Effect of dietary supplementation of phytochemicals on immunity and haematology of growing broiler chickens

Asghar Ali Kamboha, Muhammad Ammar Khan, Ubedullah Kak, Elmutaz Atta Awad, Atta Muhammad Memon, Muhammad Saeed, Nazar Ali Korejo, Manatbai Bakhetgul and Chandar Kumari

Department of Veterinary Microbiology, Sindh Agriculture University, Tandojam, Pakistan; Department of Food Science and Technology, University College of Agriculture & Environmental Sciences, The Islamia University of Bahawalpur, Bahawalpur, Pakistan; Institute of Tropical Agriculture and Food Security, University Putra Malaysia, Selangor, Malaysia; Department of Poultry Production, University of Khartoum, Khartoum, Sudan; State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, China; Department of Animal Nutrition, College of Animal Sciences and Technology, Northwest A&F University, Yangling, China; Department of Veterinary Medicine, Sindh Agriculture University, Tandojam, Pakistan; Technical Center of Xinjiang Entry–Exit Inspection and Quarantine Bureau, Urumqi, China; Graduate School of Chinese Academy of Agricultural Sciences, Beijing, China

ABSTRACT
The current study investigated the effects of phytochemicals genistein and/or hesperidin dietary supplementation on immunity and haematology of growing broilers. A total of 360 1-day-old broiler chicks (Arbor Acres, mixed sex) were randomly assigned to six treatment groups, namely T0, control; T1 and T2, supplemented with 5 and 20 mg of genistein and hesperidin; while T3, T4, and T5 diets contained 5, 10, and 20 mg of genistein + hesperidin (1:4) mixture, respectively, per kg of diet. The white blood cell count was significantly \((p < 0.01)\) increased in T1, T2, T4, and T5 compared with the control (T0) group. The haemoglobin concentration significantly \((p < 0.01)\) increased in the T5 group, while mean corpuscular haemoglobin concentration was significantly \((p < 0.05)\) higher in T4 compared with the T0 group. Antibody titres against Newcastle disease significantly \((p < 0.01)\) increased in the T5 group, while mean corpuscular haemoglobin concentration was significantly \((p < 0.05)\) higher in T4 compared with the T0 group. Antibody titres against Avian Influenza virus, as compared with the controls. At the same time, the supplemented groups had significantly \((p < 0.01)\) higher neutrophil adhesion rate and cutaneous basophil hypersensitivity test representing the cellular immune response than the controls. In conclusion, supplementation with both phytochemicals, genistein and hesperidin, positively influenced the immune parameters and haematological profile of growing broilers, thus might be considered as feed additives in broiler industry.

ARTICLE HISTORY
Received 13 September 2017
Revised 24 January 2018
Accepted 24 January 2018

KEYWORDS
Broiler chickens; genistein; hesperidin; immunity; haematology

Introduction
Phytochemicals are non-nutritive, secondary plant metabolites synthesised by organisms of the plant kingdom and contribute to the defence against infections, pests, stressful conditions, and physical damage. They are abundant in all plant parts and include several compounds, such as alkaloids, flavonoids, tannins, cyanogenic glycosides, and flavanones (Robbins 2003). The properties of phytochemicals, particularly flavonoids, are extensively studied in human as well as animal experiments dealing with health status, defence against diseases and food protection mechanisms (Acamovic and Brooker 2005). Most of these health effects have been attributed to the properties of flavonoids, that are related with anti-oxidation, anti-inflammation, immunomodulation, and gut protection (Kamboh et al. 2015). Flavonoids regulate mucosal and cellular immunity and modulate the endocrine and circulatory markers of health; dietary supplementation with flavonoids can be, therefore, used for improving immunity and health of broiler chickens.

Consumers demand for the minimisation of growth promoting antibiotics in broiler chicken production resulted in the research for natural compounds such as phytochemicals that can represent alternative natural feed additives. Phytochemicals, particularly those
having antioxidant potential, have been recognised as natural substitutes of synthetic feed additives. According to Kidd (2004), immunity and health of broiler chickens is negatively affected by stress. Al-Aqil et al. (2013) and Ahmed et al. (2015) associated the production of free radicals and reactive oxygen species (ROS) at cellular level with fear in poultry. Production of free radicals and ROS may lead to deterioration of immunity, increased susceptibility to infection, and inferior production performance. Kamboh et al. (2013) evaluated the potential of dietary antioxidants to ameliorate the deleterious effects of abiotic stress. Genistein (a soy flavonoid) and hesperidin (a citrus flavonoid) have been known to regulate mucosal and cellular immunity (Wei et al. 2012; Kamboh et al. 2016), as well as modulating the endocrine and circulatory markers of health in a positive direction (Iqbal et al. 2014). However, effect of dietary supplementation of genistein and hesperidin on immunomodulation and health performance of commercial broilers at growing stage needs to be investigated.

Therefore, the objectives of the present study were to investigate the effects of dietary supplementation with genistein, hesperidin, and their combination, on immunity and haematology of growing broiler chickens (days 1–21). The results of this study can be used to optimise feed formulations and produce broiler chickens with reduced concentrations of antibiotic residues.

**Materials and methods**

All the experimental procedures were approved by the Institutional Animal Care and Use Committee of NAU, PR China. Three hundred and sixty 1-d-old broiler chicks (Arbor Acres, mixed sex) were obtained from a commercial hatchery (Hewe Agricultural Development Co., Anhui, China), and housed in a controlled environment under 24 h light from days 1 to 21. The birds were equally divided into six treatment groups, each with four replicates – pens (15 broilers per pen) with an area of 1.6 m² for each pen. The six treatment groups were the following: T0: control, no feed additive; T1: supplementation with 5 mg of Genistein per kg basal feed; T2: supplementation with 20 mg of Hesperidin per kg basal feed; T3: supplementation with 5 mg of Genistein + Hesperidin (1:4) composite per kg basal feed; T4: supplementation with 10 mg of Genistein + Hesperidin (1:4) composite per kg basal feed; T5: supplementation with 20 mg of Genistein + Hesperidin (1:4) composite per kg basal feed. The supplemental doses and ratios for both the flavonoids were adjusted based on preliminary dosage trails (unpublished data). Feed and water were offered *ad libitum*. The birds were fed with a corn–soybean basal diet in mash form (crude protein 20.73%, metabolisable energy of 3121.9 kcal/kg) according to our earlier report (Kamboh et al. 2016). Other nutrients were adjusted as per standard requirements of broilers (NRC 1994). Purified phytochemicals (98%) genistein and hesperidin were purchased from a commercial company (Sigma Chemical Co., St. Louis, MO).

**Haematological indices determinations**

On day 21, twelve chickens per treatment group (three birds per pen) were randomly selected, and blood samples (3 mL) were collected from the wing vein into heparinised plastic tubes, and immediately stored at 4°C until further analysis. Haematological values such as haemoglobin (Hb), red blood cell (RBC), white blood cell (WBC), thrombocytes (Tb), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were determined through an automatic haematological analyser (ZC-980, Zhejiang, China). All measurements were performed in triplicate within 2 h of sample collection, to ensure their accuracy.

**Antibody titres**

On day 10, two chicks (wing-banded) per replicate pen were inoculated subcutaneously with Newcastle disease (B1 strain vaccine) virus. Similarly, two other chicks per replicate pen were inoculated subcutaneously with Avian Influenza (H5N2 strain vaccine). On day 21, the chicks were bled from wing vein and sera were collected individually in separate sterile vials. The haemagglutination inhibition (HI) test was performed to determine the antibody production response against Newcastle disease and Avian Influenza antigen as serial two-fold dilution of the reciprocal of the last dilution, according to the method of Kamboh et al. (2016).

**Cutaneous basophil hypersensitivity test**

*In vivo* cutaneous basophil hypersensitivity (CBH) test was performed according to the procedure of Corrier and DeLoach (1990). In brief, at the age of 10-d, wing-banded chicks (3 per pen) were injected with 100 µg of phytohaemagglutinin-P, suspended in a 0.1 mL of sterile PBS (Sigma Chemical Co., St. Louis, MO) into the toe web of right foot. Swelling of toe web was measured by using a Vernier calliper at 12 h after the
Values are shown as mean ± SD (n = 12).

- **WBC**: white blood cell; **RBC**: red blood cell; **Tb**: thrombocytes; **Hb**: haemoglobin; **HCT**: haematocrit; **MCV**: mean corpuscular volume; **MCHC**: mean corpuscular haemoglobin concentration.

**T0**: no feed additive, control; **T1**: supplementation with 5 mg of genistein per kg basal feed; **T2**: supplementation with 20 mg of hesperidin per kg basal feed; **T3**: supplementation with 5 mg of genistein + 10 mg of hesperidin (1:1) composite per kg basal feed; **T4**: supplementation with 10 mg of genistein + hesperidin (1:4) composite per kg basal feed; **T5**: supplementation with 20 mg of genistein + hesperidin (1:4) composite per kg basal feed.

- **Means ± SE within a row with no common superscripts are significantly different at p < .05.**

*Injection, and compared with the thickness prior to injecting the chicks. The CBH response was quantified by subtracting the mean skin thickness of the first measurements (0 h) from those at 12 h after phytohaemagglutinin-P injection.*

### Neutrophil adhesion test

Neutrophil adhesion test was performed according to the method described by Wilkinson (1978). Briefly, heparinised blood samples were analysed for total and differential leukocyte counts by using an automatic analyser (haematology analyser ZC 980, Zhejiang, China). After initial counts, the blood samples were incubated with nylon fibres (80 mg/mL) for 15 min at 37°C. The incubated blood samples were further analysed for total and differential leukocyte counts to estimate the neutrophil index of blood samples. The percent neutrophil adhesion was calculated using the following formula:

\[
\text{Neutrophil adhesion (\%) = Nlt - Nlt/Nlu} \times 100
\]

where **Nlu** is the neutrophil index of untreated blood samples and **Nlt** is the neutrophil index of treated blood samples. The neutrophil index was calculated using the following equation:

\[
\text{Neutrophil index = number of neutrophils/totall leukocyte count} \times 100
\]

### Immunological parameters

Compared with the control, supplementation with each flavonoid or their combination (Table 2) increased the antibody titre against Newcastle disease vaccination (p = .025). The combination of flavonoids was more effective than the supplementation with genistein alone (p < .05), while it was only the 10 mg/kg and 20 mg/kg combinations that resulted in a greater value compared with hesperidin alone (p < .05). Likewise, compared with the control, supplementation with each flavonoid or in combination (Table 2) increased the antibody titre against Avian influenza vaccination (p = .031). The combination of flavonoids was more effective than the individual
supplementation with genistein ($p < .05$). All the levels of combined genistein and hesperidin treatments were more effective than those of individual dietary supple-
ments with genistein or hesperidin, as indicated by significant increases in serum antibody titres against Newcastle disease, as well as influenza vaccinations, a fact that indicated an additive activity of both phytochemicals.

The cutaneous basophil hypersensitivity (CBH) response of broiler chickens was assessed after the incorporation of genistein or hesperidin alone, or as a combination into the diet (Table 2). Dietary supple-
mentation with genistein or hesperidin significantly ($p < .05$) increased the CBH response values of the broiler chickens. Moreover, the CBH response values of broiler chickens supplemented with hesperidin was significantly ($p < .05$) higher than that supplemented with genistein. Additionally, the levels of T3, T4, and T5 groups were significantly higher that of the control ($p < .05$); T5 had greater value compared with T1 and T2 groups; T4 had greater value compared with T1, a fact that indicated an additive activity of both flavo-
noids. However, no significant differences were observed between the T4 and the T5 group.

The neutrophil adhesion rates of broiler chickens expressed in percentages were assessed after the dietary supple-
mentation with genistein or hesperidin alone, as well as a mixture (Table 2). Incorporation of either genistein or hesperidin significantly ($p < .05$) increased the rates compared with the control. Moreover, the neutrophil adhesion rate percentages of T3, T4, and T5 groups were significantly ($p < .05$) higher than that of T1 and T2 groups, a fact that indicated synergism of both phytochemicals. No signifi-
cant differences ($p > .05$) were observed between the T1 and the T2 group for neutrophil adhesion rates.

**Discussion**

Circulating leukocytes are involved in immunological functions with pronounced phagocytic role against antigens of infectious nature. Supplementation of gen-
istein, as well as hesperidin into broiler diet, increased leukocytes (WBC) contents in the present study. The increased concentration of circulating mononuclear lymphocytes could be explained by rapid influx of such leukocytes into the blood from bone marrow, mainly due to stress-induced glucocorticoids in order to maintain the immunocompetent state of host (Cirule et al. 2012). The dietary phytochemicals used in this study are well-known antioxidants with proved activity both in vitro and in vivo (Kamboh et al. 2016). The raised Hb levels after the dietary supplementation with 20 mg of genistein and hesperidin mixture could be correlated with fat digestion, because dietary anti-
oxidants stimulate the fat digestion by enhancing the provision of substrates for sz-oxidation and production of succinyl-CoA through Krebs cycle (Cunningham and Klein 2005), which were associated with increased haemoglobin production (Bunn and Forget 1986). Mean corpuscular haemoglobin (MCH), an average amount of Hb per red blood cell, is a useful tool to estimate the severity of anaemia (Aguire et al. 2017). Additionally, dietary treatments of both phytochemi-
cals, genistein and hesperidin, improved the MCH index of the broiler chicken to some extent, which indicated the potential of these compounds to improve the health status of the growing birds.

Natural antioxidants play an important role in main-
taining optimal health conditions in both animals and humans, through up-regulation of immune pathways that control and ameliorate harmful effects of exces-
ve production of ROS (Puertollano et al. 2011). In the present study, dietary supplementation with the phyto-
chemicals, genistein and hesperidin, improved the antibodies productions against Newcastle disease and Avian Influenza antigens. The improved humoral immunity against Newcastle disease and Avian Influenza in this study is consistent with that reported in antibody titres of broilers dietary supplemented with genistein (Rasouli and Jahanian 2015). Similarly, Hager-Theodorides et al. (2014) reported an enhanced

---

**Table 2. Effects of dietary supplementation of genistein and hesperidin on immunological parameters in growing broilers.**

| Parameter                        | T0         | T1         | T2         | T3         | T4         | T5         | p Value |
|----------------------------------|------------|------------|------------|------------|------------|------------|---------|
| Anti-NDV titre (log2)            | 4.00 ± 0.04a | 4.50 ± 0.05d | 5.33 ± 0.05c | 5.67 ± 0.07bc | 7.00 ± 0.08b | 7.33 ± 0.08a | .025    |
| Anti-AIV titre (log2)            | 3.33 ± 0.04a | 4.83 ± 0.05d | 5.00 ± 0.05c | 5.00 ± 0.07bc | 6.00 ± 0.08b | 6.50 ± 0.08a | .031    |
| CBH response (mm)                | 0.47 ± 0.01a | 0.57 ± 0.02d | 0.68 ± 0.01bc | 0.67 ± 0.01c | 0.80 ± 0.03bc | 0.88 ± 0.01a | .011    |
| Neutrophil adhesion (%)          | 216.83 ± 3.17e | 279.00 ± 6.44d | 288.66 ± 5.31c | 334.33 ± 6.21b | 360.50 ± 5.99b | 383.33 ± 6.31a | .019    |

Anti-NDV: Anti-Newcastle disease virus; Anti-AIV: Anti-avian influenza virus; CBH: cutaneous basophil hypersensitivity. Values are shown as mean ± SE.

a–d Means ± SE within a row with no common superscripts are significantly different at $p < .05$.

T0: no feed additive control; T1: supplementation with 5 mg of genistein per kg basal feed; T2: supplementation with 20 mg of hesperidin per kg basal feed; T3: supplementation with 5 mg of genistein + hesperidin (1:4) composite per kg basal feed; T4: supplementation with 10 mg of genis-
stein + hesperidin (1:4) composite per kg basal feed; T5: supplementation with 20 mg of genistein + hesperidin (1:4) composite per kg basal feed.
IgY antibody production in chickens after the dietary administration of phytochemical quercetin. The phytochemicals used in this study possibly improved B cell activity, which are prone to oxidative damage, and thus affected immunity (Catoni et al. 2008). The plant-based flavonoids exhibited immunostimulatory, antioxidant, anti-inflammatory, as well as antimicrobial properties (Havsteen 2002; Kamboh et al. 2015), which might explain the health effects of broiler chickens observed in this study.

Neutrophil adhesion test is an indicator of marginalisation of mononuclear phagocytic cells in the blood vessels, and also of immunostimulation. Neutrophils are important components of the innate immune response, and any defect in neutrophil adhesion can result in bacterial infections, which may be either local or systemic (Yang et al. 1999). Trans-epithelial migration (chemotaxis) and adhesion of neutrophils are important components of innate immunity and are considered the most crucial factors in maintaining the equilibrium of gut bacterial flora (Zemans et al. 2009). The cytokine transforming growth factor-β (TGF-β) is recognised as a regulatory factor for such neutrophil functions, as well as for monocytes and macrophages. The molecular mechanism that controls neutrophil numbers in tissues is characterised by local production of TGF-β and expression of adhesion molecules such as ICAM-1 (Silvestre-Roig et al. 2016). The marked increase in the parameters of cellular immunity (i.e. neutrophil adhesion rate and cutaneous basophil hypersensitivity response) as a result of the dietary supplementation with genistein and hesperidin indicated cellular immune-stimulation. Neutrophils and basophils perform a wide range of functions, such as phagocytosis, chemotaxis, as well as intra- and extracellular destruction of antigen fragments ( Müller et al. 2005). It is well established that cellular immune responses are highly sensitive to oxidative damage (Catoni et al. 2008), hence antioxidants like flavonoids such as genistein and hesperidin, are potential candidates for cellular immune-stimulation. Similar results were reported in laboratory animals by dietary inclusion of flavonoids-rich plant leaves (Ghule et al. 2006) and seeds (Shukla et al. 2009) and in broilers by dietary genistein (Rasouli and Jahanian 2015).

Conclusions
Both phytochemicals improved immunity and haematological indices, and could serve as potential alternative feed additives instead of synthetic antibiotics in broiler industry. Interestingly, supplementation with the mixture of phytochemicals showed more intense effects than their individual inclusion in cases of haemoglobin and mean corpuscular haemoglobin concentration as well as parameters of cellular and humoral immunity. The phenomenon regulating these changes needs to be further investigated by taking into consideration the synergistic effect of antioxidants.

Acknowledgements
Asghar A. Kamboh highly appreciates the monetary support of his institution (SAU, Tandojam) to conduct this academic activity at NAU, Nanjing, China. All lab fellows and Prof. Zhu highly acknowledged for their support and advice during this project.

Disclosure statement
The authors certify that there is no conflict of interest with any financial organisation regarding the material discussed in the manuscript.

Funding
This work was funded by Sindh Agriculture University, Tandojam, Pakistan.

ORCID
Asghar Ali Kamboh http://orcid.org/0000-0002-5176-6685
Ubedullah Kaka http://orcid.org/0000-0002-6469-3542
Elmutaz Atta Awad http://orcid.org/0000-0002-4312-501X

References
Acamovic T, Brooker JD. 2005. Biochemistry of plant secondary metabolites and their effects in animals. Proc Nutr Soc. 64:403–412.
Aguilhe PC, Kehinde AS, Ospina-Rojas IC, Murakami AE. 2017. Comparative effect of different detoxified rubber seed meal on haematological and serum biochemical indices of broilers. J Anim Health Prod. 5:50–57.
Ahmed AA, Musa HH, Sifaldin AZ, Musa TH, Fedail JF. 2015. Hepatocyte nuclear factor 4-α, glucocorticoid receptor and heat shock protein 70 mRNA expression during embryonic development in chickens. J Anim Health Prod. 3:54–58.
Al-Aqil A, Zulkifli I, Hair Bejo M, Szilii AQ, Rajion MA, Somchit MN. 2013. Changes in heat shock protein 70, blood parameters, and fear-related behavior in broiler chickens as affected by pleasant and unpleasant human contact. Poult Sci. 92:33–40.
Bunn HF, Forget BG. 1986. Hemoglobin – molecular, genetic, and clinical aspects. Philadelphia (PA): WB Saunders Co.
Catoni C, Peters A, Schaefer HM. 2008. Life history trade-offs are influenced by the diversity, availability and interactions of dietary antioxidants. Anim Behav. 76:1107–1119.
Cîrule D, Krama T, Vrublevska J, Rantala MJ, Krams I. 2012. A rapid effect of handling on counts of white blood cells in a wintering passerine bird: a more practical measure of stress? J Ornithol. 153:161–166.

Corrier DE, DeLoach JR. 1990. Evaluation of cell-mediated, cutaneous basophil hypersensitivity in young chickens by an interdigital skin test. Poult Sci. 69:403–408.

Cunningham JG, Klein BG. 2005. Veterinary physiology. 4th ed. Philadelphia (PA): Saunders Elsevier.

Ghule BV, Murugananthan G, Nakhat PD, Yeole PG. 2006. Immunostimulant effects of Capparis zeylanica Linn. leaves. J Ethnopharmacol. 108:311–315.

Hager-Theodorides AL, Goliomytis M, Delis S, Deligeorgis S. 2014. Effects of dietary supplementation with quercetin on broiler immunological characteristics. Anim Feed Sci Technol. 198:224–230.

Havsteen BH. 2002. The biochemistry and medical significance of the flavonoids. Pharmacol Ther. 96:67–202.

Iqbal MF, Luo Y-H, Hashim MM, Zhu W-Y. 2014. Evaluation of genistein mediated growth, metabolic and anti-inflammatory responses in broilers. Pak J Zool. 46:317–327.

Kamboh AA, Arain MA, Mughal MJ, Zaman A, Arain ZM, Soomro AH. 2015. Flavonoids: health promoting phytochemicals for animal production: a review. J Anim Health Prod. 3:6–13.

Kamboh AA, Hang SQ, Bakhetgul M, Zhu WY. 2013. Effects of genistein and hesperidin on biomarkers of heat stress in broilers under persistent summer stress. Poult Sci. 92: 2411–2418.

Kamboh AA, Hang SQ, Khan MA, Zhu WY. 2016. In vivo immunomodulatory effects of plant flavonoids in lipopolysaccharide-challenged broilers. Animal. 10:1619–1625.

Kidd MT. 2004. Nutritional modulation of immune function in broilers. Poult Sci. 83:650–657.

Müller S, Hoffmann P, vd Esche U, Mach J-P, Gorczyński RM, Waelli T, Alexander C, Zähringer U, Rietschel ET, Bessler WG. 2005. A fetal sheep liver extract containing immunostimulatory substances including LPS acts as leukocyte activator in cells of LPS responder and non-responder mice. Int Immunopharmacol. 5:1809–1819.

NRC. 1994. Nutrient requirements of poultry. 9th rev ed. Washington (DC): National Academy Press.

Puertollano MA, Puertollano E, de Cienfuegos GA, de Pablo MA. 2011. Dietary antioxidants: immunity and host defense. Curr Top Med Chem. 11:1752–1766.

Rasouli E, Jahanian R. 2015. Improved performance and immunological responses as the result of dietary genistein supplementation of broiler chicks. Animal. 9:1473–1480.

Robbins RJ. 2003. Phenolic acids in foods: an overview of analytical methodology. J Agric Food Chem. 51:2866–2887.

Shukla S, Mehta A, John J, Mehta P, Vyas SP, Shukla S. 2009. Immunomodulatory activities of the ethanolic extract of Caesalpinia bonducella seeds. J Ethnopharmacol. 125:252–256.

Silvestre-Roig C, Hidalgo A, Soehnlein O. 2016. Neutrophil heterogeneity: implications for homeostasis and pathogenesis. Blood. 127:2173–2181.

Wei J, Bhatt S, Chang LM, Sampson HA, Masilamani M. 2012. Isoflavones, genistein and daidzein, regulate mucosal immune response by suppressing dendritic cell function. PLoS One. 7:e47979.

Wilkinson PC. 1978. Neutrophil adhesion test. In: Handbook of experimental pharmacology. Berlin: Springer Verlag; p. 109.

Yang X, Letterio JJ, Lechleider RJ, Chen L, Hayman R, Gu H, Roberts AB, Deng C. 1999. Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta. EMBO J. 18:1280–1291.

Zemans RL, Colgan SP, Downey GP. 2009. Transepithelial migration of neutrophils: mechanisms and implications for acute lung injury. Am J Respir Cell Mol Biol. 40:519–535.