British Journal of Multidisciplinary and Advanced Studies:

Agriculture, 4(4),1-14, 2023

Print ISSN: 2517-276X

Online ISSN: 2517-2778

Website: https://bjmas.org/index.php/bjmas/index

Published by European Centre for Research Training and Development-UK

Effects of Ethanolic Leaf Extract of *Annona Senegalensis* On Semen of Albino Rats

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doi: https://doi.org/10.37745/bjmas.2022.0235 Published: July 10, 2023

Citation: Mbaya Y.P., Bobbo A.G., Barkindo A.A., Ogwiji, M. (2023) Effects of Ethanolic Leaf Extract of Annona Senegalensis On Semen of Albino Rats, *British Journal of Multidisciplinary and Advanced Studies*: Agriculture, 4(4),1-14

ABSTRACT: *Plants with medicinal properties are frequently used to treat infertility in developing nations like Nigeria. One of such herbs, Annona senegalensis, is traditionally used to treat male fertility issues.The present study investigated its fertility enhancement claims.25 Wister rats divided into 5 groups A – E, were used for the study. Groups B – E were given 100 mg/kg, 200 mg/kg, 300 mg/kg and 400 mg/kg of the ethanolic leaf extract respectively while Group A served as control. The extract was administered for 28 days thereafter semen samples from each group were analyzed weekly for 4 weeks. At the 4th week; some enzyme, hormonal and bilirubin assays were carried out. Result showed semen characteristics such as motility, sperm concentration and normal sperm morphology were significantly (P<0.05) increased in treated Groups (B – E). Testosterone increased significantly (P<0.05) in treated Groups compared with Control. While Follicle stimulating hormone and Luteinizing hormone were lower. AST increased in numerical mean values while ALK reduced compared with the control. ALT varied insignificantly. Bilirubin did not differ significantly. These findings suggest male fertility enhancement potentials as claimed by African folklore medical practices. Hence more studies are recommended to further substantiate and elucidate its pharmacological activities.*

KEYWORDS: annona senegalensis, ethanolic leaf extract, acute toxicity, male fertility**,** semen quality

INTRODUCTION

Since the inception of medical science, humans have used herbal products, derived from plants, to support their health. The phytochemicals in plants have served as a crucial channel for pharmacological development over the past centuries (Okhale *et al*. 2016). *Annona senegalens is*

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Persoon, a member of the Annonaceae family, is one such plant having an abundance of phytochemicals (Moghadamtousi *et al*. 2015; Babalola *et al*. 2021). Annona senegalensis commonly referred to as soursop or wild custard apple, is a Senegalese fruit tree that is widespread in semi-arid to sub-humid areas of Africa and has a long history of traditional use. *A. senegalensis* has been used in a variety of food and additive applications, as well as for many ethnomedicinal purposes (Mustapha *et al*. 2013; Moghadamtousi *et al*. 2015; Okhale *et al*. 2016).

Infertility is a concern for couples, families and society (Araoye, 2003; Leaver, 2016).Numerous researches conducted during the last three decades revealed a time-related decline in semen quality, an increase in male infertility, and an increase in the occurrence of some disorders related to the male reproductive system (Mishra *et al*. 2018; Levine *et al*. 2017; Basnet *et al*. 2016; Jiang *et al*. 2014).The number and quality of male reproductive cells are frequently reduced resulting in male fertility problems. Male infertility factor can be understood by sperm parameters in males that are below the World Health Organization's established standards (Plachot *et al*. 2002). Low sperm concentration (oligospermia), poor sperm motility (asthenospermia), and aberrant sperm morphology (teratospermia) are the three most significant of these. Up to 90% of issues with male infertility are related to count. Abnormal semen characteristics often exhibit a positive association with sperm count (Sabra and Al-Harbi, 2014). Pre testicular, testicular, and post testicular factors, along with others, in disorder, ultimately leads to problems with sperm count, motility, and morphology (Iwamoto *et al*. 2007).

Despite the many different ways that *Annona senegalensis* Pers. (Annonaceae) leaves are used to cure male infertility in Nigerian folk medicine, the basis for these claims has not yet been proven by scientific research. *A. senegalensis* aqueous leaf extract could potentially have the ability to negatively impact rat testicular function, according to studies (Oladele *et al*. 2014; Nwonuma *et al*. 2015). The present study thus investigated the *in vivo* male fertility enhancement potential of ethanolic leaf extract of *Annona senegalensis* growing in Nigeria using Albino rats (Wister strains) as a model.

MATERIALS AND METHODS

Plant material
Annona senegalensis leaves were collected at Biu Local Government Area of Borno State Nigeria, in February, 2022 and identified by a plant taxonomist from the Department of Plant Science of the Modibbo Adama University Yola, Adamawa State, Nigeria. A voucher specimen was thereafter deposited at the Herbarium (PG/22/CHM/005).

Preparation of extract
The leaves were air dried under shade at room temperature (Abdalla *et al*. 2012). The dried pieces were pulverized using a BeltoneLuinohun Blender (MS-223model, Taiwan). Ethanolic extract of

the powdered leaf material was obtained according to the method described by Handa *et al*. (2008). The ethanolic leaf extract of *Annona senegalensis* was then subjected to qualitative phytochemical analysis for the presence of carbohydrates, flavonoids, alkaloids, saponins, glycosides, tannins, terpenes, resin, aloes steroids and anthraquinones using standard methods under room temperature as described by Evans (2009).

Acute Toxicity Study

Ethanolic extract of *A. senegalensis* leaf was used for this study. Using the procedure outlined by Lorke (1983), the oral acute toxicity and lethality test (LD_{50}) of *A. senegalensis* was carried out on Wister rats. The test was run in two parts, to put it briefly. Stage one involved giving the animals an oral dose of 10, 100, or 1000 mg/kg (n = 3) of an ethanolic leaf extract of A. senegalensis, and monitoring the animals for 24 hours to monitor the number of deaths. In this phase of the test, there were no deaths in any of the groups. Stage two of the test involved giving fresh animals extract doses of 1600, 3200, and 5000 mg/kg while also monitoring them for 24 hours.

Animals

A total of 25 apparently healthy male albino rats (*Rattus norvegicus*) of Wistar strain, weighing 196.38±4.4 g were used for the study. The rats were obtained from the Laboratory Animal Unit of the Department of Biochemistry, Modibbo Adama University Yola. The animals were kept in clean aluminum cages placed in well ventilated house conditions (temperature: 28–31°C; photoperiod: 12h natural light and 12h dark; humidity: 50–55%) with free access to pellets of grower's mash (Vital Feeds, Jos, Plateau State, Nigeria) and water *ad libitum*. Handling of the experimental animals conforms to international guidelines on the care and use of laboratory animals (National Research Council, 2011) and the experiment was carried out following approval from the Ethical Committee on Animal Care and Use (ECACU) of the Department of Animal Science and Range Management, Modibbo Adama University Yola.

Experimental Design

The twenty-five (25) male Wister rats were divided into five groups (A -E) of 5 rats each. The rats in groups B – E were treated with 100 mg/kg, 200 mg/kg, 300 mg/kg and 400 mg/kg of the ethanolic leaf extract by oral gavage respectively. Group A served as control and was administered clean water. The respective doses were delivered once daily for a period of 28 days before sampling commenced. Semen samples were collected on a weekly basis (every 7^{th} day) over a 4-week period. Blood samples for hormonal (Follicle stimulating, Testosterone and Luteinizing hormones), enzymes (aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase) and biochemical (Direct (conjugated) Bilirubin and Total Bilirubin) assays were taken on the 4^{th} week (i.e. day 28) of sampling, marking the end of the study.

Laboratory procedures

Semen were analyzed for count, motility and morphology as described by Abiodun *et al*. (2022). Semen incubated for 30 minutes at 37ºC. The sperms were counted using Neubauer counting chamber. The dilution of 1:20 was used and prepared by adding 50 µl of semen to the 950 µl diluent. The diluent was prepared adding 50 grams of sodium carbonate and 10 ml of 35% formalin to the distilled water to make 1000 ml. To examine the sperm morphology, semen specimens were stained with MGG. Spermatozoa stained into dark blue purple hue. To examine motility, the semen was examined microscopically to estimate motility of sperm cells. 10µl of semen was put on glass slide and covered with 22x22 mm coverslip. The slide was then examined microscopically using 40x objective lens. Following the manufacturer's instructions, a solid phase enzyme linked immunosorbent assay (ELISA) was used to measure the quantities of the hormones testosterone, luteinizing hormone, and follicle stimulating hormone in serum. While testosterone was reported in ng/ml, LH and FSH were recorded in mlU/ml. Biochemical parameters were assayed as described for alkaline phosphatase (ALP) (Wright *et al*. 1972), alanine aminotransferase (ALT) (Bergmeyer *et al*. 1986a), aspartate aminotransferase (AST) (Bergmeyer *et al*. 1986b). The levels of serum total bilirubin were measured by a timed end-point Diazo method, using automatic biochemistry profiling (Beckman Synchron LX20 Beckman Coulter Inc., CA, USA).

Statistical analysis

Data obtained were expressed as mean±standard error of mean (SEM) and subjected to two-way analysis of variance (ANOVA) using GraphPad Prism version 8.0.2 for windows to compare the effect of different doses of the extract and presented in tables. Means in different groups were compared using Bonferroni's *post hoc* test. Values of $P \leq 0.05$ were considered *significant ($P<0.05$).*

RESULTS

Phytochemical screening

The result of the phytochemical screening carried out on ethanolic leaf extract of *Annona senegalensis* is shown in Table 1. Alkaloids, Cardiac glycoside, Flavonoid, Glycoside, Saponins, Steroid, Tannins, Volatile oil were present while Anthraquinone and Saponins glycoside were absent.

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Table 1: Showing phytochemical analysis of ethanolic leaf extract of *Annona senegalensis*

Constituents	**Inference**
Carbohydrates	+
	-
Soluble starch	-
	+
Anthraquinone s	+
	+
Cardiac glycoside	-
Terpenoids	-
	-
Flavonoids	-
Tannins	
Phlobatanins	
Saponins	
Alkaloids	

+ = means present,
- = means absent
Source: (Mbaya *et al*., 2023)

Acute toxicity studies

Mortality was observed at 3200 and 5000 mg/kg which produced 100% death. There were signs of sedation, dyspnea, anorexia, coma and eventually death in the group (See Table 2). Death occurred at the maximum dose (3200 mg/kg). The LD_{50} was hence estimated as the product of the square root of the dose that recorded death and the dose that recorded no death preceding it (in this case, 1600 mg/kg dose).

Thus $LD_{50} = \frac{\sqrt{3200 \times 1600}}{1} = 2{,}262.74 = 2{,}263$

The calculated LD_{50} is therefore 2263 mg/kg.

Table 2: Acute toxicity studies on ethanolic leaf extract of *Annona senegalensis* in Wister rats

Extract dose (mg/kg)	**No. in Group**	**No. of Death**	**% Dead**
100	3	0	0
200	3	0	0
400	3	0	0
800	3	0	0
1600	3	0	0
3200	3	3	100

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Effect of ethanolic leaf extract of Annona senegalensis on semen characteristics

Table 3 shows that the Albino rats (Wister strain) which were treated with the ethanolic leaf extract *Annona senegalensis* exhibited a stepwise significant ($P<0.05$) increase in motility, sperm concentration and normal sperm morphology ($P<0.05$). However, the higher dose of 400 mg/kg did not induce any further significant($P<0.05$) improvement over the 100 mg/kg treated group.

Table 3: Effect of ethanolic leaf extract of *Annona senegalensis* on semen characteristics

				Sperm count	**Sperm morphology**	
Groups/Week	**Active**	**Sluggish**	**Dead**	**($\times 10^6$) ml**	**Normal**	**Abnormal**
Week 1						
A	45	25	30	10	45	55
B	95	3	2	35	95	5
C	75	10	15	19	75	25
D	65	20	15	19	65	35
E	70	10	20	20	70	30
Week 2						
A	70	10	20	19	70	30
B	90	7	3	29	90	10
C	78	10	12	15	78	22
D	96	3	1	35	96	4
E	95	4	1	31	95	5
Week 3						
A	85	10	5	22	85	15
B	95	4	1	35	95	5
C	90	7	3	25	90	10
D	75	10	15	25	75	25
E	45	30	25	10	45	55
Week 4						
A	87	10	3	23	87	13
B	95	4	1	32	95	5
C	95	3	2	31	95	5
D	85	10	5	27	85	15
E	70	20	10	21	70	30

Group A served as the control group. Group B was given 100 mg/kg. Group C was given 200 mg/kg. Group D was given 300 mg/kg while Group E received 400 mg/kg.

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Effect of ethanolic leaf extract of Annona senegalensis on reproductive hormones

Mean values of Testosterone in all ethanolic leaf extract of *Annona senegalensis* treated Groups were numerically higher than the non-treated (Control) Group A. Significantly (P<0.05) higher mean Testosterone values were seen in the Groups (C – E) which received 200 mg/kg and above (Table 4). Meanwhile Follicle stimulating hormone (FSH) and Luteinizing hormone (LH) were significantly (P<0.05)lower in the *Annona senegalensis* ethanolic leaf extract treated Groups B – E compared with the non-treated Control (Group A).

Table 4: Effect of ethanolic leaf extract of *Annona senegalensis* on reproductive hormones

GROUP / Hormone	**A**	**B**	**C**	**D**	**E**
TEST	0.35±0.03[a]	0.90±0.03[b]	1.05±0.02[b]	1.95±0.06[c]	1.06±0.07[b]
FSH	51.67±1.81[a]	46.10±1.82[b]	32.03±1.01[c]	34.54±1.21[c]	41.09±1.20[b]
LH	39.20±1.74[a]	36.53±1.00[a]	28.64±0.56[c]	27.79±0.89[c]	33.04±0.78[b]

[a, b, c] Means with different superscript letters across rows are significantly (P<0.05) different. Group A served as the control group. Group B was given 100 mg/kg. Group C was given 200 mg/kg. Group D was given 300 mg/kg while Group E received 400 mg/kg.

Effect of ethanolic leaf extract of Annona senegalensis on selected enzymes

Table 5 shows that AST increased numeric mean values but not significantly(P<0.05) in *Annona senegalensis* ethanolic leaf extract treated Groups B – E compared with the Control (Group A).ALT did not vary significantly (P<0.05)in all Groups B – E when compared with non-treated (Control) Group A. Meanwhile ALK reduced in mean values though not significantly in all *Annona senegalensis* ethanolic leaf extract treated Groups B – E compared with the Control.

Table 5:Effect of ethanolic leaf extract of *Annona senegalensis* on selected enzymes

	A	**B**	**C**	**D**	**E**
GOT	121.70±11.68	136.45±6.70	163.40±7.30	160.55±6.66	145.53±4.53
GPT	97.15±3.87	108.95±5.26	98.80±2.70	83.40±3.31	97.08±2.17
ALK	182.00±7.05	157.40±6.41	171.50±8.24	143.50±8.24	163.60±4.04

Group A served as the control group. Group B was given 100 mg/kg. Group C was given 200 mg/kg. Group D was given 300 mg/kg while Group E received 400 mg/kg.

Effect of ethanolic leaf extract of Annona senegalensis on bilirubin

Result of total bilirubin and direct (conjugated) bilirubin did not show any significant(P<0.05) variation across the groups (Table 6).

Table 6: Effect of ethanolic leaf extract of *Annona senegalensis* on bilirubin

	A	B	C	D	E
TB	7.02±0.22	6.10±0.26	6.70±0.29	6.67±0.37	6.69±0.15
DB	0.79±0.03	0.73±0.04	0.80±0.03	0.77±0.02	0.77±0.02

Group A served as the control group. Group B was given 100 mg/kg. Group C was given 200 mg/kg. Group D was given 300 mg/kg while Group E received 400 mg/kg.

DISCUSSION

In this study the significant($P<0.05$) increase in motility, sperm concentration and normal sperm morphology across treated groups suggests a curative potential in ethanol leaf extract of *Annona senegalensis* as claimed by folklore medical practices in Africa. Currently, sperm quality research, including an examination of seminal factors including sperm concentration, motility, and morphology, is used to diagnose male infertility (Dohle *et al*. 2005). Semen analysis is, regrettably, the best test we have to estimate male fertility, even though it is merely a rough estimate of fertility (Patela *et al*. 2018). According to several studies, the probability of infertility rises when the proportion of sperm with normal morphology, motility, and concentration declines (Guzick *et al*. 2001;Slama *et al*. 2002; Jensen *et al*. 2001).Therefore, the study's findings that the ethanolic leaf extract of *Annona senegalensis* has the potential to enhance male fertility are supported by the rise in sperm count, motility, and normal morphology.

The present study recorded significantly ($P<0.05$) higher testosterone (T) and lower Follicle stimulating hormone (FSH), luteinizing hormone (LH). It is well recognized that measuring these hormones can help in managing male infertility. Similar results have been reported by other research that investigated the connection between sperm properties and circulating sex hormones. Meeker *et al*. (2007) found that testosterone levels were significantly ($P<0.05$) favorably connected with motility while LH and FSH levels were negatively correlated with sperm concentration, motility, and morphology. According to Kumanov *et al*. (2006), testosterone levels were not linked with sperm count, motility, or morphology, whereas blood levels of LH and FSH were. According to Bassim *et al*. (2010), infertile males had significantly ($P<0.05$) higher gonadotropin (FSH and LH) levels than controls who had been scientifically shown to be fertile. According to Zhao *et al*. (2020), high levels of LH are solely connected with immature sperm that have poor motility and morphology. Jorgensen *et al*. (2016) also found an inverse relationship between serum LH levels and semen parameters. According to Bassim *et al*. (2010), FSH is required for the beginning of spermatogenesis and the maturation of spermatozoa. Higher FSH levels in infertile men are thought to be a reliable sign of germinal epithelium injury and have been linked to azoospermia and severe oligozoospermia. According to Sheikh *et al*. (2005), increased blood FSH levels are correlated with more severe seminiferous epithelial damage. By converting testosterone to oestrogens, the enzyme aromatase appears to control sperm motility (Attia and Kamel, 2011; Carreau *et al*. 2007).

Testicular functions are heavily influenced by endocrine cues from the brain, and the hypothalamus-pituitary-testis circuit is a key component of keeping endocrine balance and fertility. Gonadotropin Releasing Hormone, which is secreted by the hypothalamus, causes the pituitary gland to release the gonadotropic hormones LH and FSH. LH governs the manufacture of testosterone in Leydig cells whereas FSH controls spermatogenesis, and together, these two hormones work synergistically within the testis to build and sustain testicular function (Foresta *et al*., 2007).FSH directly affects seminiferous tubules and promotes the growth of Leydig cells in developing testes. In an immature testis, FSH increases the generation of testosterone by Leydig cells when combined with LH and HCG, but the mediating elements are yet unknown (Herbert *et al*., 2020; Smith and Walker, 2014; Appasamy *et al*., 2007). Follicle stimulating hormone (FSH) and luteinizing hormone (LH), respectively, control spermatogenesis and steroidogenesis in postpubertal males. The anterior pituitary produces LH and FSH. The gonadotropin releasing hormone (GnRH) produced by the hypothalamus promotes the production of these two hormones. The role of LH, FSH, and testosterone (T) in regulating spermatogenesis is widely understood. The spermatogenesis process must be successfully completed in order to produce testosterone. Without it, spermiogenesis suffers from an impairment in the conversion of round spermatids to spermatozoa (Hameed *et al*. 2011; Nicol *et al*. 2004). The development of spermatogonia into spermatocytes and this conversion both depend on follicle-stimulating hormone (FSH), it should be highlighted. Since testosterone and FSH do not have receptors on the germ cells, they must instead act on the Sertoli cells, which are in charge of nourishing the germ cells. The Leydig cells synthesize testosterone after receiving the luteinizing hormone signal to do so. The term "hypothalamus-pituitary-gonadal axis" (HPG axis) refers to the entire pathway.When it rises above its natural level, the anterior pituitary hormone (PRL) has a negative impact on male fertility (Singh *et al*. 2011).

The present study recorded significant($P<0.05$) no increase in AST, ALK or ALT in all *Annona senegalensis* ethanolic leaf extract treated Groups B – E, though AST had higher mean values numerically compared with the Control Group A. Sperm function is influenced by enzymes. Their involvement is thought to be crucial for ensuring the quality of the sperm (Pero *et al*. 2017). Transaminase activities in the ejaculates are good indicators of semen quality as these enzymes measure stability of the sperm membrane. AST and ALT are essential for metabolic processes, which provides energy for motility, viability, and fertilizing ability of spermatozoa (Perumal *et al*., 2015). The activities of aspartate transaminases and alanine transaminases are not significantly changed by subacute administration of the extracts at doses of 100 and 400 mg/kg ($p>0.05$) (Babalola *et al*. 2021). ALK on the other hand was lower in numerical mean values in Groups B – Ecompared with the Control Group A.

Total and direct (conjugated) Bilirubin did not change significantly ($P<0.05$) across the groups in this study. Even though there were obvious numerical differences in mean in the groups. The ethanolic leaf extract of *Annona senegalensis* treated groups had lower (numerical) mean value of

Bilirubin than the non-supplemented (Control) Group A. It has been suggested that the relationship between serum total bilirubin and normal morphology and sperm motility is inverse. According to Chen & Chen (2022), the highest quartile of serum total bilirubin is linked to normal morphology and reduced sperm motility. The non-significant ($P<0.05$) changes in the biochemical markers measured in this study between the control and treated groups are therefore indicators of healthy, functional semen ejaculates. Minor differences in numerical mean in the assay may be ascribed to metabolism of the administered extract.

CONCLUSION

In conclusion, ethanolic leaf extract of *Annona senegalensis* administration *in vivo* showed improvement in sperm motility, count and morphology after 21 days of administration in Wister rats. This suggests the ethanolic extract of the leaf possesses a male fertility enhancement property. It also showed increase in circulating testosterone, a hormone responsible for libido (sex drive) and required for the maturation of male germ cells and sperm production and quality. Enzymes (AST, ALT and ALK) and Bilirubin assayed did not differ significantly ($P<0.05$) which shows that sperm plasma membranes were undamaged and the extract was well tolerated by the liver at the doses administered.

Conflict of Interest
The authors declare no conflict of interest that may affect the outcome of the study in any way.

Acknowledgement
The authors appreciate the Modibbo Adama University Yola for the opportunity toward sourcing the Institution Based Research (IBAR) from tertiary education fund (TETFUND). We also appreciate TETFUND and the Centre for Research and Development, Modibbo Adama University Yola. We also appreciate the technical of various departments and units of the University involved int the research, particularly Mr. Ibrahim Ahmed Hayatu.

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Print ISSN: 2517-276X

Online ISSN: 2517-2778

Website: https://bjmas.org/index.php/bjmas/index

Published by European Centre for Research Training and Development-UK

Zhao, W., Jing, J., Shao, Y., Zeng, R., Wang, C., Yao, B. and Hang, D. (2020). Circulating sex hormone levels in relation to male sperm quality. *BMC Urology* 20:101 https://doi.org/10.1186/s12894-020-00674-7