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Effect of Dietary *Pongamia pinnata* on Immunity and Gut Microflora in Broiler Chickens

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AAK conceived the research project. AAC performed the experiments. NAK and RAL helped in data collection, analysis and formal write-up of manuscript, while MAC proof read the manuscript.

Key words
Broiler, Immunity, Microflora, Antibody titre, Medicinal plant

ABSTRACT

Pongamia pinnata is an indigenous medicinal plant of Asia, which has long traditional use, having anti-inflammatory, anti-pyretic, anti-nociceptive, anti-oxidant, anti-ulcer, antihyperglycaemic and anti-microbial activities. The current investigation aimed to explore the immunomodulatory and gut microflora modulatory effects of dietary *P. pinnata* in broilers. Day old broiler chicks (n=125) were purchased from local hatchery and equally divided into the five groups, i.e., C (control) that offered basal diet, while, T1, T2, T3 and T4 groups were supplemented 0.5, 1, 1.5 and 2% *P. pinnata* leaf powder (PPLP) in basal diet respectively. The experimental groups were observed for the performance parameters, antibody titer against Newcastle disease virus (NDV), relative weight of lymphoid organs (bursa, spleen and thymus) and enumeration of gut microflora. Results showed that PPLP improved ($P < 0.05$) production performance, final body weight, carcass weight and relative weight of lymphoid organs. Anti-NDV titer was improved on both 21 ($P > 0.05$) and 35 ($P < 0.05$) day in PPLP supplemented groups as compared to control. Total bacterial count and *Lactobacillus* count was increased ($P < 0.05$), while coliform count was decreased ($P < 0.05$) on day 21 in PPLP supplemented groups comparing with the control group. In summary PPLP had positive impact on performance, parameters of humoral and cellular immunity and gut microflora in a dose-dependent fashion thus suggested its' inclusion in broilers diet up to 2% level.

INTRODUCTION

It is reported that herbs and herbal products have been shown to have a beneficial effect on broiler growth performance (Lagua and Ampode, 2021). It is believed that herbs can be used as alternative to antibiotic growth promoting feed additive. Antibiotic resistance in poultry microbial strains is caused by long-term use of antibacterial products as growth promoters at subtherapeutic doses, therefore is considered threatening for humans and animals and in consequences there have been reported increased disease outbreaks, thus, many developed countries have banned the use of these products as growth promoters (Jang *et al*., 2004). Herbs have long been used to cure humans and animals, and a large percentage of the population still relies on use of natural remedies (Sudira *et al*., 2021). These natural therapeutic options have many important activities both under *in-vitro* and *in-vivo* conditions like antibacterial, antifungal, antioxidant, immune modulators, production enhancers, enzyme secretions stimuli, antispasmodic and gut environment modifiers (Refaie *et al*., 2022; Bhatt, 2015).

Pongamia pinnata is an important species of the *Pongamia* genus in the Fabaceae family. From a botanical perspective, it must be noted that the genus' nomenclature is particularly complicated because many species are synonyms in other genera (Bala *et al*., 2011). *Millettia pinnata* (L.), *Pongamia glabra* Vent., *Derris indica* (Lam.) (Scott *et al*., 2008) are some of the other names of this plant.

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P. pinnata is known by several names in many countries, including *karanja* (Bengali, Hindi, Sanskrit), *kacang kayu laut* (Malay), *ki pahang laut* (Indonesian), and *malva nut/ pongam* oil tree (English) *Sukh chain* (Pakistan). *P. pinnata* is a versatile legume tree native to the Indian subcontinent and Southeast Asia (Belide *et al*., 2010).

P. pinnata, is an Asian indigenous medicinal plant that has a long history of usage in Ayurvedic, Siddha, and Unani medical systems for treating a variety of human illnesses and disorders. Through Ayurvedic formulation various parts of this plant are used to treat bronchitis, whooping cough, rheumatic joints and to quench poly-dipsia diabetes. It used topically to treat leucoderma, leprosy, rheumatism, and foul-smelling gonorrheal sores, as well as scrofulous enlargement. It has also been discovered to have anti-inflammatory, anti-pyretic, anti-nociceptive, anti-oxidant, anti-ulcer (Jose *et al*., 2019), antihyperglycaemic and anti-microbial activities (Bajpai *et al*., 2009; Srinivasan *et al*., 2001). A bitter alkaloid is reported to be present in the plant leaves. Some of the ancient folklore claims that *P. pinnata* leaves juice is beneficial and helpful to treat several illness like diarrhea, cough, dyspepsia, flatulence, leprosy and gonorrhea (Nadkarni, 1996). It has been claimed that the leaves of *P. pinnata* are also effective as an antiparasitic and insect repellent (Heroor *et al*., 2012). In a recent study, *P. pinnata* shown to have antimicrobial, antioxidant and wound healing property (Dwivedi *et al*., 2017). Whereas, another most recent report revealed that *P. pinnata* has immunomodulatory potential by modulating the Th2 response in human peripheral blood mononuclear cells (Mathayan *et al*., 2020).

Keeping in view the health enhancing potential of *P. pinnata* current study was designed to investigate effects of dietary *P. pinnata* on immunity and gut microflora of broilers.

MATERIALS AND METHODS

Experimental plan and housing

All experimental procedures were approved by the Board of Studies, the Directorate of Advanced Studies, Sindh Agriculture University Tandojam (No. DAS/1425/ of 2021) and were carried out according to prescribed ethical standard. One hundred twenty-five (125), day old Ross 308 broiler chicks were purchased from a commercial hatchery and were distributed into five groups, each group had 25 chicks. Group A was served as control group and offered only basal diet, while groups B, C, D and E were supplemented with 0.5, 1, 1.5 and 2% of *P. pinnata* leaf powder (PPLP) with basal diet respectively. The supplemental doses of PPLP were chosen based on the results of a preliminary study conducted in our laboratory (unpublished data). The fresh water was provided *ad libitum* and feeding program was consisting of two phases, starter diet (0-21 days) and finisher diet (22-42 days). The basal diet was prepared according to National Research Council (NRC, 1994) and its ingredients and chemical composition is presented in Table I.

Table I. Ingredients and formulation of basal diet (NRC, 1994).

	Starter feed (%)	Finisher feed (%)
Ingredients		
Corn	56.80	64.76
Soybean meal (CP 44%)	35.40	27.85
Fish meal	1.00	1.00
Soybean oil	2.31	1.39
Oyster shell	1.34	1.84
Dicalcium phosphate	1.53	1.66
Common salt	0.396	0.326
Vitamin premix[1]	0.5	0.5
Mineral premix[2]	0.5	0.5
DL-Methionine	0.151	0.055
L-Lysine HCL	0.073	0.119
Total	100	100
Nutritive values		
Energy (Kcal/Kg)	2950	2960
Crude protein (%)	21.203	18.499
Arginine (%)	1.365	1.157
Lysine (%)	1.208	1.052
Methionine (%)	0.490	0.360
Met+Cys (%)	0.832	0.666
Ca (%)	0.997	1.200
Available P (%)	0.453	0.467
Na (%)	0.180	0.150

[1]Supplied per Kg of diet: vitamin A, 18,000 IU; vitamin D3, 4,000 IU; coline chloride, 500 mg; niacin, 59.4 mg; vitamin E, 36 mg; calcium pantothenate, 19.6 mg; riboflavin, 13.2 mg; vitamin K3, 4 mg; thiamin, 3.5 mg; pyridoxine, 5.88 mg; folic acid, 2 mg; vitamin B12, 0.03 mg; biotin, 0.2 mg; antioxidant, 2 mg. [2]Supplied per Kg of diet: Mn, 198.4 mg; Zn, 169.4 mg; Fe, 100 mg; Cu, 20 mg; I, 1.98 mg; Se, 0.4 mg.

The poultry house was entirely cleaned and sanitized. The birds were kept on floor housing system. Temperature was maintained around 95°F at first week and then gradually reduced by 5°F per week until it reached around 70°F. Relative humidity was maintained around 55 to 65 percent in the shed. On arrival, the chicks were provided 10% sugar solution and ground maize for the first 12 h. The water-soluble vitamins and electrolytes were added to

the drinking water for the first 2 days. The rice husk was used as litter at 2-4 inches depth for each group of broilers. Litter turning was practiced 2 times a day to minimize the gas production in the shed and assured maintenance of proper ventilation. The vaccination program was adopted according to the recommendation of Pakistan Poultry Association for time to time immunization during 6 weeks' experiment. Feed intake, weight gain was recorded on daily basis that was used to calculate feed conversion ratio (FCR) = Total feed intake (weight) / Total weight gain.

Preparation of leaf powder

P. pinnata green leaves were collected near faculty of Animal Husbandry and Veterinary Sciences, Sindh agriculture university Tandojam. The plant leaves were authenticated by a botanist/agriculturist from the Faculty of Crop Production, Sindh Agriculture University, Tandojam. The leaves were air dried in shady place to avoid the bleaching and vitamin C losses. After drying these leaves were ground and stored in airtight polythene bags in dark cool place to use for chemical analysis according to standard procedures (AOAC, 2005) till used in animal trials.

Antibody titer determination

On day 21 and 35, blood samples were collected from the wing vein with the help of 3 ml sterile syringe from five birds of each group. For the collection of chicken sera, blood samples were allowed to coagulate for 30 minutes at room temperature that were kept at -20ºC in the deep freezer until analyzed for anti-NDV antibody titers using hemagglutination inhibition test. Antibody titer was determined by hemagglutination inhibition (HI) technique using the standard method of Majiyagbe and Hitchner (1977).

Relative weight of lymphoid organs

At 21 and 42 of age, five birds from each group were sacrificed by halal Islamic method and carcass weight and weight of lymphoid organs (spleen, thymus, and bursa) was calculated using digital balance. Relative weight of lymphoid organs were determined according to the following formula:

Relative weight of immune organ = (immune organ weight (g)/BW (g) x 100

Enumeration of caecal microflora

Cecal contents were collected aseptically from the intestine of slaughtered chicks at 21 day into sterile Eppendorf tubes to enumerate the cecal microflora from each replicate. These were mixed well and stored at 4ºC in refrigerator for further analysis. For total bacterial counting and specific microorganisms e.g. Coliform bacteria, and Lactobacilli, the procedure of Memon *et al*. (2019) were adapted. Briefly, a total of five dilution tubes holding 9 mL of sterile normal saline solution were used. One mL of the sample was used to prepare a 10-fold dilution. A 0.1 ml of every tube were then cultured on nutrient agar (Oxoid, UK) plates and stored for 24 h at 37°C. After incubation, colonies were counted using a colony counter and CFU/g results were calculated using following formula:

CFU/g = (colony no. x dilution factor)/ volume plated

All bacterial isolates were recognized based on the standardized cultural, staining and biochemical properties following the Bergey's manual of systematic bacteriology (Whitman *et al*., 2012).

Statistical analysis

Results of present work were computed first using the Excel (Microsoft Inc., USA) Spread Sheets. Then the data was analyzed by one-way ANOVA using the JMP statistical software package (version 5.0.1a SAS Institute, 2000). The differences between different dietary levels were analyzed by Tukey's range test and significance level was set at 5% probability level.

RESULTS

Effect of PPLP treatment on performance

Feed intake was the highest in both day 21 and 42 in control group followed by T1, T2, T3 and T4 groups respectively (Table II). Statistical analysis revealed that both day 21 and 42, PPLP treatments reduced ($P < 0.05$) the feed intake in T2, T3 and T4 groups. Whereas, PPLP treatments improved the body weight at day 21 and 42 in broilers (Table II). At 21 days, all PPLP supplemented groups exhibited a significant ($P < 0.05$) raise in body weight as compared to control group. While, at 42 days, PPLP treatment showed significant ($P < 0.05$) improvement in body weight in T2, T3 and T4 groups as compared to C and T1 group. The best feed conversion ratio (FCR) was recorded in T4, followed by T3, T2, T1 and C groups, respectively. Statistical analysis shown that at 21 day PPLP treated groups (T1, T2, T3 and T4) showed significantly ($P < 0.05$) better FCR as compared to C group; whereas T2, T3 and T4 groups exhibited significantly better ($P < 0.05$) FCR at day 42 as compared to T1 and C groups (Table II).

Effect of PPLP on relative weight of lymphoid organs

As shown in Table III, live body weight and carcass weight was improved ($P < 0.05$) by the supplementation of PPLP in broilers. A dose-dependent effect with highest raise in T4 was recorded for both live weight and carcass weight at 21 and 42 days. The result of one-way ANOVA indicated that PPLP treatments improved ($P < 0.05$) the

Table II. Effect of PPLP treatment on performance in broiler.

Parameters/Treatment for days	Treatment groups					P-value	SEM
	C	T1	T2	T3	T4		
21 days							
Feed consumption (g)	2100^{a}	1989^{b}	1822^{b}	1766^{bc}	1688^{c}	0.0004	41.663
Body weight (g)	891.33^{b}	1019.33^{a}	1063.00^{a}	1066.67^{a}	1083.67^{a}	0.0034	27.400
FCR	2.36^{a}	1.95^{b}	1.71^{b}	1.66^{b}	1.56^{b}	0.0034	27.416
42 days							
Feed consumption (g)	3150^{a}	3077^{ab}	2998^{bc}	2911^{cd}	2852^{d}	0.0009	35.321
Body weight (g)	1623.00^{b}	1630.70^{b}	1671.00^{a}	1675.00^{a}	1706.70^{a}	0.0032	11.949
FCR	1.94^{a}	1.89^{a}	1.79^{b}	1.74^{b}	1.67^{b}	0.0020	11.990

Means showing dissimilar superscripts (a, b, c, d) in a row are significantly different at $p < 0.05$. C, Control, supplemented with 0% PPLP; T1, treated with 0.5% PPLP; T2, treated with 1% PPLP; T3, treated with 1.5% PPLP; T4, treated with 2% PPLP; PPLP, *Pongamia pinnata* leaf powder.

Table III. Effect of PPLP on average body weight, carcass weight and relative weight of lymphoid organs in broiler chickens at day 21 and 42 of age.

Parameters	Days	Treatment groups					P-value	SEM
		C	T1	T2	T3	T4		
Live body weight(g)	21 d	891.33^{b}	1019.33^{a}	1063.00^{a}	1066.67^{a}	1083.67^{a}	0.0034	27.400
	42 d	1623.00^{b}	1630.70^{b}	1671.00^{a}	1675.00^{a}	1706.70^{a}	0.0032	11.949
Carcass weight (g)	21 d	513.00^{c}	592.67^{bc}	601.00^{bc}	681.00^{ab}	716.00^{a}	0.0235	37.369
	42 d	1191^{c}	1199.00^{c}	1237.00^{b}	1248.00^{ab}	1287.00^{a}	0.0008	11.301
Bursa(g)	21 d	0.33^{d}	0.32^{c}	0.32^{c}	0.33^{b}	0.34^{a}	0.0000	0.052
	42 d	0.25^{c}	0.25^{c}	0.26^{b}	0.27^{a}	0.27^{a}	0.0002	0.060
Spleen(g)	21 d	0.09^{b}	0.09^{b}	0.08^{b}	0.08^{b}	0.10^{a}	0.0070	0.062
	42 d	0.05	0.06	0.06	0.06	0.06	0.4118	0.066
Thymus(g)	21 d	0.33^{d}	0.35^{c}	0.36^{bc}	0.39^{b}	0.42^{a}	0.0000	0.112
	42 d	0.25^{c}	0.26^{c}	0.28^{bc}	0.32^{ab}	0.35^{a}	0.0030	0.275

Means showing dissimilar superscripts (a, b, c, d) in a row are significantly different at $p < 0.05$. C, Control; supplemented with 0% PPLP; T1, treated with 0.5% PPLP; T2, treated with 1% PPLP; T3, treated with 1.5% PPLP; T4, treated with 2% PPLP; PPLP, *Pongamia pinnata* leaf powder.

relative weight of immune organs at 21 and 42 days (except spleen weight on 42 d). Bursa weight was significantly ($P < 0.05$) improved in PPLP supplemented groups as compared to control group at 21 days, while at 42 days it was raised ($P < 0.05$) in T2, T3 and T4 groups as compared to other groups. Relative weight of spleen was improved ($P < 0.05$) in T4 group as compared to control and other PPLP treated groups. Furthermore, on 21 days, the relative thymus weights was improved ($P < 0.05$) in all PPLP supplemented groups as compared to the control group; however this effect was limited to T3 and T4 groups on 42 days.

Effect of PPLP on anti-Newcastle disease virus (NDV) titre

As shown in Table IV, the T4 group exhibited the highest rise in anti-NDV antibody titer on day 21, followed by T3, T2, T1 and C groups, however, this difference was statistically non-significant ($P>0.05$). On the other hand, on day 35, PPLP treatment significantly improved the anti-NDV antibody titer in T4 group as compared to C and T1 group.

Table IV. Effect of PPLP on serum anti-Newcastle disease virus (NDV) titre in broilers.

NDV titer (log^2)	Treatment groups					P value	SEM
	C	T1	T2	T3	T4		
21 d	2.67	3.00	3.33	3.67	4.00	0.3059	0.4472
35 d	4.67^{b}	5.00^{b}	5.33^{ab}	5.67^{ab}	6.33^{a}	0.0469	0.3944

Means showing dissimilar superscripts (a, b, c, d) in a row are significantly different at $p < 0.05$. C, Control; supplemented with 0% PPLP; T1, treated with 0.5% PPLP; T2, treated with 1% PPLP; T3, treated with 1.5% PPLP; T4, treated with 2% PPLP; PPLP, *Pongamia pinnata* leaf powder.

Table V. Effect of PPLP on total bacterial counts, coliform count and *Lactobacillus* count.

Bacterial count	Treatment groups					p-value	SEM
	C	T1	T2	T3	T4		
Total bacterial count (CFU/g)	1.58E+05^{d}	1.89E+05^{c}	2.17E+05^{b}	2.47E+05^{a}	2.55E+05^{a}	0.00	6.597
Coliform count (CFU/g)	1.89E+04^{a}	1.74E+04^{a}	1.50E+04^{b}	1.40E+04^{c}	1.32E+04^{d}	0.00	3.577
Lactobacillus count (CFU/g)	1.48E+04^{e}	1.67E+04^{d}	1.93E+04^{c}	2.09E+04^{b}	2.34E+04^{a}	0.00	6.046

Means showing dissimilar superscripts (a, b, c, d) in a row are significantly different at $p < 0.05$. C, Control, supplemented with 0% PPLP; T1, treated with 0.5% PPLP; T2, treated with 1% PPLP; T3, treated with 1.5% PPLP; T4, treated with 2% PPLP; PPLP, *Pongamia pinnata* leaf powder.

Effect of PPLP on total bacterial counts, coliform count and Lactobacillus *count*

Table V exhibited a dose-dependent increase ($P < 0.05$) in total bacterial count and *Lactobacillus* count, and a dose-dependent reduction ($P < 0.05$) in the coliform count by the dietary treatment of PPLP in broilers. All PPLP supplemented groups exhibited an increase ($P < 0.05$) in the total bacterial count as compared to the control group; similar trend was recorded for the *Lactobacillus* count. On the other hand, the Coliform count of caecal contents were statistically highest ($P < 0.05$) in the control group, as compared to T1, T2, T3 and T4 groups.

DISCUSSION

In the current study, PPLP treated groups shown positive effect on weight gain and FCR when compared to the control group. However, Husna *et al.* (2017) found that 2% *P. pinnata* had no effect on body weight and feed conversion ratio in broilers up to 28th day. In addition, inclusion of 11.20-22.40% deoiled cake in the Japanese quail diet resulted in poor efficiency (Dhara *et al*., 1997). It is reported that feed efficiency is dependent upon energy and protein content, their ratio, and balance of nutrient in the diet. However, it is reported that presence of incriminated factors like tannin, trypsin inhibitors, and karanjin in any herb lead to poor FCR in chick despite of feeding uniformly balance diet (Husna *et al*., 2017; Mellen, 1984).

In current research weight of lymphoid organs (bursa, spleen and thymus) were measured to evaluate the immune status. It is well established that T- and B-lymphocytes mainly synthesized in the thymus and bursa respectively, and increased size of these lymphoid organs known to cause improved production of lymphocytes via activated mitosis (Ugur and Mueller, 2019). In a rat study, *P. pinnata* known to improve the IL-10 (an interleukin produced by macrophages and lymphocytes) production, that probably indicates the qualitative or quantitative stimulation of lymphocytes by the *P. pinnata* compounds (Dwivedi *et al*., 2017). The current study findings revealed that weight of immune organs were significantly increased in PPLP treated groups with highest weight in T4 as compare to control. It is hypothesized that increase in the weight of lymphoid organs like thymus is due to improved FCR and better nutrient digestibility (though we have not analyzed this) potential of *P. pinnata* as reported in previous studies (Krishna *et al*., 2021). By treating of *P. pinnata* with alkali or acid increased thymus weight in turns to increase antibody production, macrophages activity and increased local mucosal antibody (IgA). It is reported that, the weight of bursa, spleen and thymus is directly proportional to the immune response in broiler (Krishna *et al*., 2021). The study of Krishna *et al*. (2021) reported that weight of thymus was higher in seed karanj cake (SKC) or processed SKC as compared to control however some studies (Krishnamoorthy *et al*., 2014; Panda *et al*., 2006; Mandal, 1982) reported that, weight of lymphoid organs was similar in SKC, NaOH treated karanj cake and Ca (OH)2 treated SKC supplemented groups at age of 6 weeks.

Antibody results shows that, the antibody titre of Newcastle disease (ND) was higher in *P. pinnata* treated groups as compared to control group. The results are similar to previous study by Shinde *et al*. (2011) who reported that, aqueous extract of *Pongamia glabra* (AEPG) at the dose rate of 400 mg/kg diet (AEPG 400) produce more serum immunoglobulin followed by AEPG 200 and were lowest in control group. The results are not agreement with the previous studies conducted by Chandni and Ashwani (2017) and Krishnamoorthy *et al*. (2014), those reported a decrease in the antibody titer in model animals when fed Karanja (*P. pinnata*) seed cake. It is reported that, humoral and cell mediated immune response depends on several factors such as dosage of antigen, type of antigen, route of administration and sensitivity of an individual (Gross, 1993). Since all the previous studies were conducted on the seed cake, while, in current study we used *P. pinnata* leave powder instead of seed cake.

All PPLP supplemented groups exhibited an increase ($P < 0.05$) in the total bacterial count as compared to the control group; similar trend was recorded for the *Lactobacillus* count. On the other hand, the Coliform count of caecal contents were statistically highest in the control group, as compared to treated groups. While reviewing the available literature, it was observed that,

there was no previous report of *P. pinnata* leaf powder on the bacterial communities of chickens. However, available scientific evidences showed that *P. pinnata* have strong potential to modulate the population of microbial organisms. Like, the study of Yu *et al*. (2021) reported that phytoremediation using *P. pinnata* significantly reshaped the soil microbial communities by changing key taxa including *Actinomadura, Acidiferrobacter, Niastella, Paucibacter, Pedobacter, Terrimonas, Gemmatimonas, Chthonomonas, Dongia,* and *Pseudospirillum.* In other reports, antimicrobial activity of *P. pinnata* was also reported against bacterial (Wagh *et al*., 2007) and fungal (Kanatiwela *et al*., 2013) pathogens. Thus chicken gut microflora modulatory effects of *P. pinnata* observed in our study is a novel finding, however, it is in line with the previous work done on soil microbiota (Yu *et al*., 2021), and some in vitro studies against human pathogens (Kanatiwela *et al*., 2013; Wagh *et al*., 2007).

CONCLUSIONS

The study concluded that dietary inclusion of 0.5-2% of PPLP in broilers diet have positive effect on the production performance and carcass weight. PPLP (0.5-2%) also exhibited immunomodulatory effect by increasing the anti-NDV antibody titer and relative weight of immune organs (spleen, thymus and bursa). Dietary inclusion of PPLP increase the total bacterial and lactobacillus (beneficial) count and decrease the coliform (pathogenic) bacterial count in the intestine.

Statement of conflict of interest

The authors have declared no conflict of interest.

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