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Spermatotoxicity and Testicular Pathology in Wistar Strain Rats fed Graded Levels of Pigeon Pea Diet

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Summary: Pigeon pea is an important grain legume in the tropics and subtropics and it is a valuable source of low-cost plant protein for humans and animals, but it remains an underutilized legume. Effects of feeding graded levels of raw pigeon pea seed inclusion diets on testicular function in Wistar rats was investigated. Thirty male Wistar rats weighing between 120 and 160 g were assigned into six groups (A-F) of 5 rats each. Group A rats served as the control and were fed with standard rat feed, Group B was fed with 10% pigeon pea inclusion diet, group C: 20% pigeon pea inclusion diet, Group D: 30% pigeon pea inclusion diet, Group E: 40% pigeon pea inclusion diet and Group F: 100% pigeon pea diet. Each rat received 30 g of feeds per day for 21 days with drinking water *ad libitum*. All analyses were carried out using standard methods. The motility scores were between 34.00±2.45 and 87.00±3.00 with the control group A having the significantly highest score ($P<0.05$) compared to the other groups. Group B rats had a significantly higher ($P<0.05$) values of (76.00±6.96) than groups C, D, E, and F while groups D, E and F were lower than normal range. This same trend was observed for the sperm viability and count across the groups. No lesion was observed in the testicular histology of rats in groups A, B, and D. The testis of rats in group C showed marked expansion of the interstitium by oedema, while the testis of group E rats showed immature germ cells in the seminiferous tubular lumen and the testis of group F rats revealed slightly reduced germinal depth. It was concluded in this study that feeding of pigeon pea seed diet to rats beyond 20% inclusion level is spermatotoxic having severe adverse effects on the sperm motility, viability and count and caused some testicular lesions. However, unprocessed pigeon pea must be incorporated with caution into animal feeds, especially the male animals used for breeding.

Keywords*:* Semen, Testes, Pigeon pea, Wistar rats, Motility, Histology

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INTRODUCTION

The increasing world population particularly in the developing countries like Nigeria calls for urgent improvement in livestock production. Deficiency of animal proteins is a major problem that needs to be addressed in Nigeria and other African countries (Okah and Ibeawuchi, 2011). The level of animal proteins in diets of most Nigerians is lower than the recommended level (Okah and Ibeawuchi, 2011).

Pigeon pea (*Cajunus Cajan* L.*) is* a diploid legume crop and is a member of the family *Phaseolea. (*Devi *et al.* 2016). It is a multipurpose, hardy grain legume crop grown in several developing countries in the semi-arid tropics and sub tropics (Zu *et al*., 2006). Pigeon pea plays a vital role in human and animal nutrition as a source of dietary protein in many countries (Abdelati *et al.* 2009). *Cajanus cajan* seeds are now well-thought-out to be a non–conventional source of feed in poultry and as a valuable source of protein in feeds *(*Devi *et al*. 2016). In Nigeria, it is grown extensively in Enugu, Anambra and Benue States.

It is also cultivated in Africa and the Americas, and it has been suggested as one of Africa's drought-tolerant crops referred to as 'orphan crop' because it falls into the group of least researched crops world-wide (Odeny, 2007). Most protein supplements are very expensive and their use in ruminant nutrition competes with monogastric animals and human nutrition. There is the need to address this problem of insufficiency of dietary supplements in animal nutrition especially the protein supplements (Okah and Ibeawuchi, 2011). This can be achieved by sourcing for alternative protein feedstuff that will attract minimal competition from monogastric animals and humans.

Pigeon pea has some medicinal and nutritional benefits like antibacterial (Siyabonga *et al*., 2016), anti-oxidative effects (Wu *et al*., 2009; Muangman *et al*., 2011), reportedly used in the treatment of diabetes, dysentery, hepatitis, malaria and diet-induced hypercholesterolaemia (Oke, 2014), an important

source of low-cost vegetable protein, minerals and vitamins for humans (Fasoyiro *et al.,* 2010; Okpala and Ekwe 2013) and a good supplementation of starchy foods (Mbaeyi-Nwaoha and Onweluzo, 2013). Despite the fact that pigeon pea is a valuable and economic crop for both human and animal nutrition, pigeon pea seeds have received little attention as compared to the leaves (Nix *et al*. 2015). Phytochemical constituents of pigeon pea leaves include alkaloids, flavonoids, saponins, tannins and terpenes but anthraquinones, phlobatannins and sterols were not detected (Oke, 2014).

The effects of processed and raw unprocessed pigeon pea seed meal have been investigated on the growth, meat and egg qualities of broilers and layers (Etuk *et al*. 2003; Ahmed, *et al*. 2008; Amaefule and Obioha, 2007; Saeed *et al*. 2007), on cockerels (Yisa *et al*. 2010), in sheep (Okah and Ibeawuchi, 2011), but there is dearth of information on its effect on the sperm motility, sperm viability and sperm count of rats. It is worthy of note, however, that most of the non-conventional and also conventional legumes used in animal nutrition, contain some antinutritional factors which limit their utilization in the raw (unprocessed) state by livestock (Ahamefule *et al.* 2008). There has been report that some farmers bypass the processing of legumes used in animal feeds, so as to reduce the processing costs of animal feeds (Bawa *et al*. 2003). This calls for a need to investigate the reproductive effects of using unprocessed pigeon peas on the male rats.

This study examines the effects of feeding graded levels of raw pigeon pea seed diets on the sperm characteristics and testes of Wistar strain rats.

MATERIALS AND METHODS

Chemicals and Reagents

Bouin solution was used for fixation of the testis for studies on sperm motility, viability and count of rats.

Feed Preparation

The contents of normal feed and the graded constitutions with pigeon pea are shown in Tables 1 and 2 below.

Table 1: Composition of Normal Feed

Ingredients	Percentage (%)
Carbohydrate (Maize)	40
Protein (GNC, PKC, SB)	32 (8,16,8)
Fiber Content (Wheat Bran, Rice Bran)	18 (16,2)
Bone Meal	6
Oyster Shell	3.8
Premix (Methionine and Lysine)	0.08
Salt	0.12

** GNC- Groundnut cake, PKC- Palm kernel cake, SB-SOYA BEAN* Source: Soetan *et al*. (2017a).

Table 2: Graded levels of Pigeon pea (*Cajanus cajan*) Inclusion Diets

GROUPS	NORMAL FEED (kg)	PIGEON PEA INCLUSION (kg)	TOTAL (Kg)
A (Control)	3	-	3
B (10%)	2.7	0.3	3
C (20%)	2.4	0.6	3
D (30%)	2.1	0.9	3
E (40%)	1.8	1.2	3
F (100%)	-	3	3

The pigeon pea seeds and the rat concentrate feed (3kg each) were ground into powder form using an electric miller. The feed was then reconstituted into different percentage inclusion of pigeon pea seed diets (10%, 20%, 30%, 40%, 100%) and normal concentrate feed as control.

Experimental Animals

Thirty male Wistar strain rats (weighing between 120g and 160g) were used for this study. The animals were kept in stainless-steel individual metabolic cages (Associated Crate Ltd., England) located at the Experimental Animal Unit of the Department of Animal science, University of Ibadan, Ibadan. They were allowed to acclimatize for a period of two weeks. The animals were weighed and the weights recorded before commencement of experiment.

Experimental Protocol

The rats were assigned into six groups (A-F) of 5 rats each. Group A rats served as the control and were fed with commercial rat feed, Group B was fed with 10% pigeon pea inclusion diet, group C: 20% pigeon pea inclusion diet, Group D: 30% pigeon pea inclusion diet, Group E: 40% pigeon pea inclusion diet and Group F: 100% pigeon pea diet. All the rats were given 30 g of feed per day with water *ad libitum* for the 21 days experimental period.

Weight Assessment

The weight of each rat was taken and recorded at the start of the experiment (initial weight) and on days, 7, 14 and 21 of the study using a laboratory weighing balance.

Semen Collection and Analysis

The rats were anaesthetized with diethyl-ether before sacrifice as described in earlier studies (Ola-Davies and Ajani, 2016; Oyeyemi and Ajani, 2015). The mid caudo-ventral abdominal incision was made with sterilized pair of scissors, permitting instant access to the testis once pushed upward from the scrotum with gloved hand. The testes were then separated from the epididymis. The right and left epididymides were trimmed off the body of the testes and semen sample was collected thereafter from the tail of the epididymis through an incision made with a scalpel blade.

Percentage Sperm Motility and Viability

Sperm percentage motility was carried out using 2 to 3 drops of 2.9% warm buffered sodium citrate kept at body temperature as described by Zemjanis (1977).

Also, a drop of semen sample was placed on warm glass slide and stained with eosin-nigrosin stain for viability study after which a thin smear was then made of mixture of semen and stain. The smear was air dried and observed under the microscope (Zemjanis, 1977).

Histopathology

The testes for each animal in each group were harvested, fixed in Bouin's fluid for 24 hours and routinely processed for histology. The prepared slides were then examined using an Olympus® CX21 light microscope.

Data analysis

The data generated was analyzed using one way analysis of variance (ANOVA). SPSS Version 15 for Windows (SPSS Inc, 2006) and Microsoft Excel Professional Plus (Microsoft Corporation, 2010) were used to carry out all procedures. P values less than 0.05 were considered as significant.

RESULTS

The results of the weights of rats are shown in Table 3. There were significant (P<0.05) decreases in the weights of rats in groups E and F compared with the weights of group A rats (control).

The results for the sperm motility, viability and sperm count are shown in Table 4. The motility scores were

Table 3: Mean weights of rats in different Pigeon pea (*Cajanus cajan*) Inclusion Diets

Groups	Day 1 (g) (Initial weight)	Day 7 (g)	Day 14 (g)	Day 21 (g)
A	121.6±1.1	126.2±3.6	129.4±4.0	135.6±1.1
B	137.8±8.9	135.4±9.9	131.6±9.8	126.0±9.6
C	150.6±8.4	145.4±9.2	139.6±7.2	132.0±7.8
D	144.4±16.3	139.6±15.2	134.2±15.1	125.6±16.7
E	142.2±17.5	136.6±20.1	128±19.3	112.2±16.5*
F	144.6±13.1	134.4±14.8	125.6±16.2	102.2±18.3*

* Significant difference at P<0.05

Table 4: Mean values for percentage motility, viability, and Sperm count of albino rats in different Pigeon pea (*Cajanus cajan*) Inclusion Diets

Parameters	Group A	Group B	Group C	Group D	Group E	Group F
Motility (%)	87.00±3.00	76.00±6.96*	67.00±3.74	34.00±7.48	34.00±2.45	34.00±2.45
Viability (%)	80.00±2.74	79.00±2.92*	73.00±3.74	70.00±2.74	69.00±4.58	66.00±1.87
Sperm count ($x10^6$sperm/ml)	294.60±7.87	207.40±6.19*	193.40±7.68	192.60±6.27	187.60±6.47	176.80±2.05

* Significant difference at P<0.05

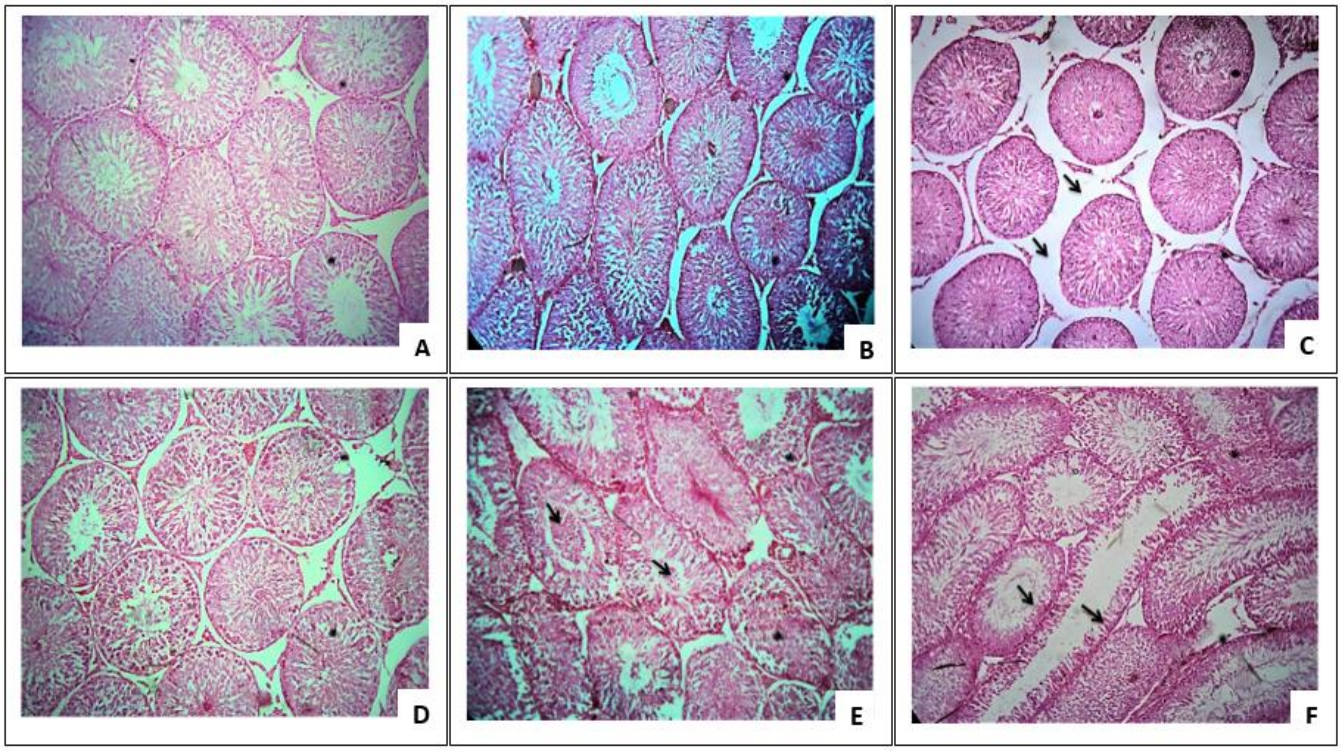

Figure 1. Transverse section of the testis of rats from groups A, B, C, D, E and F. Group C animals has marked expansion of the interstitial space (arrows) caused by oedema while group E and F showed immature germ cells in the seminiferous tubular lumen (arrows) and slightly reduced germinal depth (arrows). H&E, x150

between 34.00±2.45 and 87.00±3.00 with the control group A having the highest score (P<0.05). Group B rats fed with (10% pigeon pea inclusion diet) had a significantly higher (P<0.05) values (76.00±6.96) than groups C, D, E, and F of which mean percentage motility ranges between 34.00±2.45 and 67.00±3.74. Group E fed with 40% pigeon pea inclusion diet and group F fed with 100% pigeon pea diet had the lowest mean percentage motility of 34.00±2.45. This value was significantly lower than the mean values of groups A and B rats. The motility score was decreasing with increasing inclusion of pigeon pea. This same trend was observed for the sperm percentage viability and sperm count across the groups (Table 4).

Testicular Histopathology

No observable lesion was observed in the testicular histology of rats in groups A, B, and D (Figures 1A, 1B and 1D respectively). The testis of rats in group C showed marked expansion of the interstitium by oedema (Figure 1C). The testis of group E rats showed immature germ cells in the seminiferous tubular lumen (Figure 1E) while testis of group F rats revealed slightly reduced germinal depth (Figure 1F).

Figure 1: Group A (Control) Testis: There are no observable lesions. H&E, x150

DISCUSSION

Sperm motility, viability and sperm count are essential parameters in the evaluation of fertility potentials in the male animals (Garner and Hafez, 1993).

In this study, it was observed that the mean percentage motility values obtained for rats in groups D, E and F decreased significantly (P<0.05) with increasing inclusion levels of pigeon pea. The values obtained were below the 60.00% minimum required value for potential fertility (Garner and Hafez, 1982). This implies that feeding pigeon pea to rats beyond 20% inclusion level had adverse effect on the sperm motility and can thereby reduce the spermatozoa fertilizing capacity and consequently precipitating infertility. The results of this study is similar to the report by Soetan *et al*. (2017b) on the adverse effects of 50% raw lima beans (Phaseolus lunatus) inclusion diets, on the testes of mice. The study observed a significant (P<0.05) rise in sperm abnormalities with a drop in sperm motility and counts.

The results from this present study implies that 10-20% inclusion level of unprocessed pigeon pea fed to rats had no adverse effect on sperm percentage motility, and since this same trend was observed for the mean percentage liveability and sperm count, it connotes that the inclusion level of pigeon pea beyond 20% in rats feeds will be spermatotoxic and this will have a grave consequence on fertility potentials of the rats.

Soetan *et al*. (2014) reported significant decreases in the absolute weights of testes in rats fed with Rongai brown and Rongai white varieties of lablab beans (Lablab purpureus), when compared with the control group. Lablab purpureus-fed rats also showed significant decreases in spermatozoa motility, epididymal spermatozoa number, viability, testicular spermatozoa number and daily spermatozoa production. The percentage of morphologically

abnormal spermatozoa was significantly increased in all the rats fed with the three varieties of Lablab purpureus (Rongai brown, Rongai white and Highworth black).

Findings from our present study also corroborates earlier studies in male Wistar rats using nicotine (Oyeyemi *et al*., 2014), gossypol (Hadley *et al*., 2013) Lagenaria Breviflora Roberts; (Saba *et al*., 2009), Aloe vera gel (Oyeyemi and Ajani, 2015) and sodium arsenite (Ola-Davies *et al*., 2017).

It is likely that there are some anti-nutritional and toxic factors contained in raw pigeon pea which at higher dose might give unfavourable outcome when not adequately processed before feeding to animals. The histopathology lesions observed in testis of rats in groups C, E and F could be attributed to the presence of some toxic factors in the raw pigeon pea.

In a similar study on the effects of graded levels of pigeon pea, male rats fed with 30% raw pigeon pea inclusion diet showed *m*oderate lymphocytic infiltration in the portal areas of the liver while 100% raw pigeon pea diet produced a marked lymphocytic infiltration of the portal areas in the liver of male rats (Soetan *et al*., 2017a).

In another study on the effects of feeding three different varieties of raw lablab beans to rats, Rongai brown variety of lablab beans produced disruptions in testicular basement membrane of seminiferous tubules and loss of spermatozoa of the rats while Rongai white and Highworth black varieties caused oedema and reduction of seminiferous tubular diameters on the testis of rats (Soetan *et al*., 2014).

Immature clumps in the lumen of very few seminiferous tubules, severe congestion of interstitial vessels and a severe germinal and sertoli cell necrosis and erosion of the seminiferous lumen were observed in the testes of mice fed 50% raw lima beans inclusion diets (Soetan *et al*., 2017b) while vacuolation of secondary spermatocytes and loss of spermiogenic epithelium were reported by Ola-Davies *et al*. (2017) in the testes of male rats administered with sodium-arsenite.

In the light of the low sperm qualities observed in groups D, E, and F rats, it can be adduced that the poor sperm quality occurred during storage and maturation in the epididymis. The exact mechanism for this cannot be determined at the moment and would need to be further investigated. The exact toxic factors in the raw, unprocessed pigeon pea which caused the testicular histopathology in rats and their mechanism of action would need further study.

It was concluded in this study that feeding rats with raw pigeon pea beyond 20% inclusion level is

spermatotoxic having severe adverse effects on sperm motility, viability and sperm count. Pigeon pea in raw, unprocessed form must be incorporated with caution into animal feeds, especially the male animals used for breeding.

REFERENCES

Abdelati, K.H., Mohammed, H.A.R and Ahmed, M.E. (2009). Influence Of Feeding Processed Pigeon Pea (*Cajanus cajan*) Seeds on Broiler Performance. Int. J. Poult. Sci., 8(10): 971-975.

Ahmed, B. H., Abdel-Ati, K. A. and Elawad, S. M. (2008). Effect of Feeding Different Levels of Soaked Pigeon Pea *(Cajanus cajan)*seeds on Broiler Chickens Performance and Profitability. Research Journal of Animal and Veterinary Sciences, 1 (1): 1-4.

Amaefule, K. U. and Obioha, F. C. (2007). Pigeon pea (*Cajanus cajan* (L.) Mill sp) seed meal in layer diets: 2. Laying performance and egg quality characteristics of pullets fed raw or processed pigeon pea seed meal diets during grower and layer stages of life. International Journal of Poultry Science, 6(6): 445 - 451.

Bawa, G.S., Tegbe, T.S.B. and Ogundipe, S.O. (2003). Effect of feeding graded dietary levels of lablab seeds as a replacement for soybean on performance characteristics of young pigs. Proc. of the 28th Annual Conf. of the Nig. Soc. for Anim. Prod. 28: 230-232.

Devi, R.R., Premalatha R. and Saranya, A. (2016). Comparative analysis of phytochemical constituents and antibacterial activity of leaf, seed and root extract of *Cajanus cajan* (L.) Mill sp. International Journal of Current Microbiology and Applied Sciences 5(3): 485-494.

Etuk, E.B, Esonu, B.O. and Udedibie, A.B.I. (2003). Evaluation of toasted pigeon pea (*Cajanus cajan*) seed meal as replacement for soyabean meal and maize in broiler finisher diets. Proceedings of the 8th Annual Conference of Animal Science Association of Nigeria, September 15th - 18th Federal University of Technology, Minna, Niger State, Nigeria.

Fasoyiro, S.B., Akande, S.R., Arowora, K.A., Sodeko, O.O., Sulaiman, P.O., Olapade, C.O. and Odiri, C.E. (2010). Physico-chemical and sensory properties of pigeon pea (*Cajanus cajan*) flours. African Journal of Food Science, 4(3):120-126.

Garner, D.L. and Hafez, E.S.E. (1982). Spermatozoa and seminal plasma, In: Reproduction in farm aniumals edited by E.S.E Hafez. Lea and Febiger.

Garner D.L., Hafez E.S.E. (1993). Spermatozoa and seminal plasma. In: Hafez E.S.E (eds.) Reproduction in Farm animals (6th ed.) Lea and Febiger, Philadelphia, USA. pp. 165-187.

Hadley, M. A., Lin, Y. C. and Dym, M. (1981). Effects of gossypol on the reproductive system of male rats. Journal of Andrology, 2: 190–199. doi: 10.1002/j.1939-4640.1981.tb00615.x

Mbaeyi-Nwaoha, I.E. and Onweluzo, J.C. (2013). Functional properties of sorghum (*S. bicolor* L.) – pigeon pea (*Cajanus cajan*) flour blends and storage stability of a flaked breakfast formulated from blends. Pakistan Journal of Nutrition, 12(4): 382-397.

Muangman, T, Leelamanit, W. and Klungsupaya, P (2011). Crude proteins from pigeon pea (*Cajanus cajan* (L.) Millsp) possess potent SOD-like activity and genoprotective effect against H2O2 in TK6 cells. Journal of Medicinal Plants Research, 5(32): 6977-6986.

Nix, A., Paull, C.A. and Colgrave, M. (2015). The flavonoid profile of pigeon pea, *Cajanus cajan*: A Review. SpringerPlus 4:125DOI 10.1186/s40064-015-0906-x

Ola-Davies, O., Oloye, A. and Adeoye, A. (2017). Sodium arsenite induced reproductive perturbations in Wistar strain albino rats: Protective assessment of *Cassia fistula*. Tropical Veterinarian 35(4): 162-172.

Ola-Davies, O. and Ajani, O. S. (2016). Semen characteristics and sperm morphology of *Pistia stratiotes* Linn. (Araceae) protected male albino rats (Wistar strain) exposed to sodium arsenite. J. Complement Integr. Med. DOI 10.1515/jcim-2015-0033.

Odeny, D.A. (2007). The potential of pigeon pea (*Cajanus cajan* (L.) Millsp.) in Africa. Natural Research Forum, 31: 297-305.

Okah, P. and Ibeawuchi, C. (2011). Growth performance and haematological characteristics of West African Dwarf (WAD) sheep fed graded levels of dietary pigeon pea seed meal. African Journal of Agricultural Research, Vol. 6(11): 2461-2465.

Oke, D.G. (2014). Proximate and phytochemical analysis of *Cajanus cajan* (Pigeon pea) leaves. Chemical Science Transactions, 3(3): 1172-1178.

Okpala, L. C. and Ekwe, O.O. (2013). Nutritional quality of cookies produced from mixtures of fermented pigeon pea, germinated sorghum and cocoyam flours. European Journal of Food Research and Review, 3(1): 38-49.

Oyeyemi, M.O., Ajani, O.S (2015). Haematological parameters, semen characteristics and sperm morphology of male albino rat (wistar strain) treated with *Aloe vera* gel. Journal of Medicinal Plants Research, 9(5): 510-514.

Oyeyemi, W.A. . Kolawole, T.A Shittu, S.T Ajah R. and B.F. Oyeyemi (2014). Effects of Ascorbic Acid on Reproductive Functions of Male Wistar Rats Exposed to Nicotine. J. Afr. Ass. Physiol. Sci. 2 (2): 110-116.

Saba A. B, Oridupa, O. A, Oyeyemi, M. O. and Osanyigbe, O. D (2009). Spermatozoa morphology and characteristics of male wistar rats administered

with ethanolic extract of *Lagenaria Breviflora Roberts*. African Journal of Biotechnology Vol. 8 (7), pp. 1170-1175.

Saeed, M. S., Khadiga, A. and Ati, A. (2007). Inclusion of pigeon pea (*Cajanus cajan*) seeds in broiler chicks diets. Research Journal of Animal and Veterinary Science. 2:1-4 INSInet publication.

Siyabonga, S. J., Ayanda, M, Sydney, M.T. and Oluwole, S.F. (2016). Comparative evaluation of antibacterial activity of induced and non-induced *Cajanus cajan* seed extract against selected gastrointestinal tract bacteria. African Journal of Microbiology Research, 10(10): 319-323.

Soetan, K.O., Adedara, I.A. and Farombi, E.O. (2014). Adverse effects of three varieties of *Lablab purpureus* seeds on reproductive functions in male rats. Folia Veterinaria 58(3): 145-152.

Soetan, K.O., Akinsulie, O.C. and Tijani, M.O. (2017a). Studies on Haematology, Serum Chemistry and Histology of Liver and Kidneys of Male Wistar Strain Albino Rats Fed Graded Levels of Raw Pigeon pea (*Cajanus cajan*) Seeds. EC Nutrition 8(3): 75-84.

Soetan, K.O., Oladipupo, A.A., Akinbowale, F.D. and Aina, O.O. (2017b). Effects of Lima beans (*Phaseolus lunatus*) consumption on some reproductive indices in the male mice. Tropical Veterinarian 35(4): 225-234.

Wu, N., Fu, K., Fu, Y. J., Zu, Y.G., Chang, F.R., Chen, Y.H., Liu, X.L., Kong, Y., Liu, W., and Gu, C.B. (2009). Antioxidant Activities of Extracts and Main Components of Pigeon pea [*Cajanus cajan* (L.) Millsp.] Leaves. Molecules, 14: 1032-1043.

Yisa, A. G., Edache, J. A., Oyawoye, E. O., Diarra, S. S. and Yakubu, B. (2010). The effect of graded levels of boiled and dried pigeon pea seed meal on the carcass of cockerels. Journal of Environmental Issues and Agriculture in Developing Countries, 2(2 & 3): 125-131.

Zemjanis, R. (1977). Collection and evaluation of semen. In: Diagnostic and therapeutic technique in animal production. 3rd Edition. The Williams and Wilkins company, Baltimore, 139-180.

Zu, Y.G., Fu, Y.J., Liu, W., Hou, C.L., Kong, Y. (2006). Simultaneous determination of four flavonoids in pigeonpea [*Cajanus cajan*(L.) Millsp.] leaves using RP-LC-DAD. Chromatographia, 63:499-505.