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Studies of the Effect of Methanolic Stem Bark Extract of *Lannea acida* on Fertility and Testosterone in Male Wistar Rats

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Abstract: Objective of the study is to investigate the effect of methanolic stem bark extract of *lannaeacida* on sperm count, sperm motility, sperm morphology, serum testosterone, and histology of the testes on male Wister rats. The fresh stem bark of *lanneaacida* was collected from Ahmadu Bello University main campus the plant was cleaned and air dried at room temperature and then made into powder, then macerated using 70% methanol for 24 h a total of total of 25 adult male Wistar rats weighed between 150-200 g were randomly group into 5 different groups before oral administration of the extract. At 50 mg/kg of the group treated with *lanneaacida,* the sperm count is 34.00±1.87 m/mL while the sperm count in the control group has 29.00±1.78 m/mL, the sperm motile for the extract treated groups at 100 mg/kg is 68.20±3.56, the serum testosterone level in dose of 200 mg/kg of *Lanneaacida*is 0.64±0.11 there was no difference statistically at $p>0.05$ when compared with the control, No histological lesion was observed in all the control groups. Findings from this study shows that administration of the stem bark extract of *lanneaacida* has the tendency to enhance sperm count, morphology, motility and serum testosterone level.

Key words: Fertility, infertility, plant extract, sperm, testis

INTRODUCTION

For more than two decade the World Health Organization (WHO) has encouraged the use of traditional medicine, especially in the developing countries by promoting the incorporation of its useful elements into natural health care system (Akerele, 1987), Nigeria, with a population of close to 150 million people, has a high population growth rate and also a high rate of fertility, Available evidence also suggests that the country has high rates of primary and secondary infertility. Community based data suggest that up to 30 per cent of couples in some parts of Nigeria may have proven difficulties in achieving a desired conception after two years of marriage without the use of contraceptives (Adetoro and Ebomoyi, 1991). Regarding gender differences in the aetiology of infertility, several studies in the literature indicate that disorders in males and females account for an equal proportion of infertility, with the male factor being associated with a greater percentage of cases of primary infertility (Kuku and Osegbe, 1989).

The prevalence of infertility in a rural Nigerian community is determined by a systematic random sampling of the population. The overall prevalent rate was 30.3%, giving indices of 9.2% for primary infertility and 21.1% for secondary infertility. Primary infertility is rare after the age of 30 years and acquired causes of infertility are responsible for the high prevalence rate (Adetoro and Ebomoyi, 1991).

Africa depends on herbal remedies. The need for research and development in the field of African medicinal plants can not be over emphasized. For this reason WHO (1987), review the medicinal situation in several developing countries and made some fundamental suggestions aimed at promoting and developing the utilization of traditional medicine in order to contribute to the establishment of health care services in Africa and other developing countries (Nakajima, 1987).

Plants such as *Curcuma longa* and *Garcia kola*enhance sperm motility and decrease spermatozoa abnormality (Farombi *et al*., 2007; Adimoeja *et al*., 1995), *Asparagus racemous*, *Withaniasenticosus*, *Andrographispaniculata* and *Acanthopanaxsensticosus* (Nantia *et al*., 2009) are plants proven to improve spermatogenesis, sperm motility and morphology. Many flavonoids containing plants are know to have anti-oxidant effect (Evans, 1999).

Lanneaacida belong to the family *anacardiaceae*, in *Fulani-fulfulde (Nigeria)*faruhi and in *Hausa* is known as faàrú (Gill, 1992), The general uses of *lannaeacida*

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include Medicines: eye treatments, Products: farming, forestry, hunting and fishing apparatus, fibre, household, domestic and personal items. While the Bark is use for various Medicines purpose which include the following: anal haemorrhoids, diarrhoea, dysentery, malnutrition and debility, oral treatments, pregnancy, antiaborifacients; vermifuges, Products: exudations-gums, resins, etc., (Ellenberg *et al*., 1998).

Objective of the study:

- Investigate the effect of the *lannaeacida* on sperm count, sperm motility, and sperm morphology
- Investigate the effect of the plant extract on serum testosterone
- Investigate the effect on histology of the testes

MATERIALS AND METHODS

The study was conducted at the Department of Human Physiology, Faculty of Medicine Ahmadu Bello University Zaria in August 2009. The fresh stem bark of *lanneaacida*was collected from Ahmadu Bello University main campus and environment. It was identified by Mallam A. U. Gallah of the Herbarium section of the Department of Biological Sciences Herbarium of Ahmadu Bello University, Zaria and a voucher specimen number 384 has been deposited. The plant collected was cleaned and air dried at room temperature for 2 weeks and then made into powder using pestle and mortar. The powdered samples was then collected and stored in a clean polythene bag until required for extraction.

Method of extraction: The dried sample of the stem bark was macerated using 70% methanol for 24 h then filter with Whitman sized 1 filter paper, and the filtrate was concentrated in organ bath at about 37ºC to yield a residue of at 28 g of the methanolic extracted which was kept in a dried clean air tight container until used, the Phytochemical screening of the crude extract of *lannaeacida*was carried out using the methods of Trease and Evans (1983).

Experimental design: The methanolic stem bark extract of *lannaeacida was*administered for 14 days.

Group A: Control was treated with normal saline orally administered
Group B: Treated with 50 mg/kg of the extract orally administered
Group C: Treated with 100 mg/kg of the extract orally administered
Group D: Treated with 200 mg/kg of the extract orally administered
Group E: Treated with 400 mg/kg of the extract orally administered

Preparation of doses: 6 g of the dried aqueous methanolic stem bark extract of *lannaeacidawas* weighted and then dissolved in 15mls of distilled water to make a stock solution; the respective doses used for this experiment were prepared 50, 100, 200, and 400 mg/kg body weight of the rats. The dose of the extract to be administered to each rat was calculated as follows:

$$\text{Volume to be injected} = \text{Dose} \times \text{weight of rat} / \text{stock solution}$$

Sample collection: The rats were then euthenized by placing them into a glass chamber after which they are laid supine on a dissecting board and the limbs fastened to the board with dissecting pins and dissected exposing the thoracic cavity and blood sample was collected from each rat via the apex of the heart and stored in non-heparinzed EDTA test- tube and centrifuged, the serum was used for hormonal assay (testosterone). The testes was then expose by scrotal incision, removed and transferred into a Petri dish, the adipose tissues, and blood vessels were removed from the testes before they were washed with normal saline maintained at 37ºC, the testes were weighed in a digital weighing balance, before the epididymis were removed and weighed separately. The testes were stored in sample bottle containing 10% normal-saline (formalin 100 mL, sodium chloride 8.5 g, water 900 mL) for histological examination (Oyeyemi *et al*., 2008).

Sperm count, sperm motility and sperm morphology: The epididymis was teased into Petri dish and 1 mL of normal saline at temperature of 36ºC was added to the semen to enhance sperm survival invitro during the period of the study. The semen mixture was then sucked in to a red blood pipette to the 0.5 mark, and then diluted with warm normal saline which was sucked up to the 101 mark. The normal saline at the stem of the pipette was discarded and then the contents of the bulb of the pipette were mixed thoroughly. A drop of the semen mixture was placed on the neuber counting chamber which then spread under the cover- slip by capillary action (charging the chamber). The counting chamber was then mounted on the slide stage of the microscope and viewed under the magnification of x40. A grid system divides the counting chamber into 5 major squares using the top and right or left and bottom system of counting (Singh *et al*., 2000). The total numbers of sperm cells were counted and expressed in million per mil.

Analysis of sperm motility and sperm morphology was carried out by placing a drop of the sperm-saline mixture on two separate slide one for sperm motility (labelled A) and the other for sperm morphology (labelled B). Slide A was covered with a cover-slip and examined under the light microscope at a magnification of X40 and the sperm motility were estimated in percentage.

A smear was made on slide B by using another slide (spreader) inclined at an angle of 45 , 95% ethanol was immediately added for 2 min, followed by 1ml of Gimsa stain (Gimsa powder 0.3 g, glycerine 25 mL acetone, 25 mL free alcohol), and then allowed to stand for 10 min, after which it was washed with buffer distilled water, and allow to air dry. The sperm cells were counted by putting a drop of immersion oil and placed on a microscope at a magnification of X100. Counting was in a zigzag pattern. Both normal sperm cells (for rodents having a hook - shaped head) and abnormal cell (abnormality in head, midpiece or tail) were observed and counted, and the sperm morphology was estimated in percentage (Keel, 1990).

Hormonal assay: The stored blood serum in the non-heparinzed EDTA-test tubes were analyzed at the Department of chemical pathology Ahmadu Bello Teaching Hospital Zaria, using testosterone kit (Syntron Bioresearch, Inc. Microwell Testosterone EIA, Reference number 4410 - 96 (96 test kit). The microwell testosterone EIA is a solid- phase enzyme immunoassay utilizing the competitive binding principle. Testosterone present in the sample will compete with enzyme- labelled testosterone for binding with anti- testosterone anti body immobilized on the microwell surface. The amount of conjugate that binds to the microwell surface will decrease in proportion to the concentration of testosterone in the sample.

The unbound sample and conjugate are then removed by washing and the colour development reagents (substrates = 6.0 mL buffer hydrogen peroxide solution and 6.0 ml buffered 3, 3', 5, 5'- Tetramethylbenzidine solution) are added. Upon exposure to the bound enzyme, a color change will be observed. The intensity of the colour reflect the amount of bound enzyme- testosterone conjugate and is inversely proportional to concentration of testosterone in the sample within dynamic range of the assay. After stopping the reaction the resulting colour is measured using a spectrophotometer at 450 nm. The testosterone concentration in the sample and the control were concurrently run and was determine from the standard curve thus as follows Wilke and Utley, (1987).

- The average absorbance values (A.450) for each reference standard, control and test sample were calculated.
- A standard curve was prepared by plotting the average absorbance (A.450) versus the corresponding concentration of the standards on a log graph paper.
- Using the absorbance (A.450) value for each test sample to determine the corresponding concentration of testosterone in ng/mL from the standard curve.

Histopathological study:

(i) Preparation of tissue for histology: The technique adopted for this process are out lined by Carleton (1967)

Tissue processing: The tissue obtained after sacrificing the treated and control rats were trimmed to size and fixed in 10% formal-saline (formalin 100 mL, sodium chloride 8.5 g, water 900 mL). Using the tissue processor, the tissues were dehydrated using graded concentrations of ethanol as follows:

- 70% alcohol was used to dehydrate the tissue for 1 h twice
- 90% alcohol was used to dehydrate the tissue for 1 h twice
- Absolute alcohol was used to dehydrate the tissue for 1 h twice

They were cleared in xylene by transferring them into equal volumes of alcohol (Absolute) and xylene for 1 h; in order to avoid tissue distortion due to sharp transition from alcohol to xylene. The tissues were then passed through two changes of xylene for 1 h each.

Embedding: The tissues were embedded in paraffin wax at 55 ºC for 2 h each. This infiltration was carried out in two changes of paraffin wax for 2 h each. The tissues were later blocked out using L-shaped metal molder and subsequently mounted on a wooden block and trimmed to size for sectioning.

Sectioning: Using rotary microtome, the tissue blocks were cut into ribbons of 5 µ thickness each. The cut sections were picked with horsed - brush onto a slide. The section on the slide floated in 20% alcohol and then in warm water bath to allow for proper straightening. The sectioned were mounted on albumenized slides. They were dried in oven at 37ºC overnight.

(ii) Staining (Haematoxylin-Eosine staining method): Deparaffinizing, the sections were dewaxed using two changed of xylene for 2 min each.

Re-hydration: The sections were passed through descending graded of alcohol from absolute to 70% alcohol, each for 1 min, then washed in water followed by staining with Haematoxylin for twenty minutes then washed in water, the tissues were then differentiated with 1% acid alcohol for 5 sec. then washed with water, blued in ammonical water for 2 min, washed in water, counter-stained with 1% aqueous eosin for 2 min and rinsed in water, dehydrated in ascending grades of alcohol from 70, 90% and absolute alcohol for 1 min each, then cleared in xylene and finally mounted in DPX (distrene, tricresyl phosphate, xylene)

Statistical analysis: Result are presented as ± Standard Error of Mean (SEM). Graphs were drawn using the excel package for the drawing graphs. Statistical analysis was done using one way analysis of variance (ANOVA)

Table 1: Sperm count and % sperm motility in male Wister rats

Groups	Sperm count×10^6 cell/mL	Sperm motility (%) Motile	Non-motile
Control	29.00±1.78	55.00±2.84	45.00±2.84
50 mg/kg	34.00±1.87ns	64.40±4.61ns	36.60±4.61ns
100mg/kg	31.40±0.97ns	68.20±3.56ns	31.80±3.80ns
200mg/kg	33.2±1.98ns	56.40±1.56ns	43.60±1.56ns
400mg/kg	33.8±1.01ns	60.6±3.50ns	39.40±3.50ns

ns: Not significant

Table 2: Sperm morphology and serum testosterone level

Groups	Sperm - morphology (%) Normal	Abnormal	Testosterone ng/mL
Control	76.00±2.21	22.00±2.21	4.22±2.01
50 mg/kg	76.60±3.31ns	23.240±3.31ns	7.34±3.28ns
100 mg/kg	71.60±2.41ns	29.00±2.41ns	4.80±3.80ns
200 mg/kg	72.60±4.82ns	25.40±4.81ns	0.64±0.11ns
400 mg/kg	76.00±2.91ns	24.00±2.91ns	1.62±0.97ns

ns = not significant.

followed by a post-hoc test of Duncan. Values of $p<0.05$ was considered statistically significant (Duncan *et al*., 1977).

RESULTS

Sperm count: At 50 mg/kg of the group treated with *lanneaacida,* the sperm count is 34.00±1.87 m/mL while the sperm count in the control group has 29.00±1.78 m/mL, for the 100, 200 and 400 mg/kg treated group the sperm count is 31.40±0.97, 33.2±1.98, 33.8±1.01 m/mL but no difference statistically at $p>0.05$ as seen in Table 1.

Sperm motility: The sperm motile for the extract treated groups at 50, 100, 200 and 400 mg/kg is 64.40±4.61, 68.20±3.56, 56.40±1.56, and 60.60±3.50% there was no difference statistically at $p>0.05$ when compared with the control as seen in Table 1.

Table 1 above showed the effect of *lanneaacida* sperm count and sperm motilty there was no any significant difference between the treated and control group

Sperm morphology: The normal sperm morphology of the extract treated group 50, 100, 200 and 400 mg/kg is 76.60±3.31, 71.60±2.41, 72.60±4.82, 76.00±2.91% there was no difference statistically at $p>0.05$ when compared with the control as seen in Table 2.

Testosterone: The serum testosterone level in doses of 50, 100, 200 and 400 mg/kg of *Lanneaacida*is 7.30±3.28, 4.80±3.80, 0.64±0.11, 1.62±0.97 ng/mL there was no difference statistically ($p>0.05$) when compared with the control as seen in Table 2.

Table 2 showed the effect of *lanneaacida*sperm morphology and serum testosterone level, there was no any significant difference between the treated and control group.

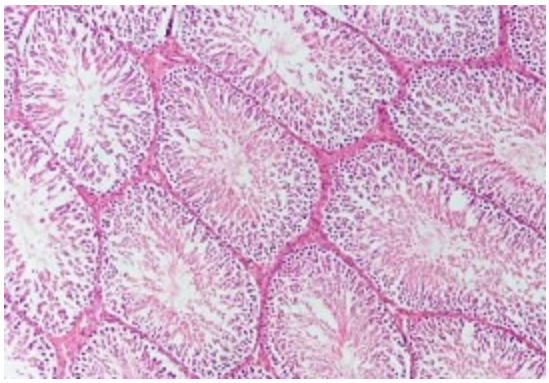

Plate 1: Photomicrograph of a section of testis of a rat control group (Group A) showed no histopathlogical lesion after H&E X 400

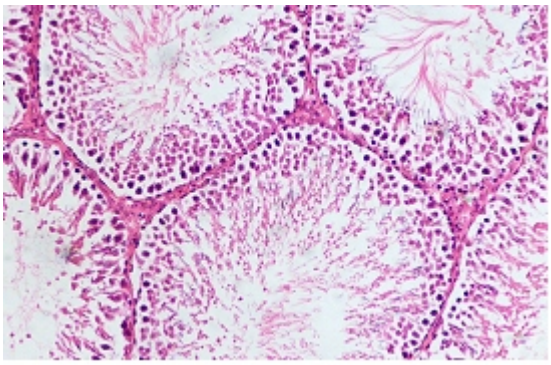

Plate 2: Photomicrograph of a section of testis of a rat treated with 50 mg/kg (Group B) showed no histopathologicallesion after H&E X 400

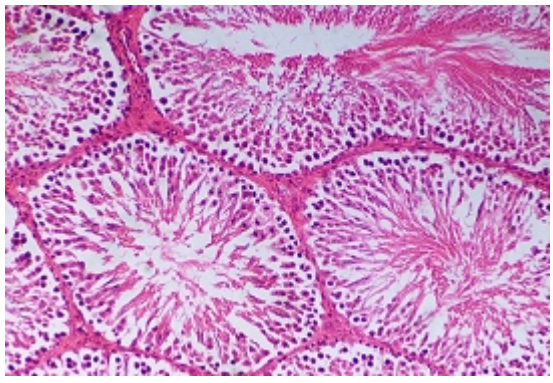

Plate 3: Photomicrograph of a section of testis of a rat treated with 100 mg/kg (Group C) linneaacida showed no histopathological lesion after H&E X 400

Histopathalogical study: The result in Plate 1 shows the result of the control, Plate 2 , 3, 4 and 5 showed the results of 50, 100, 200 and 400 mg/kg body weight treated groups, respectively.

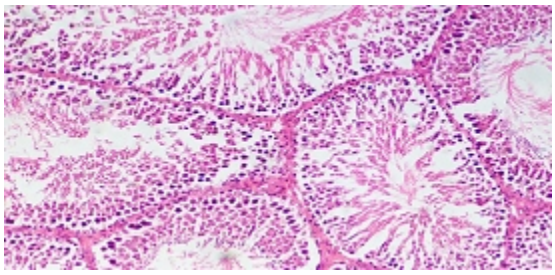

Plate 4: Photomicrograph of a section of testis of a rat treated with 200 mg/kg (Group D) showed no histopathological lesion after H&E X 400

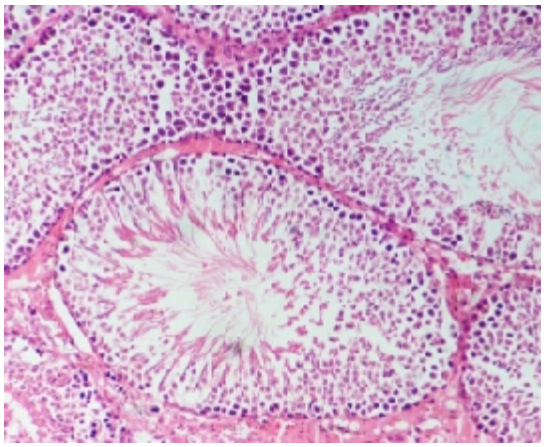

Plate 5: Photomicrograph of a section of testis of a rat treated with 400 mg/kg (Group E) showed no histopathological lesion after H&E X 400

No histological lesion was observed in the control group. The groups that were treated with the doses of 50, 100, 200 and 400 mg/kg also did not show any histological lesion when observed.

DISCUSSION

Human fertility depends on factors of nutrition, sexual behavior, culture, instinct, endocrinology, timing, economics, way of life, and emotions (Barrett, 1997). A number of things can cause impaired sperm count or mobility, or impaired ability to fertilize the egg resulting to infertility. The most common causes of male infertility include abnormal sperm production or function, impaired delivery of sperm (Swerdloff, 2009).

Sperm count: The sperm count, motility, and morphology were used in this study to evaluate the effect of prolonged administration of Methanolic stem bark extract of *lanneaacida* on male reproductive system using the wistar rats as animal model. These andrological parameters are usually evaluated to determine the fertility of a male subject (Garner and Hafez, 1993).

In this study the sperm count was observed to have slightly increases with the treated groups at different doses of 50, 100, 200 and 400 mg/kg of Methanolic stem bark extract of *lanneaacida* when compare with control group this indicate that the extract of Methanolic stem bark extract of *lanneaacida* do not inhibit spermatogenesis. But the difference was not significant at ($p>0.05$), our findings was different from (Adebowale *et al*., 2009) who found significant decrease in sperm count after administering similar doses of methethanolic extract of *LagenariaBreviflora* to male wistar rats.

Sperm motility: It was observed that there was a slight increase in the percentage motility of treated groups administered with different doses of 50, 100, 200 and 400 mg/kg of Methanolic stem bark extract of *lanneaacida* when compare with control group But the difference was not significant relevant at ($p>0.05$).

Sperm morphology: The male wistar rats in the treated group administered with different doses of 50, 100, 200 and 400 mg/kg of Methanolic stem bark extract of *lanneaacida*when compare with control group. The difference was not significant at ($p>0.05$).

Serum testosterone: Testosterone in association with follicle stimulating hormone normally acts on the seminiferous tubules to initiate and maintain spermatogenesis (Rajmil *et al*., 2007). The Increase in serum testosterone of the group administered with the dose of 50 mg/kg of the extract of lannea*acida*, Suggests that the methanolic extract might be more effective at lower dose in enhancing the release of testosterone.

Histopathology of the testes: The groups that were treated with the doses of 50, 100, 200 and 400 mg/kg of *lanneaacida* did not show any histopathological lesion as well as the control group.

CONCLUSION

Infertility is linked with several disorders that can cause impaired sperm count or mobility, or impaired ability to fertilize the ovum. Findings from this study show that administration of the stem bark extract of *lanneaacida* has the tendency to enhance sperm count, morphology, motility and serum testosterone level. This indicates that the extract could be more effective if the chemical constituent is fully studied. Therefore herbal application of extract of *lanneaacida* should be taken with caution in males and the potential of the plant as a profertility drug could be carefully explored.

RECOMMENDATION

Further studies should be carried out to isolate the active ingredients in the plant and also other route of

administration should be studied to ascertain the potency of the plant. Caution should be taken when using the plant extract as remedy in the treatment of infertility.

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