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Effects of dietary supplementation of different herbs on relative expression of Toll-like Receptors (TLRs) in poultry

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ABSTRACT

The paucity of novel antibiotics for treating human and animal infections has heightened global fears about growing drug-resistant superbugs. Antibiotic replacement approaches for food-producing animals, particularly poultry and livestock, are urgently needed by the agricultural business. The need of the hour is to develop and exploit promising antibiotic-alternative phytochemicals in order to minimize Antibiotic Growth Promoters and develop an antibiotic-free animal production system. Phytogenic plant dietary supplements have positive effects on poultry performance and immunity. Toll-like receptors (TLRs) play a key role in the innate immune system. TLRs recognize microbes that have broken through physical barriers such as the skin or the mucosa of the digestive tract, triggering immune cell responses. Supplementation of herbs in the dietary regimen of poultry had shown stimulatory effects on the immune system. Herbs such as Amla, Ashwagandha, Garlic powder, Giloy, Turmeric, Shatavari, Aloe vera, Moringa and holy basil have been shown to increase the relative mRNA expression of different toll-like receptors, thereby enhancing broiler immunological status by modulating their immune response.

Keywords: Immunity, Nutrition, Phytochemicals, Poultry, Toll-like receptor

The rising concern among people about the safety of the food they eat has led to the development of an 'organic' livestock production approach, which has accelerated the hunt for natural growth promoters that can be used instead of antibiotic growth promoters. Organic acids, fatty acids, prebiotics, probiotics and herbal preparations have all been investigated as non-antibiotic growth boosters. Herbal products have received much interest due to their ability to promote feed intake, improve endogenous enzyme secretion, activate the immune system, and have antibacterial, antiviral, and antioxidative properties (Toghyani *et al.* 2011). The use of diverse plant materials as food additives may improve the health and production of poultry (Safameher *et al.* 2012). 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 (Devegowda 1996). Changes in the intestinal microbiota, increased digestibility and nutrient absorption; enhanced nitrogen absorption, improved immune response, morphological and histological modifications of the gastrointestinal tract, and antioxidant activity are some of the possible action mechanism of herbs in the animal for growth promotion. Herbs can selectively influence the microorganisms by an anti-microbial activity or by a favourable stimulation of the eubiosis of the microflora (Singh 2018). Conventional herbal medicinal plants have been claimed to modulate the immune response, thereby augmenting non-specific immunity, essentially macrophages, granulocytes, natural killer cells and many complement functions (Hashemi and Davoodi 2012, Mirzaei-Aghsaghali 2012). Phytobiotics from herbs may be a viable alternative to conventional chemotherapy in a variety of situations by increasing the host's natural defence mechanisms by acting as immunomodulators. Several plant extracts, chemicals, and formulations, including as polysaccharides, lectins, flavonoids, peptides and tannins, have also been patented and are utilized in various *in vitro* models to test immune response (Cherng *et al.* 2008). These are generally regarded as safe for both animals and humans, ecologically sustainable, and can be used in poultry diets, thereby supporting organic livestock production (Cabuk *et al*. 2006).

Immunity and nutrition

In scientific community, the relationship between nutrition and the immune system has gotten a lot of attention. Phytochemicals have a long history of being used as immunological stimulants. Herbal extracts are already being exploited as feed supplements in intensive management systems to promote growth performance. (William and Losa 2001). The stimulation of appetite and feed intake, improvement of endogenous digestive enzyme secretion, activation of the immunological response, and

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antibacterial, antiviral, and antioxidant properties are all possible benefits of herbal extracts or active compounds in animal nutrition (Rahimi *et al.* 2011). The immune system is normally kept in a homeostatic equilibrium of immunostimulation and immunosuppression. Nutrition is a critical determinant of immune responses and malnutrition in the most common cause of immunodeficiency worldwide (Chandra 1997, Kirk 1997). Phytochemicals and their derivatives have a rich history of being used as immunostimulants. Many processes by which these plants function have been elucidated by modern herb research and new understanding of the immune system. (Hashemi and Davoodi 2012). Plant secondary metabolites are primarily responsible for herbal plants' positive effects on animal immune systems (Hashemi *et al.* 2008). TLRs (Toll like receptors) are a protein family that is crucial to the innate immune system's function. They are non-catalytic, single membrane-spanning receptors that detect structurally conserved microbe-derived compounds. TLRs sense bacteria that have broken through physical barriers such as the skin or the mucosa of the digestive tract, triggering immune cell responses. TLRs recognise a variety of pathogen-associated molecular patterns (PAMPs) (Underhill and Ozinskyn 2002). Research shows that some polysaccharides from medicinal plants can trigger the expression and activity of some TLRs and plants containing polysaccharides may assist in the initiation of system-wide enhanced immune surveillance. Polysaccharides are insoluble in alcohol and are absent in tinctures containing more than 35% alcohol. For these effects on TLR to occur, these herbs must be consumed as powders, infusions, or decoctions (Hashemi and Davoodi 2012).

Different researches which investigated different herbs like Amla (*Phyllanthus emblica*), Ashwagandha (*Withania somnifera*), Garlic powder (GP) (*Allium sativum*), Giloy (*Tinospora cordifolia*), Turmeric (*Cucurma longa*), Shatavari (*Asparagus racemosus*), Aloe vera (*Aloe barbadensis*), Moringa and Holy Basil Leaf Powder (HBLP) (*Ocimum sanctum*) incorporated diets, concluded that these herbs could increase the relative mRNA expression of toll-like receptors (TLR 2, TLR 4 and TLR 7) thus enhance immunological status of the broilers by modulating their immune response.

Effect of supplementation of different herbs on mRNA gene expression of TLRs in broilers

The effect of supplementation of different herbs on the differential expression level of TLRs, viz. TLR 2, TLR 4 and TLR 7 gene transcripts in the of VenCobb commercial broiler strains was studied by relative quantification method. The level of target mRNA was determined by comparative C_T method ($\Delta\Delta C_T$ method). TLR 2 recognizes a variety of microbial components. These include lipoproteins/ lipopeptides from various pathogens, peptidoglycan and lipoteichoic acid from Gram-positive bacteria (Takeda and Akira 2005). TLR 4 is the principal receptor for lipo-polysaccharide; a major component of outer membrane of gram-negative bacteria (Kannaki *et al*. 2010). TLR 7 family is implicated in intracellular recognition of nucleic acids. The TLR 7 recognizes some antiviral compounds and single-stranded viral RNA.

Amla

Phyllanthus emblica (syn. *Emblica officinalis*), the Indian gooseberry, or amla, is a deciduous tree of the Phyllanthaceae family and is known for its edible fruit. Amla is one of the richest sources of Vitamin C; about 700 mg (Saini *et al.* 2008). Several active tannoid principles have also been identified for their health advantages (Emblicanin A, Emblicanin B, Punigluconin, and pedunculagin) [Kim *et al.* 2005]. The fruit also contains other polyphenols like flavonoids, kaempferol, ellagic acid and gallic acid (Rehman *et al.* 2007). *Emblica officinalis* has long been utilised as a growth stimulant, immunomodulator, and antioxidant in poultry health management (Priya *et al.* 2010 and Elizabeth *et al.* 2011). Amla has been known in ayurvedic medicine for its tonifying, anti-ageing and immune enhancing properties (Lalla 2001). Animals fed on amla powder showed better ability for uptake and killing of bacteria, which might be due to the presence of tannins which stimulates phagocytic cells (Ram *et al.* 2003). The nutrigenomic expression analysis in the study conducted by Dalal (2017) of amla fruit powder supplementation revealed that relative mRNA expression of TLR 2 of broilers was found to be significantly enhanced in the treatment groups supplemented with 0.75% and 1% of the amla fruit powder and a significant downregulation 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. However, the data pertaining to the relative mRNA levels of TLR 7 in the plasma of birds revealed no significant differences in the experimental groups as compared to the control group. It was observed that dietary inclusion of amla fruit powder significantly modulates 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. The fruit of amla due to presence of saponins, phenols and tannins have potent antimicrobial activity against both Gram positive and Gram negative bacteria so this may be a cause for the relative expression of TLRs on amla supplementation in broilers.

Ashwagandha

Withania somnifera or Ashwagandha is a plant of Solanaceae family, and holds a celebrated position in the Indian *material medica*. The root has a strong pungent smell and bitter taste and contains several alkaloids (0.13 to 4.30%), which offer medicinal usages. Ashwagandha plant constitutes alkaloids and steroidal lactones, but the withanine, the main alkaloid found in its roots and leaves is thought to be responsible for its biological activity. Other constituents include saponins containing an additional acyl group (sitoindoside VII and VIII). The

herb Ashwagandha reportedly has antioxidative, antistress, anticoccidial, immunomodulatory, cardioprotective and antilipidemic effect besides playing vital role in lowering blood sugar, serum cholesterol and stress induced gastric indigestion and ulcers (Abou-Douh 2002). It has been reported that Ashwagandha significantly increases the white blood cell and erythrocyte counts (Manish *et al.* 2004 and Senthilnathan *et al.* 2006). The results of the study conducted by Jyotsana *et al* 2019; revealed that the experimental treatments containing Ashwagandha root powder in the broiler's diet at different levels, viz. 0.25%, 0.50%, 0.75% and 1.0 % had potent immune modulating activity by showing stimulatory effect on relative mRNA expression of TLR 2 and downregulation pattern of TLR 4. While, the data pertaining to the relative mRNA levels of TLR 7 in the plasma of birds revealed non-significant differences in the experimental groups as compared to the control group (without any antibiotic or ashwagandha powder supplementation). The use of Ashwagandha has mostly been linked to its immune system modulating effects (Gautam *et al.* 2004). Preparations made from this plant have proven to boost circulating antibody titer, lysosomal enzyme activity, and phagocytosis, all of which help to modulate the immunological response (Agarwal *et al.* 1999).

Giloy

Tinospora cordifolia is an herbaceous shrub which belongs to family Menispermaceae commonly known, as *Amrita* or *Guduchi* or heart moon leaved, is an important drug and is well known for its medicinal properties in Indian medicinal system. Its constituents belong to different classes such as alkaloids, diterpenoid lactones, glycosides, sesquiterpenoid, aliphatic compounds, phenolics, polysaccharides (1,4 α–D Glucan), steroids like tinosporine, tinosporide, tinosporaside, cordifolide, cordifol, heptacosanol, diterpenoid furanolactonetinosporidine, columbin and β-sitosterol (Zhao *et al.* 1991). Diets supplemented with different levels of giloy powder (0.50%, 0.75% and 1.0%) revealed that relative mRNA expression of TLR 2 of broilers was found to be significantly ($P<0.05$) enhanced. While, at the end of the experimental period broilers had significant downregulation pattern of relative mRNA expression of TLR 4 in the plasma of broilers fed diet supplemented with 0.25%, 0.50%, 0.75% and 1.0% of giloy powder in the treatment groups as compared to control. The relative mRNA levels of TLR 7 in the plasma of birds revealed non-significant differences in the experimental groups (Singh 2018). The aqueous extract of guduchi stem has shown the presence of arabinogalactan that showed immunomodulator activity (Chintalwar *et al.* 1999). The immunomodulatory, antimicrobial, antioxidant, antineoplastic, hypoglycemic, antipyretic, hepatoprotective, diuretic, anti-stress, antihyperglycemic, antidiabetic and anti-tuberculotic properties of this plant have also been reported (Sinha *et al.* 2004).

Turmeric

Cucurma longa is a perennial herb of Zingiberaceae family that is widely used and cultivated in the tropical and sub-tropical regions of the world. Its stems and rhizomes are used as food additives to improve appearance, flavour, palatability, storage condition and preservation of food (Jayaprakasha *et al.* 2005). Turmeric rhizome contains the highest active compounds (phytochemicals) than other parts in turmeric plant. Extraction process of the rhizome results in a dry yellow polyphenol-rich powder, that is oil soluble in nature (Khan *et al.* 2012). The active ingredients of turmeric rhizomes consist of volatiles and non-volatiles constituents. The major active nonvolatile substances are the coloring agent and are a rich source of phenolic compounds like cucuminoids. The major active volatile substances are curcuminoids (Toennesen 1992), ar-turmerone (Ferreira *et al.* 1992), zingiberene (Smith and Robinson 1981) and curlone (Kiso *et al.* 1983). Ahlawat (2017) observed that dietary inclusion of turmeric powder showed a potent immune modulating activity by significantly up regulatory effect on relative mRNA expression of TLR 2 and non-significant downregulation pattern of TLR 4. However, relative mRNA expression levels of TLR 7 revealed no significant differences. Active compounds found in turmeric have a wide spectrum of biological actions including antioxidative, anticarcinogenic, antihepatotoxic, anti-inflammatory and hypocholesterolemic activities (Nishiyama *et al.* 2005). Curcumin and curcuminoids possess anti-inflammatory (Ammon *et al.* 1993), antioxidative (Osawa *et al.* 1995), anticoccidials (Allen and Fetterer 2002; Abbas *et al.* 2010) and immunomodulatory (Kumari *et al.* 2007, Yarru *et al.* 2009) properties. It inhibits the lipid peroxidation, scavenges the superoxide anions and free hydroxyl radicals present in the body (Ruby *et al.* 1995) and enhances the activities of detoxifying enzymes such as glutathione-S-transferase (Piper *et al.* 1998).

Shatavari

Asparagus racemosus of order *Asparagales* belonging to family Asparagaceae and is a herbal plant. The characteristic effects of shatavari may be attributed to its concentrations of saponins (active principle), known as shatavarins which have properties like nutritive tonic, anti-stress and immunostimulant (Kamat *et al.* 2000). The root powder of *Asparagus racemosus* is used as a herbal feed additive/supplement in poultry feed. Shatavari augments the appetite and stimulates the liver. A wide range of pharmacological activities and antimicrobial activity due to essential oils has been reported in shatavari plant extract (Cowan 1999).

Garlic

Allium sativum, a member of Liliaceace family has been a subject of considerable interest as a therapeutic agent worldwide since ancient times. Garlic is known to be having

many sulphur compounds (alliin, allicin, ajoene and others), several enzymes (allinase, peroxidases, myrosinase and others), amino acids (arginine and others) and trace mineral such as selenium. Due to presence of compounds like allin, allicin, ajoene, S-allyl cysteine, diallyl sulphide and diallyl trisulfide, Garlic shows antibacterial, antiparasitic, antifungal, hypocholesterolemic and antioxidant activities (Amagase *et al.* 2001).

Aloevera

Aloe barbadensis derives its medicinal properties from more than 75 biologically active compounds present in its leaf. Aloe vera leaf consists of two sections, i.e. latex and gel. Biologically active compounds are present mainly in the dry matter of the gel (Boudreau and Beland 2006). Aloe vera also has some specific properties like antibacterial, antiviral property due presence of an alkaloid (Aloe-emodin) and antifungal activity. Aloe vera has been used in arthritis and joint related problems (Darabighane *et al.* 2011, Mmereole 2011, Eevuri and Putturu 2013).

Moringa

Moringa oleifera reduces cholesterol levels and triglycerides in body. It controls blood sugar and helps normal sugar and energy balance; offers vitamins and minerals vital for maintaining normal physiology. It offers powerful anti-aging and anti-inflammatory natural substances, many with anti-cancer properties (Marcu *et al.* 2005). Moringa leaf meal provides antioxidant compounds such as ascorbic acid, flavonoids, phenolics and carotenoids (Teixeira *et al.* 2014). Moringa leaves are known to be having prebiotic effect. They also have potentially antioxidant phytochemicals, such as chlorogenic acid and caffeic acid (Siddhuraju and Becker 2003).

The experimental treatments containing herbal feed additives (Shatavari, Garlic, Aloe vera and Moringa) in the broiler's diet at both 15% and 30% dried distillers grains with soluble (DDGS) inclusion level had 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 (Kumar 2019). This may be due to the combined effects of the herbs like: Shatavari plays a role of antiseptic, anticancer, astringent and immunomodulation (Seena *et al.* 1993); Garlic supplementation inhibits the growth of entero-pathogenic bacteria, increases villus height and thus improves nutrient utilization (Adibmoradi *et al.* 2006); Garlic powder is a natural growth promoter and it can be a potential alternative of antibiotic growth promoters in broiler chickens (Demir *et al.* 2003); Aloe vera is also reported to be having anti-inflammatory and immunity activity due to salicylic acid, which is analgesic and anti-inflammatory.

Tulsi

Ocimum tenuiflorum, Holy Basil (also Tulsi, tulasī), is an aromatic plant in the family Lamiaceae, which is native throughout the Old World tropics and widespread as a cultivated plant. Prakash and Gupta (2005) reported that tulsi contains eugenol (1-hydroxy 2-methoxy 4 allyl benzene) a phenolic compound and ursolic acid having pharmacological effects. Some of the main chemical constituents of *Tulsi* are: oleanolic acid, ursolic acid, rosmarinic acid, eugenol, carvacrol, linalool, β-caryophyllene, β-elemene (11.0%), β-caryophyllene (about 8%), and germacrene D (about 2%) (Padalia *et al.* 2011). β- Elemene has been studied for its potential anticancer properties (Li *et al.* 2009) but human clinical trials have yet to confirm its effectiveness. According to Sheoran *et al.* (2017) significant ($P<0.05$) increase was observed in the relative mRNA expression of TLR 2 of broilers in the treatment groups supplemented with 0.5% and 1% of the Holy Basil Leaf Powder (HBLP) fed individually or in combination with similar levels of garlic powder (GP). Broilers fed diet supplemented with 0.5% and 1% of the HBLP showed a significantly higher relative mRNA expression of TLR 4 in the plasma. While no significant effect was observed in the relative mRNA expression of TLR 4 in the birds supplemented with 0.5% and 1% of the garlic powder as compared to the control. Furthermore, the relative TLR 4 mRNA expression was found to be significantly higher in the treatment groups where the birds were fed a combination of GP and HBLP at 0.5% and 1% in their diet, respectively. However, the relative mRNA levels of TLR 7 in the plasma of birds revealed slightly different pattern of expression and it was found that significantly highest levels of expression was observed in treatment group supplemented at 1% of the garlic powder, followed by the treatment groups in which the broilers were fed a combination of GP and HBLP at 0.5% and 1% of their ration, respectively. In conclusion, experimental treatments containing GP and HBLP either alone or in their combination in the broiler's diet have potent immune modulating activity by showing stimulatory effect on relative mRNA expression of TLR 2, TLR 4 and TLR 7 of the commercial broilers.

Discussion

Medicinal herbs have shown to possess multiple immunomodulatory 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 same study, a significant change was observed in the relative mRNA expression of TLR cell markers on dietary inclusion of different herbs either alone or in combination, which confirmed that these herbal feed additives could alter the T cell immune system in the plasma of broiler birds. There was a significant increase in the relative mRNA expression of TLR 2 in the plasma of the broilers fed diet supplemented with graded levels of different herbs either alone or in combination with other herbs. Phytogenic additives has antibacterial, antioxidant, antistress, gut microflora manipulation, immune enhancement properties and digestive enzymes stimulation could be the probable reasons for the positive effects exerted by them on the

growth and health performance of animals (Durrani *et al.* 2007). Jamroz *et al*. (2005) reported that plant extract supplement also significantly increased the *Lactobacillus* numbers following an application of natural plant extract. Similarly Siddiqui *et al.* (2015) studied the effect of different dietary levels of *Nigella sativa* seed powder on *Escherichia coli* and total viable bacterial count in excreta of broilers. Both *E. coli* and total bacterial counts were significantly decreased by *Nigella sativa* seed powder supplemented diets irrespective of inclusion levels. Castillo *et al.* (2006) reported that the different herbs (garlic, nutmeg, cinnamaldehyde, capsicum and carvacrol) are able to enhance the growth of *Lactobacilli;* this indicates that herbs have the ability to increase the beneficial bacteria. 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 and Gupta 2005). The antimicrobial actions of essential oils in herbs are due to hydrophobicity of the 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). Feeding diets containing phytobiotics may result in inhibition of the growth and colonization of entero-pathogenic microbes in the digestive tract, thus contributing to the balance of gut microflora (Harris *et al.* 2001) and promoting the growth performance and health of birds (Adibmoradi *et al.* 2006). The study conducted by Bhardwaj *et al.* (2012) revealed that *T. Cordifolia* treated group showed marked overall promising and significant improvement in the concentration of both mean hemagglutination antibody titre and cell-mediated immune status indicating that stem extract of *Tinospora cordifolia* strengthened humoral and cell-mediated immune response in broiler chicks against Newcastle disease. Savage *et al.* (1996) reported supplementation of oligosaccharides may have a prebiotic effect through an increase in production of lactic acid, thus increasing the proliferation of beneficial bacteria and reducing the presence of Gram-negative bacteria. Turmeric powder can mediate GABAergic pathways and by this it can modulate both humoral and cell-mediated immunity. Herbs can influence selectively the microorganism by an antimicrobial activity thus favours better nutrient utilization and absorption or the stimulation of the immune system (Ahlawat 2017). Phenolic compounds show their 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). 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). Attia *et al.* (2017) reported a positive influence on cecal microflora count (decreased *coliforms* and increased lactobacilli count) by inclusion of the plant extract blend. This lowered caecal *coliform* count could be attributed to the antibacterial properties of the utilized plant extract components in the plant extract blend or to increased nutrient digestibility and subsequently less undigested nutrients available for bacterial fermentation in the ceacum. Adibmoradi *et al.* (2006) reported that garlic supplementation inhibits the growth of entero-pathogenic bacteria. Kumar (2019) has stated that aloe vera has some specific properties like antibacterial, antiviral property due presence of an alkaloid (Aloe-emodin). Goswami *et al.* (2016) reported that moringa has potent antiviral activity. Caceres *et al*. (1991) observed that the fresh moringa leaf juice inhibit the growth of microorganisms (*Pseudomonas aeruginosa* and *Staphylococcus aureus*) pathogenic to man. As TLR 2 is related with gram positive bacteria and TLR 4 with gram negative bacteria; on supplementation of different herbal feed additives there was a significant up-regulation and down-regulation in the relative mRNA expressions of TLR 2 and TLR 4 of broilers, respectively. This might be due to enhanced growth of beneficial gram-positive bacteria and decreased gram-negative bacteria in birds.

From the above reported studies, it can be inferred that, supplementation of diet with herbs, either alone or in combination; 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, give better performance and 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.

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