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K Prashanth Kumar
Ph. D Scholar, Department of Poultry Science College of Veterinary Science, P. V. N. R Telangana Veterinary University, Rajendranagar, Hyderabad, Telangana, India

V Ravinder Reddy
Professor & University Head, Department of Poultry Science College of Veterinary Science, P. V. N. R Telangana Veterinary University, Rajendranagar, Hyderabad, Telangana, India

M Gnana Prakash
Principal Scientist, Poultry Research Station P. V. N. R Telangana Veterinary University, Rajendranagar, Hyderabad, Telangana, India

Correspondence
K Prashanth Kumar
Ph. D Scholar, Department of Poultry Science College of Veterinary Science, P. V. N. R Telangana Veterinary University, Rajendranagar, Hyderabad, Telangana, India

Effect of supplementing pomegranate (*Punica granatum*) peel extract on serum biochemical parameters and immune response in broilers during summer

K Prashanth Kumar, V Ravinder Reddy and M Gnana Prakash

Abstract
This study was conducted to evaluate the effects of pomegranate (*Punica granatum*) peel extract (PPE) on serum biochemistry and immunity of broiler chicken reared during summer. The average minimum and maximum room temperatures recorded from 0-42 day were 36.8 ºC±0.25-39.7 ºC±0.25, respectively, with relative humidity ranging from 47-74%. Day old (n=160) broiler chicks were randomised to 4 dietary treatment groups containing eight replicates with five chicks each and are kept in a closed, ventilated, wire-floor battery caged broiler house under uniform conditions of temperature, humidity, and ventilation. The experimental treatment groups were: 1. Positive control (PC)-Corn soya based diet supplemented with Vit-E 70mg/kg + Selenium 0.15 mg/kg, 2. Negative control (NC)-Corn soya based diet without any antioxidant, 3. PPE50-Corn soya based diet supplemented with 50 mg PPE extract/kg, 4. PPE100-Corn soya based diet supplemented with 100mg PPE extract/kg diet. Inclusion of PPE at 50 mg/kg diet and 100 mg/kg diet decreased ($P<0.05$) the serum cholesterol levels, whereas serum protein levels were unaffected ($P>0.05$) by supplementation of PPE. Antibody titer levels in response to New castle disease vaccine and cutaneous basophilic hypersensitivity (CBH) were not significantly ($P>0.05$) influenced by supplementation of PPE in broiler diets.

Keywords: *Punica granatum,* biochemical parameters, broilers

1. Introduction
Heat stress is an increasing concern to poultry industry in hot humid climates due to reduced growth performance and increased mortality. Like mammals, Birds are homeotherms, thus they are able to maintain a near-constant body temperature. To achieve a constant body temperature, heat produced by metabolism must equal heat loss. In birds, heat losses are limited by dense coverage of feather coat and by the absence of sweat glands, thus these two characters limit their heat transfer ability from the body that generated during metabolism. The main consequence of heat exposure is a reduction in feed intake in order to reduce metabolic heat production (Gous and Morris, 2005) [1]. The optimum temperature for performance is likely to be 19 to 22 ºC for laying hens and 18 to 22 ºC for growing broilers [2]. This reduction is approximately 17 % for every 10 ºC increase in ambient temperature above 20 ºC [3]. This decreased feed intake leads to growth depression. However, the reduction in growth is often greater than the reduction in feed intake, resulting in a lower feed efficiency [4]. It has been established that high temperatures affect the development of a specific immune response in the chicken [5-7]. Thus, concerning the effects of heat stress on birds, the following findings were reported: 1) decreases in the feed consumption, bodyweight gain, as well as the total white blood cell count and antibody production [8] 2) decreases in the number of peripheral blood lymphocytes and induction of an electrolyte imbalance [9]; 3) decreases in the blood lymphocytes and spleen weight [10]; 4) decrease in macrophage activity [11]. Increase in stress induces sympatho-adrenal activity which further leads to release of corticosteroids, hormones that increase protein and lipid catabolism in turn elevating plasma cholesterol concentration [12]. Hypercholesteremia is one of the risk factors for coronary artery disease (CAD) [13]. Strategies to protect poultry from heat stress effect include i. e. i) Genetical manipulations such as introduction of dwarf gene, Naked neck chicken and frizzle gene ii) Management practices such as the use of insulation, fans, foggers and evaporative coolers. iii) Dietary manipulations such as low protein diets, antioxidant vitamins-Vit A, vit E, vit C and herbal supplements containing different properties like adoptogenic (*Withania somnifera, Ocimum sanctum*),

antioxidant (*Punica granatum, Phyllanthus emblica*) have been used to protect tissues from oxidative injury and enhances host antioxidant enzyme systems [14, 15]. Among all nutrient manipulations such as inclusion of antioxidants are most economical, practical and easiest methods to adopt. Heat stress impairs absorption of vitamins A, E and C, and, thus, increases the requirement of these vitamins [16] and reduces plasma and tissue concentrations of minerals such as Se, Fe, Zn and Cu which are related to the antioxidant and immune system [17]. Seville *et al.* [18] recently reviewed the composition of pomrgranate, health benefits, including association with antioxidant and immunomodulatory effects. The objective of this study was to evaluate the efficacy of Pomegranate *(Punica granatum)* peel extract on immunity and serum biochemical parameters in broilers reared during hot summer months.

2. Material and Methods

One day old broiler chicks were housed in open sided, well ventilated battery cages in broiler house. A total of 160 male broiler chicks from, 1-42 d old were used. The broiler chickens were constantly observed for health status and behaviour. 160 broiler chicks were randomised to 4 dietary treatment groups containing eight replicates with five chicks each and are kept in a closed, ventilated, wire-floor battery caged broiler house under uniform conditions of temperature, humidity, and ventilation. Birds were vaccinated against Newcastle (7th and 28th d) and infectious bursal disease (15th d) as per the standard vaccination schedule. Maize and soybean meal-based diets were prepared to contain 3050 and 3,150 kcal ME/kg during the starter (0 to 21 d) and finisher (22 to 42 d of age) phases, with respective crude protein contents of 21.5 and 19.5 g/100 g feed (Table 1). The experimental treatment groups were as follows:

1. Positive control (PC)-Corn soya based diet supplemented with Vit-E 70mg/kg + Selenium 0.15 mg/kg.
2. Negative control (NC)-Corn soya based diet without any antioxidant.
3. PPE50-Corn soya based diet supplemented with 50 mg PPE extract/kg.
4. PPE100-Corn soya based diet supplemented with 100mg PPE extract/kg diet.

Water and mash feed were provided *adlibitum* throughout 42 days trial period. The temperature and relative humidity of shed during trial period were 36.8 ^{o}C±0.25-39.7 ^{o}C±0.25 and 47-74% respectively.

Table 1: Ingredient and nutrient composition of basal diet

Ingredients (%)	**Starter (0-21d)**	**Finisher (22-42d)**
Maize	56.9	61.31
Soya bean meal	35.43	30.60
Vegetable oil	3.355	4.287
Salt	0.423	0.422
Dicalcium phosphate	1.602	1.542
Limestone powder	1.343	1.177
DL-methionine	0.278	0.217
L-Lysine	0.241	0.076
AB_2D_3K*	0.015	0.015
B-complex**	0.015	0.015
Trace mineral mixture***	0.100	0.100
Nutrient composition		
ME (kcal/kg)	3050	3150
Crude protein (%)	21.5	19.5
Lysine (%)	1.25	0.98
Methionine (%)	0.56	0.48
Calcium (%)	0.9	0.82
Available Phosphorous (%)	0.42	0.40

*AB_2D_3K provided per kg diet: Vitamin A 20000 IU, Vitamin B_2 25 mg, Vitamin D_3 3000IU, Vitamin K 2mg.
**Riboflavin 25mg, Vitamin B_1 1mg, Vitamin B_6 2mg, Vitamin B_{12} 40mg, and Niacin 15mg.
***Trace mineral provided per kg diet: Manganese 120mg, Zinc 80mg, Iron 25mg, Copper 10mg, Iodine 1mg.

2.1 Immunity

The humoral immunity was determined in birds by measuring antibody titre to Newcastle disease (ND) vaccine (antibody production against ND virus). At 21st and 42nd days of age blood was collected and serum was separated. Haemagglutination inhibition (HI) activity of serum was estimated and the antibody titers (log_2) were measured following the standard procedure [19].

The cell mediated immune (CMI) response was assessed by measuring cutaneous basophilic hypersensitivity (CBH) to phytohaemagglutinin phosphate (PHA-P). On 40th day of experiment, one bird per replicate was injected with 100 µg of PHA-P suspended in 0.1 ml of phosphate buffer saline (PBS) into the web between third and fourth inter-digital space of right foot, while the left web (control) was injected with 0.1 ml of PBS. The web thickness of both feet was measured by micrometer after 24 h of injection and CBH was calculated by method of Edelman [20].

2.2 Serum Bio-Chemistry

On 41st day of experiment, blood collected from one bird per replicate into a clean sterilized tube and kept in a slanted position at room temperature to facilitate separation of serum. Serum protein and cholesterol were determined by using commercially available diagnostic kits (Erba Mannheim Chemicals).

2.3 Statistical analysis

The data were analysed using General Model procedure of Statistical Package for Social Sciences (SPSS) 15th version and comparison of means was done using Duncan's multiple range test [21] and significance was considered at $P<0.05$. Data were subjected to statistical analysis under completely randomized design employing one-way analysis of variance [22].

3. Results

Table 2 shows the effect of supplementing of pomegranate peel extract on serum biochemistry in broilers reared during summer. Dietary supplementation of PPE100 and PPE50 tended to decrease ($P<0.05$) the cholesterol levels in serum compared to that of NC group. PPE100 group recorded lowest cholesterol levels. However, there was no effect ($P>0.05$) of dietary supplementation of PPE on serum protein levels. Inclusion of PPE at 50 mg/kg and 100 mg/kg had no significant ($P<0.05$) effect on mean antibody titer against Newcastle disease virus and cell mediated immune response to phytohaemagglutinin phosphate (Table 3).

4. Discussion

Increase in stress induces sympatho-adrenal activity which further leads to protein and lipid catabolism in turn elevating plasma cholesterol concentration [12]. Low cholesterol in PC compared to NC may be due to Selenium, which inhibitory effect on 3-hydroxy-3-methylglutaryl-coA (HMG-CoA) reductase, a key enzyme in cholesterol biosynthesis [23]. By quenching free radicals vit-E reduces the effects of oxidative stress there by reduces corticosteroids secretion [24]. Similar results were found by Habibian [25] by feeding vit-E at 250 mg/kg & Se at 0.5-1 mg /kg diet. In the present study decreased serum cholesterol levels by feeding PPE in diets was in agreement with Kishawy *et al*. (2016) who reported reduced cholesterol in serum by feeding PPE at 0.05% and 0.1% levels. Similarly results observed by [27, 28] Yaseen *et al*. (2016) and Sarica *et al*. (2016) by feeding PP at 15/kg and PPE at 0.1g/kg, 0.2g/kg respectively. PPE exerts inhibitory effect on pancreatic lipase activity that inhibit fat absorption from intestinal tract and increase faecal excretion of fat [26, 29] this suggests the reduced serum total cholesterol levels of PPE fed groups in present study. Supplementation of PPE did not influence the immune parameters in present study. However, improved immune response by feeding pomegranate seed pulp and polyphenol rich grape seed [30-32]. Improvement in immune responses might be due to presence of linoleic acid, n-3 fatty acids of seeds, which are known to increase immunoglobulin’s and weight of immune organs [33, 34].

Table 2: Effect of dietary inclusion of pomegranate peel extracts on serum biochemical profile in broiler chicken at 42d of age.

Treatments	Cholesterol (mg/dl)	Total Protein (g/dl)
PC	148.6^{ab}	3.26
NC	164.4^{a}	2.85
PPE50	142.3^{b}	3.08
PPE100	135.9^{b}	3.52
SEM	2.283	0.076
N	8.0	8.00
P-Value	0.012	0.167

A b c d Mean bearing at least one common superscript in a column do not differ significantly ($P>0.05$)

In addition, some polyphenols with dihydroxy groups can conjugate metals, preventing metal-catalyzed free radical formation [35]. Essentially it is iron, as well as other redox active metals (e. g. copper and manganese) catalyze the decomposition of hydrogen peroxide into hydroxyl radicals which is one of the most powerful oxidant species, being able to initiate free radical chain reactions by abstracting hydrogen from almost any molecule (Fatty acids i. e linoleic acid). *Ellagitannins* which present in PPE also have immunomodulatory function by stimulating growth of lymphocytes [36].

Table 3: Effect of dietary inclusion of pomegranate peel extracts on immune response of broiler chicken

Treatments	NDV titers (log_2)		*PHA-P response (thickness index)	Spleen weights (% live weight)
	3rd week	6th week	6th week	
PC	3.25	3.75^{a}	164.1	0.092
NC	3.50	2.75^{ab}	144.3	0.078
PPE50	2.88	2.38^{b}	151.2	0.091
PPE100	2.50	3.00^{ab}	155.4	0.097
SEM	0.133	0.132	2.355	0.005
N	8.00	8.00	8.0	8.000
P-Value	0.678	0.103	0.452	0.880

A b c d Mean bearing at least one common superscript in a column do not differ significantly ($P>0.05$).

High ambient temperatures was reported to reduce weights of lymphoid organs [37] in broilers and heat stress during experimental period might be responsible for decreased circulating macrophages and antibodies [8-11, 38, 39]. This reduction could be indirectly due to stimulation of hypothalamic pituitary adrenal (HPA) axis which results in release of corticosterone that inhibits antibody production [40, 41].

We speculate that reduced immunity in the present study might be due to low doses of PPE and high temperatures (36.3 °C-39.7 °C) during experiment. Temperature effect can be correlated with reduced spleen weights in this study.

5. Conclusion

The results of present study indicated that supplementation of Pomegranate peel extract at 100mg/kg diet significantly decreased serum cholesterol levels without any deleterious effects on performance and did not show any significant effect on immunity.

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