Journal of Animal and Veterinary Advances 11 (10): 1681-1687, 2012
ISSN: 1680-5593

The Immunotoxicity Studies on *Jatropha curcas* Kernel Meal in Young Broilers

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Abstract: *Jatropha curcas* Kernel Meal (JKM) is used in animal diet as the source of vegetable protein but it is toxic to animal. The aim of this study was to evaluate the toxic effect of JKM on immune functions in young broilers. A total of 875; 1 day old male Arbor Acres chickens were fed diets containing 0, 3, 6, 9 and 12% JKM for 21 days, respectively. In the entire experimental period, the level of serum lysozyme showed no significant difference compared with control ($p>0.05$). Dietary JKM linearly decreased the contents of serum IgA and IgG ($p\leq0.05$) and markedly increased the contents of serum IgM ($p<0.05$) and also significantly decreased the percentage of CD3+, CD4+ and CD8+ in blood ($p<0.01$) on day 7, 14 and 21. Histopathological studies on 21 days old broilers showed that dietary JKM caused the lesions of thymus, spleen and bursa of Fabricius, exhibiting widely diffuse hemorrhages. These results suggest that dietary JKM at 3-12% could result in immunosuppression or immunodepression in young broilers and the responses followed a dose dependent manner.

Key words: Young broilers, *Jatropha curcas* kernel meal, toxic, T-lymphocytes, immunoglobulin, chicken

INTRODUCTION

Jatropha curcas is low-growing and drought-tolerant multipurpose small tree of significant economic importance because of its several industrial and medicinal uses (Makkar *et al*., 2007; Chivandi *et al*., 2004; Aiyelaagbe *et al*., 2007; Agbelusi *et al*., 2007). This plant grow in heavy rainfall regions also survives on barren, eroded land and under harsh climatic conditions (Becker and Makkar, 2008). *Jatropha curcas* seeds are rich in oil and protein. The oil is used as fuel directly or to make high quality bio-diesel by the transesterified form. In order to use the oil as biodiesel, large scale cultivations of this plant has planned or developed in India, China, Madagascar, Myanma, Philippines and many other developing countries (Francis *et al*., 2005). This is resulted in increasing quantity of byproducts that can be used as livestock feed.

Jatropha curcas Kernel Meal (JKM) being a byproduct that obtained after extraction of oil in *J. curcas* seeds is good source of protein. This meal (full defatted) contains approximately crude protein of 60% in dry matter (Makkar *et al*., 2008) and 90% of which is present in the form of true protein (Makkar and Becker, 1997). The levels of all essential amino acids except lysine, in JKM protein are higher than those of FAO reference protein for a growing child of 2-5 years of age are also higher or similar compared to those of Soybean Meal (SBM) (Kumar *et al*., 2010a, b; Makkar and Becker, 1997). However, the seeds and kernel meal contain high levels of anti-nutritional factors such as trypsin inhibitor, lectins and phytate (Goel *et al*., 2007) and the toxic components phorbol esters (Makkar and Becker, 1997) which prevent their utilization as feed ingredients. Heat-labile anti-nutrients such as protease inhibitor and lectin are easy to inactivate by heating treatment whereas phorbol esters can not be destroyed by heating at temperature of 160°C for 30 min (Aderibigbe *et al*., 1997; Makkar and Becker, 1997; Makkar *et al*., 1997).

In previous studies, raw *J. curcas* seeds as well as deshelled seed meal were reported to be highly toxic to human and animal. Adam and Magzoub (1975) reported Nubian goats fed diet containing raw *J. curcas* seeds at doses ranging from 0.25-10 g/kg/day caused poisonous with mortality occurring between 2 and 21 days (Adam and Magzoub, 1975). The accidental consumptions of raw *J. curcas* seeds by children (age group of 4-8 years) showed adverse signs and symptoms such as abdominal pain, vomiting, diarrhea and burning sensation in the throat (Rai and Lakhanpal, 2008; Singh *et al*., 2010). Vomiting, diarrhea and acute abdominal pain began to occur gradually after 10 min in ingestion of seeds. Five cases of raw *J. curcas* seeds poisoning in adults were reported with same symptoms above reported (Shah and Sanmukhani, 2010). Toxic studies on raw *J. curcas* seeds in brown Hisex chicks for 4 weeks showed growing depress and widespread hemorrhage and congestion. It was also observed that there were a increase in serum

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glutamate oxaloacetate transaminase activities and potassium and phosphorus concentrations, a decrease in total protein and calcium concentrations (Devappa *et al*., 2010; El-Badwi and Adam, 1992). The diets containing 0.5% raw *J. curcas* seeds resulted in a high mortality in Brown Hisex chick (El-Badwi *et al*., 1995). These results indicate higher susceptibility of Hisex chicks to raw *J. curcas* seeds and chickens can be a reliable animal model to assess the toxicity of *J. curcas* or to investigate value in use for JKM (Devappa *et al*., 2010; Makkar and Becker, 2009).

The Immune System plays a crucial role in maintaining body health which protects the host from potentially pathogenic agents including microorganisms (viruses and bacteria), parasites and fungi to eliminate neoplastic cells and to reject foreign compounds. Consequently, this system can be the target for immunotoxic effects caused by a variety of foreign substances. The information concerning the immune system toxic effects of JKM used in animal have been scanty. Therefore, the objective of this study was to evaluate the toxic effects of JKM in diets on the immune system in young broilers.

MATERIALS AND METHODS

This study was carried out in appliance with the ethical guidelines for experimental animals by Sichuan Agricultural University (Sichuan, China).

Preparation of JKM: *Jatropha curcas* seeds were obtained from Huili county in Sichuan province (China). The seeds were dehulled by the small sheller and obtained seed kernels and then the kernel and shell were mixed with the kernel to shell ratio (w/w) of 10:1. The mixture was pressed in a power press to obtain JKM under following conditions, feed and press temperature of 110 and 161°C, respectively moisture of 5%. The meal was stored in cool place, determined the proximate composition and essential amino acids (Table 1) and used for the process of diets.

Diet formulation: Soybean meal, rape oil (food grade) and other feed ingredients were obtained from local market. The basal (control, JKM-free) (Table 2) was formulated according to the Nutrient Requirements of Broiler (NY/T 33-2004, China) and the diet containing 12% JKM (JKM_{12}) was formulated with JKM to substitute the SBM based on the same true digestible amino acid, isonitrogen and isoenergy (The ratio of true available amino acid of JKM were measured by the lab, published) and then the JKM_{12} diet and the control diet was mixed on the basis of the different proportion to get the other diets containing JKM of 3 (Control: JKM_{12} = 3:1, JKM_3), 6 (Control: JKM_{12} = 1:1, JKM_6) and 9% (Control: JKM_{12} = 1:3, JKM_9), respectively. All diets were fed in mash form.

Table 1: The proximate compositions and amino acid of JKM

Items	JKM
Proximate (%)	
Moisture	5.23
Crude protein	36.14
Crude lipid	23.50
Crude ash	8.09
Phosphorus	0.35
Calcium	0.81
Gross energy (kcal g^{-1})	5.19
Phorbol esters (g kg^{-1})	1.73
Essential amino acids (g kg^{-1})	
Arginine	40.72
Histidine	10.13
Isoleucine	16.34
Leucine	22.77
Lysine	14.08
Phenylalanine	16.01
Methionine	9.61
Threonine	13.41
Valine	13.92

Table 2: Ingredients and nutrient content of experimental diets (control and JKM_{12})

Ingredients	Control[1]	JKM_{12}[2]
Corn	51.820	51.700
Soybean meal	39.500	29.700
JKM	0.000	12.000
Rapeseed oil[3]	4.200	2.200
Dicalcium phosphate	1.800	1.840
Limestone	1.350	1.160
L-Lysine HCl	0.050	0.160
DL-Methionine	0.200	0.160
Salt (NaCl)	0.400	0.400
Choline chloride (50%)	0.150	0.150
Vitamin premix[3]	0.030	0.030
Mineral premix[4]	0.500	0.050
Total	100.000	100.000
Calculated nutrient content[5]		
ME (kcal kg^{-1})	2,999.000	3,002.000
CP (%)	21.500	21.500
True digestible lysine (%)	1.104	1.103
True digestible methionine (%)	0.481	0.487
True digestible threonine (%)	0.733	0.714
Calcium (%)	1.008	1.007
Available phosphorus (%)	0.457	0.457

[1]Control: Corn and soybean meal basal diet without any JKM; [2]KJM_{12}: The diet containing 12% JKM; [3]The vitamin premix provided the following per kilogram of complete diet: retinyl acetate, 12,000 IU; cholecalciferol, 3,000 IU; Vitamin E, 7.5 mg; Vitamin K, 1.5 mg; thiamine, 0.6 mg; riboflavin, 4.8 mg; niacin, 10.5 mg; pantothenic acid, 7.5 mg; pyridoxine, 1.8 mg; biotin, 0.135 mg; folic acid, 0.15 mg and Vitamin B_{12}, 0.01 mg; [4]The mineral premix provided the following per kilogram of complete diet: Cu ($CuSO_4 \cdot 5H_2O$) 8 mg, Mn ($MnSO_4 \cdot H_2O$) 120 mg, Zn ($ZnSO_4 \cdot 6H_2O$) 100 mg, Fe ($FeSO_4 \cdot 6H_2O$) 100 mg, I (KI) 0.70 mg, Se (Na_2SeO_3) 0.30 mg; [5]Nutrient contents were calculated values according to China Feed Database Information Web Centre in 2009

Birds, management and experimental design: A total of 875, 1 day old male Arbor Acres chickens with an initial BW of 41.27±0.11 obtained from commercial hatchery were used in the study. The experiment was conducted as a

completely randomly experimental design. The chickens were randomly divided into five groups by BW with seven replicates of twenty five chickens in a metal battery cage (1×2 m). The 1 group designated as the Control (C) group was fed with the basal diet (control, JKM-free) and other four groups was fed with the diets containing JKM levels of 3, 6, 9 and 12%, respectively. Room temperature was maintained at 35°C during the 1st 3 days of life and then was decreased gradually according to age until attaining 24°C at 21 days. Artificial lighting was continuous. Chickens were free access to feed and water throughout trial. Mortality, except sampled chickens was recorded during the experiment. Three birds of average BW per each replicate on day 7th and 2nd birds of average BW per each replicate on day 14th and 21st were sampled blood from jugular vein after a 12 h overnight fast and then killed by exsanguinations. Peripheral blood from one bird was transferred into an EDTA-coated tube. Blood of the other was centrifuged at 1500 g for 5 min to obtain serum at room temperature which was stored at -20°C for further analysis. At 21 days of age, the spleen, thymus and bursa of fabricus in broiler were removed and fixed in 4% of paraformaldehyde solution for histopathological studies.

Proximate analysis: Samples of JKM were analyzed for dry matter, ash, crude protein and lipid by the standard methods of the Association of Official Analytical Chemists (AOAC, 2000). Gross energy of JKM was determined with bomb calorimeter. The content of phorbol esters was measured using HPLC according to the method of Rakshit *et al*. (2008).

Amino acid analysis: The amino acid compositions of JKM were determined using HPLC after hydrolyzing the samples with 6M Hcl for 24 h at 110°C. The sulphur-containing amino acids were oxidised with performic acid before the acid hydrolysis. But tryptophane was not analyzed.

Blood analysis: Serum IgA, IgG and IgM serum were measured by double-antibody sandwich ELISA using commercial kit (R and D Systems). Serum lysozyme activity (LZM, A050) was measured spectrophotometrically with commercial diagnosed kit (Jiancheng Bioengineering Institute, Nanjing, China). The peripheral blood of seven birds in each group was taken to determine the percentages of CD3+, CD4+ and CD8+ T-cells by the flow cytometry at 7, 14 and 21 days of age. FCM Method Anti-clotting peripheral blood (1 mL) was put in a test tube containing 1 mL 0.1 mol L^{-1} (pH7.4) Phosphate-Buffered Saline (PBS) and then transferred to centrifuge tube containing 2 mL lymphocyte separation medium and centrifuged at 200 g min^{-1} for 20 min. Approximately, 0.5 mL lymphocyte layer was collected, transferred to another centrifuge tube and then 2 mL PBS added and centrifuged at 200 g min^{-1} for 5 min. The supernatant was discarded. The cell concentration was determined using the normal counting method of blood cells and then diluted to 1.0×10^6 cells mL^{-1} with PBS. Lymphocytes were adjusted to a concentration of 1.0×10^6 cells mL^{-1} with PBS. The aforementioned 1 mL cell suspension was transferred to another centrifuge tube and centrifuged at 200 g min^{-1} for 5 min. The supernatant was discarded. The cells were respectively stained with 10 μL mouse anti-chicken CD3-FITC (clone CT-3, Southern Biotech), mouse anti-chicken CD4-FITC (clone CT4, Southern Biotech) and mouse anti-chicken CD8a-RPE (clone CT-8, Southern Biotech) for 15-20 min at room temperature and then 2 mL PBS added and centrifugal. The supernatant was discarded. The cells were resuspended in 0.5 mL PBS and examined by fluorescence activated cell sorter (FACS, Coulter Corp., USA).

Histopathological studies: The fixed tissues were dehydrated and embedded in paraffin and serial sections were prepared then stained with haematoxylin and eosin for histopathological study.

Statistical analysis: All data were subjected to a one-way Analysis of Variance (ANOVA) according to the GLM procedure of SAS (Version 9.2e, Institute Inc., Cary, NC). The significance of difference between means was tested by Duncan's multiple range test ($p<0.05$). The effect of supplemental levels of JKM was determined using orthogonal polynomials for linear and quadratic effects.

RESULTS AND DISCUSSION

Mortality of chicks at the whole experimental period: As shown in Table 3, the death of chicks occurred in a dose dependent manner. The higher the addition level of JKM, the sooner the chicks died and no birds was survival in JKM_9 and JKM_{12} before the 3rd week. But some birds fed diets of JKM_3 and JKM_6 were survived.

Lysozyme activity: The serum lysozyme contents in different experimental groups are shown in Table 4. No significant difference in lysozyme activity were observed in the entire experimental period ($p>0.05$).

Table 3: Mortality rate of broiler chicks at per week of the whole experimental period

	JKM level in diets (%)				
Mortality weeks (%)	0	3	6	9[1]	12[1]
1st	0	1.70	7.43	35.43	56.00
2nd	0	10.29	43.43	44.57	27.43
3rd	0	6.29	12.00	-	-

[1]All broiler chicks in JKM_9 and JKM_{12} treatment groups except for selected chicks to be sampled, died before the 3rd week

Serum immunoglobulin A, G, and M contents: Serum immunoglobulin contents of broilers were affected by dietary JKM level (Table 5). Compared with control with the increase of dietary JKM level, the contents of serum IgA and IgG linearly reduced in throughout experiment ($p \leq 0.05$), moreover, IgA contents quadratically decreased on day 14th and 21st ($p<0.05$) and serum IgG contents also quadratically decreased on day 7th and 14th ($p<0.01$). In entire experimental period, the concentration of serum IgM linearly and quadratically increased when birds were fed diets containing different level of JKM ($p<0.05$).

Peripheral blood T-lymphocyte subpopulations: Dietary JKM level had a dose response effect on peripheral blood T-lymphocytes subsets in broilers (Table 6). When dietary JKM increased, the percentage of CD3+, CD4+ and CD8+ T-lymphocytes decreased linearly and quadratically on day 7th, 14th and 21st ($p<0.01$).

Histopathology studies: Histopathological studies of the organs showed different experimental diets produced pathological changes in thymus, spleen and bursa of Fabricius in broiler chickens. Severely pathological changes, widely hemorrhages and exudates in thymus were observed in all of JKM treatment groups (Fig. 1), the similar clinical signs were also seen in spleen (Fig. 2) but the hemorrhage and hyperemia in bursa of Fabricius were only found in the chicks fed 6% JKM (Fig. 3). The thymus, spleen and bursa of Fabricius in the control

Table 4: Effects of dietary JKM level on serum lysozyme contents in broiler chickens

	JKM level in diets (%)					
Lysozyme contents ($\mu g\ mL^{-1}$) (days)	0	3	6	9	12	SEM
7	1. 31^{ab}	1.25^{ab}	1.49^{a}	1.16^{b}	1.33^{ab}	0.04
14	1.30^{a}	1.38^{a}	1.43^{a}	1.30^{a}	1.37^{a}	0.02
21	1.42^{a}	1.51^{a}	1.54^{a}	-	-	0.03

Values are Means (n = 7). Means value in the same row with different superscript differ significantly ($p<0.05$)

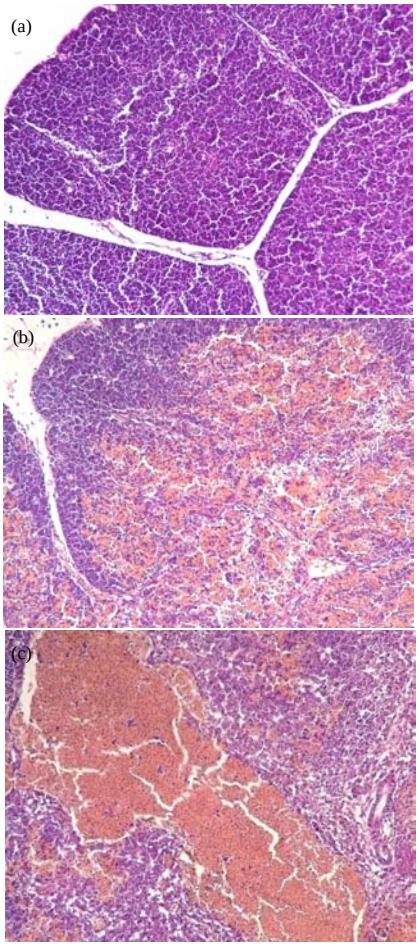

Fig. 1: Pathological change of thymus observed in; a) Control (Normal); b) JKM_3 and c) JKM_6 treatments. b and c widely and severely hemorrhages. H and E x200

Table 5: Effects of dietary JKM level on serum immunoglobulin A, G and M contents in broiler chickens

	JKM level in diets (%)						p-value[1]	
Items (days)	0	3	6	9	12	SEM	L	Q
IgA ($\mu g\ mL^{-1}$)								
7	10.29^{ab}	12.60^{a}	9.62^{ab}	9.42^{ab}	8.05^{b}	0.52	0.04	0.61
14	11.04^{ab}	12.46^{a}	10.58^{ab}	9.28^{b}	9.12^{b}	0.40	<0.01	0.02
21	11.64^{a}	12.28^{a}	10.20^{b}	-	-	0.31	0.05	<0.01
IgG ($ng\ mL^{-1}$)								
7	673.65^{a}	618.50^{ab}	556.55^{b}	552.65^{b}	530.55^{b}	16.89	<0.01	<0.01
14	619.28^{a}	568.21^{a}	444.81^{b}	465.70^{b}	471.83^{b}	17.05	<0.01	<0.01
21	529.87^{a}	459.59^{ab}	452.23^{b}	-	-	15.68	0.04	0.07
IgM ($ng\ mL^{-1}$)								
7	235.14^{a}	275.46^{ab}	292.34^{b}	285.17^{b}	292.31^{b}	7.31	0.01	0.01
14	249.74^{a}	278.35^{ab}	309.12^{b}	290.82^{b}	310.36^{b}	6.51	<0.01	<0.01
21	218.47^{a}	266.37^{b}	259.97^{b}	-	-	8.33	0.04	0.03

Values are means (n = 5), mean values in the same row with different superscript differ significantly ($p<0.05$), [1]Orthogonal contrasts: L = Linear and Q = Quadratic effect of supplemental JKM

Table 6: Effect of dietary JKM level on blood T-lymphocyte subpopulations in broiler chickens

	JKM level in diets (%)						p-value[1]	
Item (days)	0	3	6	9	12	SEM	L	Q
CD3+ (%)								
7	10.83^{a}	5.26^{b}	3.03^{c}	3.51^{c}	2.05^{C}	0.57	<0.01	<0.01
14	17.77^{a}	12.26^{b}	6.70^{c}	5.45^{c}	2.98^{c}	0.12	<0.01	<0.01
21	22.72^{a}	9.10^{b}	5.10^{c}	-	-	1.75	<0.01	<0.01
CD4+ (%)								
7	7.50^{a}	4.92^{b}	3.23^{bc}	3.57^{bc}	1.88^{c}	0.40	<0.01	<0.01
14	12.42^{a}	8.11^{b}	6.72^{bc}	5.56^{bc}	3.25^{c}	0.72	<0.01	<0.01
21	19.06^{a}	8.94^{b}	5.44^{b}	-	-	1.47	<0.01	<0.01
CD8+ (%)								
7	8.42^{a}	2.84^{b}	3.67^{b}	4.68^{b}	2.32^{b}	0.60	<0.01	<0.01
14	9.26^{a}	2.70^{b}	3.27^{b}	2.32^{b}	2.99^{b}	0.67	<0.01	<0.01
21	10.67^{a}	3.78^{b}	3.20^{b}	-	-	0.86	<0.01	<0.01

Values are means (n = 5), means value in the same row with different superscript differ significantly ($p<0.05$), [1]Orthogonal contrasts: L = Linear and Q = Quadratic effect of supplemental JKM

group didn't show any of pathological changes. In the present study, all diets containing 3-12% JKM caused broilers mortality. The higher the JKM level was in the diet, the faster the animal died. These results indicate JKM obtained by pressing at temperature of 161°C are highly toxic to broilers. Rakshit *et al.* (2008) reported whether treated or untreated using chemical and heating, all groups occurred in mortality of rats. The rats fed with the treated meal exhibited delayed mortality compared to untreated meal fed rats (Rakshit *et al.*, 2008). The major toxicity in *J. curcas* seeds are indentified as the phorbol esters (Makkar *et al.*, 1998; Makkar and Becker, 1997) which can not be completely removed from *J. curcas* seeds or kernel meal by chemical and mechanical methods (Aregheore *et al.*, 2003; Kumar *et al.*, 2010a; Rakshit *et al.*, 2008).

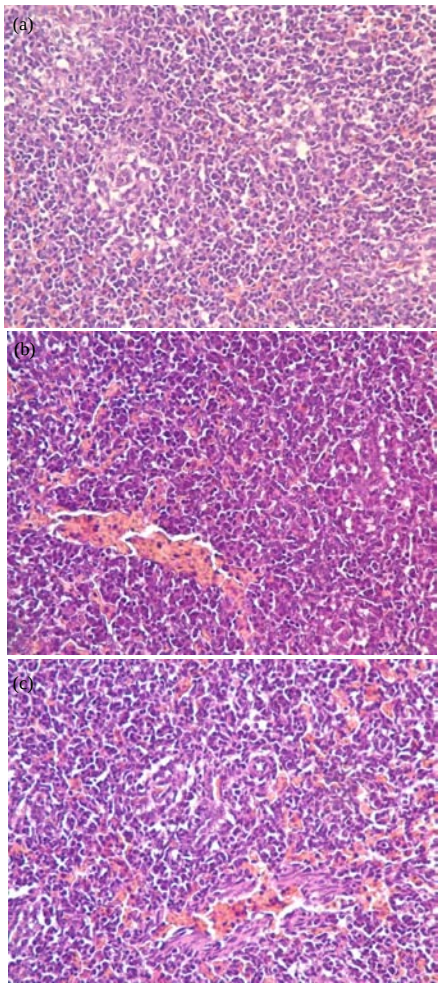

Fig. 2: Pathological change of spleen observed in; a) Control (Normal); b) JKM_3 and c) JKM_6 treatments. b and c widely hemorrhages. H and E x400

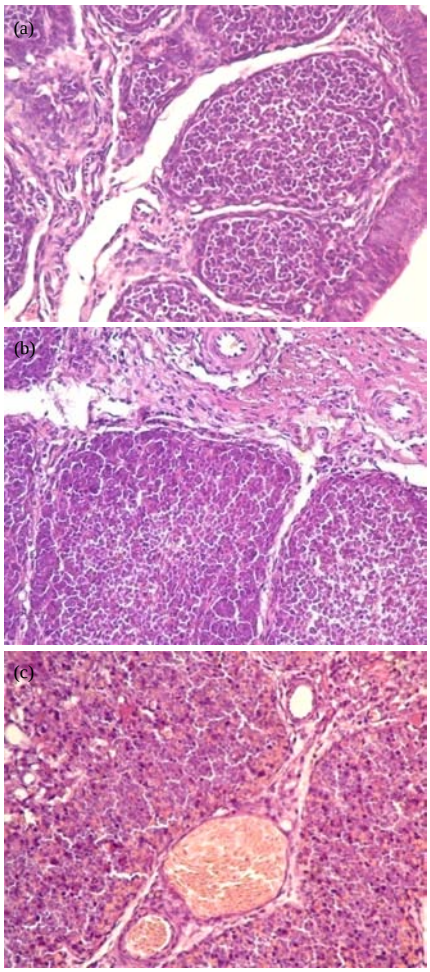

Fig. 3: Pathological changes of bursa of Fabricius observed; a) Control; b) JKM_3 Normal bursa of Fabricius and c) JKM_6 widely diffuse hemorrhages and hyperemic vein. H and E x400

The phorbol esters content depends on the residual oil present in the cake or the meal after processing (Rakshit *et al*., 2008). The immune system plays a central role in guarding health against foreign substances and protects from invasion of potential pathological agents. It is composed of two major subdivisions, the innate or nonspecific immune system and acquired or specific Immune System. In this study, dietary JKM did not affect the lysozyme activity in serum which is mainly secreted by phagocytes and is a nonspecific immune indicator. The bursa of Fabricius in birds which is equivalent to bone marrow in mammals is the primary organs of lymphopoiesis and plays pivotal for the normal development of B-lymphocytes (Glick, 1991; Glick *et al*., 1956; Pavelka and Roth, 2005; Ratcliffe, 2006). B-lymphocytes represent the major cells of the antibody-mediated humoral immunity.

In this study, dietary JKM supplementation by in large, decreased the contents of serum immunoglobulin, suggesting that the toxin in JKM may suppress the responses of the humoral immune system and the immuosupression or immunodepression may be a result of the direct histological damage to the bursa of Fabricius. The alteration and histological lesion in bursa of Fabricius which resulted in immunosuppression or immunodepression were also observed in birds (Ezeokoli *et al*., 1990; Qureshi and Edens, 1997). The percentage of CD3+, CD4+ and CD8+ T-lymphocytes can be used to assess the immune status of birds owing to their importance role in cell immunity (Arstila *et al*., 1994; Erf, 2004; Lillehoj and Trout, 1994).

In the present study, the proportions of CD3+, CD4+ and CD8+ T-lymphocytes in broilers were decreased linearly and quadratically when the dietary JKM supplemental levels increased indicating that the effect of dietary JKM on subpopulations of peripheral blood T-lymphocytes exhibits a dose suppressive response to cell immunity of birds which may be caused by the toxin in JKM directly damaging to thymus (Fig. 1).

CONCLUSION

The data obtained in the present study exhibited that dietary JKM caused the serious histological damage to immune organs and decreased the counts of the serum immunoglobulin and blood T-lymphocytes. The effect of dietary JKM on the immune functions including humoral immunity and cell immunity was a dose suppressive manner.

ACKNOWLEDGEMENTS

This study was supported by the international scientific corporation project of the Ministry of Science and Technology of China (2007DFA30920) and the specific research supporting program for academic sustentation research team in Sichuan Agricultural University.

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