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Detoxification Characteristics of Dietary Neem Kernel Meal Treated Under Carbon Dioxide Environment and Lyle: Effects on Haematology, Histology and Biochemical Indices in Swine

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Target Audience: Nutritional biochemists, livestock producers, feed producers

Abstract

Investigations on the phytochemical content, protein-nature and feeding potential of neem kernels of Nigerian origin were conducted. Having subjected the whole kernel meal to serial treatments in carbon dioxide environment and lyle the treated and untreated meal were mixed in diets at graded levels. 16-weanling piglets from a commercial strain (Large white x Duroc), averaging 12± 0.5 kg were used for the trial which lasted 21 days. Results showed that raw neem kernel meal contained high concentrations of the azadirachtin, salannin, nimbin and desacetyl-salannin, was very low in protein-nitrogen (3%) and also low in some essential amino acids namely lysine and threonine, 3.4g/16g nitrogen and 3.2g/16gN respectively. Treatments decreased the phytotoxin levels to negligible amounts while increasing the level of protein, nitrogen and amino acids. Treatments however failed to eliminate the bitter taste of neem alkaloids. Data on groups of pigs fed untreated or raw dietary neem kernel meal presented abnormal values on haematological indices, biochemical determination namely tissue soluble enzymes, serum total protein, albumin, globulin, urea, bilirubins and organ histology which were adversely affected relative to the groups receiving treated neem kernel meal and the standard diets ($p < 0.05$). Correlation of enzyme activities in piglets fed raw neem in diets as a functional test for nutritional adequacy of the test feedstuff revealed that the activities were also adversely affected compared with the treated neem kernel meal or the control diet ($p< 0.05$). Enzymes activities on the treated neem based diet was normal similar to those on the control diet ($p > 0.05$). If the neem kernel is processed to eliminate anti-nutritional factors and is supplemented with the deficient amino acids, it may provide an alternative energy and protein source for livestock.

Key words: Neem kernel meal, treatments, feeding potential, weanling pigs.

Description of Problem

Many plants useful to man and animal as foodstuff contain natural chemical compounds referred to as phytotoxins. These toxic factors are capable of inducing adverse effects, especially in monogastric animals when consumed. The toxicants can be thermo-labile or heat insensitive. The latter constitute more problems than the former. Neem, *Azadirachta indica* A. Juss, is known to harbour a wide variety of the thermostable natural chemical

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compounds. Preliminary studies (1) revealed that neem (margosa) contains secondary metabolites collectively called azadirachtins made of nimbin, nimbidin, nimbinin, nimbosterol, sugiol, nimbiol. Subsequent works (2, 3, 4, 5,) showed that neem harbours the alkaloids, salannins, heat-stable phenols and related analogues. Numerous investigations have been conducted on the effects of neem products on invertebrates and vertebrates including man. However, there exist doubtful reports on the toxicitity of azadirachtins including the other chemical compounds of neem in higher vertebrates. Another area of interest for research is to correlate the *in vivo* enzyme activity in vertebrates with the metabolism of the chemical compounds of neem. This is to ascertain the functional test of the feedstuff for its nutritional adequacy for higher vertebrates. Toxicological studies with vertebrates using neem seed extracts (6, 7,) found no toxic effects on rats and no toxicity was observed in albino rats and mice fed nimbidin at 2000mg/kg or 100mg/kg. On the other hand, extract of fresh neem leaves caused 67 - 75% mortality in guinea pigs and 80 - 90% death in experimental rabbits (8). Neem extract was found to be spermatocidal and was 100% effective in preventing pregnancy in rhesus monkeys and human beings (9), reduced blood pressure while increasing respiratory rate in dogs. It was reported (2) that oil from neem is bitter but not poisonous. Neem oil is used to treat beans before storage against pests (10). Neem fruits/seeds are recommended for humans in Gambian medical practice (11). As opposed to these reports, a case of human poisoning with fatal results has been reported (12) and it was suspected that margosa, neem fruit was responsible. It would appear, judging from the controversial reports on neem that its potential use in nutrition is not worthwhile. It is against this background that attempts have been made in this study to improve the nutritional value of neem kernels which abound every where in Nigeria - with promising potential as cheap alternative feedstuff for livestock especially monogastric animals that share similar foodstuffs with humans.

Materials and Method

Procurement and treatment of neem kernels

Ripe neem fruits were harvested by stripping from branches or collecting from the ground. Treatment of the fruits was initiated by soaking in water for 24h to soften the pulp and the kernels obtained by

Table 1. Percent composition of the experimental diets on as fed basis.

Dietary treatments	1	2	3	4
Ingredients	Contrl.	Raw	Raw	Treated
Maize	78.58	71.40	67.40	67.40
Soybean meal	18.82	6.00	-	-
Neem kernel meal	-	20.00	30.00	30.00
Dicalcium phosphate	0.84	0.84	0.84	0.84
Ground limestone	0.80	0.80	0.80	0.80
Sodium chloride	0.40	0.40	0.40	0.40
*Min.-vit. premix	0.40	0.40	0.40	0.40
Antimicrobial premix	0.16	0.16	0.16	0.16
Total	100	100	100	100
Nutrient content (%)				
DE intake, MJ/day	14524	14351	14170	14171
ME intake, MJ/day	13646	13499	13356	13356
Protein intake (as fed)	15.00	14.50	13.90	14.00

*Mineral-vitamin premix supplies 2.5kg/tonne of feed, 10,000000IU vitamin A; 300, 0000IU vitamin D3; 8000IU vitamin F; 2000mg vitamin K; 2000mg vitamin B1; 5500mg vitamin B2; 12mg vitamin B12; 10,000mg niacin; 100mg selenium; 1200mg vitamin B6; 10,000mg vitamin C; 6,000mg antioxidant; 7000mg pantothenic acid; 600mg folic acid; 500,000mg choline chloride; 60,000mg iron; 80,000mg manganese; 8000mg copper; 50,000mg zinc; 450mg cobalt; 2000mg iodine; 30mg biotin and 100,000mg magnesium.

working the softened fruits mechanically. The kernels were soaked in water overnight, removed to discard the water and the process repeated twice after which they were sun-dried then milled. The meal was subjected to anaerobic fermentation followed by lyle (an-unconventional alkali) treatment following the laid down procedures (13).

Experimental procedures

Sixteen weanling piglets from a commercial source (Large white x Duroc), with mean weight of 12± 0.5kg were used for the experiment. They were housed individually in a stall. Four iso-energetic and isonitrogenous diets were prepared: a corn-soy reference diet and three diets with 20 and 30% raw neem kernel meal (RNKM) or 30% treated neem kernel meal (TNKM). The experiment was designed as a single-factor. Each of the four treatments had four replicate stalls with a piglet/ replicate. Distribution of piglets to rations was done at random. Animals were fed *ad libitum* diets presented in Table 1 over a feeding trial that lasted for 21d.

At the termination of the feeding trial, blood samples were collected from the jugular vein of the pigs from each replicate. Blood samples for analysis of total protein, albumin, globulin, urea, bilirubins including tissue soluble enzymes were centrifuged at 3020 rpm relative centrifugal force for 15min to obtain clear sera while samples for haematocrit (Hct), erythrocyte (Rbc), leucocyte (Wbc) or haemoglobin (Hb) counts were taken in sterile heparinised vacutainer tubes. Histopathological studies were conducted by euthanising the pigs and the organs, brain, liver and jejunum were taken. Tissues were fixed for 24h before embedding in wax. Slides were prepared and studied using the Ortholux Leitz Wetzlar microscope.

Chemical analyses

The nutrient content of diets, proximate composition of raw neem kernel meal(RNKM) and treated neem kernel meal (TNKM) were determined according to the conventional procedures of AOAC (14). The quantification of azadirachtins, desacetylsalannins, nimbins and salannins was performed using high performance liquid chromatography, HPLC reversed phase procedure in which the quantities of the phytotoxins of neem kernels were achieved through the use of external standards and valley-to-valley integration (15). Amino acids profile of both RNKM and TNKM was determined employing the Eppendorf (Biotronik) LC 3000 amino acids analyser. Percentage protein-nitrogen in raw and processed neem samples was carried out using the automated FP-2000 protein-nitrogen analyser (Leco Corp.). Blood samples were analysed for Hct, Rbc, Wbc and Hb as described by Dacie and Lewis (16). Serum total protein, albumin and globulin were determined as outlined by Wootton(17), while the blood metabolites, urea and bilirubins were estimated using the laid down methods of Scott and Searcy(18, 19). The enzyme activities of alanine amino transferase (ALT; EC 2.6.1.2), acetyl cholinesterase, alkaline phosphatase (AP; EC 3.1.3.1) and total acid phosphatase were determined according to the new conventional methods in UITH (20).

Statistical analysis

Data were subjected to analysis of variance using the model for a one-way classification design. Significant differences between means were compared by Student-Newman-Keuls test. SAS program (21) was used for statistical analysis.

Results and Discussion

Table 2. Summary of evaluation of the HPLC results on determination of treated & untreated neem kernel meal chemical constituents

Determination mg/g	Raw	Treated
Azadirachtin A		
Mean value	**2.380**	**0.141**
Standard deviation	0.044	0.001
Desacetyl-salannin		
Mean value	**0.812**	**0.040**
Standard deviation	0.009	0.001
Nimbin		
Mean value	**1.077**	**0.049**
Standard deviation	0.001	0.001
Salannin		
Mean value	**3.484**	**0.125**
Standard deviation	0.042	0.001

The summary of evaluation of the chemical constituents in RNKM and TNKM is presented in Table 2. The native RNKM gave high values of azadirachtin A, salannins, nimbin and desacetylsalannin. Treatments methods used reduced the concentrations of the neem toxins to negligible values. The toxicants in the untreated neem kernel have been reported to elicit adverse effects on livestock especially the tritiated azadirachtins followed by salannins (8, 9,) though there seems to be species differences in the mode of reaction to these compounds. The influence of neem chemical compounds on animals is also shown to be source dependent. Reduction in quantity of the toxins caused by treatments in this study suggests an improvement in the nutritional value of the meal as exemplified in the data (comparable to the control diet) on performance of swine fed TNKM in diet compared to the control diet. Similar benefits of detoxification on performance have been documented (5).

Results of analyses of raw and treated NKM for this study showed that treatment increased the protein content of the meal from 14.98 in raw meal to 19.75 % in treated meal and the mineral content (ash) from 2.02 to 2.65mg/100g. Treatment with lyle achieved reduction in the fibre content of neem kernel meal from 23.01 in raw meal down to 22.00% in lyle treated meal. Alkali treatment has been shown to make nutrients available or increase nutrient digestibility by breaking down the alkali-labile bonds (22, 23). The decrease in fibre content observed is of nutritional advantage to the monogastrics since they lack enzymes to handle high dietary fibre. Alkali treatment also has been shown to improve palatability since it was reported to improve the taste following alkali treatment of water-washed neem seed cake for cattle (24). Swine with a high sense of taste cannot tolerate the extreme bitter taste of neem products if fed in the raw form.

Data obtained on the protein nature of neem kernel indicated that raw neem kernel is very low in protein-nitrogen (3%). Amino acid profile of the neem kernel meal also showed that the kernel is grossly deficient in some essential amino acids, lysine (3.4g/16gN) as compared (25) to the reference standard.

The chemical score (26) of neem kernel meal protein is 42 as compared with 100 for whole egg.

Table 3. Amino acid profile of raw & treated neem kernel meal & FAO/WHO (1973) reference pattern

Amino acid	Raw	Raw	Treated	Treated	FAO/WHO
Essential	mg/g	g/16gN	mg/g	g/16gN	
Isoleucine	4.33	3.38	5.50	3.55	4.00
Leucine	8.31	6.49	10.62	6.86	7.00
Lysine	4.80	3.10	5.26	3.40	5.50
*Methionine	2.05	1.32	2.08	1.34	
*Cystine	3.34	2.16	3.54	2.69	
*Phenylalanine	4.43	3.46	6.14	3.97	
*Tyrosine	2.47	1.93	3.27	2.11	
Threonine	3.77	2.94	5.01	3.24	4.00
Tryptophan	1.46	0.91	1.55	0.94	1.00
Valine	6.36	4.97	8.36	5.40	5.00
Non-essential					
Aspartic acid	10.38	8.11	14.83	9.58	
Glutamic acid	29.36	22.94	40.10	25.90	
Alanine	5.06	3.95	6.78	4.38	
Arginine	7.38	5.77	13.64	8.81	
Glycine	5.17	4.04	6.31	4.08	
Histidine	1.88	1.47	3.16	2.04	
Proline	5.12	4.00	6.69	4.32	
Serine	5.37	4.19	5.37	4.22	

FAO/WHO (1973) requirement for methionine+cystine is 3.50g/16gN and for phenylalanine+tyrosine is 6.00g/16gN

The low nutrient content of RNKM explains the reason why incorporation direct in diet is always accompanied by poor performances and other adverse effects in experimental animals. Treatments of the kernel meal for this study brought accretion of protein-nitrogen (from 3.0 to 5.3%) and some essential and non-essential amino acids. Thus, if the antinutritional factors are removed and the neem products supplemented with the deficient amino acid, the kernel meal may provide an economic supplement feedstuff.

Table 4. Influence of untreated & treated neem kernel meal based diets on some haematological indices in swine

Dietary treatments	1	2	3	4	SEM
Haematological indices					
Hct(%)	38.66[a]	27.66[b]	24.00[b]	35.66[a]	1.4
RBC ($x10^{12}$/L)	261.33[a]	195.00[b]	179.66[c]	259.00[a]	2.6
WBC ($x10^{9}$/L)	18.06[a]	10.88[c]	8.86[c]	15.67[b]	0.66
Hb(%)	62.66[a]	41.00[b]	38.33[b]	58.67[a]	2.7

Treatment means in rows followed by different letters are significantly different ($p < 0.05$)
Hct, hematocrit; Rbc, red blood cells; Wbc, white blood cells; Hb, hemoglobin

Table 4 presents Hct, RBC, WBC and Hb counts from pigs given raw meal in diets compared with the treated meal and the control diets. RNKM based diets decreased the counts on blood corpuscles relative to the TNKM and the standard diets ($p < 0.05$). Decreased blood cells count on raw kernel meal diets suggested injurious action of the neem phytotoxins and a dysfunction in haematopoiesis. Raw neem kernel contains high levels of the toxins, tritiated azadirachtins, salannins, nimbin, nimbidin, desacetyl-salannin which have been implicated in the pathology in the fed animals. Another deleterious effect of feeding raw neem has been reported by workers (9) who demonstrated that the anti-feedant fraction «G» extract from neem kernel lowered blood-sugar and exhibited male contraceptive properties. Effects on haematology following intake of dietary raw neem meal in this study are in line with related works (27) which reported progressive degradation of blood corpuscles during intoxication of lectins. Decline in blood corpuscles, particularly Wbc manifests a fall in production of defence mechanism to fight infection thereby lowering the animal's immunity to infection. Result on blood cellular constituents recorded on diet with treated neem kernel meal was normal comparable to that on the reference diet. Improved leucocyte count accompanying feeding of treated neem kernel meal in diet indicated an improvement in disease resistance for the pigs maintained on such a diet. Since haematological indices vary under different nutritional and pathological studies, certain biochemical determination such as total protein, albumin, globulin, urea, bilirubins and tissue enzyme activities were investigated to provide further information to fully assess the feeding value of treated and untreated neem meal in diet.

Table 5. Influence of untreated & treated neem kernel meal based diets on some biochemical indices in swine

Dietary treatments	1	2	3	4	SEM
Biochemical determination					
TP(g/L)	70.00[a]	48.00[b]	44.00[b]	68.33[a]	2.0
ALB.(g/L)	31.66[a]	21.66[b]	18.66[b]	30.33[a]	1.5
TG(g/L)	29.03[a]	17.66[b]	14.66[c]	28.33[a]	0.72
BUN(mmol/L)	1.13[a]	4.52[c]	8.06[d]	3.33[b]	0.55
TB(µmol/L)	0.13[a]	2.56[b]	3.79[c]	0.74a	0.31
CB(µmol/L)	0.092[a]	0.048[b]	0.027[c]	0.08[a]	0.0057
FB(µmol/L)	1.10[a]	2.84[b]	3.67[b]	1.28[a]	0.40

Treatment means in rows followed by different letters are significantly different ($p < 0.05$)
TP, total protein; ALB., albumin; TG, total globurin; BUN, blood urea nitrogen; TB, total bilirubin; CB, conjugated bilirubin; FB, free bilirubin

Table 5 shows the influence of untreated and treated neem kernel meal diets on some biochemical determination in swine. Total serum protein and albumin levels on TNKM diet were normal and similar to those on the control diet. Total serum protein and albumin values on RNKM diets were low and inferior to those of the TNKM or standard diet ($p < 0.05$). Total protein is known to be a measure of storage amino acids. Enhancement of

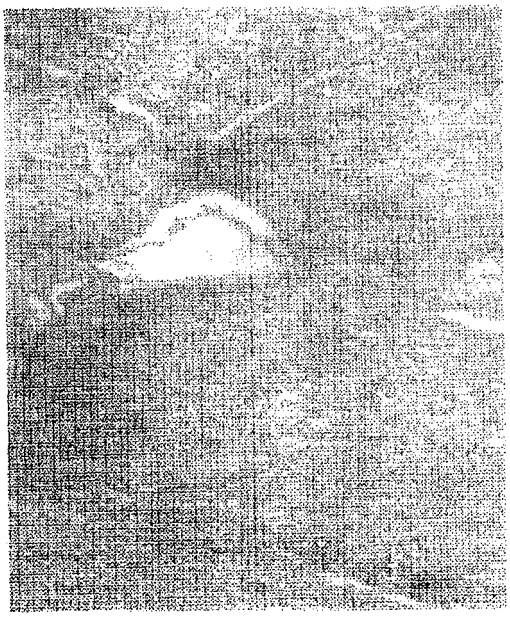
this implies a rise in amino acid absorption and utilisation and vice-versa. Reduced total protein or albumin manifests an alteration in normal systemic protein metabolism, attributed in part to interference in protein utilisation. Previous reports (8, 9) had indicated that the chemical compounds in neem products may be the major factors affecting protein synthesis or utilisation in fed animals. Raw neem contains trypsin inhibitors. The trypsin inhibiting activity of neem kernel meal is 15 units/mg protein (8). The turnover of total globulin in pigs receiving treated neem meal diet was as good as the control diet while total globulin in pigs fed raw neem meal diets was reduced ($p < 0.05$). Reduction in total globulin may indicate that the gamma globulin fraction is automatically reduced with a concomitant reduction in humoral immunity. Observations on blood metabolites, urea nitrogen, total, conjugated and free bilirubins showed high concentrations on diets containing raw neem kernel meal relative to the treated meal or control diet ($p < 0.05$). Concentrations of the metabolites on treated neem meal diet as well as the standard diet were low or normal. Similar works (28) reported increases in metabolic wastes in rats dosed with raw beans in diets. These authors showed that the increased activities of amino acids degrading enzymes, arginase and ornithin-trans-carbamoylase are responsible for elevated levels of blood metabolites. Blood levels of these substances are used as an index of severe pathology and poor protein metabolism. Accumulation of these substances, beyond the normal threshold is considered dangerous to the body. Elevated level of blood urea for instance, is an index of high ammonia production which is extremely toxic, especially in monogastrics. It is also an indication of poor quality dietary protein. Increased blood metabolic wastes observed could be mediated via the high level phytotoxins in untreated neem products which reduced protein utilisation while increasing the catabolism of amino acids that were subsequently degraded into these metabolites. The imbalance amino acid of neem kernel especially with regards to lysine could as well cause elevation in the blood metabolites observed (29).

Table 6. Influence of dietary treated & untreated neem kernel meal on certain enzymes activities in swine

Dietary treatments	1	2	3	4	SEM
Enzyme activity (IU/l,37°C)					
ACE	27.70a	0.53b	0.41b	27.33a	0.66
ALT	18.33a	12.67b	14.44b	20.00a	0.91
AP	26.33a	19.11b	22.9b	28.00a	1.40
TAP	4.01a	2.50b	2.71b	4.35c	0.69

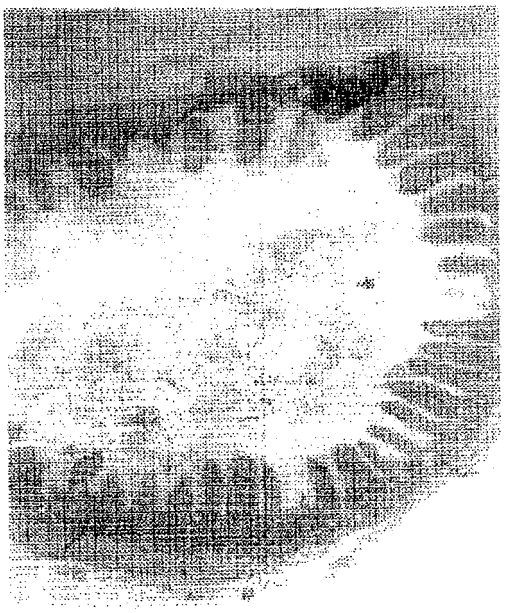
Treatment means in rows followed by different letters are significantly different ($p < 0.05$)
ACE, acetyl-cholinesterase; ALT, alanine amino transferase; AP, alkaline phosphatase; TAP, total acid phosphatase

Effects of dietary treated and untreated neem kernel meal on some tissue soluble enzymes in swine are shown in Table 6. Acetyl cholinesterase (ACE) activity in pigs fed raw neem kernel meal in diets decreased significantly ($p < 0.05$). ACE activity on treated neem kernel meal and the control diets was normal. Piglets offered RNKM rations had reduced alanine amino transferase (ALT), alkaline phosphatase (AP) and total acid phosphatase (TAP) relative to the control or treated neem kernel diet ($p < 0.05$). Increasing dietary level of raw neem meal from 20 to 30% did not influence the activities of ALT, AP or TAP. The correlation of activity of the enzymes with the metabolism of neem products is important for supplying extra information on their interaction within whole body system. The primary physiologic role of ACE is the regulation of acetyl-choline, a chemical transmitter of synapses. ACE is usually present in high concentrations at terminals of cholinergic neurons (30). Decreased concentrations of ACE in pigs dosed with RNKM in diets suggest that the regulation and functions of neurons and other complex functions of the CNS in general, that depend on ACE will be adversely affected. ALT is essential in the metabolism and energy processes of cells. The decrease in ALT activity observed in this study might be indicative of decreases in cellular metabolism and energy in the body of the fed animal. AP is a membrane associated enzyme present in most animal tissues. The organs with



Micrograph 1. Liver of a Piglet fed treated NKM diet with normal hepatic structure as control

Micrograph 1. Liver of a Piglet fed treated NKM diet with defects



Micrograph 3. Jejunum of a Pig offered treated NKW diet depicting normal, tail villi compare to the control. Mag: x 400

Micrograph 4: Jejunum of a Pig fed raw NKM die with inflammation, ulceration or sloughing villi. Mag: x 400

high AP activity are those involved in active transport mechanism like the liver, kidney, heart, intestine (31). In the present study, reduced or low AP activity was recorded from pigs on native neem kernel meal based diets as against the groups maintained on the treated neem meal diet and the standard diet, indicating that the hydrolysis of monophosphoric esters in the organs assayed was concomitantly depressed (32). The presence of AP in amounts determined by the body's physiological needs is essential for proper functioning of organs. However, very low or extremely high AP activity can precipitate a threat to the life of body cells which depend on phosphate esters for vital processes (33). Very low AP activity observed might be due to the deleterious effects of raw neem phytotoxins acting in concert with other antinutrients to produce the adverse effects on the activity of the enzymes in the body of animal. Like AP, a given amount of TAP is required in the body cells for destruction of unwanted materials. Lower TAP activity as observed might create malfunctioning of the body cells concerned. It can be stressed from the present study that neem products play a negative role in the activities of the enzymes/esterases studied.

Micrographs 1-4 show results on the histological alterations that occurred in the liver and intestine of pigs fed diet with treated or raw neem kernel meal respectively. The livers of piglets fed treated neem kernel meal diet showed normal hepatic architecture similar to those receiving the control diet (Micrograph 1). Pigs receiving diets with unprocessed neem kernel meal showed livers with necrosis, lesions or congestion (Micrograph 2). The jejunums of pigs offered treated NKM diet depicted normal, tall villi comparable to those on the standard diet (Micrograph 3). Ingestion of diets containing raw neem kernel meal caused inflammation and ulceration or sloughing off of villi epithelial (Micrograph 4). The histological studies showed that pigs fed untreated neem kernel meal elicited defective organs while those given treated-NKM meal diet produced normal organs. These findings are in line with those of early studies which reported gastrointestinal tract lesions following ingestion of large doses of dietary phytotoxins (34). Similarly, it has been demonstrated that continued intake of sub-lethal doses of phytotoxins caused corrosion of organs (35). In conclusion, the optimum utilisation of neem products as cheap alternatives to the conventional energy and protein feedstuff will be possible if the neem product is given adequate treatment especially treatments that involve removal of the inherent phytotoxins and the extreme bitter taste followed by supplementation with the limiting amino acid, lysine.

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