Studies on toxicity to cockerel chicks of raw full-fat neem (*Azadirachta indica* A. Juss) seed kernel

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ABSTRACT

Toxicity to cockerel chicks of raw full-fat neem seed kernel (RFK) was tested. Graded doses of 0, 75, 150, 225 and 300 g RFK/kg diet replaced similar levels of peanut. Each diet was offered to 30 chicks *ad libitum* for 35 days. The RFK depressed (P<0.05) appetite and severely (P<0.01) decreased mass gain of chicks independent of the doses used. It also induced haemorrhagic anaemia (P<0.01) and leucocytosis (P<0.05) at 150 g or more RFK/kg diet. The leucocytosis largely resulted from heterophilia. At an inclusion rate of 150 g or more RFK/kg, aspartate aminotransferase activity was elevated (P<0.05) while cholesterol concentration was lowered (P<0.05). However, activity of alanine aminotransferase and concentrations of bilirubin, creatinine and uric acid did not differ significantly among the cockerels. Lesions associated with RFK included emaciation and paleness of carcasses, kidney congestion and mucous enteritis. Microscopically, there was coagulative necrosis of mucosal cells involving the submucosal glands and sloughing of intestinal villi, focal hepatocytic drop-out and haemorrhagic and oedematous nephritis. The overall effect was a highly significant (P<0.01) mortality of cockerels, which was dose-dependent; and all birds fed on 300 g RFK/kg diet died before day 35 of feeding.

Key words: raw full-fat neem seed kernel, toxicity, cockerel chicks

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Introduction

Shortages of poultry feedstuffs in the underdeveloped countries have led nutritionists to investigate the possibility of using locally produced oilseed meals in poultry rations.

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One such oilseed is neem (*Azadirachta indica* A. Juss) seed. The neem tree is known for its high resistance to drought and the ability to grow in poor soils, hence the reasons for its massive propagation in the semi-arid zone of Nigeria. Emphasis of current research efforts on neem seed kernel is the assessment of its nutritive value (GOWDA et al., 1996; NAGALASHMI el al., 1996; VERMA et al., 1998). De-oiled neem kernel meal contains a high level of protein (47.9%) (JAMES et al., 1997) similar to that of peanut meal (46%) (OYENUGA, 1968) and should, theoretically, make a good protein supplement in poultry rations. But growth depression and poor feed conversion efficiency have been reported in broiler chicks fed on rations containing de-oiled raw neem cake (100 g/kg) (REDDY and RAO, 1988). These effects were attributed to triterpenoids present in neem kernel cake (DEVAKUMAR and SUKH-DEV, 1993). To our knowledge, no study has been reported on the feeding value of raw full-fat neem kernel (RFK) in chicks' diet. In addition, specific lesions and/or signs in birds associated with RFK are not clearly defined. Consequently, the present studies were designed to investigate growth response of cockerel chicks and some specific toxic effects of RFK.

Materials and methods

Feeding trial. Ground raw full-fat neem seed kernel (RFK) was incorporated into standard cockerel chicks' rations at the rates of 0, 75, 150, 225 or 300 g/kg diet as substitutes for similar levels of peanut meal. The rations were isonitrogenous (210 g crude protein/kg), isoenergetic (11.5 MJ/kg) and fed as mash *ad libitum* to chicks for 35 days. One hundred and fifty, 7-day-old, Shika-Brown cockerel chicks were weighed individually (43.1 ± 0.3 g; mean $\pm$ SD) and randomly distributed into five dietary groups of 30 chicks on a weight equalization basis. Each treatment had 3 replicates of 10 chicks. The chicks received vaccination against Newcastle and infectious bursal diseases at the appropriate ages. Determination of body mass changes and food consumption was carried out weekly.

Haematology and plasma chemistry. At termination of the feeding (day 35) birds were deprived of only feed for 6 hours before blood collection. Three birds were randomly selected from each group and bleeding was by exsanguination following cervical dislocation. Each bird was bled into two sets of bottles containing disodium ethylene diamine tetra-acetate (EDTA) (1.0 mg/mL) and heparin (0.2 mg/mL blood) for cell count and plasma chemistry, respectively. Haemoglobin concentration, erythrocyte (RBC) count and leucocyte (WBC) differential count were done according to the method of BROWN (1976); whereas total WBC count and haematocrit (PCV) determination were performed with QBC_{11} machine (Centrifugal Hematology System, Becton Dickinson Co. USA). The heparinized blood was centrifuged ($12,800 \times g$ for 5 min.) immediately after collection and plasma stored at -80 ^{0}C for 48 hours before use. Biochemical analyses were done to assess changes that might not be evident histologically. The plasma samples were analyzed for

aspartate and alanine aminotransferases, total and conjugated bilirubin, cholesterol, uric acid, and creatinine using commercial assay kits (Randox Laboratories Ltd., Crumlin, U. K.) with the aid of an auto-analyser (SMA 12/60 Technicon Auto-analyser, Terrytown, N.Y. USA). All the birds fed on 300 g RFK/kg died before day 35 of the experiment; hence, only post-mortem examination was carried out.

Histopathology. The sacrificed birds were eviscerated and portions of liver, kidneys and segments of small intestine were fixed in 10% neutral buffered formalin for 72 hours. Tissues were trimmed, dehydrated in a series of graded ethanol, cleared in xylene and embedded in paraffin wax. Sections of 5 μm thickness were cut using a rotary microtome and stained with haematoxylin and eosin for light microscopy.

Statistical analyses. All data were subjected to one-way analysis of variance. Treatment means were separated using Duncan's New Multiple Range Test (SAS Institute, 1992) where significant differences were detected.

Results

Chicks' performance. Severe growth depression (P<0.01), low (P<0.05) feed ingestion and its efficiency were associated with RFK, irrespective of the neem inclusion rates. On the other hand, mortality of chicks increased (P<0.01) with increasing rates of RFK (Table 1).

Table 1. Mass gain, food ingestion and mortality of cockerels offered RFK (mean ± s.d)

Performance trait	Concentration of RFK (g/kg diet)				
	0	75	150	225	300
Mass gain (g/bird)**	198.5 ± 7.9[a]	166.6 ± 9.8[b]	166.7 ± 8.5[b]	152.3 ± 9.1[b]	127.0 ± 8.8[c]
Food ingestion (g/bird)*	758.4 ± 22.7[a]	711.2 ± 24.2[b]	733.9 ± 29.3[b]	729.0 ± 26.4[b]	684.9 ± 24.3[b]
Mortality of cockerels (%)**	3.3[d']	20.0[c]	56.7[b]	66.6[b]	100.0[a]

Means in a row with different superscripts differ. *(P<0.05); **(P<0.01)

Haematology and plasma chemistry. Values of parameters from blood samples of birds fed on the test diets were altered by RFK compared to the reference group (Table 2). Haematocrit percentages were highly (P<0.01) reduced in birds fed 150 g or more RFK/kg diets. Erythrocyte counts were also reduced (P<0.01) at 225 g RFK/kg. Conversely, progressive heterophilia was observed as RFK in diets increased, and the counts were significantly (P<0.05) higher in birds on diets containing 150 g or more of RFK/kg.

Table 2. Haematology of chicks offered reference and test diets

Blood constituents	Diets			
(g RFK/kg)	(0)	(75)	(150)	(225)
Haematocrit (%)**	32.5 ± 2.6^a	30.6 ± 1.8^a	22.0 ± 2.0^b	19.7 ± 1.5^b
Erythrocytes ($\times 10^6$/mm^3)**	2.6 ± 0.2^a	2.3 ± 0.1^a	1.8 ± 0.2^{ab}	1.6 ± 0.1^b
Haemoglobin (g/dL)**	10.2 ± 0.5^a	9.8 ± 0.3^a	7.6 ± 0.4^b	7.3 ± 0.3^b
Total leucocytes ($\times 10^3$)*	19.9 ± 1.1^a	20.5 ± 1.4^b	23.7 ± 1.2^b	25.2 ± 1.8^a
Lymphocytes (%)*	56.3 ± 2.5^a	52.8 ± 2.1^a	48.2 ± 3.0^b	45.5 ± 2.3^b
Heterophils (%)*	26.8 ± 1.7^b	32.0 ± 1.9^{ab}	37.1 ± 1.8^a	38.9 ± 2.1^a
Monocytes (%)	10.2 ± 0.6	9.3 ± 6.3	10.0 ± 0.7	11.4 ± 0.5
Eosinophils (%)	3.4 ± 0.0	3.9 ± 0.2	2.9 ± 0.1	3.0 ± 0.2
Basophils (%)	1.5 ± 0.0	2.0 ± 0.1	1.8 ± 0.0	1.2 ± 0.1

[ab] Means in a row with different superscripts differ significantly.*(P<0.05); ** (P<0.01).

Table 3. Some plasma metabolites from chicks fed on reference or test diets

Plasma metabolites	Diets			
(g RFK/kg)	(0)	(75)	(150)	(225)
Alanine aminotransferase (U/L)	10.3 ± 2.8	9.6 ± 2.2	11.2 ± 2.5	9.7 ± 2.0
Aspartate aminotransferase (U/L)	49.2 ± 3.1^b	55.5 ± 3.6^b	95.8 ± 3.4^a	76.6 ± 3.3^a
Cholesterol (mg/dL)	152.6 ± 28.1^a	148.1 ± 25.3^a	105.3 ± 19.9^b	100.0 ± 22.6^b
Total bilirubin (mg/dL)	$0.54 \pm .07$	$0.56 \pm .09$	$0.48 \pm .10$	0.58 ± 0.07
Conjugated bilirubin (mg/dL)	$0.14 \pm .03$	0.13 ± 0.1	$0.14 \pm .02$	$0.15 \pm .04$
Uric acid (mg/dL)	3.1 ± 0.6	3.2 ± 0.4	$2.3 \pm .05$	2.8 ± 0.05
Creatinine (mg/dL)	1.0 ± 0.2	0.9 ± 0.1	0.8 ± 0.1	1.2 ± 0.2

[ab]Means in a row with different superscripts differ (P<0.05).

Monocyte count was slightly (P>0.05) increased by RFK at the rate of 225 g/kg. Similarly, eosinophilic and basophilic counts were not significantly influenced by RFK content of the diets. Of the plasma metabolites examined (Table 3), aspartate aminotransferase activity increased (P<0.05), and cholesterol concentration significantly (P<0.05) decreased with increasing dietary contents of RFK. However, none of the treatments resulted in significantly altered alanine aminotransferase activity. In addition, no differences (P>0.05) in plasma bilirubin (total or conjugated), uric acid and creatinine concentrations were found among birds on the test or control diets.
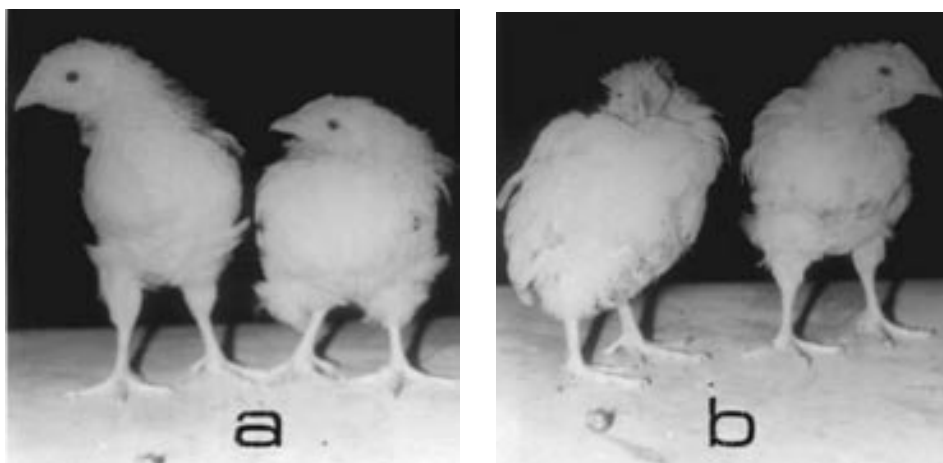
Fig. 1. Shows cockerels on control diet (a) and on diet containing 225 g RFK/kg (b). Note the toxicity symptoms, including weakness of the neck and pupilary constriction of eyes of experimental birds.
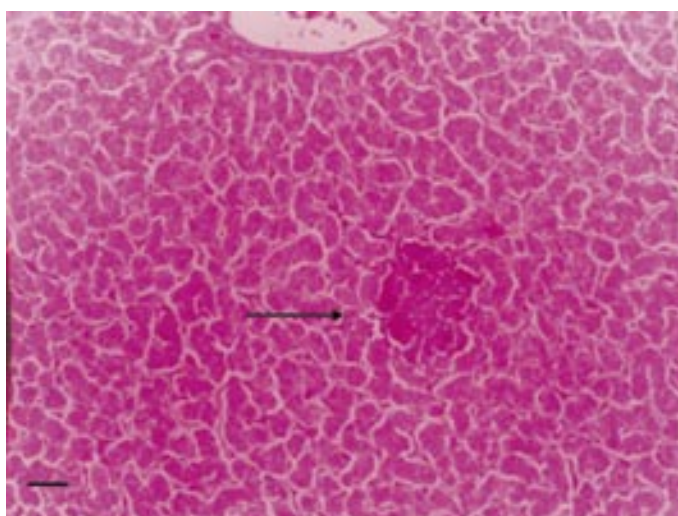

Fig. 2. Liver of cockerel on 225 g RFK/kg diet. Note the coagulative necrotic focus at periportal area (arrow) (H&E; ×200, scale bar = 40 μm)
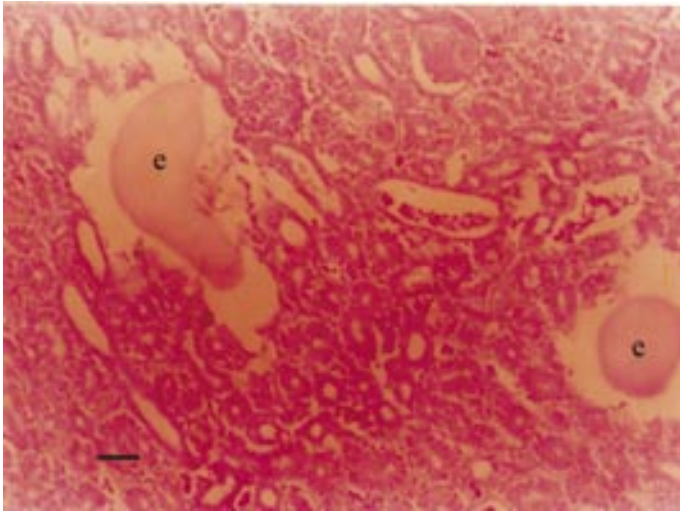
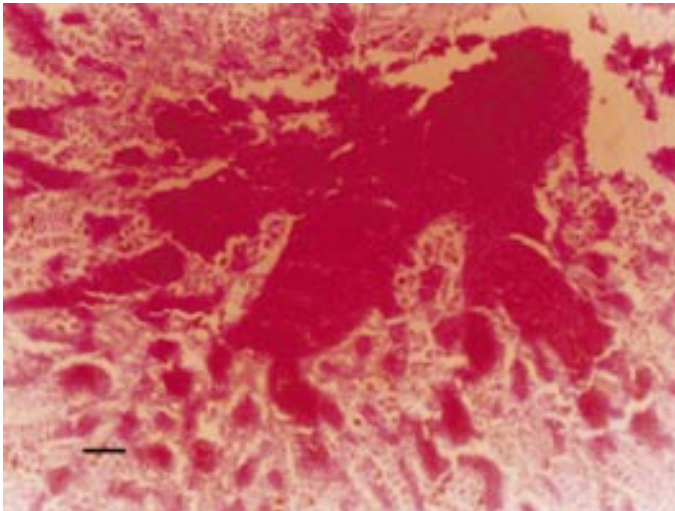Fig. 3. Kidney of cockerel on 225 g RFK/kg diet. Note the oedema (e) (H&E; × 200; scale bar = 40 µm).

Fig. 4. Kidney of cockerel on 225 g RFK/kg diet showing severe congestion and haemorrhagic spots (H&E; × 200; scale bar = 40 µm).
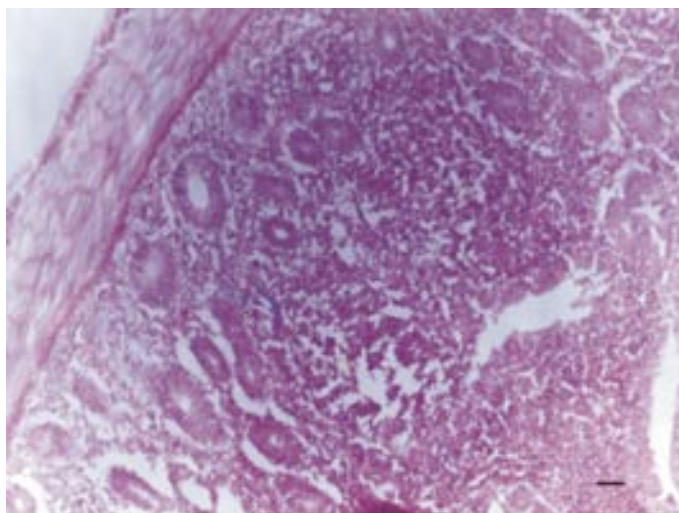
Fig. 5. Small intestine of cockerel exposed to 225 g RFK/kg diet. Note the necrotic enteritis which extended to the sub-mucosa (H&E; × 200; sclale bar = 40 μm).

Pathology. Clinical examination of birds fed 150 g or higher levels of RFK/kg diet revealed loss of body mass and the keel bone became very prominent; ruffled appearance of feathers, constriction of eye pupils and head retraction (Fig. 1b) especially in birds that were apparently ill. Autopsy showed marked pallor of the carcasses. Catarrhal enteritis was observed in all the birds on the test diets. Jejunal segment of the small intestine of birds given the high doses (150 or 225 g/kg) of the test material was haemorrhagic. The liver and kidneys of the test groups were enlarged and congested compared to those of the reference group. Histological evaluation of the tissues revealed the following changes: In the liver the most consistent finding was a high incidence of focal coagulative necrosis (Fig. 2). The sinusoids were extensively dilated and congested. Kupffer cells were hypertrophic and their cytoplasm vacuolated. Focal nephritis with marked oedema was common in the kidneys (Fig. 3). Degeneration of tubular lining cells and the accompanied scattered heterophilic infiltration were extensive. Also, marked vascular congestion with areas of haemorrhages were regular features in the kidney (Fig. 4). In the small intestine, severe mucosal necrosis, which extended to the sub-mucosal glands, resulted in sloughing of the villi (Fig. 5). There was also extensive acute inflammatory cell infiltration of the necrotic areas. These lesions were milder in birds on a lower rate (75 g/kg) of RFK.

Discussion

The necrotising lesions caused by RFK in the small intestine destroyed the intestinal villi responsible for absorption of feed, which is needed for good performance of chicks. Hence, much of the nutrients consumed in feed by the chicks on the test diets were wasted and could not be converted into poultry products. The severe anaemia induced by RFK was, at least in part, haemorrhagic in nature due to extensive damage of the vascular capillary network, as was evident from the gross and microscopic investigations. Severe haemolysis as a contributory factor to the blood loss in this study was unlikely because no significant differences were found in bilirubin concentrations between the chicks on the reference or test diets. The significant rise in total leucocyte counts of birds on the test diets resulted from heterophilia due to acute inflammatory lesions and massive tissue necrosis. Lymphopenia seemed to be exaggerated by the proliferative response of heterophils to the increasing toxic challenges from increased dietary levels of RFK, but absolute lymphocyte counts did not differ significantly among the dietary treatments. The low plasma cholesterol levels of birds fed 150 g or more RFK/kg diet was attributed to poor absorption from the digestive tract and/or decreased hepatic lipogenic activity. RIVETZ et al. (1977) previously observed a decrease in serum cholesterol in domestic fowl with Borrelia anserina infections, which they ascribed to decreased intestinal absorption due to enteritis or decreased liver synthesis. The higher aspartate aminotransferase activity in birds with damaged liver is consistent with other reports in which increased enzyme activity had been associated with hepatocellular damage in chickens, turkeys, caged birds (primarily psittacine) and ducks (MOLANDER et al., 1957; CORNELIUS et al., 1959; ROZMAN et al., 1974; LOHR, 1975; RIVETZ et al., 1977; PEARSON et al., 1979; ROSSKOPF et al., 1982). Alanine aminotransferase activity appeared stable in all the treatment groups contrary to the observations of NAGALAKSHMI et al. (1996) who reported significantly lower activity of the enzyme in broiler chicks fed on alkali-treated neem kernel cake. Indeed, the enzyme activity has been reported to be low in body tissues (heart and skeletal muscles, liver and lung tissues) of chickens and geese (CURTIS et al., 1980; CAMPBELL and COLES, 1986) and may not be a good diagnostic tool in poultry diseases. The discrepancy, arose, perhaps, from the different neem products used. The inflammatory, haemorrhagic and necrotising effects of RFK accounted for the high mortality of chicks in dose-dependent relationship. The neem triterpenoids were probably responsible for the RFK toxicity, although this proposition was not tested in the present study. However, studies with related triterpenes of Lantana camara origin, especially lantadene A, produced lesions similar to those of neem. For instance, triterpene has been reported to produce severe acute zonal necrosis of the liver, hepatocellular enlargement and fine cytoplasmic vacuolation, together with some degree of bile accumulation in canaliculi, hepatocytic cytoplasm, and nonspecific renal tubular necrosis (KELLY, 1993). On the other hand, the cholestatic activity of Lantana triterpenes with extensive icterus of carcasses and organs of animals (KELLY, 1993) was not

obvious in this study. In conclusion, RFK induced necrotizing and haemorrhagic lesions. Neem toxins appeared to have an affinity for fast growing cells such as epithelium of the digestive tract, vascular endothelial cells and hepatocytes. The ability to incorporate neem seed kernel in poultry diet will, therefore, depend on the processing techniques that will eliminate neem toxins, especially the triterpenoids.

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SAŽETAK

Istražena je toksičnost sirovih punomasnih sjemenki biljke *Azadirachta indica* za pjetliće. Količine od 0, 75, 150, 225 i 300 g sjemenki/kg nadomještene su za istu količinu kikirikija u obroku koji je davan skupini od 30 pjetlića *ad libitum* u tijeku 35 dana. Sirove punomasne sjemenke smanjile su apetit (P<0,05) i tjelesni prirast (P<0,01) pjetlića bez obzira na primijenjenu količinu. U količini 150,0 g/kg i više, sjemenke su također izazvale hemoragijsku anemiju (P<0,01) i leukocitozu (P<0,05). Leukocitoza je bila uglavnom rezultat heterofilije. Pri dodatku količine od 150,0 g/kg obroka i više, aktivnost aspartat aminotransferaze bila je povećana (P<0,05), a koncentracija kolesterola smanjena (P<0,05). Aktivnost alanin aminotransferaze te koncentracije bilirubina, kreatinina i mokraćne kiseline nisu bile značajno različite u pjetlića različitih skupina. Među poremećajima koji se mogu dovesti u svezu s uporabom sjemenki u hrani opaženi su mršavi i blijedi trupovi, kongestija bubrega i upala sluznice crijeva. Mikroskopski je uočena koagulacijska nekroza sluzničnih stanica, uključujući submukozne žlijezde te ljuštenje crijevnih resica, žarišno propadanje jetrenih stanica i hemoragijski odnosno edematozni nefritis. Ukupni učinak očitovao se u značajno (P<0,01) većoj smrtnosti pjetlića povezanoj s količinom dodanih sjemenki, a svi pjetlići hranjeni količinom od 300,0 g/kg obroka uginuli su prije 35. dana od početka davanja sjemenki.

Ključne riječi: punomasne sjemenke, *Azadirachta indica*, toksičnost, pjetlići