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Histomorphological Studies of Methanolic and Aqueous Leaf Extract of *Annona Squamosa* on the Testis and Epididymis of Male Sprague Dawley rats

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ABSTRACT
Male reproductive capacity continues to be adversely affected by environmental, industrial and pharmaceutical toxins. The producing power of sperm cells in the testis and the sperm modification activities in the epididymis are fundamental in establishing male capacity to reproduce itself. Medicinal plants are of immense value in the field of prevention and treatment of diseases. The use of plant extracts as fertility enhancer in human is now in the increase because of the shifting of attention from synthetic drugs to natural plant. *Thus, t*he focus of the present study is to examine the fertility enhancing properties of the leave extract of *Annona Squamosa* in male Sprague-Dawley rats. Twenty-one rats were divided equally into three groups of seven rats each. Group 1, the control group received distilled water, Group 2 received 300mg of *Annona squamosa* methanol leaves extract and group 3 received 400mg of *Annona squamosa* aqueous leaves extract. The administration was done orally for 21 days. There was a significant increase in the percentage of progressive sperm motility and sperm counts in the aqueous and methanol extract treated rats when compared with the control.There were no damage to the seminiferous tubules in the treated rats with a well outlined tubules interspersed with seminiferous epithelium. There was consistent presence of numerous sperm cells in the lumen of the epididymis of the treated groups which was comparable with the control. There is structural evidence that aqueous and methanol leaves extracts of the *Annona squamosa* enhanced sperm morphology and function in both the testis and epididymis in rat.

Keywords: Sperm, *Annona squamosa*, Reproduction, Fertility

INTRODUCTION
Male reproductive capacity continues to be adversely affected by environmental, industrial and pharmaceutical toxins which open the overall male reproductive health to agent that will help revitalize the reproductive system[1].Male reproductive capacity is established on the number sperm cell produced, the number of abnormal sperm cell, erectile and ejaculatory dysfunction. Therefore, producing power of sperm cells in the testis and the sperm modification activities in the epididymis are fundamental in establishing male capacity to reproduce itself [2]. Plants have created the foundation of health care system throughout the world. Medicinal plants are of immense value in the field of prevention and treatment of diseases. The use of plant extracts as fertility enhancer in human is now in the increase because of the shifting of attention from synthetic drugs to natural plant products as it is cheap and readily available [3].

Annona squamosa Linn. also known as custard apple belongs to the family Annonaceae. It is a small evergreen tree with thin, simple and alternate leaves [4,5]. It is widely cultivated in India as an ornamental plant with a delicious edible fruit.Different parts of *Annona squamosa Linn*are used in folkloric medicine for the treatment of various diseases. Different parts of the plant have been researched and have confirmed its medicinal properties. The aqueous extract of leaf of Annona squamosa was found to demonstrate significant anticancer activity on human epidermoid carcinoma cell line and colon cancer cell line [6]. Extract obtained from leaves of *Annona squamosa* is useful in maintaining healthy blood sugar and cholesterol levels as it reverses the abnormal lipid profile seen in diabetic animals [7,8].The results obtained from in vitro studies clearly suggest that the methanol, chloroform, and aqueous extracts of *Annona squamosa* leaves possess antifungal and antioxidant activity. The extracts were found to express dose-dependent inhibition against all tested fungi strains in both agar well diffusion and broth dilution methods [9].

In reproduction and fertility, treatment with *A. squamosa* during the pre-implantation period has been reported to show no signs of toxicity, and no alteration in the corpora lutea, implantations and embryo in terms of development numbers in pregnant rats when

investigated [10]. There is paucity of information in the literature on the effect of *Annona squamosa* on the testes and epididymis or any evaluation on male reproductive capacity despite the fact that it is well researched on other areas. Thus, the present study aimed at evaluating the reproductive activities of *Annona squamosa* leaf extract in male Sprague Dawley rats. The focus of the present study is to examine the fertility enhancing properties of the leave extract of *Annona squamosa* in male Sprague-Dawley rats through analysis of sperm cell number, sperm motility and morphology and histology of the testis and the epididymis.

MATERIALS AND METHODS

Plant material

Annona squamosa leaves were obtained from the department of Anatomy garden, College of Medicine of the University of Lagos and was identified and authenticated at the Botany department of the University of Lagos, Nigeria.

Preparation of the extracts

The leaves was air-dried for 20days and then grinded to become powder. 200g of powdered *Annona squamosa* was added into 2litres of methanol and another 200g into 2litres of distilled water. The mixture was soaked for 48hours and then filtered. The filtrate sample was placed inthe water bath and the rotary evaporator to remove the solvent; after the solvent had been removed the residue was kept inside refrigerator to preserve it and to make it freeze. It was then transfer into the petri-dish. The residue inside the petri-dish was finally placed inside the freeze dryer for 24hours in order to remove the moisture from the sample and to make it dry. Each processed residue yielded 10ggreenish-brown colored extracts. It was stored in a very cool place, and the fresh solution was prepared in distilled water when required.

Experimental design

Twenty-one male Sprague Dawley rats of average weight 150g. The animals were housed in a well ventilated room at the animal house in Osun State University Osogbo Nigeria. The rats were allowed to acclimatize to laboratory conditions (12 hday/12 h night; 23 ± 3°C) for two weeks and they were being fed with commercially available rat chow and had free access to tap water *ad libitum.* The rats were divided equally into three groups of seven rats each. Group 1, the control group received distilled water, Group 2 received 300mg of *Annona squamosa* methanol leaves extract and group 3 received 400mg of *annona squamosa* aqueous leaves extract. The administration was done orally for 21 days and the rats were sacrificed twenty(24) hours after the last administration.

Animal Sacrifice and Sample collection

Semen collection

The left testis was removed along with its epididymis. The caudal epididymis was separated from the testis and lacerated to collect semen with a microscope slide for sperm evaluation.

Procedure for semen analysis

The right caudal epididymis was excised and minced to allow the sperms to swim out into 1mls of normal saline and allowed to stay for 10 min in the medium. This was done in order to evaluate concentration count, sperm morphology, and sperm motility.

Sperm concentration count analysis

The concentration and total count of spermatozoa was estimated using a Neubauer haemocytometer. A fixed volume of the sample was withdrawn with a micro-pipette and delivered onto the edges of Neubauer chamber of haemocytometer. Both chambers of haemocytometer were scored and the average count was calculated. The count was done under a light microscope at ×400 magnifications and expressed as ×10^6/ml

Sperm Motility Analysis

To determine the percentage of motile sperm, a drop of the sperm suspension was drawn using a rubber Pasteur pipette and placed on a Neubauer chamber, covered by a 22×22 mm cover slip and the percentage of motile sperm was evaluated.

Sperm Morphology Analysis

Moreover, to examine the sperm morphology, a drop of the sperm suspension was placed on a glass slide and a smear was prepared. The smear was fixed in ethanol for 1 h, stained with haematoxylin and eosin, washed, dried, and examined with a light microscope at a magnification of ×100. Spermatozoa were counted and the percentage of abnormal sperm determined. Morphologic abnormalities of spermatozoa were categorized as Head defect, including large, small, amorphous, vacuolated, double heads or any combination of these Neck defects, including distended/irregular/bent mid piece, abnormally thin mid-piece or any combination of these. Tail defects, including absent tail, short, multiple, hairpin, irregular width, or coiled tails, tails with terminal droplets, or any combination of these. These finding were expressed as percentage (%) of morphological abnormal sperm

Histology

Testes and epididymis were fixed in Bouin's fluid. The tissues were then placed in ascending grades of alcohol 50, 70, 90, 95% and then absolute alcohol for about 2 hours in each of the alcohol preparations. The tissues were infiltrated twice with xylene then molten wax at a constant temperature of 58°C in an oven before finally embedded in molten paraffin wax to form tissue block. The tissues blocks were sectioned at 5µ using a rotary microtome. One surface of a slide was made adhesive by rubbing it with a drop of egg albumen. A tissue section was then placed in the center of the slide and immersed in a water bath (53°C). Water was drained

and the slides were placed in an incubator at 37–40°C overnight. Each section was deparaffinized in xylene for 1min before immersed in absolute alcohol for another 1 min and later in descending concentrations of alcohol, for about 30 s each so as to hydrate it. The slides were then rinsed in water and immersed in an aqueous solution of hematoxylin. The slides were dipped in 1% aqueous eosin for 30seconds and rinsed in water, then immersed in 70%, 90% and twice in absolute alcohol for 30 s each to dehydrate the preparation. The preparation was cleared of alcohol by dipping it in xylene for 1 min. Each slide was then cleaned, blotted and mounted in Canada balsam under a cover slip and examined under the microscope (×40 and ×100 magnifications).

Statistics

Results were analyzed statistically, to see the correlation between the results using the Graphpad prism version 5.0.3 (GraphPad Software, Inc., CA 92037 USA). The results were presented as mean ± standard error of mean with significant level at $P < 0.05$.

RESULTS

Semen analysis

There was a significant increase in the percentage of progressive sperm motility and sperm counts in the aqueous *(A. squamosa* 400mg) and methanol (*A. squamosa* 300mg) extract treated rats when compared with the control (Table 1 & 2) respectively.

Significant decrease in the total number of abnormal sperm morphologywas observed in the aqueous extract treated groups (*A. squamosa* 400mg) when compared with the Control group (Table 3).

	SPERM MOTILITY (%)	
GROUPS	**MOTILE**	**NON-MOTILE**
Control	81.50 ± 1.32	18.48 ± 1.40
A. squamosa 300mg	83.50 ± 1.55*	16.50 ± 1.20*
A. squamosa 400mg	86.50 ± 1.71*	13.39 ± 1.32*

Table 1: Shown result for Control group, *A. squamosa* methanol extract treated group (A. squamosa300mg) and A. squamosa aqueous extract treated group (*A. squamosa* 400mg) sperm motility (motile and non motile).

	Total Sperm count ($x10^6$/ml)
GROUPS	
Control	69.75 ± 3.40
A. squamosa 300mg	76.25 ± 3.47*
A. squamosa 400mg	84.75 ± 2.17*

Table 2: Shown result for Control group, *A. squamosa* methanol extract treated group (*A. squamosa* 300mg) and *A. squamosa* aqueous extract treated group (A. squamosa400mg) total sperm count ($x10^6$/ml).

	SPERM MORPHOLOGY (%)	
GROUPS	**NORMAL**	**ABNORMAL**
Control	85.75 ± 2.84	14.25 ± 2.84
***A. squamosa* 300mg**	81.25 ± 1.60	18.75 ± 1.60
***A. squamosa* 400mg**	90.50 ± 1.32	11.69 ± 2.32*

Table 3: Shown result for Control group, *A. squamosa* methanol extract treated group (*A. squamosa* 300mg) and *A. squamosa* aqueous extract treated group (A. squamosa 400mg) sperm morphology (normal and abnormal).

Histology of the Testis and eididymis

Figure 1, 2 and 3 shown photomicrographs of the testis of adult male Sprague-Dawley rats stained with H&E at magnification x40 and x100. There were no damage to the seminiferous tubules in the treated rats with a well outlined tubules interspersed with seminiferous epithelium (SE). The aqueous and methanolic treated groups showed normal interstitial cell (IC) alignment and the Sertoli cells (SC) that appeared intact. The lumen (L) and the presence of thin basement membrane (BM) were observed which shows no characteristic presentation of spermatogenic arrest.

Figure 4, 5 and 6 shown photomicrographs of the testis of adult male Sprague-Dawley rats stained with H&E at magnification x40 and x100. The epididymis, mature sperm cells (MSC), Basal cells (BC), Columnar cells (CC), Connective tissue (CT) and Microvilli (MV) are well demonstrated across all the study groups. There was consistent presence of numerous sperm cells in the lumen of the epididymis of the treated groups which was comparable with the control. Pseudo-stratified columnar epithelium is pronounced in the control group with large principal and basal cells, while this same feature was seen in the treated groups.

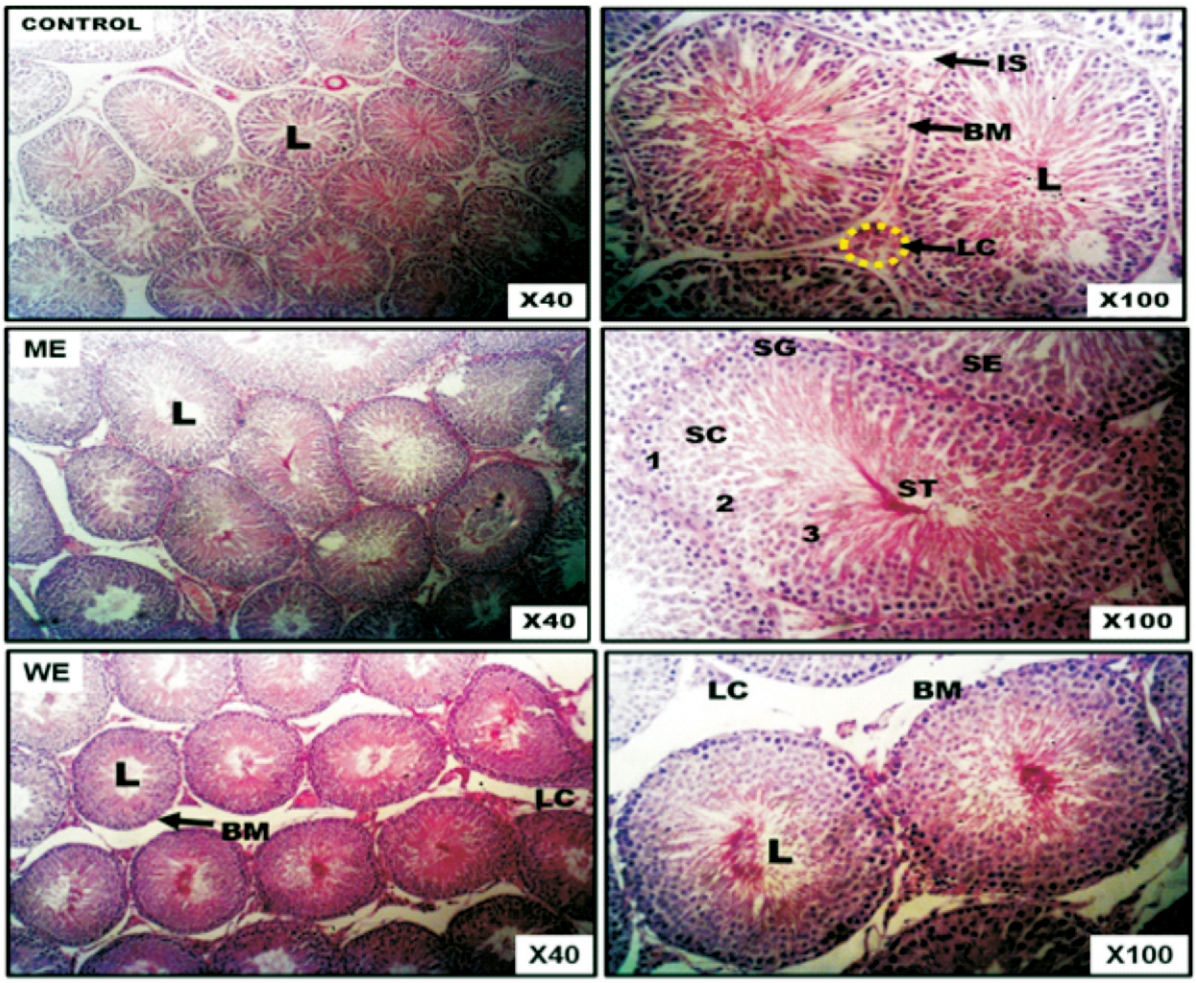

Figure 1, 2 & 3: Shown photomicrographs of the testis of adult male Sprague-Dawley rats stained with H&E (x40 and x100).Figure 1, 2 & 3 are Control group treated with distilled water, 300mg of *Annona squamosa* methanol leaves extract (ME) treated group and 400mg of *Annona squamosa* aqueous leaves extract (WE) treated group respectively. Legends: seminiferous epithelium (SE), basement membrane (BM), lumen (L), interstitial space (IS), interstitial cells (IC), Sertoli cells (SC), seminiferous tubules (ST).

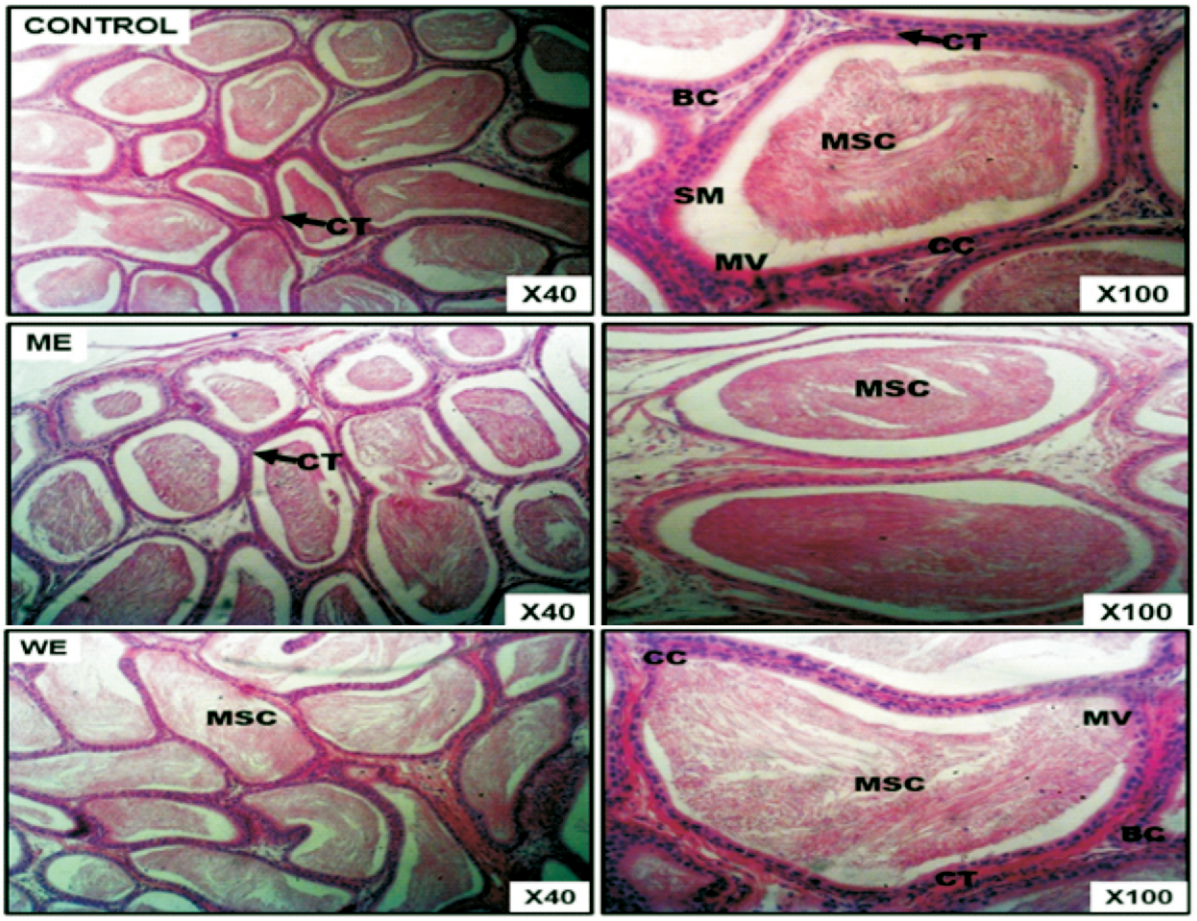

Figure 4, 5 & 6: Shown photomicrographs of the epididymis of adult male Sprague-Dawley rats stained with H&E (x40 and x100). Figure 4, 5 & 6 areControl group treated with distilled water, 300mg of *Annona squamosa* methanol leaves extract (ME) treated group and 400mg of *Annona squamosa* aqueous leaves extract (WE) treated group respectively. Legends; Mature sperm cells (MSC), Basal cells (BC), Columnar cells (CC), Connective tissue (CT) and Microvilli (MV)

DISCUSSION

The results of the present study showed that both the aqueous and methanol extracts of the *Annona squamosa* leaves improved reproductive capacity of male rats as a marked increase in sperm quality and quantity were observed. This is characterized by a variety of semen components such as; morphology, motility, normal sperm and life sperm. The histological study showed well outlined interstitial cells, without any observable loss of interstitial cells nor degenerating Sertoli cells and seminiferous tubules appear intact with seminiferous epithelium in both treated groups. There is consistent presence of numerous sperms cells in the lumen of the epididymis of the treated rats. The concentrations of sperm cells in the lumen seem to be higher in the treated groups when compared with the control group. Lysing of the interstitial connective tissue is not observable across the treated groups. This shows that the leave extracts of *Annona squamosa* did not have any negative effect on the testis and epididymis. The results of this study suggested that the aqueous and methanol leaves extracts of the *Annona squamosa* has characteristics that could improve male reproductive health. This is projected as marked increase in sperm quality and quantity were observed in male rats. Increased sperm structure and function are basis for the improved reproductive capacity.

The phytochemical screening of *Annona squamosa* leaves extract showed the presence of sterol such as cholesterol which is vital to cell membranes structure and function as a precursor to fat-soluble vitamins and steroid hormones [11]. The fertility enhancing property could be attributed to its sterol content. The importance of sterol compounds in sperm production and maturation are well established and it is supported by the modulation of sterol composition in spermatozoa membranes. The development of spermatozoa requires extensive remodeling of membrane structure; involving stage-specific changes in membrane permeability and fluidity[12] thereby creating an effective passage for substance across the membrane wall as

membrane dynamics is essential for cellular life growth and development.

CONCLUSION

There is structural evidence that aqueous and methanol leaves extracts of the *Annona squamosa* enhanced sperm morphology and function in both the testis and eididymis in rat. This effect has been attributed to its sterol content as membrane remodeling agent for efficient passage of substance across the cellular wall needed for normal development. The findings from this study depict clearly that aqueous and methanol leaves extracts of the *Annona squamosa* is a promising substance in the improvement of fertility in male. The results from this study would serve as a preliminary template for further comparative studies and subsequent research work in higher animals and in human.

CONFLICT OF INTEREST STATEMENT

We had no conflict of interest

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