levels of amla fruit powder on differential gene expression level of toll like receptors (TLRs) in broilers. Three hundred, one-day-old broiler chicks were used for the study. The chicks were randomly distributed into 30 subgroups i.e., 6 dietary treatments with five replicates per treatment and each replicate has 10 birds. The first group was kept as a control (T_1) and given basal diet without antibiotic while second (T_2) basal diet with antibiotics, third (T_3), fourth (T_4), fifth (T_5) and sixth (T_6) groups were dietary supplemented with amla fruit powder @0.25%, 0.5%, 0.75% and 1%, respectively. At the end of feeding trial (6th week), blood samples were collected from one broiler per replicate, making five samples per treatment and thus a total of 30 samples were analysed. Total RNA was isolated from blood samples by using TRIZOL method; cDNA was prepared, and analysis of temporal differential gene expression profile was carried out using Step I plus real-time PCR system. The differential expression level of TLRs that includes TLR 2, TLR 4 and TLR 7 gene transcripts in birds was studied by using relative quantification method and level of target mRNA was determined by comparative C_T method ($\Delta\Delta C_T$ method). Significantly highest level of increase in mRNA expression of TLR 2 was observed in treatment group 0.75% amla-supplemented group (T_5) and significant down regulation of TLR 4 was observed in all amla supplemented group.

HIGHLIGHTS

- Inclusion of amla powder in the diet has desirable effects on the immunological parameters.
- Dietary amla powder supplementation has a significant effect on the expression of the toll-like receptor.

Keywords: Amla powder, broilers, completely randomized design, gene expression, natural anti-oxidants, toll-like receptors

Poultry industry in India has emerged as one of the fastest growing segment of the agriculture sector. The broiler sector has been the most dynamic sector in poultry due to its marginal investments and quick returns. The low productivity is mainly due to poor management, inadequate nutrition and health coverage. Furthermore, the success of poultry industry depends upon its fast growth and low mortality during first two weeks of its life, which can be managed by good hygienic and feeding conditions. The consumers are now becoming more aware of safety and quality of food products consumed by them. The production of safer poultry products without any chemical and microbial residues is the order of the day. Over a period of time, extensive efforts have been made to lower down the cost of production by lowering the expenses on feed. Feed additives are one of the important tools used for improving feed conversion ratio, growth rate and disease resistance. This has leads to widespread use of a

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number of "feed additives". Feed additives are commonly described as non-nutrient substances which accelerate growth, efficiency of feed utilization, beneficial for health or metabolism of the animals. The range of feed additives used in animal production industry is very broad ranging from growth promoters to disease preventing agents. Supplementations of these agents in poultry nutrition are mainly aimed to improve digestibility and bioavailability of various nutrients, thereby, enhancing economic gains by reducing the input costs. The additives that hold great promise in the feeding of poultry comprise of antibiotics, coccidiostats, antioxidants, enzymes, hormones, probiotics, buffers, organic acids, mould inhibitors, herbal products, synthetic micronutrients etc.

Antibiotics affect the metabolism of the microorganisms and suppress microbial growth in the gut. Use of antibiotics has negative effects on animal health and its production such as residues in tissues, withdrawal period and development of resistance in microorganisms (Botsoglou and Fletouris, 2001). Recently, the emphasis is being directed towards the search of herbal formulations which could be effective for amelioration of stress and leads to increase in production of birds. Several Indian herbs are reported to possess adaptogenic, antistress and immunomodulator properties (Wadhwa *et al*., 2007). New additives of phytogenic origin have been proposed to livestock producers. Herbs, spices and various plant extracts have received increased attention as possible antibiotic growth promoter replacements. In this view, the plants identified with properties of secondary metabolites became interesting due to their antimicrobial, antioxidant effects and their stimulating effects on animal performance and digestive enzymes. At present, there are large numbers of Natural Growth Promoters (NGPs) available in the market including herbs, probiotics, prebiotics and synbiotics etc. The use of naturally occurring compounds like herbs, herbal preparations and other botanicals are preferred over chemical compounds to satisfy consumer concerns over safety and toxicity (Makkar *et al*., 2007).

A number of studies have reported the positive effect of herbs and their active components on digestion process. They have been shown to stimulate bile salt secretion and digestive enzyme activities of intestinal mucosa and of pancreas (Platel and Srinivasan, 2004). Herbs could be expected to serve as feed additives due to their suitability and preference, lower cost of production, reduced risk of toxicity, minimum health hazards and environment friendliness. Recent research works on herbal formulations as feed additives have shown encouraging results as regards weight gain, feed efficiency, lowered mortality and increased liveability in poultry birds (Mishra and Singh, 2000). Amla belonging to family Euphorbiaceae, is a medicinal plant known to be of therapeutic importance since the dawn of civilisation. It is widely available in most of the tropical and sub-tropical countries. In poultry health management, Emblica officinalis has been widely used as growth promoter, immunomodulator (Priya *et al*., 2010) and antioxidant (Priya *et al*., 2010 and Elizabeth *et al*., 2011). The key bioactive principles of amla fruit are flavonoids, phyllemblin, ascorbic acid, gallic acid, alkaloids and tannins. Vitamin C, tannins and flavonoids are found in maximum concentration and exhibit antioxidant action (Kaur *et al*., 2002). The fruit of amla due to presence of saponins, phenols and tannins have potent antimicrobial activity against both Gram positive and Gram negative bacteria. The tannins present in the fruit of amla are reported to have potent antimicrobial activities. Keeping in view the above biochemical properties of amla and its beneficial effects on various physiological processes, the present study is to evaluate the effect of supplementing different levels of amla fruit powder on differential gene expression of toll like receptors.

MATERIALS AND METHODS

Ethical approval

The experimentation with the birds was conducted in accordance with guidelines approved by the Institutional Animal Ethics Committee, 12/CPCSEA Dated 6.2.2017 in the Department of Animal Nutrition, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar.

Birds

Three hundred, one-day-old broiler chicks were purchased from a local commercial hatchery. The chicks were individually weighed, wing banded and randomly distributed into 30 subgroups means 6 dietary treatments with five replicates per treatment and each replicate has 10 birds.

Experimental diet

The first group was kept as a control (T_1) and given the basal diet without antibiotic while second (T_2) basal diet with antibiotic, third (T_3), fourth (T_4), fifth (T_5) and sixth (T_6) groups were supplemented with amla fruit powder @0.25%, 0.5%, 0.75% and 1%, respectively in the diet. Birds were vaccinated against F1 strain of Ranikhet disease on 3rd day and Infectious Bursal Disease on 14th day.

Feeding, watering and housing

The experimental chicks were reared under deep litter system. The floor of the pens was thoroughly cleaned, disinfected before scattering of the bedding material. Well chopped dry wheat straw was used as bedding material to form the litter. The straw was evenly spread upto 5 cm thickness. The litter was regularly racked to avoid any lump formation. Wooden brooders fitted with bulb in the centre were used in each pen for brooding. During the initial period of growth extra care was taken to assure efficient feeding and watering of the chicks so that they could be well introduced and acclimatized. The feeding programme consisted of a starter diet until 28 days and a finisher diet from 29 to 42 days of age. Weighed amount of feed was offered on paper sheets for first 3 days and thereafter, in the automatic feeders up to 28 days of age. Afterwards, the feeds were offered through hanging feeders maintained at appropriate heights. The chicks were provided *ad libitum* clean drinking water through the plastic waterers during first two weeks of the experiment. Thereafter, bigger plastic waterers were used till the end of the experiment.

Experimental design

Completely Randomized Design (CRD) was used as experimental design at uniform and standard management practices.

Feed evaluation and experimental diet composition

All feed ingredients, additives and supplements used in the experiment were procured in one lot before the start of the experiment. The ingredients, additives and supplements used in the diet formulation were maize, soybean meal, vegetable oil, fish meal, mineral mixture, vitamins, coccidiostat, lysine, DL- methionine and amla fruit powder. The sources, composition and mixing rate of additives/supplements used in ration formulations are presented in Table 1.

Table 1: Ingredient composition of experimental diets during different phases of growth

Ingredient (kg /100 kg of feed)	0-4 wks	4-6 wks
Maize	58	60
Soybean meal	30	25
Fish meal	7	7
Vegetable oil	3	6
Mineral mixture	2	2
Feed additives (g/100 kg feed)		
Spectromix	10	10
Spectromix BE	20	20
Veldot	50	50
Choline chloride	50	50
Lysine	50	50
DL-methionine	150	150

Evaluation of feed ingredients

Feed ingredients used for ration formulations were evaluated for proximate nutrients (AOAC, 2016). The evaluated and measured values of feed ingredients used in preparing the experimental diets are presented in Table 2.

Table 2: Chemical composition of feed ingredients used in ration formulation

Ingredient	CP (%)	CF (%)	EE (%)	TA (%)	Lysine* (%)	Methionine* (%)	ME* (kcal/kg)
Maize	9.11	2.44	3.44	2.25	0.18	0.15	3300
Soybean meal	45.15	3.93	3.16	8.47	2.57	0.76	2230
Fish meal	47.40	1.79	5.16	26.62	1.42	1.42	2210

*Calculated values Singh and panda.

Figuring and composition of diets

Basal ration was formulated as per BIS (2007) to fulfil the metabolizable energy (ME) and crude protein requirements of birds. Level of crude protein in starter (0-4 weeks) and

finisher (4-6weeks) ration was 22 percent and 20 percent, respectively. The respective ME content was 3000 and 3200 KCal/kg are presented in Table 3.

Table 3: Chemical composition (% DM basis) of experimental diets in different growth phases of broiler chicks

Attributes	0-28 d	29-42 d
Dry Matter (DM)	88.79	88.59
Crude Protein (CP)	22.92	20.32
Ether Extract (EE)	3.32	3.62
Crude Fibre (CF)	5.12	5.19
Ash Content	9.15	9.25
Nitrogen Free Extract (NFE)	59.49	61.62

Experimental diets

The experimental dietary treatments were as under:

- T_1 : Basal diet without antibiotic
- T_2 : Basal diet with antibiotic
- T_3 : Basal diet + 0.25 % amla fruit powder
- T_4 : Basal diet + 0 .50 % amla fruit powder
- T_5 : Basal diet + 0.75 % amla fruit powder
- T_6 : Basal diet + 1.00 % amla fruit powder

Composition, sources and rate of mixing of feed additives/supplements

1. **Spectromix:** Powder (Ranbaxy Animal Health, New Delhi). Each gm. contained Vitamin A-82,500 IU, Vit D3-12000 IU, Vit B2-50 mg and Vit.K-10mg.Mixing rate: 10 g/100Kg of feed.
2. **Spectromix BE:** Powder (Ranbaxy Animal Health, New Delhi). Each gm. Contained Vit. B1- 8 mg, Vit. B6- 16 mg, Vit. B12- 80 mg, niacin-120 mg, calcium pentothenate-80 mg, Vit. E-160 mg, Lysine hydrochloride-10 mg, DL-methionine-10 mg and calcium 260 mg. Mixing rate: 20 g/100 kg of feed.
3. **Veldot:** Venkeys- Dinitro-O-Toluamide (Coccidiostat). Mixing rate: 50 g/100 kg of feed.
4. **Choline chloride:** Contain 60 percent choline. Mixing rate: 50g/100kg of feed.
5. **Lysine:** Contained 98% lysine. Mixing rate: 50 g/100 kg of feed.
6. **DL-methionine:** Contained 98% methionine. Mixing rate: 150 g/100 kg of feed.

Observations and sampling

The following recording and sampling procedures were followed during the experimental period.

Feed intake

Weekly feed intake and residue left per replicate was measured throughout the experiment to calculate the feed consumption per bird.

Body weight gain

The birds were weighed individually fortnightly interval to calculate body weight gain.

Feed conversion ratio

Feed conversion ratio (FCR) for each replicate was calculated as follows:

$$FCR = \frac{\text{Total feed consumed (g)}}{\text{Total body weight gain (g)}}$$

Blood collection and analysis

At the end of the feeding trial (6^{th} week), blood samples were collected from one broiler per replicate, making five samples per treatment and thus a total of 30 samples were analysed. About 2 ml of blood was collected from each bird via brachial wing vein puncture using sterilized syringes and 5 ml scalp vein needle set into vacutainer containing ethylene diamine tetra acetic acid (EDTA) for haematology and TLR mRNA expression.

Reverse transcription (cDNA synthesis); RNA extraction and preparation of cDNA

Total RNA was isolated from blood samples by using Trizol® as per the manufacturer's instruction. In brief, 1 ml of Trizol® reagent, 200 µl of chloroform was added to 600 µl of blood followed by centrifugation for phase

separation and precipitation with isopropanol. Total RNA extracted was dissolved in 20 µL Nuclease Free Water and quantified using Qubit® 2.0 fluorometer (Invitrogen). Reverse transcription was carried out with total reaction volume of 20 µL using cDNA synthesis kit (Promega A5000). Briefly, NFW (7.3 µL), 5X RT buffer (4 µL), $MgCl_2$ (1.2 µL), 10 mMdNTPs (Promega A5000) (1 µL), total RNA (5 µL), Random hexamer (1 µL), RNAase in (0.5 µL). The polymerase chain reaction (RT-PCR) cyclic conditions were as initial incubation at 25°C for 5 min, reverse transcription at 42°C for 1 h, extension temperature is optimized between 37 - 55ºC and deactivation at 70°C for 15 min in thermal cycler (Applied Biosystem). The cDNA was stored at −20°C till further use.

Real time PCR

For the analysis of the temporal expression profile of different genes, real-time PCR was carried out using Step I plus real-time PCR system. For the real-time PCR reaction, SYBR Green dye-based PCR master mix (Affymetrix) was used, and all the instructions were followed as per the manufacturer. The reaction for the target gene, TLRs (TLR 2, TLR 4, and TLR 7), and the endogenous control, β-actin gene was carried out in triplicate along with non-template control as a negative control for each sample. The reaction mixture used to carry out the real-time PCR reaction for TLRs 2, 4 and 7; and β-actin gene contains 2X SYBR green PCR mastermix (Affymetrix, 12.5 µL), primers (forward and reverse 0.5 M each) (Table 4), NFW (variable), and template (3 µL). The cyclic conditions used for amplification were according to the instructions of the manufacturer. Amplification was done with denaturation for 15 min at 95°C, followed by 40 cycles of denaturation for 5 s at 95°C, and annealing/elongation for 30 s at 60°C, and a final melting curve analysis.

Relative quantification by comparative CT method (ΔΔCT method)

The average CT (Threshold cycle) value obtained for the TLRs 2, 4 and 7 (target) gene were normalized to β-actin (endogenous control). The data obtained were subjected to a comparative CT method for the analysis of the expression levels of the targeted TLR gene and endogenous control. The sample at 26 h of incubation was selected as a calibrator.

Sequencing of product

Amplicons were sequenced using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) on an automatic ABI 3130 xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). The sequence obtained shows 100% nucleotide identity with the TLR sequence of chicken available in the global database.

Statistical analysis

The resultant data were statistically analysed according to the procedure laid down by Snedecor and Chochran, (1994). The values are given as means ± standard errors and $p<0.05$ was set as level of significance. Gene expression patterns of the genes by means of qRT-PCR were analysed

Table 4: Oligonucleotide sequences of sense and antisense primers for real-time PCR products determined

Gene[1]	Primer	Primer sequence[2]	Accession No.	Product size (bp)
β-Actin	Sense	5′-GAGAAATTGTGCGTGACATCA-3’	L08165	152
	Antisense	5′-CCTGAACCTCTCATTGCCA-3’		
TLR 2	Sense	5′-CATTCACCATGAGGCAGGGATAG-3’	AB046533	157
	Antisense	5′-GGTGCAGATCAAGGACACTAGGA-3’		
TLR 4	Sense	5′-TTCAGAACGGACTCTTGAGTGG-3’	AY064697	131
	Antisense	5′-CAACCGAATAGTGGTGACGTTG-3’		
TLR 7	Sense	5′-TTGCTGCTGTTGTCTTGAGTGAG-3’	AJ627563	182
	Antisense	5′-AACAACAGTGCATTTGACGTCCT-3’		

[1]TLR 2 = Toll-like receptor 2; TLR 4 = Toll-like receptor 4; TLR 7 = Toll-like receptor 7; [2]Primers for Toll-like receptors and β-actin were described by Sato *et al*. and Bai *et al*. respectively; bp-base pair.

using 2-DDCT method and were compared using student test. The values of qPCR were given as means ± standard errors and p<0.05 was set as the level of significance.

RESULTS

Performance of broiler chicks

Average feed intake

Data pertaining average feed intake of the experimental birds under different dietary treatments are presented in table 5. Average feed intake during 0-14 days ranged from 501.5 (T_6) to 527.0 (T_1) and intake did not differ significantly in amla supplemented group as compared to control. Feed intake during 15-28 days of age ranged from 1416.5 (T_5) to 1544.2 (T_1) and intake was decreased in amla supplemented group as compared to control (T_1) and significantly lower feed intake was found in 0.50%, 0.75% and 1% amla supplemented group as compared to control group as well as from antibiotic supplemented group.

Table 5: Average feed intake (g/bird) during different growth periods under different dietary treatments

Treatments	0 to 14 d	15 to 28 d	29 to 42 d	0 to 42 d
T_1	527.0 ± 1.1	1544.2^{a} ± 15.8	1988.0^{b} ± 14.5	4059.0 ^{a} ± 6.4
T_2	519.5 ± 3.2	1507.6^{b} ± 8.4	1916.4^{d} ± 9.9	3943.5 ^{b} ± 5.0
T_3	517.1 ± 14.5	1495.0 ^{b} ± 14.0	2031.0^{a} ± 3.9	4042.3 ^{a} ± 5.8
T_4	508.1 ± 9.8	1423.9^{c} ± 4.4	1970.8^{bc} ± 14.8	3902.9^{c} ± 5.5
T_5	524.4 ± 4.1	1423.2^{c} ± 8.9	1929.2^{d} ± 5.2	3876.0^{d} ± 4.6
T_6	501.5 ± 8.9	1416.5^{c} ± 8.4	1942.4^{cd} ± 3.8	3860.4^{d} ± 6.5

Means bearing different superscripts in a column differ significantly (P<0.05).

Average feed intake during 29-42 days ranged from 1916.2 (T_2) to 2031.0 (T_3). Feed intake was significantly higher at 0.25% amla supplemented group (T_3) as compared to other groups and lowest feed intake was found in antibiotic supplemented group (T_2), 0.75% (T_5) and 1% (T_6) amla supplemented group. Feed intake was decreased as the level of amla fruit powder inclusion increases. Over all feed intake ranged between 3860.4 (T_6) to 4059.0 (T_1) and lowest feed intake was found in 0.75% and 1% amla supplemented group followed by 0.5% (T_4) amla supplemented group and this differ significantly as compared to control. There were significant (P<0.05) differences among the groups fed with higher levels of amla fruit powder as compared to control with regard to feed intake. High feed consumption was recorded in the control group and in 0.25% amla supplemented group.

Average body weight gain

Data pertaining average body weight gain of the experimental birds under different dietary treatments are presented in table 6. Mean body weight gain at the age 0 to 14 days ranged from 290.7 (T_1) to 314.5 (T_6) and body weight gain did not differ significantly in amla supplemented group as compared to control. Body weight gain at age of 15 to 28 days ranged from 873.4 (T_1) to 920.7 (T_5) and significantly higher body weight gain was found in the antibiotic and amla supplemented group as compared to control group.

Table 6: Average body weight gain (g) under different dietary treatments

Treatments	0 to 14 d	15 to 28 d	29 to 42 d	0 to 42 d
T_1	290.7 ± 3.6	873.4^{c} ± 5.5	941.7 ^{d} ± 5.8	2105.8 ^{d} ± 8.4
T_2	298.9 ± 5.9	911.2^{ab} ± 4.0	985.0^{bc} ± 5.5	2195.2 ^{b} ± 6.1
T_3	292.6 ± 5.3	894.7^{b} ± 7.8	985.3^{bc} ± 3.3	2172.6 ^{c} ± 4.6
T_4	298.4 ± 5.9	907.3^{ab} ± 8.7	975.4 ^{c} ± 6.5	2181.2^{bc} ± 5.1
T_5	314.5 ± 11.0	920.7 ^{a} ± 5.6	1005.1^{ab} ± 8.5	2240.4 ^{a} ± 5.5
T_6	305.5 ± 11.6	910.3^{ab} ± 5.8	1016.7 ^{a} ± 8.1	2232.7 ^{a} ± 6.6

Means bearing different superscripts in a column differ significantly (P<0.05).

Mean body weight gain from 29 day to 42 day of growth period ranged from 941.7 (T_1) to 1016.7 (T_6) and significantly higher gain was found in 0.75% and 1% amla

supplemented group followed by 0.5% (T_4), 0.25% (T_3) amla supplemented group and antibiotic supplemented group as compared to control group (T_1). Over all body weight gain at the end of growth period ranged from 2105.8 (T_1) to 2240.4 (T_5) and significant higher body weight gain was found in 0.75% (T_5) and 1% (T_6) amla supplemented group as compared to control (2240.4) and (2232.7) respectively followed by 0.25%, 0.50% and antibiotic supplemented group as compared to control group.

Feed conversion ratio

Data pertaining to FCR of the experimental birds under different dietary treatments are presented in table 7. Feed Conversion Ratio during 0-14 days of the experiment was higher in the T_1 and lowest FCR was obtained in the T_6 groups but these do not differ significantly. FCR during 15-28 days ranged from 1.54 to 1.76 the difference was statistically significant ($P<0.05$) and improved FCR was found as the level of inclusion of amla fruit powder was increased as compared to control and antibiotic supplemented group.

Table 7: Feed conversion ratio under different dietary treatments

Treatments	0 to 14 d	15 to 28 d	29 to 42 d	0 to 42 d
T_1	1.81 ± .12	1.76 ^{a} ± .01	2.11 ^{a} ± .01	1.92^{a} ± .01
T_2	1.74 ± .09	1.65 ^{a} ± .01	1.94 ^{d} ± .01	1.79 ^{c} ± .00
T_3	1.76 ± .05	1.67 ^{b} ± .02	2.06 ^{b} ± .00	1.86 ^{b} ± .01
T_4	1.70 ± .07	1.56 ^{c} ± .00	2.02 ^{c} ± .00	1.78 ^{c} ± .02
T_5	1.67 ± .02	1.54 ^{c} ± .01	1.92 ^{d} ± .02	1.73 ^{d} ± .01
T_6	1.65 ± .06	1.55 ^{c} ± .01	1.91 ^{d} ± .01	1.72 ^{d} ± .01

Means bearing different superscripts in a column differ significantly ($P<0.05$).

FCR during 29-42day of the growth period antibiotic and 0.75% and 1% amla supplemented group results in better FCR. This differs significantly from the control group followed by 0.25% and 0.50% amla supplemented group. At the age of 6 weeks highest (1.92) FCR was obtained in the basal diet (T_1) and difference was significantly higher in comparison to amla supplemented groups and lowest FCR was obtained in 0.75% and 1% amla supplemented groups (T_5) and (T_6).

Expression level of TLRs

The nutrigenomic expression analysis as presented in Table 8 revealed that relative mRNA expression of TLR 2 of broilers was found to be ($p<0.05$) enhanced in the treatment groups T_5 and T_6 supplemented with 0.75% and 1% of the amla fruit powder respectively. While, as presented in Table 7, at the end of the 6 weeks of experimental protocol broilers had significant down regulation pattern of relative mRNA expression of TLR 4 in the plasma of broilers fed diet supplemented with 0.25%, 0.50%, 0.75 % and 1% of amla fruit powder in the treatment groups T_3, T_4, T_5 and T_6 respectively and in antibiotic supplemented group. However, the data pertaining to the relative mRNA levels of TLR 7 as shown in Table 8 represented in the plasma of birds revealed non-significant differences were observed in the experimental groups T_2, T_3, T_4 and T_5 and T_6 as compared to the control group and antibiotic supplemented group. In nutshell, experimental treatments containing amla powder in the broiler's diet have potent immune modulating activity by showing stimulatory effect on relative mRNA expression of TLR 2 and down regulation pattern of TLR 4 of the commercial broilers.

Table 8: Relative quantitation expression analysis of the toll like receptors (TLR, TLR4 and TLR 7) with reference to the endogenous reference gene β actin

Sample Name	Target Name	Cт Mean	Cт SD	ΔCт Mean	ΔΔCт	RQ
T_1	TLR 2	25.71	0.10	11.5	0	1
T_2		25.15	0.48	11.44	-.06	1.04
T_3		25.73	0.44	12.15	0.65	.63
T_4		25.55	0.34	12.13	0.62	.65
T_5		25.74	0.24	9.63	-1.87	3.66
T_6		24.89	0.26	10.60	-0.89	1.86
T_1	TLR 4	15.5	.03	-1.61	0	1
T_2		14.69	0.15	.97	2.58	.17
T_3		14.56	0.23	.98	2.59	.16
T_4		15.30	0.13	.87	2.48	.18
T_5		15.90	0.05	.79	2.40	.19
T_6		15.08	0.23	.79	2.39	.19

T_1	TLR 7	16.37	0.14	2.16	0	1
T_2		15.91	0.18	2.20	.04	.97
T_3		15.67	0.16	2.10	-0.06	1.04
T_4		15.51	0.10	2.09	-0.06	1.04
T_5		17.42	0.04	2.31	-0.06	.9
T_6		16.38	0.09	2.10	-0.06	1.04
T_1	β actin	14.21	1.1	—		—
T_2		13.71	0.28	—		—
T_3		13.58	0.43	—		—
T_4		13.42	0.38	—		—
T_5		15.11	0.29	—		—
T_6		14.29	0.62	—		—

Effect of supplementation of amla fruit powder on relative mRNA expression of toll-like receptors TLR 2, TLR 4 and TLR 7 in the plasma of broilers are studied. Significantly highest level of increase in the mRNA expression of TLR 2 was observed in the treatment group 0.75% amla supplemented group (T_5) and significant down regulation of TLR 4 was observed in all amla supplemented group as compared to control group. While, non-significant difference in the mRNA expression of TLR 7 was observed in amla supplemented group as compared to control group.

DISCUSSION

Average feed intake

Average feed intake during 0-14 days ranged from 501.5 (T_6) to 527.0 (T_1) and intake did not differ significantly in amla supplemented group as compared to control. Feed intake during 15-28 days of age ranged from 1416.5 (T_5) to 1544.2 (T_1) and intake was decreased in amla supplemented group as compared to control (T_1) and significantly lower feed intake was found in 0.50%, 0.75% and 1% amla supplemented group as compared to control group as well as from antibiotic supplemented group. Average feed intake during 29-42 days ranged from 1916.2 (T_2) to 2031.0 (T_3). Feed intake was higher at 0.25% amla supplemented group (T_3) as compared to other groups and lowest feed intake was found in antibiotic supplemented group (T_2), 0.75% (T_5) and 1% (T_6) amla supplemented group. Feed intake was decreased as the level of amla fruit powder inclusion increases. Over all feed intake ranged between 3860.4 (T_6) to 4059.0 (T_1) and lowest feed intake was found in 0.75% and 1% amla supplemented group followed by 0.50% (T_4) amla supplemented group and this differ significantly as compared to control. Higher feed consumption was recorded in control group and in 0.25% amla supplemented group. The lower feed consumption at higher levels of amla supplemented group than control group might be due to better utilization of nutrients. The feed intake of all the chicks receiving amla fruit powder was lower than control and there was a linear decrease with the level of addition. Similarly, decrease in feed consumption as above was also reported by Wadhwa *et al*. (2007) supplemented amla powder in broiler ration. However few reports are available that significant improvement in feed consumption is noticed in supplementation with natural Vitamin C (amla) as compared to synthetic Vitamin C.

Average body weight gain

Mean body weight gain at the age 0 to 14 days ranged from 290.7(T_1) to 314.5 (T_6) and body weight gain did not differ significantly in amla supplemented group as compared to control. Body weight gain at age of 15 to 28 days ranged from 873.4 (T_1) to 920.7 (T_5) and significantly higher body weight gain was found in antibiotic and amla supplemented group as compared to control group. Mean body weight gain from 29 day to 42 day of growth period ranged from 941.7 (T_1) to 1016.7 (T_6) and significantly higher gain was found in 0.75% and 1% amla supplemented group followed by 0.5% (T_4), 0.25% (T_3) amla supplemented group and antibiotic supplemented group as compared to control group (T_1).Over all body weight gain at the end of growth period ranged from 2105.8 (T_1) to 2240.4 (T_5) and significant higher body weight gain was found in 0.75% (T_5) and 1% (T_6) amla supplemented group as compared to control (2240.4) and (2232.7) respectively followed by 0.25%, 0.50% and antibiotic supplemented group as compared to control group. The higher body weights observed in amla supplemented groups may be attributed to anabolic and antioxidant effect of ascorbic acid, gallic acid and tannic acids present in *E. officinalis*. Wadhwa *et al.* (2007) showed increased body weight gain during 0-4 weeks. Enhancement of intestinal activities of trypsin, lipase and amylase and improved gut morphological characteristics are the major mechanisms through which phytoadditives exert their beneficial effect on the nutrient

digestibility. The antioxidant activity of the bioactive compounds like carvacrol, cineol, thymol and pinene and also the improved activities of enzymes in the alimentary tract helps in the stimulation of useful microbial activity and inhibition of pathogenic microflora, which is mainly because of the beneficial impact and influence of various phytogenic feed additives which also helps in performance improvement and feed conversion ratio. Apart from this, the phytogenic additives have other beneficial properties that includes anti-stress, immune enhancement properties, antibacterial, gut microflora manipulation, antibacterial and stimulation of digestive enzymes which helps in enhancing the health, growth and productive performances of the animals.

Feed Conversion Ratio

Feed Conversion Ratio during 0-14 days of the experiment was higher in the T_1 and lowest FCR was obtained in the T_6 groups but these do not differ significantly. FCR during 15-28 days ranged from 1.54 to 1.76. The difference was statistically significant ($P<0.05$) and improved FCR was found as the level of inclusion of amla fruit powder was increased as compared to control and antibiotic supplemented group. FCR during 29-42 day of the growth period antibiotic and 0.75% and 1% amla supplemented group results in better FCR this differ significantly from the control group followed by 0.25% and 0.50% amla supplemented group. At the age of 6 weeks highest (1.92) FCR was obtained in the basal diet (T_1) and difference was significantly higher in comparison to amla supplemented groups and lowest FCR was obtained in 0.75%, and 1% amla supplemented groups (T_5) and (T_6) The feed conservation efficiency was improved as level of amla increased. The feed intake of all the chicks receiving amla was lower than of control and there was a linear decrease with level of addition (Kumari *et al.,* 2012). The results showed that the cumulative feed conversion efficiency at the end of experiment was better in group T_6 and T_5 followed by T_4 and T_2. Similarly broilers fed with Amla, Tulsi and Turmeric either alone or in combination @ 0.25% and 0.50% levels resulted in better feed efficiency.

Toll-like receptors expression

The differential expression level of TLRs, viz. TLR 2, TLR 4 and TLR 7 gene transcripts in the of Ven Cobb commercial broiler strains was studied by relative quantification method. The level of target mRNA in different treatment groups was determined by comparative CT method (ΔΔCT method). The nutrigenomic expression analysis as presented previously in Table 8 revealed that relative mRNA expression of TLR 2 of broilers was found to be ($p<0.05$) enhanced in the treatment groups T5 and T6 supplemented with 0.75% and 1% of the amla fruit powder respectively. While, as presented previously in Table 8 at the end of the 6 weeks of experimental protocol broilers had significant down regulation pattern of relative mRNA expression of TLR 4 in the plasma of broilers fed diet supplemented with 0.25%, 0.50%, 0.75 % and 1% of amla fruit powder in the treatment groups T3, T4, T5 and T6 respectively and in antibiotic supplemented group. However, the data pertaining to the relative mRNA levels of TLR 7 Table 8 in the plasma of birds revealed no significant differences were observed in the experimental groups T2, T3, T4 and T5 and T6 as compared to the control group and antibiotic supplemented group. In nutshell, experimental treatments containing amla powder in the broiler's diet has potent immune modulating activity by showing stimulatory effect on relative mRNA expression of TLR 2 and down regulation pattern of TLR 4 of the commercial broilers.

Our study for TLR2 are in consonance with finding of Sheoran *et al*. (2017) concluded that the addition of garlic powder and holy basil leaf powder at higher level of 1% of the feed either alone or in combination in the diet of the broilers increased the relative mRNA expression of TLR 2, 4 and 7. Our study in case of expression of TLR4, TLR 7 are in contrary to Sheoran *et al*. (2017) Medicinal herbs has shown to possess multiple immune modulatory actions like phagocytosis, modulation of immunoglobulin and cytokine secretion, cellular co-receptor expression, class switching, lymphocyte expression, and histamine release (Mahima *et al*., 2012). In current work, it was observed that dietary inclusion of amla fruit powder significantly modulate the relative mRNA expression of TLR cell markers; this confirmed that these herbal feed additives could stimulate the T cell immune system in the plasma of broiler birds. In the present investigation, we found that there was a significant increase in the relative mRNA expression of TLR 2 in the plasma of the broilers fed diet supplemented with 0.75% amla fruit levels powder. TLR 2 recognizes a variety of microbial components. These

include lipoproteins/lipopeptides from various pathogens, peptidoglycan and lipoteichoic acid from Gram-positive bacteria (Takeda *et al*., 2005).

TLR 4 is the principal receptor for lipopolysaccharide, This is a major component of outer membrane of gram-negative bacteria (Kannaki *et al*., 2010). Several studies have shown that the essential oils and biologically active compounds in herbs are effective against bacteria such as *E. coli, Shigella* spp. Salmonella typhi, and Pseudomonas aeruginosa (Prakash *et al*., 2005). The antimicrobial actions of essential oils in herbs are due to phenolic compounds present in them. They exert membrane damaging effects to microbial strains and stimulate leakage of cellular potassium this is responsible for a lethal action related to cytoplasmic membrane damage (Mahamood, *et al*., 2008). They show its immunomodulatory effect by increase in interferon-γ, interleukin-4, T-helper cells, NK cells (Mondal, *et al*., 2011). Thus, reducing total bacterial count, increasing neutrophil and lymphocyte count and enhancing phagocytic activity and phagocytic index. Another study revealed that the ethanol and methanol extracts of O. sanctum had the ability to inhibit the growth of all test bacteria including *E. coli* and *P. aeruginosa* (Pathmanathan, *et al*., 2010). Herbs can influence selectively the microorganism by an antimicrobial activity thus favors better nutrient utilization and absorption or the stimulation of the immune system (Wenk, 2003).

From the above reported studies and our result findings, it can be inferred that, supplementation of diet with 0.75% amla improved performance, suppressed the growth of harmful organisms like Coliforms, thereby creating a conducive environment for the growth of the beneficial microbes like *Lactobacillus*, *Bifidobacteria* spp. and thereby, aid in digestion and give better performance. Result findings related to the relative mRNA gene expression of TLR 2 and TLR 4 in the present study it can be inferred that amla given at 0.75% and 1% of feed showed better results as compared to control group. TLR 7 family is implicated in intracellular recognition of nucleic acids. The TLR 7 recognizes some antiviral compounds and single-stranded viral RNA. In this study, supplementation of diet with amla powder did not significantly increase the relative mRNA expression of TLR 7 in the plasma of the broiler birds. Researchers are focusing on an extract of *A. sativum* called Ajoene. This appears to protect CD+ cells from attack by HIV early in the viral life cycle. Allicin present in the A. sativum can protect against plasmodium infection by enhancing the host innate as well as innate immunity (Feng *et al*., 2012). Based up above study it can be concluded that the supplementation of amla fruit powder at higher levels 0.75% and 1% results in up regulation of TLR2 and down regulation of TLR4. This might be due to enhanced growth of beneficial gram positive bacteria and decreased gram negative bacteria in birds.

Yitbarek *et al*. (2013) concluded that the supplementation of broiler chickens diet with yeast derived macromolecules has shown the possible role of yeast extract as a nutritional supplement to enhance gut health in chickens' possible modulation in epithelial cell turnover as well as immunomodulation. Supplementation of broiler diets with Yeast derived macromolecules resulted in both local and systemic immune responses where mainly TLR2 was involved locally, whereas only TLR4 was involved systemically with the production of both pro- and anti-inflammatory cytokines. Sheoran *et al*. (2017) concluded that the addition of garlic powder and holy basil leaf powder at higher level of 1% of the feed either alone or in combination in the diet of the broilers increased the relative mRNA expression of TLR 2, 4 and 7, although, among the two feed additives HBLP fed at 1% of diet was found to have enhance the overall growth performance and immune status of birds by augmenting the T cell mediated immune response and thereby protects them from disease without decreasing performance traits.

CONCLUSION

Based upon the above study, it can be concluded that amla fruit powder can be effectively supplemented as an alternative to antibiotics growth promoter in poultry ration. The best results were obtained at 0.75% amla supplementation level (T_6). The differential expression level of TLRs, viz. TLR 2, TLR 4 and TLR 7 gene transcripts in the of was studied by relative quantification method. The relative mRNA expression of TLR 2 was found to be enhanced ($P<0.05$) in the treatment groups T_5 and T_6 supplemented with 0.75% and 1% of the amla fruit powder, respectively. This might be due to enhanced growth of beneficial gram positive bacteria and decreased gram negative bacteria in birds. Broilers had significant down regulation pattern of relative mRNA expression of TLR 4 in the plasma of broilers fed diet supplemented

with 0.25%, 0.50%, 0.75 % and 1% of amla fruit powder in the treatment groups T_3, T_4, T_5 and T_6 respectively and in antibiotic supplemented group. The relative mRNA levels of TLR 7 in the plasma of birds revealed non- significant differences.

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