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Effect of *Eugenia jambolana* and *Psidium guajava* Leaf Meal Mixture Supplementation on Antioxidant Indices and Immune Responses in Broiler Chicks

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ABSTRACT

This experiment was conducted to study the effect *Eugenia jambolana* and *Psidium guajava* leaf meal mixture (LMM) supplementation on antioxidant and immune indices of broilers. One-hundred-and-twenty day-old unsexed broiler chicks were randomly distributed into 4 groups of 30 birds per treatment, each having 3 replicates (10 chicks / replicate) in an experiment based on completely randomized design (CRD). All the birds were fed standard rations to meet their nutrient requirements. Rations of the birds of groups T_1, T_2, T_3 and T_4 were supplemented with LMM at 0, 2.5, 5.0 and 7.5 % of diet, respectively. Feed intake during 0-3, 4-6 and 0-6 weeks were significantly (P<0.05) higher in T_1 followed by T_2, T_3 and least in T_4 groups. Weight gain during 0-3, 4-6 and 0-6 weeks of broiler chicks were significantly (P<0.05) higher in T_1 as compared to T_2, T_3 and T_4 groups. Feed conversion ratio (FCR) during 0-3 weeks among groups was statistically non-significant (P<0.05), while, at 4-6 weeks, it was found to be lowest in T_4, whereas, during 0-6 weeks FCR was significantly (P<0.05) lower in T_1 than that of other groups. LMM supplementation decreased (P<0.068) average body weight during metabolism trial in T_4 than that of T_1 group. Nitrogen (N) intake and retention were significantly (P<0.021, P<0.001) lower and N excretion was significantly higher (P<0. 006) in T_3 and T_4 than that of T_1 and T_2. Mean haemoglobin and packed cell volume were significantly (P<0. 034, P<0.002) higher in T_1 and T_2 than that of T_3 and T_4. The LMM supplemented groups showed better antioxidant indices (P<0.05), cell mediated (P<0.05) and humoral immune responses (P<0.05) as compared to control. It was concluded that LMM supplementation (2.5 %) improved antioxidant status, immune response and growth performance of broiler chicks.

Key words: Antioxidant indices, Broilers, Immune response, Leaf meal mixture

INTRODUCTION

Phytogenic feed additives are the products of plant origin used in livestock and poultry feeding as non-nutritive compounds to improve their nutritional and health status, and their productive performance. Now a days, application of these phytogenic feed additives in the ration of poultry gained importance due to the ban of synthetic drugs as growth promoters by European Union (Windisch *et al.,* 2008). Leaf meal (LM) and/ or leaf meal mixture (LMM) of *Eugenia jambolana* and *Psidium guajava* containing plant secondary metabolites (PSMs) especially condensed tannins (CT) are promising alternatives to synthetic drugs in the poultry ration. The CT's are naturally occurring water soluble poly-phenolic compounds with varying molecular weight. Presence of large number of phenolic hydroxyl groups in CTs enables them to show the beneficial effects (Pathak, 2013; Pathak *et al.,* 2013a,b,c; Pathak *et al.,* 2016, 2017). Possible mechanisms of antioxidant activity of CT are free radical scavenging activity, chelation of transition metals and inhibition of pro-oxidative enzymes (Decker, 1997). Condensed tannins are also known to prevent superoxide formation and lipid peroxidation (Pathak, 2013; Pathak *et al.,* 2017). Additionally, LMM are good source of various nutrients and excellent source of antioxidants (Atawodi *et al.,* 2013; Zargar *et al.,* 2016; Zargar *et al.,* 2017; Pathak *et al.,* 2017).

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To date, some tree leaves and their extracts have been evaluated for possible antimicrobial, anti-parasitic and antioxidant activities (Daing *et al.,* 2017a; Daing *et al.,* 2017b; Pathak *et al.,* 2013; Pathak *et al.,* 2014; Zargar *et al.,* 2016; Zargar *et al.,* 2017) and compounds isolated from LMM have shown great potential against infectious agents such as pathogenic bacteria, gastrointestinal nematodes and coccidian spp., *etc. Psidium guajava* (PS) is used as a traditional medicine in all over the world. It is traditionally used to treat diarrhoea and dehydration, gastroenteritis, dysentery, stomach pain, diabetes mellitus, and wounds. In addition, PS showed anthelmintic, antioxidant, antibacterial, anti-inflammatory properties as well as growth promoting potential.

Eugenia jambolana, commonly known as black plum or Jamun is an important medicinal plant. The leaves are potential source CTs. Different parts of the *Eugenia jambolana* are also reported for anthelmintic, antioxidant, anti-inflammatory, neuro-psycho-pharma cological, anti-microbial, anti-bacterial, and antifugal, nitric oxide scavenging, free radical scavenging, anti-diarrheal, anti-fertility, gastro-protective, anti-ulcerogenic and radio-protective activities. Keeping all these points in view, the present study was planned to investigate the dietary incorporation of graded level of tanniferous LMM comprising *of Eugenia jambolana and Psidium guajava on* antioxidants indices, immune status and growth performance of broiler chickens.

MATERIALS AND METHODS

The experimental study was conducted in the Division of Animal Nutrition, Faculty of Veterinary Sciences & AH, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, R.S. Pura, Jammu. Fresh leaves of *Eugenia jambolana* and *Psidium guajava* were harvested from Faculty premises of R.S. Pura, Jammu. Both tree leaves were air-dried in the shed for 12-15 days. The dried tree leaves of *Eugenia jambolana* and *Psidium guajava* were milled in the laboratory hammer mill. The ground tree leaves were thoroughly mixed in proper ratio (50:50) on the cemented floor and suitable LMM was prepared for *in-vivo* trial.

A total of 120 day-old unsexed and healthy commercial broiler chicks of Cobb-K strains were purchased from Kashmir Valley Poultry Breeding Farm and Hatchery, Jammu They were randomly distributed into four dietary treatment groups (T_1, T_2, T_3 and T_4) of 30 birds per group, and brooded on a partitioned deep litter house at the experimental site. Each group was sub-divided into three replicates of 10 birds per replicate in an experiment based on CRD. On arrival chicks were given water and feed *ad libitum,* small amount of feed was sprinkled on papers inside brooding area for easier consumption and recognition of feed by chicks up to ten days of age. Temperatures were kept at 32^{o}C for the first 7 days. Water and respective diets were supplied *ad libitum i.e.* birds in T_1 group were given control diet (0% LMM) to meet their nutrient requirements, while the birds in T_2, T_3 and T_4 groups were given diet containing LMM of *Eugenia jambolana* and *Psidium guajava* at 2.5, 5.0 and 7.5 %, respectively. Broiler starter and finisher diets were prepared as per BIS (1992). Ingredient and chemical composition of experimental diets are presented in the Table 1.

The parameters measured were feed intake, body weight gain, FCR, haematological profile, antioxidant indices and immune response of broiler birds. Daily feed intake per bird was calculated every morning by subtracting the amount of feed left in the feeding trough from what was offered the previous day and then dividing it by the number of birds in the replicate. Chicks in a pen were weighed individually with a digital weighing scale and the previous weight was subtracted from the current weight to determine the body weight gain per bird for the week. FCR was calculated by dividing the feed consumed by the live weight gain.

Samples of tree leaves, LMM, feed offered and residue left were analyzed for various proximate principles *viz.*, dry matter (DM), organic matter (OM), crude protein (CP), ether extract (EE), nitrogen-free extract (NFE) and total ash as per AOAC (1995) and calcium (Talapatra *et al.,* 1940) and phosphorus (AOAC, 2000). Excreta samples were analysed for N, Ca and P.

The CT content of tree leaves and LMM was estimated by using butanol-HCl method (Makkar, 2000). Metabolizable energy (ME) values of experimental diets were calculated according to the formula given by Pauzenga (1985).

Blood samples were collected from each bird at the time of slaughter. Two birds were randomly selected from each replicate for bleeding and slaughter. Out of 6 ml of blood, about 2.0 ml of blood was collected into labelled sterile universal bottles containing ethylene-diamine-tetra-acetic acid (EDTA) at 1 mg/ml of blood as anticoagulant for estimating haematological profile. Hemoglobin (Hb) and packed cell volume (PCV) were estimated in whole blood immediately after the collection of blood by cyanomethhaemoglobin method (Dacie and Lewis, 1969) and Wintrobe's tube (Jain, 1986), respectively. Another 2 ml of blood was collected in tubes containing acid citrate dextrose (ACD) at 1.5 ml/10 ml blood as anticoagulant, centrifuged at 2000 rpm for 15 min at 4°C, followed by separation of plasma and

Table 1. Ingredients and chemical composition of experimental broiler diets

Attributes	Experimental diets							
	Broiler starter diets				Broiler finisher diets			
	T_1	T_2	T_3	T_4	T_1	T_2	T_3	T_4
Ingredient composition (%)								
Maize	51.00	48.50	46.00	43.50	57.00	54.50	52.00	49.50
Rice polish	3.75	3.75	3.75	3.75	6.50	6.50	6.50	6.50
Soybean meal	35.00	35.00	35.00	35.00	27.50	27.50	27.50	27.50
MBM	5.00	5.00	5.00	5.00	4.00	4.00	4.00	4.00
Soybean oil	1.75	1.75	1.75	1.75	2.00	2.00	2.00	2.00
DCP	1.16	1.16	1.16	1.16	1.00	1.00	1.00	1.00
LSP	1.00	1.00	1.00	1.00	0.70	0.70	0.70	0.70
Salt	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
*Premix	0.47	0.47	0.47	0.47	0.45	0.45	0.45	0.45
Lysine	0.31	0.31	0.31	0.31	0.30	0.30	0.30	0.30
Methionine	0.16	0.16	0.16	0.16	0.15	0.15	0.15	0.15
LMM	0.00	2.50	5.00	7.50	0.00	2.50	5.00	7.50
Chemical composition (% DM)								
OM	92.55	92.43	92.11	91.99	92.14	92.08	91.97	91.88
CP	22.93	22.83	22.97	23.07	20.01	20.13	19.81	19.80
EE	4.45	4.40	4.23	4.64	5.04	5.12	5.22	5.07
CF	4.42	4.53	4.69	4.82	4.63	4.38	4.72	4.83
NFE	60.75	60.67	60.22	59.46	62.46	62.45	62.20	62.18
Total ash	7.45	7.57	7.89	8.01	7.86	7.92	8.03	8.12
AIA	2.43	2.61	2.78	2.92	2.50	2.43	2.76	2.91
Calcium	1.67	1.64	1.77	1.83	1.73	1.56	1.83	1.77
Phosphorus	0.76	0.77	0.73	0.70	0.69	0.67	0.63	0.61
ME ($KcalKg^{-1}$)	2818	2790	2762	2734	2925	2897	2869	2841
CT (% of diet)	0.0	0.18	0.37	0.53	0.0	0.19	0.39	0.54

*Premix (vitamins &minerals) added at the rate of 470 g/100 kg feed which contained: vitamin A, 5000 IU; vitamin D, 1000 IU, vitamin E, 10 mg; vitamin K, 850 mg; vitamin B1, 600 mg; vitamin B2, 2 mg; vitamin B6, 1.8 mg; vitamin B12, 4 mcg; niacin, 1.2 mg; folic acid, 2 mg; d pantothenic acid, 3.2 mg, Cu, 2.6 mg; Mn, 19.2 mg; Zn, 16 mg; Se, 0.45 mg; Fe, 9.5 mg; Co, 0.25 mg and I, 0.55 mg buffy coat. The resulting erythrocyte pellet was washed thrice with 250 mOsm/litre (pH-7.4) of phosphate buffered saline (PBS) as per Yagi *et al.* (1989) to determine the various antioxidant enzymes activity, while remainder of about 2 ml of blood sample was collected in sterile centrifuge tube for collection of serum to determine humoral immune response against previously sensitized birds with sheep RBC (SRBC) as antigen.

Various antioxidant indices *viz.* reduced glutathione (GSH; μ mol/mg Hb), glutathione-S-transferase (GST; μ mol/mg Hb), super oxide dismutase (SOD; U/mg Hb), catalase (Cat; U/mg Hb), lipid peroxidise (LPO; μ mol/mg Hb), total-thiol (T-SH; μ mol/ml), protein-thiol (TP-SH; μ mol/mg Hb) and non-protein thiol groups (NP-SH; μ mol/ml) were determined by using standard protocols. The GSH was estimated by DTNB method of Prins and Loos (1969), whereas, GST activity was determined as per the method described by Habig *et al.* (1974). Catalase was assayed in erythrocytes by the spectrophotometric method as described by Bergmeyer (1983). The SOD activity in erythrocytes and LPO level in RBC haemolysate were determined as per the methods described by Marklund and Marklund (1967) and Placer *et al.* (1966), respectively. Total thiol (T-SH) groups in the RBC haemolysate were determined following the method of Sedlak and Lindsay (1968).

The effect of CT containing LMM supplementation in broiler chicks on CMI response (foot pad thickness) was assessed through *in vivo* cutaneous delayed-type hypersensitivity (DTH) reaction against phytohaemagglutin-P (PHA-P). Towards the beginning of experimental feeding of 6th weeks, two birds from each replicate were used. The foot pad area of tested birds was cleaned with cotton swab containing sprit and the thickness of the foot pad was measured with the help of digital Vernier callipers, which would represent the basal (0 h) value. All the tested birds were injected intradermally with 25 μl of PHA-P solution. The thickness of the foot pad was subsequently measured at 24 h and then at 48 h and 72 hours.

Assessment of humoral immune response was assessed through haemagglutination (HA) titre on 24 broiler finisher chicks selected at random at 32 days. Venous blood from sheep was collected aseptically in equal volume of Alsever's solution and stabilized for one week. Finally, a suspension of 0.5 % sheep red blood cells (SRBC) was adjusted with phosphate buffer saline (PBS) solution. For immunization, 100 μl of this suspension was injected intravenously in wing vein of each tested bird. Blood from immunized birds was collected after 10 days for collection of serum. Total SRBC antibody titres were assayed in the serum of each bird according to the agglutination method described by Hudson and Hay (1991). Antibody titters were reported as $\log_2$ of the highest dilution of serum that agglutinated an equal volume of a 0.5% SRBC in PBS solution.

The results obtained were subjected to analysis of variance and treatment means were ranked using Duncan's multiple range test (DMRT). The periodic alterations in CMI response was analyzed using General linear model (GLM) procedures. Significance was declared at $P<0.05$ unless otherwise stated. All the statistical procedures were performed as per Snedecor and Cochran (2004).

RESULTS AND DISCUSSION

The ingredients and chemical composition of experimental diets of broiler chicks has been presented in the table 1. The proximate compositions of all the experimental diets during both starter and finisher phases were within the range as suggested by BIS (1992). The CT content of LMM was 7.69 percent.

Data pertaining to cumulative feed intake, body weight gain and feed conversion ratio (FCR) are presented in table 2. Feed intake (g) during 0-3, 4-6 and 0-6 weeks were significantly ($P<0.05$) higher in T_1 followed by T_2, T_3 and least feed intake was recorded in T_4 groups. Lower feed intake in T_3 and T_4 groups may be due to high percentage of CT which is having astringent property. At higher level, CTs bind with glycoprotein of the saliva and may also form complex with other nutrients that may causes a sensation in the oral cavity, resulting in reduced palatability and decreased feed intake in broiler birds. Present results are contradictory to the findings of Rahman *et al.* (2013 who reported that *P. guajava* leaf meal up to .

reduced (P<0.05) PCV but not below the level considered normal for birds.

Data pertaining to antioxidant indices are presented in table 3. Mean values of catalase, and GST were significantly (P<0.05) higher in CT containing LMM supplemented groups (T_2, T_3 and T_4) as compared control (T_1) group. The highest GSH activity was evident in T_4 followed by T_3 and T_2, whereas the least GSH activity was observed in T_1. Significantly (P<0.006) higher SOD activity was obtained in T_3 as compared to T_1, T_2 and T_4. The LPO activity (µ mol/ mg Hb) drastically (P<0.001) reduced as the level of CT containing LMM increased and it was found to be lowest in T_4 followed by T_3, T_2 and the highest LPO activity was observed in T_1. Significantly (P<0.012) higher T-SH activity was evident in T_4 as compared to T_1, T_2 and T_3 groups, while NP-SH activity was found to be highest in T_4 followed by in T_3, T_2 and least NP-SH activity was noted in T_1.

It clearly indicated that tanniferous LMM supplementation significantly (P<0.001) increased NP-SH activity in broiler chicks. However, P-SH did not differ significantly among groups. Results of the present study showed that CT containing LMM supplementation at 2.5, 5.0 and 7.5 % of the broiler diets significantly (P<0.05) improved the antioxidant indices as indicated by increased levels of catalase, GSH, GST, SOD, T-SH and NP-SH and decreased level of LPO. Further CT prevents superoxide formation and lipid peroxidation (Lau and King, 2003). The presence of catecholic B-ring and free hydroxyl groups in CT structures are the key factors responsible for their potent antioxidant activities. The antioxidant activity is further enhanced by the polymerization of flavan-3-ols. Thus, the CT acts as potent antioxidant via H-atom transfer or single –electron transfer mechanism. Several earlier studies in ruminants have demonstrated the antioxidant properties of CT containing LM and/or LMM (Dutta *et al.,* 2012; Dey and De, 2014; Dey *et al.,* 2015; Pathak *et al.,* 2017). It was reported that dietary supplementation of CT through LMM improved activity of GSH, GST, SOD, catalase, T-SH and P-SH and significantly reduced LPO levels in CT supplemented groups.

The CMI response against PHA-P as antigen injected intra-dermally in the foot pad of broiler chicks is illustrated in the Fig. 1. The CMI response (foot pad thickness) of broiler chicks increased significantly

Table 2. Effect of *Eugenia jambolana* and *Psidium guajava* leaf meal Mixture supplementation on cumulative body weight gain, feed intake and FCR in broiler chicks

Time	Groups				SEM	P Value
	T_1	T_2	T_3	T_4		
Body weight gain (g)						
0-3 weeks	627^{b}	593^{ab}	592^{ab}	589^{a}	6.29	0. 038
4-6 weeks	1247^{d}	1132^{c}	1075^{a}	1106^{b}	15.34	0.001
0-6 weeks	1874^{d}	1725^{c}	1667^{b}	1694^{a}	16.89	0.001
Feed intake (g)						
0-3 weeks	1085^{d}	1052^{c}	1035^{b}	997^{a}	2.93	0.001
4-6 weeks	2935^{d}	2866^{c}	2831^{b}	2684^{a}	8.40	0.001
0-6 weeks	4021^{d}	3918^{c}	3866^{b}	3681^{a}	11.29	0.001
Feed conversion ratio (FCR)						
0-3 weeks	1.75^{b}	1.80^{c}	1.76^{b}	1.72^{a}	0.02	0.546
4-6 weeks	2.38^{b}	2.58^{c}	2.68^{d}	2.50^{a}	0.03	0.011
0-6 weeks	2.16^{a}	2.30^{c}	2.34^{d}	2.20^{b}	0.02	0.005

[a,b,c,d]Means with different superscript within a row differ significantly (P<0.05)

(P<0.05) with increasing level of LMM. Significantly higher CMI response was observed in T_4 followed by T_3, T_2 and the lower CMI response was recorded in T_1 group. The maximum foot pad thickness was recorded after 24 h onward and up to 72 h post inoculation of PHA-P in broiler chicks.

It is clearly indicated that the dietary supplementation of CT containing LMM in broiler diets significantly improved CMI response. The birds fed red sorghum (containing tannins) exhibited higher immune-responsiveness than their reconstituted counterparts assessed through cellular (footpad index) immune response (Kumar *et al.,* 2007). The role of tanniferous LMM on immune response can be understood as flavonoids, CTs and microelements have been suggested to act as antioxidants and exert their antioxidant activity by scavenging the free radicals and preventing lipid peroxidation (Yuting *et al.,* 1990). Latshaw (1991) have suggested that cellular integrity is very important for receiving and responding to messages needed to coordinate an immune response. The improved total thiol group and decrease in lipid peroxidation in the present study might have improved cellular integrity so that CMI response was increased. In an earlier report (Yokozawa *et al.,* 1997), green tea tannin eliminated oxidative stress in rats, which might be the reason for improved immune performance observed in LMM supplemented broiler chicks than that of un-supplemented control.

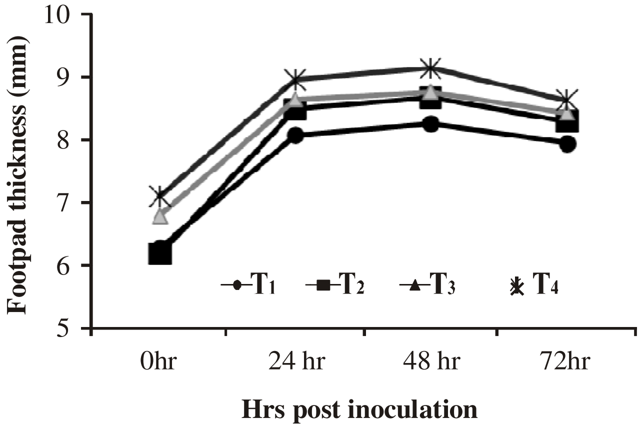

Fig. 1. DTH response to PHA-P (foot pad thickness) in broiler chicks fed on LMM supplemented diets

Humoral immune response (HA titre, log2 value) against sheep RBC (SRBC) in broiler chicks fed CT containing LMM supplemented diets is depicted in

Table 3. Effect of *Eugenia jambolana* and *Psidium guajava* leaf meal mixture supplementation on haematological profile and antioxidant indices of broiler finisher chicks

Parameters	Groups				SEM	P Value
	T_1	T_2	T_3	T_4		
Haematological profile						
Haemoglobin (g/dl)	10.73^{b}	10.54^{ab}	11.00^{b}	10.02^{a}	0.13	0.034
PCV (%)	29.83^{ab}	30.83^{bc}	31.17^{c}	28.83^{a}	0.27	0.002
Antioxidant indices						
Catalase (U/mg Hb)	2.50^{a}	3.61^{b}	3.88^{b}	3.78^{b}	0.21	0.043
GSH (μ mol/mg Hb)	1.29^{a}	1.53^{a}	1.64^{ab}	1.96^{b}	0.09	0.017
GST (μ mol/mg Hb)	4.51^{a}	6.29^{b}	6.61^{b}	6.99^{b}	0.32	0.005
SOD (U/mg Hb)	6.45^{a}	7.52^{a}	11.94^{b}	7.32^{a}	0.74	0.006
LPO (μ mol/mg Hb)	1.75^{d}	1.04^{c}	0.58^{b}	0.26^{a}	0.17	0.001
TP-SH (μ mol/mg Hb)	1.14^{a}	2.35^{a}	2.42^{a}	4.92^{b}	0.49	0.012
NP-SH (μ mol/ml)	0.64^{a}	1.72^{b}	1.77^{b}	3.15^{c}	0.29	0.001
P-SH (μ mol/ml)	0.51	0.64	0.66	1.77	0.26	0.311

^{a,b,c}Means with different superscript within a row differ significantly (P<0.05)

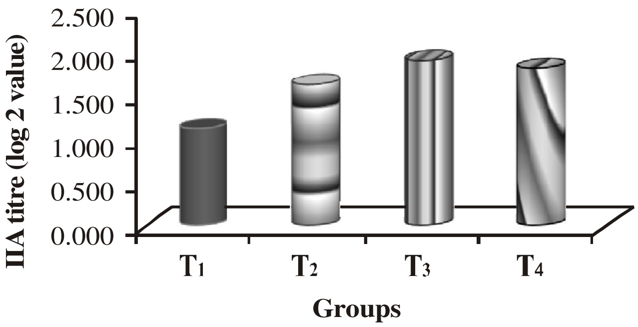

Fig. 2. Humoral immune response (HA titre, log2 value) of broiler chicks fed LMM supplemented diet

Figure 2. Dietary supplementation of tanniferous LMM in broiler chicken significantly (P<0.001) increased the HA titre (log2) value in LMM (T_2, T_3 and T_4) supplemented groups as compared to un-supplemented control (T_1) group, however, HA titre (log2) values not differ significantly among three treatment groups (T_2, T_3 and T_4). Similarly, Durrani *et al.* (2008) reported the immune-stimulatory effects of neem (*A. indica*) leaves infusion in broiler chicks. They found that *A. indica* infusion successfully improved antibody titre at the level of 50 ml/liter of fresh drinking water in broilers. Present findings are also in line with Sadekar *et al.* (1998), who fed dry leaves powder of *A. indica* to broilers (2g/kg), which significantly enhanced the antibody titers against IBD and ND virus antigens. Subapriya and Nagini (2005) also reported antiviral properties of neem leaf and its constituents. The immune-stimulating properties of LMM as observed in the present study might be due to presence of CT. Many previous studies also reported immune-stimulating properties of CT containing LMM (Dubey *et al.,* 2012; Pathak *et al.,* 2014; Singh *et al.,* 2015).

CONCLUSION

It was concluded that *Eugenia jambolana* and *Psidium guajava* leaf meal mixture (50:50) supplementation (2.5 %) in broiler chicks improved antioxidant status, cell mediated and humoral immune responses without affecting performance.

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