ASSESSMENT OF ACUTE AND SUB-CHRONIC TOXICOLOGICAL EFFECTS OF *NEOCARYA MACROPHYLLA* SEED CAKE ON WISTAR RATS

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Abstract
The acute and sub-chronic toxicological effect of Neocarya macrophylla seed cake (NMSC) on wistar rats were investigated in a view to determine its suitability as an additive, food ingredient or nutrient supplement in human diet. The proximate analysis of NMSC showed that it had high protein and crude fibre values of 56.04±0.00 % and 7.41±0.01 % but low carbohydrate and ash contents of 13.19±0.49 % and 6.79±0.02 %respectively. The result of the phytochemical analysis revealed that NMSC contained tannins, flavonoids and alkaloids. Forty wistar rats with an average weight between 40-60 g were randomly allotted to four dietary treatments and fed with commercial feed (group I) and 0 %, 10 % and 20 % of NMSC diet for groups II to IV respectively for four weeks. At the end of the experiment, the rats were sacrificed and hematological parameters, plasma biochemical parameters and histopathological examination were carried out. The rats, when monitored weekly had good physical appearance and neither mortality nor any sign of acute and sub-chronic toxicity was recorded throughout the experimental period. There was no significant difference between most of the hematological parameters of test group rats when compared with those of the control groups. Histopathological analysis of the organs of the test rats showed no visible lesion in both the kidney and heart. The liver of the rats fed with 10 % diet had no visible lesions but there was a mild diffuse hydropic degeneration of hepatocytes at 20 % inclusion. NMSC seemed to have potential of being used as feed supplement.

Keyword: *Neocarya macrophylla*, seed cake toxicity, haematological parameters, biochemical parameters

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1. INTRODUCTION

The rapidly growing world protein requirement has directed major attention to plant proteins. Oilseeds are valuable sources of lipid and basically processed for their edible oils leaving behind a lot of protein-rich meal. Proteins are usually recovered from the meals and marketed as food ingredients in developed countries. The most produced oilseeds worldwide are, in decreasing order, soybean, rapeseed, cotton, groundnut and sunflowers, amongst others (FAO, 2009 and Radha *et al*., 2015). The over dependence on only few plant species for food and food ingredients has made food security a challenge and a major concern in the world. This has led to several calls for investigation into alternative food sources to improve food security worldwide. Conservation, domestication and utilization of many indigenous wild fruit and vegetable species can contribute to hunger reduction and improved nutrition and health (Ekue *et al*., 2010). During periods of grain shortage, people increase their reliance on wild plant foods to supplement their diets (Glew *et al*., 2005). Several studies (Taehee *et al*., 1997; Freiberger *et al*., 1998) have shown that these wild products are an alternative source of oil and protein for human and animal feeding. In many countries, the supply of animal protein is inadequate to meet the protein needs of the rapidly growing population and the consumption of high amount of meat increases the risk of cardiovascular diseases and some types of cancer. This has also necessitated contemporary research that is directed towards studying the food properties and potential utilization of protein from locally available food crops, most importantly from underutilized or neglected high protein oilseeds and legumes (Enujuihya *et al.*, 2000; Singh *et al*., 1991).

N. macrophylla (Gingerbread plum) is one of the seeds that is rich in protein, but lesser known and underexploited. The trees are found in the arid and semiarid regions mainly in the Western part of Africa and Central America particularly Panama. Gingerbread plum, purely West African species was formerly *Parinari macrophylla* Sabine but now *N. macrophylla* (Sabine). It belongs to Chrysobalanaceae family which has 17 genera and 350 species (Irvine, 1961 and Tijani *et al*., 2011). The fruit are used in variety of ways. Many are eaten fresh or boiled with cereals. Some are consumed as snacks, others mixed into cooked dishes while some are roasted and enjoyed like cashew or almonds. The kernel is an excellent source of oil which is composed of oleic acid 40 %, eleostearic acid 31 %, linoleic acid 15 %, palmitic acid 12 % and stearic acid 2 %. The plant was reported to treat snakebite, pain and inflammations; traditional healers usually boil the leaves or the stem bark in water and serve the snakebite victim (Yusuff *et al.*, 2015).

Previous studies have reported that the seeds are of high food value with about 40-60 % oil and 21-25 % protein content (Amza *et al.*, 2010). The defatted seed meal contains 61 % protein. The seeds are good source of certain amino acids such as lysine, valine and phenylalanine and others which are important for balancing the deficiency of these essential amino acids in cereal-based diets (Amza *et al.*, 2010). Research conducted on gingerbread plum fruit revealed its high nutritional values (Cook *et al.*, 2000; Audu *et al.*, 2005). The living tree provides villagers with shelter, dye, glue, fodder, firewood, soap, structural materials, and even termite repellents (in the Gambia). The leaves are used medicinally for toothache and mouthwash while the kernels are usually roasted and enjoyed like cashews or almonds (NRC, 2008).

Neocarya macropylla is a plant employed in traditional medicine to manage pain conditions in Northern Nigeria. It is commonly known as Gawasa or Farar rura in Hausa language in Nigeria. It is a shrub or small tree (6-10 m) high with densely pubescent and russet brown stems and alternate or ovate leaves (10-25 cm) long (Arbonnier, 2004; Yusuff *et al*., 2015). It is also used in treating diarrhoea, asthma, dysentery, skin infections, cancer, pulmonary troubles, ear and eye infections, tooth decay, snakebite, pain, inflammation and skin infections (Warra *et al*., 2013). Some preliminary phytochemical screening and physico-chemical studies of the seed oil (Warra *et al.*, 2013) as well as the antimicrobial studies on the fruit and root bark of the plant (Audu *et al*., 2005) were previously undertaken. The leaves have anthelminthic activities (Barnabas *et al.*, 2011). The nutritional and anti-nutritional profiles of the seeds have also been reported (Mohammad *et al.*, 2015). Acute toxicity studies and evaluation of analgesic property of the methanol stem bark extract of *N. macrophylla* were previously studied (Yusuff *et al*., 2015). The protein isolate obtained from it might fount major application in protein fortification for a variety of food products and may also be a potential food ingredient (Amza *et al.*, 2011). Utilization of *N. macrophylla* seed cake in livestock diets and other food supplements has not been investigated. This study therefore aims at evaluating the acute and sub-chronic toxicological effect of the *N. macrophylla* seed cake, incorporated into rat diet at different percentages, using wistar rats as a case study in a view of ascertaining its potential as a suitable food ingredient or nutrient supplement in human diet.

2. MATERIAL AND METHODS

Preparation of *N. macrophylla* seed cake

N. macrophylla seeds were obtained from Junju town, Niger and were identified and authenticated at the Biological Sciences Department, Bayero University, Kano (BUK). The seeds were selected, cleaned and kept at room temperature. They were air dried and milled using a laboratory scale hammer miller prior to extraction. The kernels were removed and pulverized to fine powder to increase the extent of extraction. Oil was extracted from the seed flour by Soxhlet extraction method using n-hexane as the solvent. *N. macrophylla* seed

cake (NMSC) obtained after the oil extraction was then air dried, pulverized again and passed through a 200 mesh size and stored for analyses. All reagents used for the chemical analysis were of analytical grade.

Proximate composition analysis

The ash content of NMSC and the compounded feed were determined by heating 2 g of sample in a vector muffle furnace at 550^{o}C for five hours (Ajayi, 2009). Moisture, crude fat and crude fibre contents were determined following the standard methods of Association of Official Analytical Chemists (AOAC, 2006). Nitrogen content was estimated using the micro-Kjeldahl method as described by AOAC (2006) and crude protein content was calculated with a conversion factor of 6.25. The available carbohydrate content was determined by difference. The calorific energy value was obtained by multiplying the values of carbohydrate, protein and crude fat with Atwater factors of 17, 17 and 37 respectively (Olaofe *et al.*, 2009). Determinations were made in triplicate.

ANIMAL AND DIETS

Feed compounding

A basal diet was formulated to meet the entire nutrient requirement for young rats. The diet was prepared according to the formula and procedure used by Souza *et al.* (2007) and Ajayi *et al*. (2015) with slight modifications. The total quantity of diet used for the experiment was 3000 g and the basic ingredients used for the formulation were: maize (40%), soybeans (18.20%), groundnut cake (14.20%), palm kernel cake (7.10%), corn bran (7.10%), wheat (7.10%), bone (3.30%), oyster shell (2.20%) and salt (0.80%). The above formulation was used for the 0 % diet (Group II); 10% and 20 % were taken from each feed ingredient and replaced withNMSC for the test diets (Groups III and IV respectively). Group I rats were fed with commercial feed. (Groups I and II were used as the Control groups in this research work). Each of the diet was pelletized, dried for two days and packed in four separate transparent plastic buckets for analyses.

Experimental animals

Forty wistar rats weighing between 70-100 g, purchased from Anatomy Department, Faculty of Veterinary Medicine, University of Ibadan, Nigeria and housed in their experimental animal house were used for the acute and sub-chronic toxicity profile of NMSC. The rats were divided into four groups (Group I, Group II, Group III and Group IV) of 10 rats each and kept in plastic cages at room temperature. The rats had access to feed and water *ad-libitum* for a period of four weeks and maintained under standard conditions of humidity, temperature, and 12 h light/dark cycle before sacrifice. They were acclimatized for a week before the commencement of the study. A standard protocol was drawn up in accordance with current guidelines for the care for laboratory animals and ethical guideline. They were fed according to their group levels with different percentages of NMSC diets. Their feed intake was monitored daily.

Weekly body weight measurement, mortality and clinical signs

During the four-weeks feeding period, all the animals were observed daily for clinical signs, morbidity and mortality patterns once before feeding, immediately after feeding and up to 24 h after feeding. Any abnormality in terms of behavioral and physical changes such as eyes, skin, posture, fur, and response to handling were taken note of. The time the sign started and how long it lasted, if any, were also recorded. The weight of all the experimental groups and the control were recorded weekly using a sensitive balance, during the acclimatization period, once before commencement of feeding and once weekly throughout the experimental period as described by Raphael *et al.* (2014); the final body weights were measured a day to the end of the experiment (28th day).

Organs collection

After 24 h of commencement of the experiment process, three rats from each group were sacrificed for assessment of sub-acute toxicological effect of the NMSC incorporated into the diets. At the end of the four weeks, five rats from each group were allowed to fast

overnight and sacrificed to assess the sub-chronic toxicological effect of the seed cake. The rats were sacrificed in both cases by cervical dislocation. Blood samples were collected into EDTA bottles to prevent blood coagulation and used for haematological and biochemical studies. The organs collected were the heart, brain, kidney, liver, lungs and spleen. These organs were weighed immediately after collection and preserved in 10 % formalin solution for pathological studies to one decimal place and used for the calculation of organ weight ratio (Akthar *et al.*, 2009).

$$Organ\ weight\ ratio = \frac{\text{Organ weight (g)}}{Body\ weight\ (g)}$$

Determination of haematological parameters

Blood samples were collected by the orbital technique and stored into EDTA bottles for haematological analyses. The sample bottle was shaken gently to mix up the blood with EDTA to prevent clotting. The values of the red blood cells (RBCs) count, total and differential white blood cells (WBCs) count, packed cell volume (PCV), erythrocyte sedimentation rate and haemoglobin (Hb) mean corpuscular volume and mean corpuscular haemoglobin concentration were determined according to the methods of Jain (1986). The quantities of RBCs and WBCs were determined with the improved Neubauer Haemocytometre (Mbaka *et al.*, 2010). All haematological parameters were determined at room temperature.

Determination of biochemical parameters

Part of the blood sample collected was kept at room temperature for 30 min to clot. The test tube containing the clotted blood sample was centrifuged using a table centrifuge to enable a complete separation of the serum from the clotted blood (Raphael *et al.*, 2014). The clear serum supernatant was aspirated with syringe and needle and stored in a clean sample bottles for biochemical analysis. The values of total serum protein, serum albumin, serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), serum alkaline phosphatase (ALP), serum urea and serum creatinine were determined following standard laboratory procedures. Albumin and globulin were determined by colorimetry. The albumin/globulin ratio was obtained by dividing the calculated albumin value by the calculated globulin value. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were also determined (MAFF, 1984 and Zimmerman, 1983).

Histopatological examination

The rats were dissected and examination of the heart, liver, kidney, lungs and brain were carried out. Tissue samples were stored in 10 % formalin for histological analyses. These tissues were fixed and put through series of dehydration in graded concentration of xylene. They were embedded in wax, sectioned at 5 µ and transferred to clean glass slides. The thin sections were stained with haemotoxylin and eosin (H and E) dyes for examination under light microscope for histological changes following the method described by Jain (1986) and Raphael *et al*. (2014).

Statistical analysis

All experiments were conducted at least in triplicate. Numerical data were expressed as mean values ± standard error. Statistical analyses were performed with SPSS Inc. software (version 2010). Two ways analysis of variance (ANOVA) was used to determine significant differences between means, with the significance level taken at a probability less than 5 % ($p \leq 0.05$). Duncan test was used to perform multiple comparisons between means.

3. RESULTS AND DISCUSSION

The effect of NMSC incorporated in rat diets was investigated on the haematology, histopathology and liver profile of wistar albino rats as part of a wider study to evaluate the nutritional potential of the seed cake and thereby evaluating its safety in functional foods.

Proximate composition of *N. macrophylla* cake and compounded diets

NMSC has a very high protein content (56.04±0.00 %), high value of ash content (6.53±0.06 %) and low carbohydrate content of

13.19±0.49 % (Table 1). This protein content obtained is higher than that of *Balanite aegyptiaca* seed cake (49.32±0.13 %) (Ajayi *et al*., 2015); ground nut cake (50.90±1.27 %) (Fekria *et al.*, 2012); soy bean meal (44.03 %) and wheat bran (14.98 %) as (Ahmed *et al*., 2009) but lower than (78.70±0.40 %) for defatted *Cucumeropsis mannii* as reported by Eunice *et al.* (2012). The ash and moisture content are quite low. The values of the protein, moisture and ash content obtained in this study were similar to those reported previously for defatted gingerbread seed flour (Amza *et al.*, 2011). The ash content of 6.53±0.06 % obtained is higher than 4.40±0.02 % for *Balanite aegyptiaca* seed cake (Ajayi *et al.*, 2015) and 2.55±0.12 % for *T. tetraptera* (Aguomo *et al.*, 2011). Carbohydrate is a major source of energy supply in the food system. The carbohydrate content of NMSC is 13.19±0.49 %. This value is higher than 6.04±1.2 % reported for NMSC (Amza *et al.*, 2011); this difference might due to the geographical location of the plant seed. However it is lower than the values of some conventional seed flours. The result showed a significant difference in protein content at 10 % and 20 % substitution of NMSC. The carbohydrate content on the other hand showed significant decrease with increase in NMSC with a range of 55.91±0.72 - 50.73±0.16 while 13.19±0.49 was obtained in NMSC. This trend in increase in protein content and decrease in carbohydrate content with increasing NMSC is expected since NMSC is rich in proteins (Amza *et al.*, 2010). The calculated metabolic energy value obtained is 317.48±1.89 Kcal/10 g for NMSC, 317.96±0.67 for group II diet (0 %; control II), 322.82±0.54 Kcal/100g and 314.10±0.16 Kcal/100g respectively for the experimental feed. This is an indication that NMSC could be a source of energy.

Effect of acute and sub-chronic toxicity of *N. macrophylla* seed cake on the general behavior and body weight of the rats and the mean weight of organs of rats

The effect of acute and sub-chronic toxicity of NMSC on the general behavior and body weight of the rats is shown on Table 2. No sign of toxicity (both physical and clinical) and mortality were observed on the rats used for the control and experimental groups during the period of study. All the animals used were male and survival rate was 100 % for test and control groups.

Table 1: Diet and proximate composition of NMSC, control I, 10 % and 20 % diets

Ingredients	Percentage (%)	Group II (g)	Group III (g)	Group IV (g)
Diet composition				
Maize	40.00	1200.00	1080.00	960.00
Soybeans	18.20	546.00	491.40	436.80
Groundnut cake	14.20	426.00	383.40	340.80
Palm kernel cake	7.10	213.00	191.70	170.40
Corn bran	7.10	213.00	191.70	170.40
Wheat	7.10	213.00	191.70	170.40
Bone	3.30	99.00	89.10	79.20
Oyster shell	2.20	66.00	59.40	52.80
Salt	0.80	24.00	21.60	19.20
NMSC	0.00	0.00	300.00	600.00
Total	100.00	3000.00	3000.00	3000.00
Proximate analysis				
Parameter	**NMSC**	**Group II**	**Group III**	**Group IV**
Moisture	12.32 ± 0.53^{a}	10.52 ± 0.23^{b}	10.09 ± 0.14^{b}	10.46 ± 0.10^{b}
Crude protein	56.04 ± 0.00^{a}	16.23 ± 0.06^{d}	18.84 ± 0.01^{c}	20.58 ± 0.01^{b}
Crude fat	4.51 ± 0.01^{a}	3.40 ± 0.00^{b}	3.19 ± 0.01^{c}	3.23 ± 0.06^{c}
Crude fibre	7.41 ± 0.01^{c}	8.03±0.06	9.00 ± 0.00^{a}	9.00 ± 0.01^{a}
Ash	6.53 ± 0.06^{a}	6.00 ± 0.01^{b}	4.20 ± 0.00^{c}	6.07 ± 0.06^{b}
CHO	13.19 ± 0.49^{d}	55.91 ± 0.72^{a}	54.68 ± 0.14^{b}	50.73 ± 0.16^{c}
Dry matter	87.68 ± 0.53^{a}	89.28 ± 0.14^{a}	89.91 ± 0.14^{a}	89.61 ± 0.07^{a}
CEV (kcal/100 g)	317.48 ± 1.89^{b}	317.96 ± 0.67^{b}	322.82 ± 0.54^{a}	314.10 ± 0.16^{c}

CHO: Carbohydrate content, CEV: Calorific Energy Value

The weights of the rats were found to be increasing gradually. The percentage weight gain was found to be 73.3 % in the group of rat fed with standard rat feed (Vital Feed, Nigeria); this is higher than 59.80 % obtained for the test rats fed with feed compounded with 20 % NMSC. The 20 % test rats had a highest body weight gain when compared with 10 % counterparts. Organ to body weight ratios are indices which are often used in toxicological evaluations (Michael *et al.,* 2007). The results in this study indicate that these indices were not significantly altered by sub-acute treatment. This lends credence to the absence of injuries to the liver, heart and kidney. Fig. 1a & b show the histogram of the mean weight of organs of rats in acute toxicity and sub-chronic toxicity assessment. The weight of the kidney, heart and spleen of the test rats were found to be comparable both in the acute and sub-chronic toxicity with those of the control rats. Organ weight is an important index of physiological and pathological status in animals. The relative organ weight is fundamental to establish whether the organ was exposed to the injury or not. The heart, liver, kidney, spleen, and lungs are the primary organs affected by metabolic reaction caused by toxicant. The liver, being a key organ in the metabolism and detoxification of xenobiotics, is vulnerable to damage induced by any huge variety of chemicals (Jothy *et al.,* 2001 and Rapael *et al.,* 2014). The absence of any significant difference in the parameters obtained in this experiment is an indication that the NMSC did not affect the weight of the organs.

Effect of acute and sub-chronic toxicity of *N. macrophylla* seed cake on the haematological parameters of the rat blood

Hematological parameters are important indices of the physiological and pathological status for both animals and humans (Adeneye *et al*., 2006). The effect of NMSC on the haematological parameters of the rats' blood is shown on Table 3. After Day 3 for the acute toxicity, WBC was found to be higher in the control rats than in the experimental rats. The lymphocyte was also found to be higher in the control rats than in the experimental rats. There was no significant ($P>0.05$) effect on the basophil, eosinophil, monocyte and neutrophil count in the experimental rats compared to the control ones. RBC, Hb, PVC and platelets were not significantly different ($P>0.05$) in the test rats compared with the control rats. After the Day 28, PCV, Hb concentration, WBC and platelets increased significantly ($P<0.05$) in experimental rats when compared with the control rats. There was an increase in RBC concentration but not significantly. However total WBC, basophil, eosinophil, lymphocyte, monocyte, neutrophil were not significantly ($P>0.05$) affected in the test rats compared with the control ones. Analysis of blood parameters is relevant to risk evaluation and the changes in the haematological system have a higher predictive value for human toxicity, when the data are translated from animal studies (Ibahim *et al.,* 2010). The assessment of haematological parameters could be used to reveal the deleterious effect of foreign compounds on the blood constituents of animals (Odeyemi *et al.*, 2009). They can also be used to determine possible alterations in the levels of biomolecules, metabolic products, haematology, normal functioning and histomorphology of the organs (Jothy *et al.,* 2001). The increase in erythrocytes WBC, PCV, platelets and Hb after the 28 day study may be due to over production of haematopoietic regulatory elements by the stroma cells and macrophages in the bone marrow (Raphael *et al.*, 2014 and Kafaie *et al.*, 2012). This study has also shown that NMSC in rats feed did not cause any change in haematological parameters. Haematological changes such as anaemia are often accompaniments of bone marrow toxicity (Flanagan and Dunk, 2008) among other causes. The lack-of-effect on neutrophil levels indicates that the extract may not have induced any inflammatory process since these cells are usually elevated in the course of inflammations (Formela *et al.*, 1995).

Table 2: General behavior and body weight of control and test rats

Group/Concentration	Sex	Number of rat/mortality	Mortality Ratio	Physical signs of toxicity
First Phase				
Group I: Control I	Male	10/0	00	None observed
Group II: Control II	Male	10/0	00	None observed
Group III: 10 % NMSC	Male	10/0	00	None observed
Group IV: 20 % NMSC	Male	10/0	00	None observed
Total number of rats		40	00	None observed
Second Phase				
Group I: Control I	Male	7/0	00	None observed
Group II: Control II	Male	7/0	00	None observed
Group III: 10 % NMSC	Male	7/0	00	None observed
Group Iv: 20 % NMSC	Male	7/0	00	None observed
Total number of rats		28	00	
Body weight of rats (g)	**Group I**	**Group II**	**Group III**	**Group IV**
0 week	74.56±12.71	82.61±10.21	92.68±10.67	82.48±6.78
1 week	82.06±12.86	92.24±19.24	102.12±17.47	97.74±12.76
2 weeks	95.64±14.94	111.18±21.97	117.20±18.17	113.62±17.09
3 weeks	95.90±23.30	122.65±19.93	129.46±17.82	113.24±16.96
4 weeks	134.38±20.72	128.63±26.92	129.24±22.00	131.20±21.63
Weight gain (g)	54.60±14.10	45.95±22.80	39.16±14.43	49.32±16.38
Weight gain (%)	73.23	55.62	42.25	59.80
Organ weight ratio	0.04	0.05	0.04	0.05

Values are mean ± standard deviation; week 0, n = 10; weeks 1-4, n = 7

Table 3: Haematological analysis of blood samples of control and test rats

Parameter	Group I	Group II	Group III	Group IV
Acute toxicity	**(24 h)**			
PCV (%)	32.33±2.31^{b}	38.00±1.73^{a}	41.00±1.73^{a}	38.33±2.89^{a}
Hb (mg/dl)	10.83±0.75^{b}	12.93±0.76^{a}	13.87±0.55^{a}	12.90±0.14^{a}
RBC (10^{6}/µl)	5.35±0.03^{b}	6.36±0.21^{a}	6.84±0.32^{a}	6.25±0.73^{a}
WBC (10^{3}/µl)	11416.67±1587.71^{b}	12883.33±2706.17^{ab}	16233.33±1342.88^{a}	15650.00±1685.97^{a}
Platelets	183666.67±13279.06^{a}	115000.00±73749.58^{a}	129000.00±11269.43^{a}	115666.67±22678.92^{a}
Lymphocyte (%)	68.33±2.89^{a}	68.00±3.00^{a}	58.67±6.11^{b}	64.00±2.65^{ab}
Heterophile (%)	24.33±2.31^{a}	26.67±3.79^{a}	30.67±3.79^{a}	29.00±2.65^{a}
Monocyte (%)	2.33±0.58^{a}	3.33±0.58^{a}	3.00±1.00^{a}	3.33±1.53^{a}
Eosinophil (%)	4.33±0.58^{a}	1.67±1.16^{a}	3.67±2.31^{a}	3.67±1.16^{a}
Basophil (%)	0.67±0.58^{a}	0.33±0.58^{a}	0.67±0.58^{a}	0.00±0.00
Sub-chronic toxicity (4 weeks)				
PCV (%)	35.67±3.06^{a}	36.67±6.35^{a}	40.33±5.51^{a}	37.00±3.61^{a}
Hb (mg/dl)	12.17±1.19^{a}	12.37±1.80^{a}	13.70±1.73^{a}	12.57±0.90^{a}
RBC (10^{6}/µl)	3.50±0.63^{a}	3.92±0.38^{a}	4.19±0.60^{a}	3.97±0.13^{a}
WBC (10^{3}/µl)	18083.33±2345.92^{ab}	12900.00±4171.63^{b}	18450.00±1956.40^{a}	14316.67±1575.07^{ab}
Platelets	112000.00 ±30805.84^{a}	136666.67±38630.73^{a}	150333.33±37846.18^{a}	136000.00±48538.64^{a}
Lymphocyte (%)	63.00±6.00^{a}	56.00±1.00^{a}	52.67±3.79^{a}	60.33±12.86^{a}
Heterophile (%)	31.00±5.57^{a}	35.67±1.16^{a}	40.00±2.65^{a}	34.00±12.29^{a}
Monocyte (%)	3.33±1.16^{a}	4.00±1.00^{a}	3.67±0.58^{a}	4.33±1.16^{a}
Eosinophil (%)	2.33±1.53^{a}	4.00±1.73^{a}	3.67±1.53^{a}	2.00±1.00^{a}
Basophil (%)	0.33±0.58^{a}	0.33± 0.58^{a}	0.00 ±0.00^{b}	0.67±0.58^{a}

Values are mean ± standard deviation of triplicate results
Values in the same row with the same superscript are not significantly different at P > 0.05

Table 4: Biochemical analysis of serum of blood of control and test rats

Parameter	Group I	Group II	Group III	Group IV
Acute toxicity	**(24 h)**			
Total protein (g/l)	6.70±0.17^{a}	6.83±0.35^{a}	7.00±0.20^{a}	7.10±0.17^{a}
Albumin (g/l)	1.60±0.17^{b}	1.77±0.29^{ab}	2.33±0.51^{a}	2.33±0.15^{a}
Globulin (g/l)	5.10±0.00^{a}	5.07±0.15^{a}	4.33±0.55^{b}	3.77±0.06^{c}
A/G ratio	0.27±0.06^{c}	0.33±0.06^{bc}	0.47±0.15^{ab}	0.57±0.06^{a}
AST (U/L)	185.67±4.62^{a}	190.33±3.51^{a}	181.00±3.61^{a}	185.00±12.29^{a}
ALT (U/L)	22.00±1.73^{a}	22.33±2.52^{a}	22.33±2.52^{a}	25.67±1.53^{a}
ALP (U/L)	89.67±6.35^{a}	108.67±8.12^{a}	101.67±9.45^{a}	91.33±16.07^{a}
BUN (mg/dl)	17.03±0.64^{a}	16.03±0.57^{a}	15.77±1.08^{a}	15.73±1.10^{a}
Creatinine (mg/dl)	0.43±0.06^{a}	0.50±0.10^{a}	0.50±0.10^{a}	0.50±0.10^{a}
Sub-chronic toxicity (4 weeks)				
Total protein (g/l)	7.93±0.06^{bc}	8.47±0.15^{a}	8.17±0.29^{ab}	7.63±0.12^{c}
Albumin (g/l)	2.87±0.12^{a}	3.23±0.49^{a}	2.93±0.51^{a}	2.60±0.26^{a}
Globulin (g/l)	5.47±0.12^{b}	5.57±0.15^{a}	5.67±0.06^{a}	5.10±0.30^{a}
A/G ratio	0.52±0.01^{a}	0.56±0.03^{a}	0.53±0.11^{a}	0.51±0.08^{a}
AST (U/L)	197.00±1.73^{a}	217.33±31.94^{a}	194.00±2.65^{a}	209.33±15.50^{a}
ALT (U/L)	26.67±2.08^{a}	25.33±2.08^{a}	27.33±1.53^{a}	27.67±2.52^{a}
ALP (U/L)	95.33±4.04^{a}	124.67±5.57^{a}	134.00±3.46^{a}	139.67±40.53^{a}
BUN (mg/dl)	16.10±0.69^{a}	16.60±0.53^{a}	15.83±0.57^{a}	16.07±0.67^{a}
Creatinine (mg/dl)	0.50±0.00^{b}	0.63±0.06^{ab}	0.67±0.06^{a}	0.63±0.12^{ab}

Values are mean ± standard deviation of triplicate results
Values in the same row with the same superscript are not significantly different at P > 0.05
A/G ratio:Albumin/Globulin ratio; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatise; BUN: blood urea nitrogen

Table 5: Histopathological analysis of organs of control and test rats

Organ	Group I Acute toxicity (24 h)	Group II	Group III	Group IV
Heart	No visible lesions seen.	No visible lesions seen.	No visible lesions seen.	No visible lesions seen.
Kidney	No visible lesions seen.	No visible lesions seen.	No visible lesions seen.	No visible lesions seen.
Liver	There is a severe diffuse vacuolar degeneration of hepatocytes.	There is a severe portal congestion, with severe diffuse vacuolar degeneration of hepatocytes.	There is marked periportal congestion with mild cellular infiltrates at the portal region.	There is a severe portal congestion. The hepatocytes appear shrunken.
	Sub-chronic toxicity (4 weeks)			
Heart	No visible lesions seen.	No visible lesions seen.	No visible lesions seen.	No visible lesions seen.
Kidney	No visible lesions seen.	No visible lesions seen.	No visible lesions seen.	No visible lesions seen.
Liver	There is a mild diffuse hydropic degeneration of hepatocytes, with moderate periportal cellular infiltration.	There is a severe diffuse vacuolar degeneration of hepatocytes.	No visible lesions seen.	There is a mild diffuse hydropic degeneration of hepatocytes.

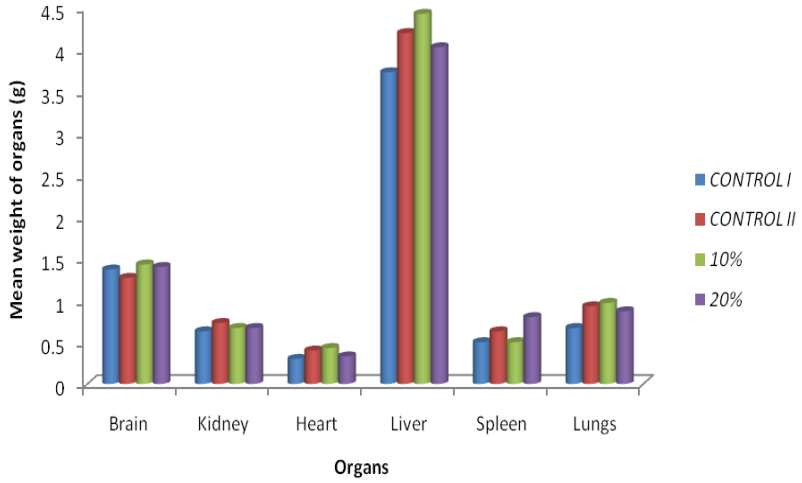


Fig. 1a: Histogram showing the mean weight of organs of rats in acute toxicity assessment

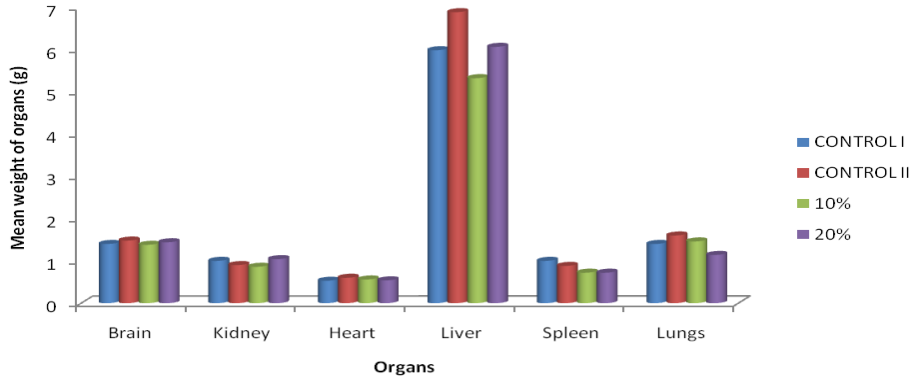
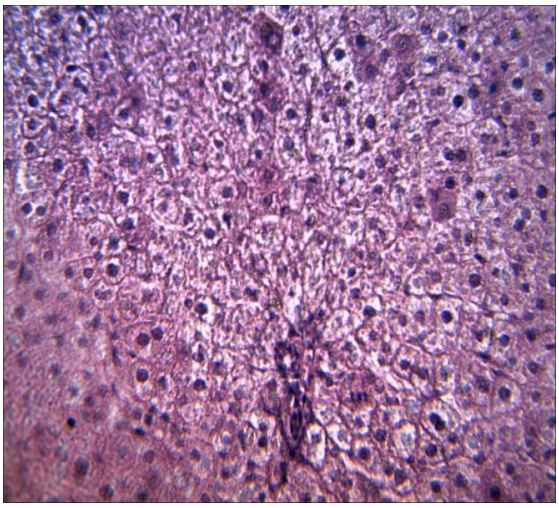


Fig. 1b: Histogram showing the mean weight of organs of rats in sub-chronic toxicity assessment

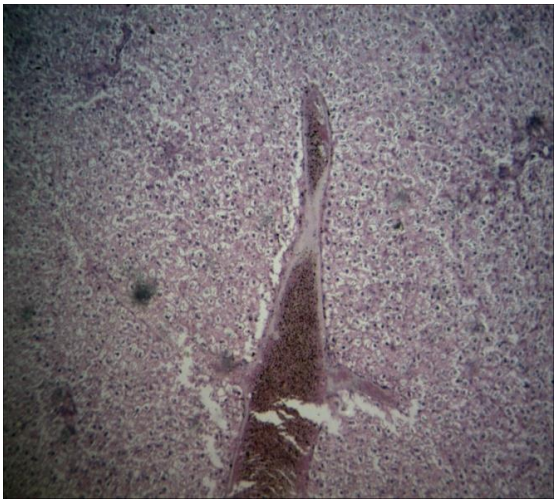
Effect of acute and sub-chronic toxicity of *N. macrophylla* seed cake on the biochemical parameters of the rat blood

The results of the biochemical parameters such as Serum Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP), Total protein, albumin, globulin and creatinine of rats fed with raw NMSC at different percentage levels of inclusion in the feed showed that after the day 3 of the experiment, there was no significant difference ($P<0.05$) in the liver enzymes (AST, ALT, ALP and others) in the test rats as compared to the control ones (Table 4). There was a significant difference ($P<0.05$) in albumin and globulin concentration in the rats from both groups. There was also a significant ($P<0.05$) different in the ratio of albumin to globulin in the experimental rats compared to the control rats. After the 28 days of experiment, there was no statistically significant ($P>0.05$) difference in urea, creatinine, albumin, globulin of rats from both groups. The ratio of albumin to globulin was comparable in all the treated groups. The assessment of the activities of enzymes such as ALT, AST and ALP provides powerful information on the liver function. ALT is a cytosolic enzyme found in very high concentration in the liver, and an increase of this specific enzyme indicates hepatocellular damage; AST is less specific than ALT as an indicator of liver function. Alkaline phosphatase is membrane bound and its alteration is likely to affect the membrane permeability and produce

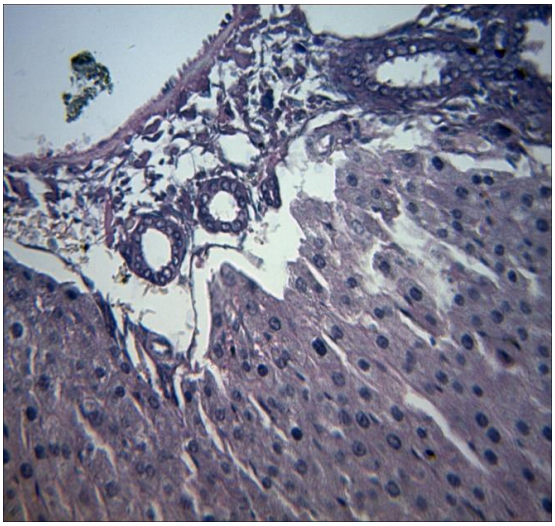
derangement in the transport of metabolites. A rise in serum ALP level is usually a characteristic finding in cholestatic, infiltrative liver disease as well as hepatitis and bone diseases (Aliu *et al.*, 2006 and Angelico *et al.*, 2010). Urea and creatinine are considered as a suitable prognostic indicator of renal dysfunction and kidney failure for any toxic compounds (Gnanami *et al.*, 2008). In this study, the absence of significant differences in these parameters after 24 hours and 28 days of experiment suggests that NMSC probably did not have harmful effect on the kidney and the liver.



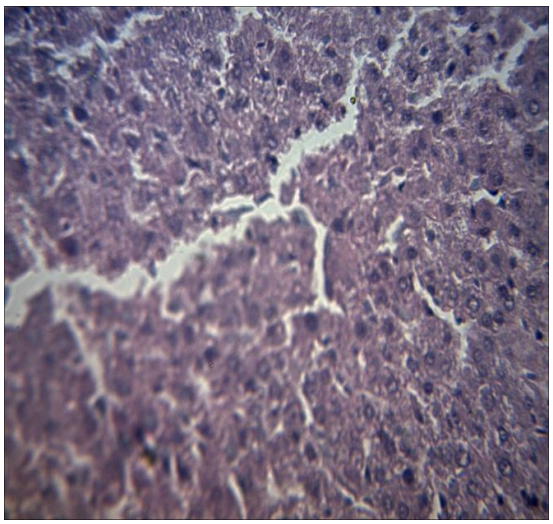
(2a). Group I showing a severe diffuse vacuolar degeneration of hepatocytes



(2b). Group II showing a severe portal congestion and severe diffuse vacuolar degeneration of hepatocytes



(2c). Group III showing marked periportal congestion



(2d). Group IV showing severe portal congestion (the hepatocytes appear shrunken)

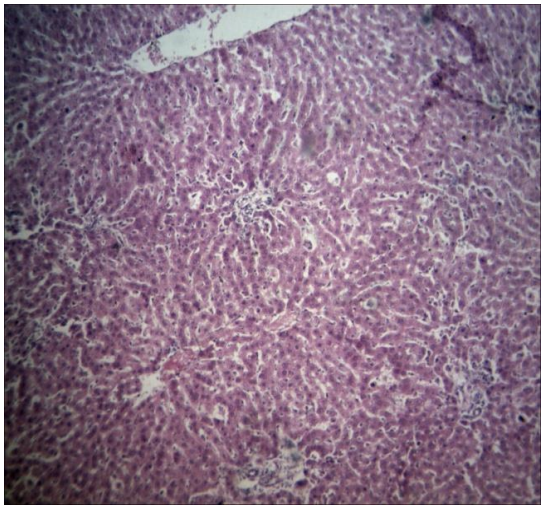
Fig. 2: Photomicrograph sections of the liver cells of rats in acute toxicity assessment

Results of histopathological analysis of organs

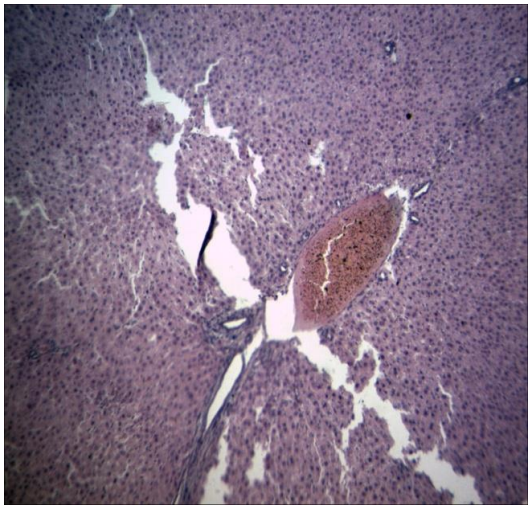
The result of the histopathological analysis of organs of rat fed with diet compounded with NMSC is presented on Table 5.

Fig. 2&3 illustrates the photomicrograph of liver section for the experimental rats within both the acute and sub-chronic toxicity levels. There was no abnormal activity (no visible lesion) seen around the kidney and the heart of

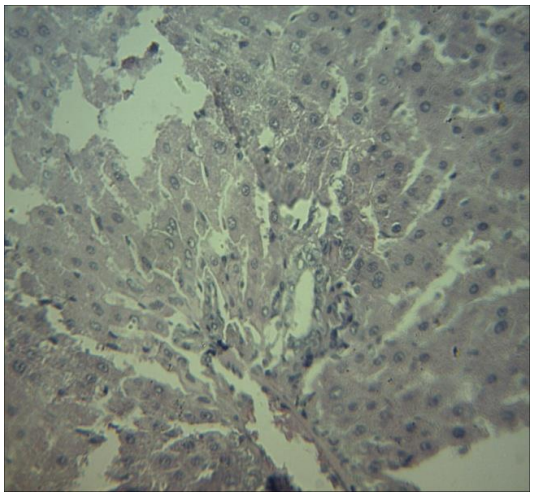
both the experimental and control rats at the level of toxicity studied. After 24hours of feeding the rats with NMSC there is a severe diffuse vacuolar degeneration of hepatocytes on the liver of rats in Group I; a severe diffuse vacuolar degeneration of hepatocytes on the liver of rats in Group II; a marked periportal congestion on the liver of rats in Group III and severe portal congestion on the liver of rats in Group IV.



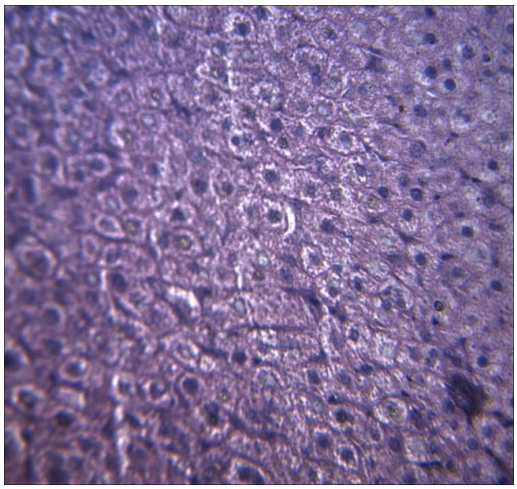
(3a). Group I showing moderate periportal cellular infiltration



(3b).Group II showing a severe diffuse vacuolar degeneration of hepatocytes



(3c). Group III showing no visible lesions



(3d). Group IV showing a mild diffuse hydropic degeneration of hepatocytes

Fig. 3: Photomicrograph sections of the liver cells of rats in sub-chronic toxicity assessment

These observed lesions in the liver in the acute toxicity assessment cannot be attributed to the seed cake as they were seen in both control and experimental groups. This might be due to the commercial feed used to feed the rats in the process of acclimatization. After 28 days of feeding the rats with NMSC there is a mild diffuse hydropic degeneration of hepatocytes on the liver of rats in Group I; a severe diffuse vacuolar degeneration of hepatocytes on the liver of rats in Group II; no visible lesions was seen on the liver of rats in Group III and a mild diffuse hydropic degeneration of hepatocytes on the liver of rats in Group IV. The assessment of the activities of enzymes such as ALT, AST and ALP provides powerful

information on the liver function. There was no significant difference (P<0.05) in the liver enzymes (AST, ALT, ALP and others) in the test rats as compared to the control ones (Table 4). The changes observed in the histological properties of the liver of rats after the 28 days of experiment as compared to those in the acute study may suggests that NMSC has no toxicological effect on the liver of the rats under this study.

4. CONCLUSION

The protein and carbohydrate content of the seed, when processed could be helpful in reducing nutritional related problems (such as protein-calorie malnutrition) in Africa; *N. macrophylla* seed cake has a potential of being utilized successfully as a substitute for protein in food formulations for man and livestock. There was no significant change in the haematological and biochemical parameters of the test rats. In conclusion, the results of the present study clearly showed that the NMSC under the conditions studied, did not induce any acute or sub-chronic toxic effects in Wistar rats. NMSC was well tolerated and did not induce toxic effects even at 20% dietary level as evidenced by absence of any ill effects on growth, body weight gain, organ weight, histology, hematology or clinical enzymes in Wistar rats. This result suggests therefore that *Neocarya macrophylla*seed cake (NMSC) could be successfully used as a substitute for protein in food formulations for man and livestock and might also be potentially safe for human consumption. NMSC could probably also be used as raw materials for the pharmaceutical and food industries.

Conflict of interest statement

We declare that we have no conflict of interest in the cause of this experiment.

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