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Evaluation of Raw and Detoxified Neem Cake in the Diets of Wistar Rats (*Rattus norvegicus*) as Partial Protein Supplement

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ABSTRACT

Present experiments were conducted to test raw and detoxified neem cake as a replacement of soybean meal in the diets of Wistar rats, Replacement of up to 25% soybean meal with DNC only resulted in positive growth rate (0.60 g/d) in rats compared to control (1.55 g/d). Low levels of DNC supplementation (12.5% in experiment 1 and 25% in experiment 2) caused similar nutrient intake and digestibility compared to control group. The supplementation of DNC did not exert any abnormal changes in blood profiles. Serum testosterone levels were lower (P<0.05) in rats that received DNC at 50% and RNC at 25%. Mild changes in intestinal villi were observed in DNC 25% group. Feeding of DNC was better (P<0.05) than RNC and replacement of SBM nitrogen with DNC up to 25% did not affect growth, feed consumption, blood profiles and histopathology.

Key words: Detoxification, Digestibility, Neem seed cake, Protein Supplement, Rats

INTRODUCTION

Raw neem seed cake (RNC) as a residue after oil extraction is high in crude protein (36- 38%) and its estimated availability is around 0.9 million tonnes per annum in India alone (Radhakrishna, 2003). The RNC, being used as fertilizer-cum-pesticide, was found unsuitable for animal feeding due to presence of bitter and toxic triterpenoids (azadirachtin, salannin, nimbin, nimbidiol *etc.*). The bitterness of neem is attributed to limonoids which are the triterpenoids and through water washing of cake converted it into a wholesome protein substitute (Nath *et al.,* 1983; Agrawal *et al.,* 1986; Nath *et al.,* 1989; Sastry and Agarwal, 1992) which led to a loss of 22% DM due to washing. To avoid such loss, processing the cake in alkaline medium without water washing was tried either by soaking it in water (1: 5 w/v) containing either NaOH (2% w/w) for 24 h or by ensiling with 2.5% urea (w/w) for 5-6 d (Nagalakshmi *et al.,* 1996; 1999).

The sundried and ground alkali treated and urea ammoniated neem seed kernel cake was found to be suitable for feeding of growing lambs (Musalia *et al.,* 2000), kids (Anandan *et al.,* 1999) and broiler chicken (Nagalakshmi *et al.,* 1996 and 1999) without affecting their growth, nutrient utilization, blood profile, rumen/caecal fermentation pattern, physical, chemical characteristics of carcass including organoleptic sensory score and gross and histopathology of vital organs. Variations were observed in chemical composition and incriminating factors (azadirachtin and salannin) of neem seeds/kernels collected from different parts of Karnataka (Kaushik *et al.,* 2007). The process of detoxification of neem seed cake was standarized (Saxena *et al.,* 2010). Resultant detoxified neem seed cake (DNC) was evaluated. Hence, this study was conducted to see the effect of inclusion of both RNC and DNC in diets of rats on growth rate, nutrient digestibility, metabolic profiles and gross changes in vital organs.

MATERIALS AND METHODS

The rats were kept in well aerated laboratory cages (43 × 27 × 15 mm). Paddy husk was used as a bedding material. The cages were covered with stainless steel cage grill having provision to keep experimental feed pellets and water bottle. Clean water was provided *ad lib.* There was 12 h light and dark cycle with a light period between 06: 00 and 18: 00 h. In experiment 1, 54 Wistar male albino rats of 6 weeks age weighing between 100-130 g were purchased from recognized animal breeder. Initially, all the rats were offered a control diet (Group I) for a week. Then graded levels

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(12.5, 25.0, 37.5% replacing soybean meal nitrogen) of DNC containing pelleted diets were introduced (Group II, III, IV). Rats in Group III and IV lost weights due to poor intake. Hence, further incremental levels (50.0 and 62.5 %) were not introduced and switched to control diet.

The results of first experiment formed the basis for second experiment. Further, raw neem cake was also introduced at lower level for comparison. Two levels of replacement of DNC (25 and 50%) and one level of RNC (25%) were fed to rats. For this purpose, 32 rats were randomly divided into 4 groups of eight each and allotted to control (Group I), DNC 25, DNC 50 and RNC 25 (Group II, III and IV). The diets were continued for a period of six weeks. Two rats were kept in each cage.

A hand driven noodle making machine was procured from Mathura, Uttar Pradesh, India and 8 mm die was prepared locally. The ingredients of rat diet such as maize (*Zea mays*) grain, soybean (*Glycine max*) meal, bengal gram (*Cicer arietinum*) flour, soybean oil, milk powder and mineral vitamin premix were procured from a single source. The ingredients were thoroughly ground (in case of maize grain and soybean meal), mixed and moistened with water to make dough and compacted through hand-driven machine. The resultant pellets were sundried and stored for feeding rats. The composition of diets has been given in Table 1. The rats were fed as suggested (NRC, 1978). At the end of two weeks of experimental feeding, digestibility trials were conducted in both the experiments. The faecal pellets were carefully handpicked from each cage every day in the morning and frozen (-20°C). Pooled diets and faecal samples were dried, ground and analyzed for DM, OM, CP and EE (AOAC, 1995). The ADF content was estimated (Van Soest *et al.,* 1991). Blood was collected in both the experiments by cardiac puncture in heparinised vials and plasma was stored at -80°C till analyzed. The levels of total protein, albumin, urea, glucose and hormones (testosterone and insulin like growth factor; IGF I) were estimated in plasma. The biochemical profile was determined using kits from

Table 1. Ingredient composition (%) of experimental diets for rats

Ingredient	Group			
	dNC 0	**dNC 12.5**	**dNC 25.0**	**dNC 37.5**
Experiment-1				
Maize flour	52	53	54	56
Bengal gram flour	24	23	22	20
Soybean meal	16			
Soybean oil	2	2	2	2
Milk powder	4	4	4	4
Mineral vitamin premix	2	2	2	2
Neem detoxified cake	-	2	4	6
Experiment-2				
Ingredient	**dNC 0**	**dNC 25.0**	**dNC 50.0**	**rNC 25.0**
Maize flour	42	44	47	44
Bengal gram flour	24	22	19	22
Soybean meal	16	12	8	12
Ghee (Clarified butter)	2	2	2	2
Milk powder	4	4	4	4
Jaggery	10	10	10	10
Mineral vitamin premix	2	2	2	2
Neem detoxified cake/ neem raw cake	-	4	8	4

M/s Span Diagnostic Limited, Surat, Gujarat, India. Hormones were estimated by Radio-Immuno-Assay using kits (Immunotech, France). All the rats were sacrificed humanely by using anaesthesia after six weeks of feeding. At post mortem, organs like liver, kidney, adrenals, spleen, intestine and testis were collected in 10% formal saline immediately. The tissues were dehydrated, cleared, embedded and processed for paraffin block making. Sections of 5 µm thickness was made by using microtome and stained with haematoxylin and eosin stain (Robinson and Gray, 1996). The slides were observed for histological changes under light microscopy (Nikon, Japan).

The data were subjected to one way analysis of variance (Snedecor and Cochran, 1994). The statistical software SPSS (SPSS Inc., Chicago, IL, USA) was used for analysis of data and analysis variance assuming for independent constant variance structure with post-hoc Turkey to find the pair wise significance between treatments.

RESULTS AND DISCUSSION

The chemical composition of diets has been presented in Table 2. The CP content ranged from 17.89 to 18.07% in experiment 1 and from 16.43 to 16.69% in experiment 2. The diets fulfilled the requirements (NRC, 1978) for rats for growing purpose. The calculated ME (Kcal/kg diet) ranged from 3509 to 3551 in experiment 1 and from 3533 to 3592 in experiment 2.

The ADG during the period of three weeks was found to be 0.5 g in rats that were supplemented with DNC replaced at 12.5% level with soybean meal. In other groups (25 and 37.5% replaced diets), the ADG was on negative side. The rats in other cages almost returned to control during 3 weeks period. In the second experiment, ADG increased to 0.60 g in 25% DNC replaced diets compared to 0.54 g in first experiment. At higher replacement level (50%), negative ADG was observed. Similarly, when RNC replaced SBM even at 25% level, negative ADG was observed (Table 3). The DMI was found to be at par with control when DNC was incorporated replacing 25% SBM. DMI reduced ($P<0.05$) when DNC was incorporated at higher levels (37.5 and 50%). However, RNC supplementation reduced DMI even at 25% replacement level. Results indicated that DNC could overcome the negative effects of RNC on intake and ADG to a limited extent up to 25% replacement of SBM. In contrast to *in vitro* results, results of rat trials indicated a completely different picture. Even though, DNC used in the study had non-detectable levels of azadirachtin and salannin, beyond certain levels of replacement (12.5% in case of first trial and 25% in case of second trial) resulted in adverse effect of growth performance of rats. Replacement of soybean meal with water washed NSC caused depression in growth rate compared to solvent (methanol or ethanol) extracted

Table 2. Chemical composition (%) of experimental diets for rats

Attribute	NC 0	dNC 12.5	dNC 25.0	dNC 37.5
Experiment-1				
DM	93.37	93.12	93.78	93.36
OM	95.55	95.15	94.73	94.89
CP	18.07	18.06	18.00	17.89
EE	5.31	5.16	5.35	5.21
ADF	3.68	3.36	4.21	3.10
Experiment-2	**dNC 0**	**dNC 25.0**	**dNC 50.0**	**rNC 25.0**
DM	93.15	93.27	94.78	93.46
OM	95.55	94.73	94.89	94.73
CP	16.59	16.69	16.66	16.43
EE	4.99	5.00	4.99	5.00
ADF	3.68	3.36	4.21	4.50

Table 3. Growth performance of rats in different groups

Attribute	dNC 0	dNC 12.5	dNC 25.0	dNC 37.5	dNC 0 (Reversal)	SEM
Experiment-1						
Initial BW (g)	139.64^{b}	122.93^{b}	122.92^{b}	105.00^{b}	85.11^{a}	5.86
Final BW (g)	157.64^{b}	132.36^{ab}	111.49^{ab}	87.73^{a}	154.05^{b}	8.00
ADG (g)	0.86^{a}	0.45^{a}	-0.54^{a}	-0.82^{a}	3.29^{b}	0.43
DMI (g/d)	11.59^{c}	8.17^{bc}	5.28^{ab}	3.45^{a}	-	1.01
Experiment-2	**dNC 0**	**dNC 25.0**	**dNC 50.0**	**rNC 25.0**		**SEM**
Initial body weight (g)	186.33	187.70	199.45	189.90		3.98
Final body weight (g)	251.45^{c}	212.68^{b}	150.70^{a}	155.61^{a}		8.84
ADG (g)	1.55^{c}	0.60^{b}	-1.16^{a}	-0.82^{a}		0.21
DMI (g/d)	13.72^{b}	13.85^{b}	6.61^{a}	7.21^{a}		1.26

^{a,b,c}Means bearing different superscripts in a row differ significantly (P<0.05)

NSC indicating superiority of solvent extraction over water washing (James *et al.,* 2009), however, any of the treatments used did not yield comparable results over control diet which received soybean meal.

In experiment 1, the intakes of DM, OM and CP (g/d) were found to be higher (P<0.05) in control and DNC 12.5% followed by DNC 25.0% and 37.5% with similar intakes between 12.5 and 25.0% groups. In the second experiment, the intakes of DM, OM and CP were found to be similar in DNC groups replaced at 25% level to that of control compared to DNC replaced at 50% level and RNC at 25% level where low (P<0.05)

Table 4. Effect of feeding detoxified neem cake on intake and nutrient digestibility in Wistar rats

Attribute	Group				
	dNC 0	**dNC 12.5**	**dNC 25.0**	**dNC 37.5**	**SEM**
Experiment-1					
DMI (g/d)	11.59^{c}	8.17^{bc}	5.28^{ab}	3.45^{a}	1.01
OMI (g/d)	11.07^{c}	7.78^{bc}	5.00^{ab}	3.28^{a}	0.97
CPI (g/d)	2.28^{c}	1.48^{b}	0.96^{ab}	0.59^{a}	0.21
DMD	79.59^{c}	76.96^{bc}	69.85^{ab}	69.39^{a}	1.50
OMD	82.98^{c}	81.02^{bc}	75.56^{ab}	75.00^{a}	1.16
CPD	84.43	81.59	79.38	81.63	0.93
EED	84.33^{c}	78.13^{ab}	73.55^{a}	75.95^{ab}	1.48
ADFD	54.06^{c}	43.89^{bc}	21.88^{ab}	8.28^{a}	5.89
Experiment-2					
	dNC 0	**dNC 25.0**	**dNC 50.0**	**rNC 25.0**	
DMI (g/d)	13.72^{b}	13.85^{b}	6.61^{a}	7.21^{a}	1.26
OMI (g/d)	13.11^{b}	13.110^{b}	6.27^{a}	6.83^{a}	1.20
CPI (g/d)	2.276^{b}	2.311^{b}	1.102^{a}	1.184^{a}	0.211
DMD	74.40^{b}	74.83^{b}	63.60^{a}	63.71^{a}	1.76
OMD	78.57^{b}	78.45^{b}	68.33^{a}	69.03^{a}	1.52
CPD	67.79^{b}	66.04^{b}	55.47^{a}	54.52^{a}	1.99
EED	81.66^{b}	78.89^{b}	68.65^{a}	66.09^{a}	2.30
ADFD	73.60^{b}	38.84^{b}	25.02^{a}	20.21^{a}	3.04

^{a,b,c} Means bearing different superscripts in a row differ significantly (P<0.05)

levels were observed (Table 4). At low levels of replacement (12.5% of soybean meal), neem seed cake did not exert any adverse effect on digestibility of DM, OM, EE, NDF and ADF compared to control. However, CP digestibility was not affected by graded levels of neem seed cake. In the second experiment, digestibility values were found to be similar up to 25% level of replacement. Feeding of RNC at 25% and DNC at 50% level resulted in reduction in nutrient digestibility when compared to control group. The reason for low palatability and low growth performance of test rats could be due to change in taste, smell and texture of diet compared to control and not attributed to its protein quality (Aregheore *et al.,* 2003). There was a declining trend for nutrient digestibility when DNC and RNC were included at levels higher than 25%. This could be due to presence of bitter principles present in DNC or RNC which might not have been inactivated during the detoxification process. Another reason for low intake and digestibility could be due to cold pelleting process that does not involve any steam or extrusion. It was found that steam processing using pelleting or extrusion expansion increased DMI in lambs fed water washed urea ammoniated NC followed by pelleting and expanded extrusion (Madhavi *et al.,* 2006). Histopathological observations especially in intestine confirmed poor performance of rats compared to control. Similar to our results, poor performance of cockerels was observed (Uko and Kamalu, 2006) fed raw neem seed kernel as well as autoclaved neem seed kernels compared to reference diet which could be due to combined effects of poor metabolizablity of nutrients and reduced intake of feed and water. Decreased CP digestibility was observed in goats (Anandan *et al.,* 1999) fed water

Table 5. Effects on certain blood profiles and hormones

Particular	Group				
	dNC 0	**dNC 12.5**	**dNC 25.0**	**dNC 37.5**	**SEM**
Experiment 1					
Total Protein (g/dL)	6.80	7.03	6.40	5.96	0.25
Albumin (g/dL)	4.12	4.25	4.28	3.62	0.16
Globulin (g/dL)	2.69	2.77	2.12	2.34	0.19
A:G Ratio	1.57	1.71	2.29	1.57	0.16
ALT (IU/L)	17.19	19.06	21.43	19.69	1.00
AST (IU/L)	249.24	218.72	222.33	405.59	29.65
Glucose (mg/dL)	133.56	134.56	98.43	93.79	9.20
Urea (mg/dL)	41.08	38.85	42.27	52.18	2.35
Testosterone (ng/mL)	0.87	0.17	0.11	0.14	0.13
IGF-1 (ng/mL)	5.60	3.82	6.00	5.47	0.85
Experiment-2					
	dNC 0	**dNC 25.0**	**dNC 50.0**	**rNC 25.0**	
Total protein (g/dL)	6.60	6.33	6.67	6.50	0.21
Albumin (g/dL)	3.98	4.33	3.03	3.98	0.24
Globulin (g/dL)	2.62	2.00	3.63	2.53±0.52	0.24
A: G ratio	1.77	2.41	0.89	1.72	0.22
ALT (IU/L)	20.39	16.13	20.47	19.12	1.44
AST (IU/L)	158.36	220.75	189.67	219.89	13.77
Glucose (mg/dL)	130.98	93.05	99.86	78.45	5.91
Testosterone (ng/mL)	1.25^{b}	0.63^{ab}	0.16^{a}	0.24^{a}	0.13
IGF-1 (ng/mL)	5.00	8.41	3.87	5.07	1.30

[a,b]Means bearing different superscripts in a row differ significantly (P<0.05)

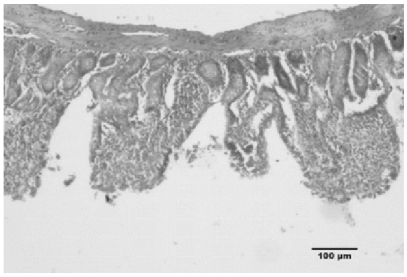

Fig 1

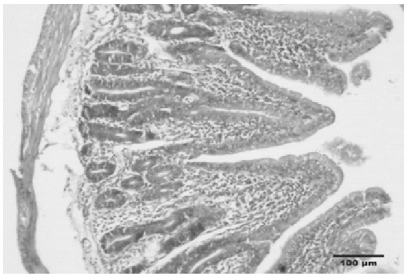

Fig 2

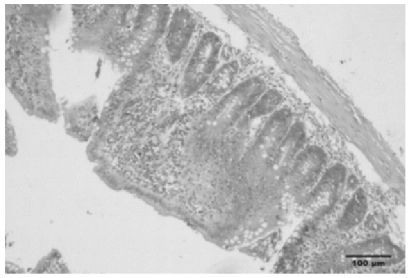

Fig 3

Fig: 1. 37.5% DNC fed group showed fusion of villi and crypt elongation with damage to the mucous membrane intestine (Experiment-1); Fig 2, 25% DNC fed group showed fusion of villi and mild damage to the intestinal mucous membrane (Experiment-2); Fig 3, 25 % RNC Intestine showed loss of mucosal epithelium, fusion of villi to large extent with mononuclear cell infiltration and elongation of crypts.

washed neem seed cake followed by urea ammoniation which could be due to binding of some nitrogen on account of ammoniation thus making it unavailable for the host animal (Solaiman *et al.,* 1979; Borhami and Johnson, 1981).

The levels of protein, albumin, globulin, A: G ratio, ALT, AST and glucose were found to be similar in different groups in both the experiments indicating no adverse effect of feeding detoxified neem seed cake in Wistar rats (Table 5). There were no differences in blood biochemical profiles like serum and liver enzymes (ALT, AST and ALP), serum bilirubin, cholesterol and protein of rats exposed to different doses of azadirachtin (Srivastava and Raizada, 2007).

Reduced sperm counts and increased frequency of spermatozoa with abnormal head morphology were observed (Awasthy, 2001) in mice exposed to alcohol extracts of neem leaves (500, 1000 and 2000 mg/kg body weight). The deleterious effects on reproduction and teratogenicity are manifested by significant changes in survival and growth index of pups and skeletal malformation by the azadirachtin (Biegel *et al.,* 1998). The reduced testosterone concentrations in rats fed DNC and RNC groups observed in the present study corroborated with reduced fertility levels (Awasthy, 2001). There was reduction in semen velocity parameters like curvilinear velocity, straight-line velocity, average path velocity and type-A spermatozoa. Such indices were reduced in detoxified karnaja (*Pongamia pinnata*) cake (dKC) feeding to rams replacing 75% of SBM (Dienshkumar *et al.,* 2011a) and further supported by reduced testosterone levels (Dineshkumar *et al.,* 2011 b).

The rats fed DNC and RNC were apparently normal in both groups. The weights of liver and testes expressed as percent of body weight was lower in 37.5% replaced group in first experiment compared to other groups whereas it was similar in all the groups in second experiment (Table 6). The weight of kidneys expressed as percent of body weight increased at higher level of replacement (37.5% DNC in first experiment and 50% DNC and 25% RNC in second experiment). On gross examination, the organs revealed no changes except in two of four rats of DNC 50% group in second experiment showed congestion in testes and enlarged adrenals. RNC feeding at 25% level did not cause any changes in gross pathology of organs.

In experiment 1, 12.5% DNC fed group showed mild degenerative changes in the liver, intestine showed increased goblet cells and catarrhal inflammation of mucous membrane. The 25% NSC fed group showed mild degenerative changes in liver and interstitial fibrosis in the kidney. Fusion of villi, elongation of crypts was observed in the intestine. Spleen showed reticular cell hyperplasia and testis showed increased spermatogenesis activity. The 37.5% NSC group showed degenerative changes in the liver and intestine showed fusion of villi and crypt elongation with damage to the mucous membrane (Fig. 1). The effects on liver, kidney, spleen, testis and intestine were dose dependent

Table 6. Weight of vital organs (% of BW) of rats fed neem seed cake

Particular	Group				
	dNC 0	**dNC 12.5**	**dNC 25.0**	**dNC 37.5**	**SEM**
Experiment-1					
Liver	4.05[ab]	4.51[b]	4.39[ab]	3.90[a]	0.09
Heart	0.42	0.43	0.41	0.52	0.02
Kidney	0.78[a]	0.87[a]	0.90[ab]	1.10[b]	0.04
Testes	2.60[b]	2.81[b]	2.48[ab]	1.72[a]	0.14
Experiment-2					
	dNC 0	**dNC 25.0**	**dNC 50.0**	**rNC 25.0**	
Liver	3.53	3.23	3.75	3.40	0.09
Heart	0.39	0.37	0.40	0.37	0.01
Kidney	0.53[a]	0.66[ab]	0.82[b]	0.76[ab]	0.04
Testes	1.13	1.24	1.23	1.28	0.11

[a,b]Means bearing different superscripts in a row differ significantly (P<0.05)

changes. In experiment 2, 25% DNC fed group showed fusion of villi and mild damage to the intestinal mucous membrane (Fig. 2). The DNC 50% group showed testicular edema in testis and crypt elongation and fusion of villi in the intestine. RNC 25% group showed mild mononuclear cell infiltrations in liver and kidney. Testis showed testicular degeneration and loss of seminiferous tubular epithelium. Intestine showed loss of mucosal epithelium, fusion of villi to large extent with mononuclear cell infiltration and elongation of crypts (Fig. 3). Liver and kidney showed focal mononuclear cell infiltration. The changes observed in the intestine and testis were in agreement with the other changes observed in this study.The damage in the intestine might be due to the effect of toxin which reduced absorption and result in reduced body weight gain in rats which was observed in high dose group. The DNC 12.5 and 25% groups showed mild changes without affecting other parameters.

CONCLUSIONS

Feeding of DNC was better than RNC and upto 25% replacement of SBM nitrogen and did not exert adverse effect on growth, feed consumption, metabolic profiles including hormones, gross and histo-pathology of vital organs.

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