Journal of Medicinal Plants and By-products (2022) Special Issue: 59-65

Original Article

Effect of *Trigonella foenum-graecum* L. on Immune Function and Liver Activity of Ross 308 Broiler Chickens

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Article History: Received: 01 December 2020/Accepted in revised form: 16 January 2021

Abstract

In this study, the effect of fenugreek (*Trigonella foenum-graecum* L.) seed powder and aqueous extract on growth performance, immunity, and liver activity in the ROSS 308 broiler were investigated. A total of 270 day-old broiler chicks were randomly divided into 9 groups (n=30), with three replicate, T_1 (control group) fed with a basal diet, T_2 fed with basal diet added to Immunofin®, and T_3 fed with basal diet added to HEPARENOL® in drinking water based on a producer recommended methods. Other 6 groups, were fed with basal diet added to 3 different level of fenugreek seed powder: T_4 (0.1%), T_5 (0.2%), T6 (0.3%) and 3 different level of extract in drinking water: T_7 (0.1%), T_8 (0.2%) and T_9 (0.3%) and All groups were used during 42 days of rearing. To evaluate antibody titers against Newcastle disease, Avian Influenza, and Gambro vaccines, Hemagglutination Inhibition and Enzyme-Linked Immunosorbent Assay tests were performed. Administration of fenugreek seed extract and powder according to recommended doses had no significant effect on growth performance; however, it had a significant effect on the antibody levels and the levels of Alanine Aminotransferase and Aspartate Aminotransferase enzymes that highly increase in liver disorder, were significantly decreased by aqueous extract of fenugreek seed in 0.2% and 0.3% ($P \leq 0.05$). Overall, the present study showed that aqueous extract and seed powder of fenugreek could be used as a new approach to increase the level of immunity and to improve liver activity in broilers.

Keywords: Fenugreek Seed, Newcastle diseases, Avian Influenza, Immunity, Liver activity

Introduction

Currently, feed additives play an important role in poultry farming. Many studies conducted to improve the feed conversion ratio (FCR) in food animals [1]. Feed additives are used in the poultry feed industry for many purposes, such as FCR reduction, growth promotion, disease resistance, and most importantly for improvement of vaccination efficacy and their immunomodulatory effects [1,2]. Herbal medicine is one of the important and highly practical feed additives. Although antibiotic growth promoters have been used to promote the growth and efficacy, their side effects and bacterial resistance have made them unusable in the feed industry [3].

Now days, herbal medicine is widely used for many purposes and its application is expected to be increased in future with improved knowledge on its effects and functions. Fenugreek (*Trigonella foenum-graecum* L.) is a well-known medicinal plant that is mainly cultivated in India, Pakistan, Iran, and China [2,4]. Fenugreek (*T. foenum-graecum* L.) seed has been used traditionally for diabetes treatment [5]. Important components of fenugreek are alkaloids, flavonoids, steroids, and Saponins [4]. In continual researches, other roles of fenugreek including anti-atherosclerotic effect, antioxidant properties, and immunity stimulation have been confirmed [4]; therefore, the present study aimed to investigate cellular and humoral responses, immunomodulatory potential, liver tonic effect, growth

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promotion, and FCR improvement of Fenugreek seeds in broilers.

Material and Methods

The present study was carried out in an educational broiler farm in Shahmirzad School of Veterinary Medicine, Semnan University from May to August 2019. A total of 270 ROSS 308 day-old broiler chicks were randomly divided into 9 treatment groups (T_1-T_9) with 3 repetitions for each treatment (n=10).

Rearing and Experimental Treatments

All groups had free access to food and water (ad-libitum) and reared in a battery cage with ROSS 308 recommended catalog (Ross Broiler Management Handbook-Aviagen). Their basal diet analysis was shown in Table 1. T1 was the control group fed with a basal diet without any additives in drinking water, T2 was positive control group fed with a basal diet added to Immunofin® (Pars ImenDaro) the main compound of Immunofin® is Echnacea, its dosage is 8 hours daily with 1/1000 concentration in drinking water. The T3 group was positive control group, fed with basal diet added to HEPARENOL® (M.C.I Santé Animale). It is composed of Sorbitol 35 g, Acetyl methionine 10 g, Choline chloride 7.5 g, Betain 6 g, Lysine HCI 2 g per liter.its dosage is 1/1000 concontration in drinking water. Immunofin® and HEPARENOL® were used as a positive control for immunomodulatory and liver tonic effects of fenugreek seed, respectively. T4, T5 and T6 groups were fed with three levels of Fenugreek seed powder premix for the first three days of life and three days before and after vaccination with 0.1%, 0.2%, and 0.3% concentrations, respectively. T7, T8, and T9 groups were fed with basal diet, and three-level of Fenugreek seed aqueous extract in drinking water for the first three days of life and three days before and after vaccination with 0.1% (1 ml/liter), 0.2% (2 ml/liter), and 0.3% (3 ml/liter) concentrations, respectively. All the chickens were weighted individually at the start of the experiment and at weekly intervals thereafter. Weekly feed intake and body weight of treatment groups were recorded and feed conversion ratio (FCR) was also calculated.

The maintenance and treatment of the animals was in accordance with the guidelines for the care and use of the laboratory animal's committee of Semnan University and we received the code E-95-01 from the ethics committee in biological research.

Fenugreek Seed Premix and Extract Preparation

In the present study, the fenugreek seeds were obtained from Agricultural Research Center of Semnan Province (Seed code: 003-008-073 According to the Iranian Herbal Pharmacopoeia.). Seeds were soaked to prepare the aqueous extract. There for, 3 kg of fenugreek was washed and dried at room temperature in the shade and then grinded by an electric mill. In addition, every 300 grams of the seed dissolved in 4 liters of distilled water to obtain a mucilage compound. The liquid was removed from the surface of the tube and dried on Ben Marie. Appropriate concentrations of the extract were prepared before administration. The aqueous extract of fenugreek seed was added with doses of 1, 2 and 3 ml/liter of drinking water. Fenugreek seed powder was then added to the diet by 0.1%, 0.2%, and 0.3% as premix.

Vaccination Schedules

As shown in Table 2 and Figure 1, all of 9 treatment groups were vaccinated based on vaccination schedule in Iran broiler flocks.

Humoral and Cellular Immunity and Enzyme Measurement

Vaccination response to Newcastle Disease (ND) and Avian Influenza (AI) was evaluated by HI test in accordance with OIE protocols in days 21, 30 and 42 of rearing period. For Infectious Bursal Disease (IBD), antibody levels were analyzed by indirect ELISA kit CK113 IBD using BioChek on 42th day of rearing.

The average number of white blood cells in five 40× microscopic fields multiplied by 4,000 provides the total white blood cell count (TWBC; per mm^3) estimate, after a modification of a method described by Campbell and Ellis [6].

Fig. 1 Eye drop vaccination in 9-day old chickens

A minimum of 200 leukocytes per slide were sorted into categories: small or medium lymphocytes, monocytes, Heterophiles (typical, variant, and classic types), basophils, or eosinophils. Morphological criteria for sorting [6].

Table 1 Analyze of the experimental basal diets used in treatments groups

	Starter	Grower	Finisher 1	Finisher 2
Age Fed (day)	0-10	11-24	25-39	40- finish
Energy ME, kcal/kg	3000	3100	3190	3200
Crude Protein %	23	21.5	19.7	18.3
Lysine Total %	1.44	1.29	1.15	1.05
Methionine Total %	0.56	0.51	0.47	0.43
Methionine+Cystine total %	1.08	0.99	0.9	0.84
Calcium %	1	0.9	0.85	0.83
Available Phosphore %	0.5	0.46	0.44	0.42

Table 2 Vaccination schedule in treatment groups, IBV: Infectious Bronchitis Virus

Vaccine	Name &producer	Vaccination method	Age of vaccination (day)
ND+IBV	CEVAC® VITABRON L	Spray	1
ND	Izovac ND Clone	Eye Drop	9
ND+AI	Gallimune 208 ND+ Flu H9 ME	Injection	9
IBD	Nobilis® Gumboro D78	Drinking Water	14
ND	Izovac ND Clone	Drinking Water	19
ND	AvinewNeO, Merial	Drinking Water	25

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were also measured by commercial kits [Pars Azmoon, Tehran, Iran] using an autoanalyzer (BS Mindray 1200) at 42th day.

Statistical Analysis

The data were statistically analyzed by analysis of variance (ANOVA) using General Linear Model procedure in SPSS (10.0) software. The replicates were considered as experimental units and the values were expressed as means ± standard error (SE). Duncan's multiple range test (Duncan 1955) was also used to evaluate the significant difference between the means ($P \leq 0.05$).

Results

Humoral Immunity

For immunomodulatory effects, T_3 group were excluded and T_2 treatment was investigated as the positive control for immunomodulatory effects. The results are shown in Table 3.

HI-ND Results

On 21th day, T_9 treatment group showed the best antibody response against the ND vaccine with a significant difference ($P \leq 0.05$) with T_2 group, followed by T_4 and T_8 treatment groups (Table 3). On 30th day, the best responses were observed in T_6, T_8, and T_9 treatment groups with a significant difference with the control group. On 42th day, the best antibody response was observed in the T_5 treatment group. It is while all treatment groups showed significant increase in antibody responses compared to the control group (T_1), with no significant difference between each other.

HI-AI Results

On 21th day, no significant difference was observed in the treatment groups (Table 3). On 30th and 42nd days, T_8 group showed the best response with a significant difference with the control group ($P \leq 0.05$).

ELISA-IBD Results

IBD antibody levels were shown in Table 3. On 42th day, 28 days after IBD vaccination, all treatment groups, except T_6, T_8, and T_9 showed a significant difference compared to the control group ($P \leq 0.05$), thus a lower level of antibody was observed in T_9, T_8, and T_6.

Cellular Immunity

For cellular immunity, similar to the humoral immunity, T_3 group were excluded and T_2 was investigated as the positive control for immunomodulatory effects. The highest level of leucocyte was observed in T_8 group, and the best heterophile/lymphocyte ratio (H/L) in T_2 group (Table 4).

Enzyme Level

For the hepatic enzyme activity, T_2 group was excluded and T_3 was investigated as the positive control for hepatic enzyme activity and liver tonic effects (Table 4).

ALT

On 42th day, a significant difference was observed in the treatment groups compared to the control group ($P \leq 0.05$). There was no significant difference between the T_8 and T_3 (positive control, HEPARENOL®) groups. Compared to premix treatments, drinking water treatments had the best hepatic function.

AST

Similar to ALT, in AST, all treatment groups showed a significant difference with the control group. In other words, positive control treatment improved hepatic function and T_8 and T_9 treatments significantly ($P \leq 0.05$) decreased the AST level compared to that of other treatments.

Body Weight and FCR

No significant differences were observed in the treatment groups regarding the body weight and FCR. The live body weights and FCRs were aligned with ROSS 308 broiler performance manual with no mortality in all treatment groups for whole rearing duration. Detail on body weights and FCRs were shown in Table 5, 6, and Fig. 2 respectively.

Discussion

In the present investigation, the addition of Fenugreek seeds premix and aqueous extract to drinking water at recommended doses had no significant effect on production performances (Table 5 & 6), including live body weight and FCR ($P \leq 0.05$), which was consistent with the results of a study by Beghoul *et al*. [7]. In another study, fenugreek seeds were tried on with a premix level of 0.5%, 1%, 2% and 4% in broiler feed, in which 2% and 4% aqueous extract treatment groups showed a significant decrease in body weight and feed intake [8].

Table 3 Humoral antibody responses against ND, AI and IBD with HI and ELISA test.

Treatment Group	HI – ND			HI – AI			ELISA – IBD
	21th D	30th D	40th D	21thD	30thD	40thD	42thD
T1	4±0.89 c	4.85±0.85 cb	4.5±1.04 d	5.5±0.75 a	6±1.41 bc	5.5±1.04 cb	2615±131 ab
T2	4.5±0.04 c	5.5±1.04 abc	6.66 ± 0.81 ab	5.66±0.51 a	6.1±0.75 abc	6.33±0.81 ab	2722.13±295 ab
T3	-	-	-	-	-	-	-
T4	5.8±0.75 ab	5.6±1.21 abc	6.5±1.04 ab	5.66±0.51 a	6.5±1.05 ab	6.1±1.16 abc	2650.35±207 ab
T5	4.5±1 c	6.16±0.98 a	7.17±1.04 a	5±0.89 a	6.5±1.05 ab	6.5±1.04 ab	2598.13±251 b
T6	4.5±1 c	6±1.09 ab	5.66±1.03 bc	5.66±0.51 a	5.66±0.52 bc	5.66±0.51 cb	2142.63±127 c
T7	4.6±1.03 bc	5.8±0.81 abc	5.66±0.81 bc	5.66±0.81 a	6.2±0.84 abc	6.1±0.75 abc	2815±148 a
T8	5±0.89 abc	6.16±0.75 a	6.16±0.75 ab	5.5±0.83 a	7.17±0.75 a	7.16±0.75 a	2085.88±204 cd
T9	6.1±0.98 a	6.63±0.81 a	6.5±0.83 ab	5±0.63 a	6.5±1.05 ab	6.5±1.37 ab	1918.63±156 d

HI: Hemagglutination Inhibition, ND: Newcastle Disease, AI: Avian Influenza, ELISA: Enzyme-Linked Immunosorbent Assay. 1th day ND, AI and IBD titer were 7.4 ± 0.55, 9.2 ± 0.55 and 4180 ± 635 respectively, mean ± SE. Column contains different letter differ significantly ($P \leq 0.05$). T2 and T3 treatments, were investigated as the positive control for immunomodulatory effects and liver tonic activity respectively.

Table 4 Enzyme Level and Cellular Immunity Component.

Treatment Group	ALT (U/L)	AST (U/L)	T. Leuc (103/ µl)	Heterophil (%)	Lymphocytes (%)	H/L
T1	36.5±2.5 a	450.5±1.5 a	8.750±0.25 bc	34.6±2.01 a	65.3±2.31 bc	0.527 a
T2	-	-	7.500±0.5 c	10.8±1.44 c	61.1±3.14 d	0.177 c
T3	7±1 e	140±1 e	-	-	-	-
T4	13.5±0.5 bc	180±1 d	9.300±0.55 ab	34.4±1.81 a	51.2±4.22 cd	0.672 a
T5	13±2 c	205±0.5 c	7.500±0.5 c	23.6±2.24 b	66.6±3.73 cd	0.355 b
T6	14.5±1.5 bc	230±1 b	9.375±1.125 ab	31.6±2.83 a	61.6±4.26 bc	0.514 a
T7	16±1 b	200±1 cd	8.900±1.35 bc	25.7±1.63 ab	74.8±3.18 b	0.343 b
T8	9.5±1 de	140±1 e	10.500±0.75 a	25.3±3.29 ab	74.6±6.03 a	0.339 b
T9	10±1 d	150±2 e	9.875±5.87 ab	32.3±2.67 a	67.6±4.97 b	0.478 a

Column contains different letter differ significantly ($P \leq 0.05$). T2 and T3 treatments, were investigated as the positive control for immunomodulatory effects and liver tonic activity respectively. Mean ± SE.ALT and AST: Unit / Liter. Total leucocyte: 10^3/ microliter

The reason for this difference with our results seems due to fenugreek seeds concentration; premix concentration in their study is up to 20 times as much as that of our study. Inclusion of fenugreek seeds more than 1% is not cost-effective, too. Overall, ND and AI vaccine efficacy and immunity response in 3^{th} - 5^{th} weeks of broiler life are very important. Any immunomodulatory agent able to increase antibody response and vaccination efficacy could be effective for the whole broilers rearing period. In the present study, on 21^{th} day, the best response was observed in T_9 treatment group for HI - ND, with a high level of the antibody and a significant difference with other experimental groups, especially positive control treatment, Immunofin® (Table 3). Regarding the vaccine efficacy, the maximum immune response in the least possible time is very important, especially in broilers, because of their high sensitivity and immature immune system. On 30^{th} and 42^{th} days, all treatment groups showed acceptable responses for HI - ND and HI - AI with a significant difference with the control group ($P\leq 0.05$); however, T_8 and T_9 groups showed the best results. There is no other study on fenugreek seed to compare with the present study except that limited researches fenugreek alone. In a diet with 1% fenugreek premix, antibody level against ND at 24^{th} and 34^{th} day significantly increased, which is attributed to ingredients such as flavonoids, steroid and saponin found in fenugreek [9].

In short, fenugreek seed could be effective in immune response in 0.2%, 0.3%, extract and 0.2% premixes. Regarding antibody response against IBD, the T_9 treatment group showed the weakest response (Table 3). Interestingly, this is exactly the opposite of the last findings on ND and AI immunity in the present study. This might be attributed to the immunomodulatory effects of fenugreek seed protecting bursa of Fabricius and a low level of challenge due to IBD vaccination. As described in IBD pathogenicity, with the increased challenges in bursa of Fabricius by either live vaccine or field virus, high amount of IgG against IBD was found in serum [10] However, regarding the bursa of Fabricius protection, T_9, T_6 and T_8 groups had the best results, respectively. Interestingly, treatment groups with low IBD antibody levels showed a high ND antibody levels, emphasizing on the bursa protection and immunomodulatory effects of fenugreek seeds.

ALT and AST are two important biochemical markers indicating the normal function of liver. Both ALT and AST are intracellular enzymes and their concentration increase by cellular injuries such as hepatocyte necrosis and cell membrane permeability dysfunction [11,12]. AST is found in liver, cytoplasm, and mitochondria of skeletal and cardiac muscles [12]. In the present study, all efforts were made to avoid muscle damage in the chicken throughout the rearing period. ALT levels, in all treatment groups significantly decreased compared to the control group. However, the best response (Table 4) were observed in T_3 (HEPARENOL®), T_8 and T_9 groups ($P\leq 0.05$). Broiler diet formulation composed of less than 5% fat concentration and the liver normal function is crucial in lipogenesis induced by all tissues, including the liver itself. Many factors could disturb liver function, which is the major detoxification organ in the body. The most important potential toxins include heavy metals, antibiotics, and drugs. Detoxification is done through oxidation, reduction, hydrolysis, and conjugation. The liver changes these toxins to polar by-products, which are then eliminated via urinary system and gall bladder [13]. Metabolic disorders like fatty liver syndrome or liver malfunction are very important, which almost could not be diagnosed in their primitive stages [14]. Liver and its normal function is very vital in broilers, particularly in layers. Saponin, vitamins A, B1, C, nicotinic acid, and alkaloids are nutritional ingredient that may act as immunomodulators and liver tonic ingredients. Alkaloids, including trigonelline, gentianine, and carpine compounds are the most important alkaloids in fenugreek seeds [15].

Table 5 Body weight (g) weekly intervals.

Treatment	7^{th} D	14^{th} D	21^{th} D	28^{th} D	35^{th} D	42^{th} D
T1	187±5	471±12	896±29	1425±78	2005±95	2586±145
T2	188±9	485±32	914±35	1482±65	2000±123	2455±138
T3	184±16	478±25	905±32	1449±55	2043±144	2540±161
T4	176±20	462±40	885±44	1421±83	1924±102	2510±129
T5	171±7	455±13	876±27	1409±59	1940±133	2558±138
T6	168±11	445±27	864±33	1398±81	2010±98	2522±119
T7	182±21	465±31	879±29	1406±56	1905±139	2499±167
T8	179±17	452±19	873±50	1408±55	1915±118	2505±138
T9	174±22	445±27	861±41	1399±69	1960±115	2520±148

D: day, there was no significant difference in all treatment groups ($P\leq 0.05$). Mean ± SE.

Table 6 All treatment's FCR in weekly intervals.

Treatment	7th D	14th D	21th D	28th D	35th D	42th D
T1	0.893	1.24±0.11	1.35±0.12	1.51±0.13	1.74±0.13	1.87±0.14
T2	0.895	1.27±0.10	1.36±0.11	1.54±0.18	1.76±0.14	1.88±0.19
T3	0.896	1.29±0.09	1.35±0.05	1.53±0.09	1.74±0.01	1.89±0.20
T4	0.897	1.28±0.11	1.37±0.12	1.52±0.16	1.75±0.07	1.87±0.13
T5	0.894	1.30±0.13	1.39±0.06	1.52±0.12	1.76±0.11	1.88±0.18
T6	0.892	1.27±0.09	1.36±0.06	1.53±0.10	1.75±0.11	1.87±0.09
T7	0.895	1.28±0.07	1.37±0.10	1.54±0.15	1.73±0.04	1.89±0.11
T8	0.887	1.27±0.15	1.37±0.11	1.52±0.17	1.74±0.07	1.88±0.05
T9	0.890	1.28±0.11	1.35±0.04	1.51±0.09	1.73±0.10	1.88±0.10

D: day. There was no significant difference in all treatment groups ($P \leq 0.05$). Mean ± SE.

Fig. 2 broiler chicken in the end of rearing period.

It seems vitamins A and B1 component of seeds are effective in liver function and could decrease ALT and AST enzyme levels. Also, since the best responses were observed in the high dosage of aqueous extract treatments (T_8 and T_9), one is safe to say that their effect on liver function is dose dependent. The humoral and cellular immune responses are developed together by broilers in response to the vaccination. Cellular immunity plays the essential role in vaccination and its antiviral immunity effects [16]. Heterophiles (H) and lymphocytes (L) constitute the mass of circulating immune cells and H/L ratio specifically shows stress conditions. In case of high heterophile, lymphocytes count could indicate high glucocorticoid levels [17]; however, paying attention only to H/L ration is not recommended because in some cases, heterophile amount may be the same and the lymphocyte count decreases. Bacterial Lipopolysaccharide can increase H/L ratios and glucocorticoid with a different pattern. As shown in Table 4, T_2 treatment showed the best H/L ration and was significantly different from all other treatment groups. Although there was a significant difference between the T_8 and T_9 groups and other treatment groups regarding the heterophile percentage, it was not as much satisfied as T_2 group. In other words, unlike Immunofin® (T_2), heterophile level was not decreased in T_8 and T_9 groups; however, in 0.2% and 0.3% (T_8 & T_9) concentrations, it was more effective than other treatments.

Conclusion

Fenugreek (*T. foenum-graecum* L.) seed aqueous extract was found to be more effective than the powder premix and basal diet. Additionally, fenugreek seed had no effect on FCR and body weight, though significantly increased humoral immunity level and was considered as immunomodulatory agents. Fenugreek seed aqueous extract in 0.2% and 0.3% concentrations had a good immunomodulatory and liver tonic effect, in addition to bursa of fabricius protection. Moreover, it had the same and to some extent better performance than control positive treatments.

Acknowledgements

The results described in this paper were part of student thesis. This study was funded by the Deputy Research of Semnan University (Semnan, Iran) for educational purposes.

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