Int. J. Morphol.,
31(2):662-667, 2013.

The Histo-morphometric Evidences of *Vernonia amygdalina* Leaf Extract-induced Testicular Toxicity

Evidencias Histomorfométricas de la Toxicidad Testicular Inducida por el Extracto de la Hoja de *Vernonia amygdalina*

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SAALU, L. C.; AKUNNA, G. G. & OYEWOPO, A. O. The histomorfometric evidences of *Vernonia amygdalina* leaf extract-induced testicular toxicity. ***Int. J. Morphol., 31(2)***:662-667, 2013.

SUMMARY: *Vernonia amygdalina* (bitter-leaf) is native to West Africa but occurs mostly in its cultivated form in various parts of central and southern Nigeria. It is a dark-green leafy vegetable commonly used in cooking and as folkloric medicine for the management of several diseases in Nigeria. In the present study, the effects of varying doses of ethanolic leaf extract of *Vernonia amygdalina* on the rat testis histo-morphometry were investigated. Forty male wistar rats were divided into groups of four. Group A, as the control was given 10 ml/kg/day/oral distilled water while Group B, C and D subsequently treated with 100, 300 and 600 mg/kg/day/oral route *Vernonia amygdalina* leaves extract respectively for 56 days. Results showed that Group B rats had normal testis histology comparable to the control group. However, rats in Group C and D exhibited dose-dependent poor testes histo-morphometric profiles, with the higher dosage-group (D) providing a worse feature. Thus, there was a statistically significant ($p < 0.05$) reduction in the tubular diameter, cross-sectional area of the tubules, number of tubular profiles per unit area and the mean numerical density of seminiferous tubules of Group C and Group D animals compared to the control group. Our results therefore indicated that, while the lower dose (100 mg/kg) of Vernonia amygdalina leaves extract can be accommodated by rat testis, the higher doses (300 mg/kg and 600 mg/kg) demonstrate testicular toxicity in the rat.

KEY WORDS: *Vernonia amygdalina*; Histology; Morphometry; Testis; Wistar rat.

INTRODUCTION

It is paramount to be acquainted with the fact that accurate morphometric information may not only provide answers to important questions about the spermatogenic process but may also correlate with physiological and biochemical findings, leading to a complete understanding of infertility.

Globally, infertility affects about 50 to 80 million couples at some point of their reproductive lives with a variety of biological and behavioral determinants (WHO, 2003).

Various medicinal plants ranging from *Quassia amara* (Raji & Bolarinwa, 1997), *Carica papaya* (Lohiya *et al*., 1994), *Ruta graveolens* (Khouri & El-Akawi, 2005), *Terminalia catappa* (Ratnasooriya & Dharmasiri, 2000), *Ricinus communis* (Raji *et al*., 2006), *Garcinia kola* (Akpantah *et al.*, 2003) and *Vernonia amygdalina* (Oyeyimi *et al*., 2008) have been implicated in male infertility.

Fortunately, several countries in the world are gifted with plant biodiversity, and there is currently an emanating awareness about the significance of plant remedies in health care delivery system. In many parts of the world, efforts are now being aimed at investigating therapeutic efficacy of locally available medicinal herbal plants.

Researching to the efficacy of herbs used in traditional practice would be valuable in establishing standard dosages for herbal preparations and to scrutinize their toxicity (Saalu *et al.*, 2008; Saalu *et al.*, 2010; Akunna *et al.*, 2012). One of such herbs is *Vernonia amygdalina*.

Vernonia amygdalina, popularly known as "bitter leaf" is a shrub of 2-5 m tall with petiolate leaves of about 6.0mm wide. It is widely distributed in Nigeria especially in the south-eastern part of the country. "Bitter leaf" is popular in Nigeria because of its dietary and diverse medicinal uses (Ojiako & Nwanjo, 2006). It is known as Onugbu in eastern

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part of Nigeria were it is renowned for its flavoring ability, as Ewuro in Western part and Chusar Doki in Northern part of Nigeria. The leaf decoction of *Vernonia amygdalina* is used traditionally in treatment of diabetes, malaria, fever, hiccups, and gastric discomfort (Dalziel, 1937; Bever, 1960). It has been reported to exert schistosomicidal, anti-plasmodial, antitumorigenic and anti-cancer effects (Hamowia & Saffaf, 1994; Ojiako & Nwanjo; Oyeyimi *et al.*).

Phytochemically, *Vernonia amygdalina* consist of several stigmasranerype saponins, such as vernonioside A, Bl, 42, A3, 82, D3, A.4, and C (Jisaka *et al.*, 1993). Sun dried leaves of *Vernonia amygdalina* has been reported to contain saponins, tannins, iron, zinc, magnesium, sodium, potassium, caicium and phosphorus (Akindahunsi & Salawu, 2005).

In 1993, Jisaka *et al.*, reported that the leaves contain vernodaline and vernolide which were evident by elicit antitumoural activity in leukaemia cell P-388 and C l2l0. Igile *et al.*, (1994) demonstrated the anti-oxidant activities of luteoiin-o-p-glucosidfela vonoid compounds present in the leaves of *Vernonia amygdalina* using the united oxidation of ß-carotene linoleic.

Although the use of herbs has more than tripled over the last decade, little scientific support exists to aid in regulation of the dosage of plant extract in folkloric medicine which may be valuable in forestalling a prospective testiculo-toxic effect.

This study consequently investigated the aftermath of different doses of fresh *Vernonia amygdalina* leaves extract on rat testis histo-morphometry.

MATERIAL AND METHOD

Procurement of the plant material. The leaves of *Vernonia amygdalina* were purchased in a local market at Bariga Lagos State, Nigeria. They were authenticated by a staff in the herbarium of the Department of Botany, University of Lagos, Lagos, Lagos state, Nigeria, where a voucher specimen was deposited for reference. The leaves were thoroughly washed in sterile water and the water was then drained from the leaves.

Plant extraction. The leaves of *Vernonia amygdalina* was dried on a laboratory table for 8 days and reduced to powder. One hundred grams of the powder was macerated in 95% ethanol (300 ml) for 72 hours. The liquid extract obtained was concentrated in voacuo at 40oC. The yield was 3.77%. The extract was stored in a refrigerator at 4oC until used.

Experimental etiquette. The protocol of experimentation was approved by the Departmental Ethical Committee responsible for the use of laboratory Animals, Lagos State University. The study is consistent with the standard of the use of laboratory animals (World Medical Association & American Physiological Society, 2002).

Forty adult male Sprague-Dawley rats (10 to 11 weeks old) weighing 175-180 g were used for this research work. The rats were randomly divided into four groups (A-D) of ten rats each such that the average weight difference between and within groups did not exceed ±20% of the average weight of the sample population. Rats in group A which served as control were given 10 ml/kg/day of distilled water for 56 days. Rats in groups B– D served as the treatment groups and were orally treated with 100, 300 and 600 mg/kg/day of the extract, respectively, for 56 days, the extent of spermatogenesis in rat being 51.6-56 days (Heller & Clermont, 1964; Jegou *et al*., 2002). The appropriate quantity of the ethanolic extract was given orally through an orogastric cannula into the stomach via the esophagus (Prakash, 1981). The extract was administered once daily by 12 noon for six days (Monday to Saturday) within a week.

Animal sacrifice and sample collection. The rats were at the time of sacrifice first weighed and then anaesthetized by placing them in a closed jar containing cotton wool soaked with chloroform anaesthesia. The abdominal cavity was opened up through a midline abdominal incision to expose the reproductive organs. Then the testes were excised and trimmed of all fat. The testes weights of each animal were evaluated.The testes were weighed with an electronic analytical and precision balance (BA 210S, d=0.0001-Sartoriusen GA, Goettingen, Germany). The testes volumes were measured by water displacement method using Archimedes principle (Acott, 1999). The two testes of each rat were measured and the average value obtained for each of the two parameters was regarded as one observation. Testes of each animal were fixed in 10% formol-saline for histological examination.

Determination of morphometric parameters. Histological slides were prepared from the formol-saline fixed testes. However, before embedding, it was ensured that the sections were orientated perpendicular to their long axes, and chosen as "vertical sections". For each testis, five vertical sections from the polar and the equatorial regions were sampled (Qin & Lung, 2002) and an unbiased numerical estimation of the following morphometric parameters was estimated using a systematic random scheme (Gundersen & Jenson, 1987):

Diameter (D) of seminiferous tubules. The diameter of seminiferous tubules with profiles that were round or nearly

round were estimated for each animal and a mean, D, was determined by taking the average of two diameters, D1 and D2 (Perpendicular to one another). D1 and D2 were taken no more than when D1/D2 ≥ 0.85.

Cross-sectional area (AC) of the seminiferous tubules. The cross-sectional areas of the seminiferous tubules was estimated from the formula AC = πD2/4 (where π is equivalent to 3.142 and D the mean diameter of the seminiferous tubules).

Number of profiles of seminiferous tubules in a unit area of testis (NA). The Number of profiles of seminiferous tubules per unit area was determined using the unbiased counting frame anticipated by Gundersen (1977). Using this frame, in addition to counting profiles completely inside the frame we counted all profiles with any part inside the frame provided they do not intersect the forbidden line (fulldrawn line) or exclusion edges or their extension.

Numerical Density (NV) of seminiferous tubules. This is the number of profiles per unit volume and was determined by using the modified Floderus equation: NV =NA/ (D +T) (Gilliland *et al.*, 2001) where, NA is the number of profiles per unit area, D is the diameter and T the average thickness of the section.

The evaluation of the diameter was done with calibrated eyepiece and stage grids mounted on a light research microscope. Estimation of volume density of testicular components and number of seminiferous tubules were done on a computer monitor onto whom a graph sheet was superimposed and on which slides were projected from a research light microscope (Model N -400ME, CEL-TECH Diagnostics, Hamburg, Germany).

Statistical analysis. The data were statistically analyzed and expressed as Mean ± SD. Analysis was carried out using analysis of variance (ANOVA) with Scheffe's post hoc test. The level of significance was considered at $p < 0.05$.

RESULTS

As shown in Figure 2, the group of animals that were administered 100 mg/kg of the *Vernonia amygdalina* leaves extract showed normal testis histology comparable to the control group (Fig. 1). However, figures 3 and 4 showed that the group of animals that were given 300 mg/kg and 600 mg/kg of the extract respectively exhibited dose-dependent poor testes histo-morphometric profiles, with the higher dosage providing a worse feature as shown in figure 4.

As shown in Table I, the mean seminiferous tubular diameters of rats treated with 300 and 600 mg/kg were: 113.42±10.12b and 100.21±7.13b mm respectively. These values showed a significant decline ($p < 0.05$) compared to the tubular diameter (160.41±8.22 mm) of control rats. However, there was a significant increase in the tubular diameter (210.24±11.13a) of animals treated with 100 mg/kg of the extract compared to tubular diameter (160.41±8.22 mm) of the control rats.

The disparity in the cross-sectional area of the tubules, the number of tubular profiles per unit area and the mean numerical density of seminiferous tubules treaded a similar pattern as the tubular diameter (Table I). However, there was a significant increase in the cross-sectional area of the tubules, the number of tubular profiles per unit area and the mean numerical density of seminiferous tubules treated with 100 mg/kg of the extract (Table I).

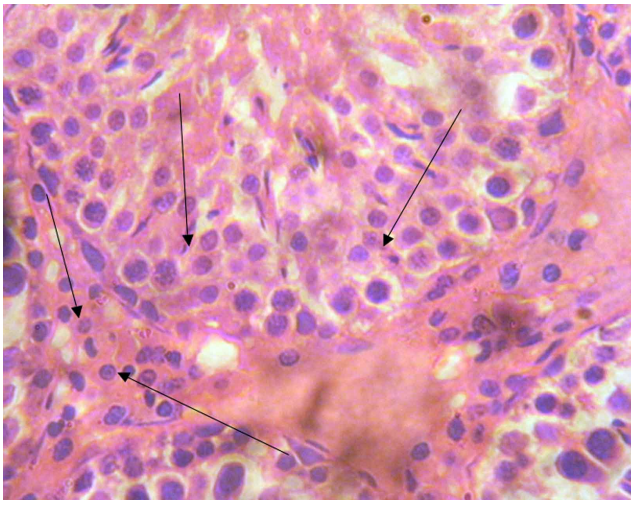

Fig. 1. Photomicrograph of group A rat testes stained with H&E. X 400 S; seminiferous tubules L; lumen, l; Leydig cell.

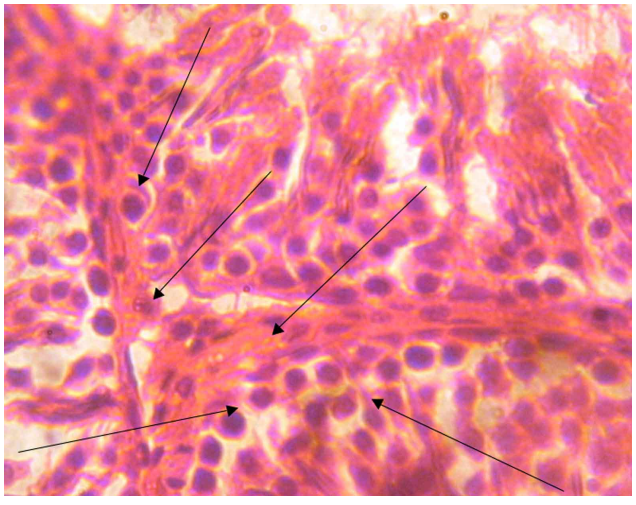

Fig. 2. Photomicrograph of group B rats testes stained with H&E. X 400 S; seminiferous tubules L;lumen, l; Leydig cell.

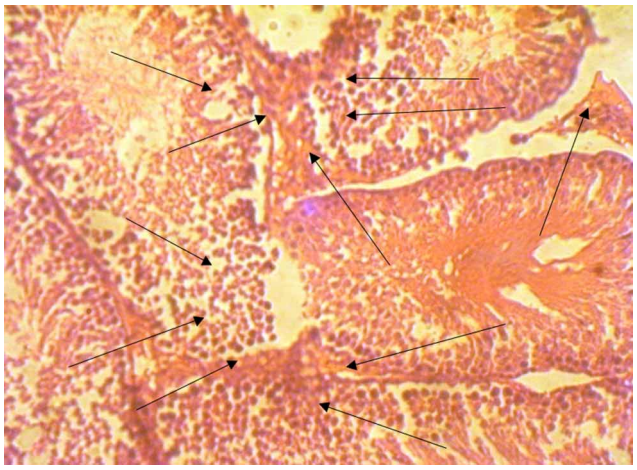

Fig. 3. Photomicrograph of group C rat testes stained with H&E. X 400 S; seminiferous tubules L;lumen, l; Leydig cell

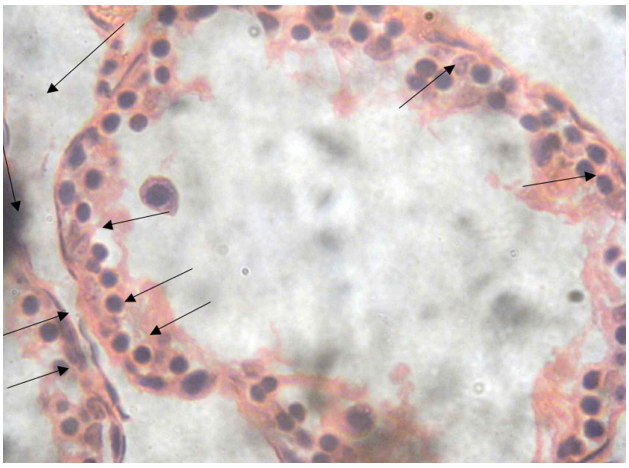

Fig. 4. Photomicrograph of group D rat testes stained with H&E. X 400 S; seminiferous tubules L;lumen, l; Leydig cell.

Table I. Seminiferous tubular diameter, cross sectional area, numerical densities of seminiferous tubules and number of profiles per unit area in experimental and control Sprague-Dawley rats.

Treatment	**D** (μm)	$\mathbf{A_c}$ ($x10^3 \mu m^2$)	$\mathbf{N_A}$ ($X10^{-8} \mu m^{-2}$)	$\mathbf{N_v}$ ($x10^{-10} \mu m^{-3}$)
A	160.41±8.22	27.51±8.13	24.21±5.13	11.13±4.75
B	210.24±11.13a	43.54±5.12a	33.52±4.12a	19.53±4.23a
C	113.42±10.12b	18.40±6.24b	14.45±3.33b	7.63±5.57b
D	100.21±7.13b	15.35±1.12b	11.42±1.43b	5.13±3.82b

a, b represent significant increase and decrease respectively at $P<0.05$ when compared to control values. All values are expressed as Mean ± Standard deviation (n=10). A= 10ml/kg/day/oral distilled water (Control), B= 100mg/kg/day/oral *VA* extract, C= 300mg/kg/day/oral *VA* extract, D= 600mg/kg/day/oral *VA* extract.

DISCUSSION

Describing histological sections with stereological methods helps to unravel some essential issues allied with qualitative microscopic investigation.

In this study, analysis was carried out on randomly sampled vertical histological sections using stereological method in a bid to uphold accuracy in favor of the investigation.

The results showed a dose dependent significant ($P<0.05$) decrease in the mean seminiferous tubular diameters, cross-sectional area of the tubules, number of tubular profiles per unit area and the mean numerical density of seminiferous tubules of rats treated with 300 and 600 mg/kg of *Vernonia amygdalina* leaf extract. The significant reduction in these geometric values was supported by the distortion in the morphological features of the seminiferous epithelium and interstitium (Figs. 3 and 4), which is liable for the outstanding production of the luminal fluid (Okanlawon & Ashiru, 1998).

Avalanche of studies have reported the important roles played by germinal epithelium in production of luminal fluid which is a precursor for spermatogenesis (Fisher, 2002; Syed & Hecht, 2002). The leydig cell determines the size of the interstitium thus several reports (Castro *et al*., 2002; Saalu *et al.*, 2011) correlates the level of testicular testosterone with the number of Leydig cell. The reduction in morphometric values coupled with the degenerated epithelium in the present study only bolstered previous report (Fisher, 2002; Castro *et al*., 2002, Saalu *et al.*, 2011). In 2008, Oyeyemi *et al*., reported a significant reduction in sperm count, sperm motility, percentage of normal sperm and an increase in the percentage of abnormal sperm post-administration of 250 and 500 mg/kg/body weight of aqueous extract of *Vernonia amygdalina*. The dose-reliant decline in the testicular volume and diameter of the tubules from our study would only imply a significant decline in the interstitium due to the destruction of the Leydig cells. This suggests that the extract affected the process of spermatogenesis as reflected by a dwindle in the mean

seminiferous tubular diameters, cross-sectional area of the tubules, number of tubular profiles per unit area and the mean numerical density of seminiferous tubules of rats treated with 300 and 600 mg/kg of *Vernonia amygdalina* leaf extract.

However, the group of animals that were treated with 100 mg/kg of the *Vernonia amygdalina* leaves extracts showed normal histo-morphometric profiles comparable to the control group (Fig. 1). The report indicated that lower dose (100 mg/kg) of *Vernonia amygdalina* leaves extracts had no deleterious effect on the testis. This result is in conformity with several reports (Ohigashi *et al.*, 1991; Jisaka *et al.*; Igile *et al*.; Oyeyemi *et al.*).

Three dimensional deductions obtained from the present study provide quantitative evidence that the ethanolic leaf extract of *Vernonia amygdalina* is testiculotoxic at 300 mg/kg and 600 mg/kg of dosage. Due to the quantity of spermatogenic cells in the basal layer and the Sertoli-Sertoli cell barrier which determines the number of cells in the adluminal compartment (Osinubi, 2006); we would be unable to conclude based on the histo-morphometric alterations in the present study. Nevertheless, the three-dimensional evaluations obtained in this study are a sound conclusion of the histo-morphometric characteristic of the rat testis.

The results of this study therefore indicate that, while the lower dose (100 mg/kg) of *Vernonia amygdalina* leaves extract had no harmful effect on the rat testis; the higher doses (300 mg/kg and 600 mg/kg) indeed demonstrate testicular toxicity in the rat.

CONCLUSION. A regulation of the dosage of this extract in folkloric medicine may therefore be beneficial in forestalling a possible reproductive impairment. This is even more significant when one considers that the vulnerability of man to toxic substances would be higher than that of rodents since rodents posses more efficient xenobiotic biotransformation system than man (Lüllmann *et. al*., 1973).

SAALU, L. C.; AKUNNA, G. G. & OYEWOPO, A. O. Evidencias histomorfométricas de la toxicidad testicular inducida por el extracto de la hoja de *Vernonia amygdalina*. ***Int. J. Morphol., 31(2)***:662-667, 2013.

RESUMEN: La *Vernonia amygdalina* (hoja amarga) es originaria de África Occidental, pero se produce mediante cultivo en varias partes del Centro y Sur de Nigeria. Es una verdura, una hoja color verde oscuro, común en la cocina y como medicina alternativa en el manejo de varias enfermedades en Nigeria. Se investigaron los efectos de diferentes dosis de extracto etanólico de la hoja de *Vernonia amygdalina* para estudiar los efectos sobre la histomorfometría testicular en la rata. Cuarenta ratas Wistar macho se dividieron en 4 grupos. Grupo A, como control con el suministro de 10 ml/kg/día/agua destilada vía oral, y los Grupos B, C y D tratados con 100, 300 y 600 mg/kg/día/ vía oral del extracto de *Vernonia amygdalina*, durante 56 días. Los resultados mostraron que las ratas del grupo B tenían una histología testicular normal y comparable con el grupo control. Sin embargo, las ratas del grupo C y D mostraron bajos perfiles histomorfométricos testiculares, dependientes de la dosis, y con la dosis más elevada, grupo (D) se observaron característica aún menores. Hubo una reducción estadísticamente significativa ($p<0,05$) en el diámetro tubular, área de sección transversal de los túbulos, número túbulos por unidad de superficie y densidad numérica media de los túbulos seminíferos del Grupo C y D en comparación con el grupo de control. Nuestros resultados indicaron que dosis bajas (100 mg/kg) del extracto de *Vernonia amygdalina* pueden ser aceptables y no alteran el testículo de rata, pero con dosis altas (300 mg/kg y 600 mg/kg) se observa toxicidad testicular.

PALABRAS CLAVE: *Vernonia amygdalina*; Histología; Morfometría; Testículo; Rata Wistar.

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Received: 08-08-2012
Accepted: 04-01-2013