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Evaluation of *Moringa oleifera* (L) Aqueous Seed Extracts on Aphrodisiac, Gonadal and Epididymal Sperm Reserves of Wistar Rats

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ABSTRACT
This study was designed to evaluate the effects of *Moringa oleifera* (L) aqueous seed extract on aphrodisiac, gonadal and epididymal sperm reserves of Wistar rats. Twenty-five male and fifteen female Wistar rats aged two months weighing 150 – 200 g were purchased and housed in cages at the Faculty of Pharmaceutical Science, Ahmadu Bello University Zaria. The Wistar rats were provided with a 12 hours light and dark cycle, fed with pellets of broiler starter and drinking water were provided *ad libitum*. The rats were acclimatized for 14 days and they were randomly divided into 5 groups A, B, C, D and E. Group B, C and D as treatment groups, whereas, group A and E were negative and positive controls, respectively, with 5 rats in each group and each was kept singly in separate cage. Groups A and E received 1 ml of distilled water and 5 mg of sildenafil citrate orally respectively. Groups B, C and D received suspension of *Moringa oleifera* aqueous seed extract orally at the dose rate 100, 200 and 300 mg/kg respectively, between 9:00 - 10:00 am daily for 21 days. Female rats were paired with males at a ratio of 1:1, and mating behaviour recorded. Group C and E male rats showed a significant ($p < 0.05$) increase in mounting frequency (MF), respectively. Intromission frequency (IF) was significantly ($p < 0.05$) increase in group C and E, respectively. Gonadal and epididymal sperm reserves were significantly different ($p < 0.05$) between the *M. oleifera* treated and control groups.

Keywords: Aphrodisiac: Gonadal sperm reserves: *Moringa oleifera* aqueous seeds extract; Fertility

INTRODUCTION

Semen characteristic is one of the criteria use for the evaluation of the reproductive capacity of matured male animals (Moradpour, 2019). Reproduction is one of the most important factors affecting livestock production and its success greatly depends on factors like genetic merit, physical environment, nutrition and management (Thornton, 2010). Evidence from literature suggests that nutritional factors are the most vital ingredient that has direct effect on reproductive performance and the potential to moderate the effects of other factors (Alabi, 2005; Kheradmand *et al*., 2006; Diskin and Kenny, 2014; Ibtisham *et al*., 2018).

Herbal therapy can be used to alleviate male infertility, irrespective of the etiology (Anthony *et al*., 2006). A large number of plants have been tested for their possible fertility regulatory properties (Bhatia *et. al.,* 2010). Some medicinal plants are extensively used as aphrodisiac to relieve sexual dysfunction or as fertility enhancing agents (Fatoba *et al*., 2013). They provide a boost of nutritional value thereby improving sexual performance and libido in livestock (Yakubu *et al*., 2007; Sumalatha *et al*., 2010). Many factors such as infectious and non-infectious diseases, drug treatments, chemotherapy, toxins, air pollution, and insufficient vitamin intake may have harmful effects on spermatogenesis (Iliyasu, *et al*., 2014a). Stress is known to compromise reproductive function especially oxidative stress resulting from the production of oxygen free radicals in excess of the antioxidant capacity of the stressed tissue (Ayobami *et al*., 2013). Any oxidizing radical is a potential agent of oxidative stress (Ayobami *et al*., 2013). The major antioxidant enzymes in mammals are superoxide dismutase (SOD), catalase and glutathione peroxidase, which are all endogenous (Ighodaro and Akinloye, 2018). Substances like vitamins A, C, flavonoids and carotenoids are examples of exogenous antioxidants found in food (Asma *et al*., 2005; Musa-Azara *et al*., 2014).

Moringa oleifera (Horse-radish tree or Drumstick or Magic tree) is an important medicinal plant, belonging to the family Moringaceae. *Moringa oleifera* is an ancient tree commonly found in northern India and Africa, in places like Pakistan, Nepal and Nigeria, Niger and Ghana respectively, deemed medicinal in all its constituents (leaves, seeds, flowers, and bark) (Taher *et al*., 2017). *M. oleifera* is small or medium sized trees grow up to 10 m tall, with thick, soft, corky, deeply fissured bark, growing mainly in semiarid, tropical and subtropical areas (Anwar and Bhanger, 2003). The

leaves, seed and the pods are known to have high content of protein, minerals and vitamins (Gopalakrishnan *et al*., 2016). *Moringa oleifera* leaves (Asma *et al*., 2005) seeds (Lalas and Tsaknis, 2002) and roots (Ashok and Pari, 2003) are excellent sources of antioxidants. The stem bark of *M. oleifera* is used as an abortifacient and antioxidant (Ghasi *et al*., 2000).

There is paucity of information on the use of *M. oleifera* aqueous seed extract in Wistar rats as aphrodisiac and gonadal reserves enhancing agents. Hence the need to determine the effects of aqueous seed extract of *M. oleifera* on gonadal and epididymal sperm reserves, mounting frequency (MF), intromission frequency (IF) and ejaculation frequency (EF) in Wistar rats.

MATERIALS AND METHOD

Ethical Statement

All experimental protocols were subjected to the Approval of Institutional Animal Ethics Committee of ABU Zaria.

Plant Collection and Identification

The plant leaves and fruits were collected in dry season (November- March) from Anguwan Yusi, Sabongari Local Government Area of Kaduna state. The plant was authenticated and assigned with Voucher number 0571 by a taxonomist at the Herbarium, Department of Biological Sciences, Faculty of Science, ABU, Zaria, Nigeria.

Aqueous Extraction of *Moringa oleifera* Seed

The seeds were obtained from the fruits that dried under the shade for 14 days to ease the shedding of the seeds. The dried seeds were ground into powdered form (40 g) and weighed using a weighing balance and transferred into one liter beaker. Three hundred milliliter (300 ml) of distilled cold water was added to the powder and allowed to stand for 48 hours. Thereafter it was heated on a water bath at (60 ^{0}C) for 3 hours. Hot water was added continuously to the residue and subsequently filtered. The procedure was repeated three times at 10-15 minutes interval and then the filtrate was evaporated to dryness on water bath at (60 ^{0}C). The liquid extract was concentrated to dryness *in vacuo* at 40 ^{0}C using a rotary evaporator. The dried extract was stored at 4 ^{0}C until required.

Phytochemical Screening

The presence of various constituents in the seed extract of *M. oleifera* was determined by preliminary phytochemical screening as described by Thimmaiah (2004). The tests for tannins, phlobatanins, cardiac glycosides, carbohydrates and flavonoids, anthraquinones, saponins and cyanogenetic glycosides were carried out using procedures described by Trease and Evans (1983). Phytochemical screenings were done to detect the presence of metabolites such as tannins, cardiac glycosides, carbohydrates, flavonoids, anthraquinones, by method described by Trease and Evans (1983).

Animal Procurement and Experimental Design

A total of Fifty-two rats were used for the acute toxicity and Libido test. The rats weighing 150 - 200 g were purchased from the Department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine, Ahmadu Bello University Zaria. The rats were housed in polypropylene cages and maintained under environmental room temperature of approximately 25 ^{0}C and were provided with a 12 hours light and dark cycle. They were fed on pellets of broiler starter (Hybrid Feeds®, Nigeria) and drinking water was provided *ad libitum*. The rats were acclimatized to laboratory environment for 15 days before experimentation

Acute Toxicity Study

The acute toxicity effects of the aqueous extract of the seeds of *M. oleifera* was determined by the method described by Lorke (1983). The experiment was done in two phases. In phase I trial; here nine rats were randomly divided into three groups of three rats each. Groups 1, 2 and 3 were given *M. oleifera* seeds extracts at dose rates of 10, 100 and 1000 mg/kg respectively, orally. In the second phase (II), three rats were placed at random into three groups of one rat each. The groups were individually treated with three different doses of *M. oleifera* seeds extract based on the outcome of the initial trial. The median lethal dose (LD_{50}) of *M. oleifera* seeds extract as an indication of its acute toxic effect was determined by taking the geometric mean of the highest dose that did not produce death and the lowest dose that produced death.

Libido Test

Libido test was done according to the method described by (Akmar *et al*., 2019). Twenty-five male Wister rats weighing (150 - 200 g) that are showing brisk sexual activity were selected for the study. They were divided into 5 groups with 5 rats each and each was kept singly in separate cages throughout the experimental period. Groups A and E were the negative and positive control groups which received 1 ml/kg of distilled water and 5 mg of sildenafil citrate orally respectively. Groups B, C and D received suspension of *Moringa oleifera* aqueous seeds extract orally at the dose rate 100, 200 and 300 mg/kg respectively, daily for 21 days between 9:00 - 10:00 am. Since the male Wistar rats were not be tested in unfamiliar circumstances the animals were brought to the laboratory from the cages and exposed to dim light between 4:00 pm to 6:00 pm daily for 6 days before the experiment. Fifteen female rats were made receptive by administration of suspension of ethyl estradiol (Lynoral® tablets Organon Pharm) orally at the dose of 100 µg /rat 48 hours prior to the pairing. The receptivity of the female animals was confirmed before the test by exposing them to male Wister rats, other than the control, the animals were accustomed to the testing condition. Each male Wister rat was placed individually in a cage with the receptive female rats in the same cage. The receptive female rat was introduced into the cages of the male at a ratio of 1 female to 1 male. The observations for mating behaviours commenced immediately and continued for 3 hours as described by (Kimchi *et al*., 2007). The test would be terminated if the male failed to evince sexual interest. If the female does not show receptivity, she will be replaced by another female from the cluster of the female Wistar rats. The male Wister rats would be observed for mounting frequency (MF) intromission frequency (IF) and ejaculation frequency (EF).

Determination of Gonadal and Epididymal Sperm Reserves

Gonadal and epididymal sperm reserves were determined as described by Zemjanis (1977) and Olukole *et al*. (2010). Similarly, individual sperm motility were determine according to the method described by Yan *et al*. (2007), animals were deeply anaesthetized with ketamine (100 mg/kg bodyweight) and sacrificed on Day 21 of the experiment. Semen samples were collected from the cauda epididymides. Thereafter, the testicular parenchyma was sliced and homogenized with a high-speed blender for two minutes with 100 µl of 0.9 % NaCl solution containing antibiotics (sodium penicillin G, 100 IU/ml and streptomycin sulphate 1 mg/ml) to prevent bacterial growth, for determining the epididymal sperm reserves. The caput, corpus and cauda epididymides were isolated, and minced with a pair of scissors separately in 20 µl of 0.9 % NaCl solution. All tissues were removed immediately and the samples were filtered according to the method described by Türk *et al*. (2008) and examined. One millilitre of the gonadal and epididymal filtrates was pulled together in 20 µl of normal saline solution. Sperm concentration of the testicular and epididymal sample were determined using haemocytometer and light microscope as described by Ozegbe and Omirinde (2012).

Statistical Analyses

Data generated from different groups were presented in mean ± SEM and subjected to one-way ANOVA. $P < 0.05$ was considered significant. All the data were analyzed using GraphPad Instat version 5 (2000).

RESULTS

A total of 6.67 kg of the dried *Moringa oleifera* seed (MOS) powder yielded 918.8 g of the crude extract. The extract was a golden brown, oily liquid. Preliminary phytochemical screening for seed extract of *M. oleifera* revealed the presence of alkaloids, flavonoids, saponins, steroids and tannin (Table 1). Phytochemical bioactive quantification revealed the presence of components and its quantity as summarised in (Table 2).

Table 1: Extraction and Phytochemical Analysis of Aqueous Seed Extract of *Moringa oleifera*

Phytochemical /Components	Presence or Absence of Secondary Metabolites
Flavonoids	++
Glycosides	++
Steroids	++
Tannins	+
Saponins	+
Reducing sugar	++
Phenolics	–
Tarpenoids	–
Carbohydrates	++
Alkaloids	++
Eugenols	+

Keys: (+) = slightly present; (++) = largely present; (-) = absent

Table 2: Quantitative analysis of secondary metabolites in aqueous seed extracts of *Moringa oleifera*

Phytoconstituents	Quantity (%)
Alkaloid	9.3 ± 0.04
Glycosides	11.3 ± 0.4
Flavonoids	13.4 ± 0.03
Reducing sugar	10.3 ± 0.2
Steroids	14.4 ± 0.3
Carbohydrates	18.6 ± 0.1
Eugenols	1.4 ± 0.03
Saponins	12.8 ± 0.06
Tannins	8.6 ± 0.03

First Phase Evaluation of LD_{50} of *Moringa oleifera* Aqueous Seeds Extract

No death was recorded after oral administration of *Moringa oleifera* aqueous seed extract in any group of the Wistar rats as presented in (Table 3).

Second Phase Evaluation of LD_{50} of *Moringa oleifera* Aqueous Seeds Extract

No death was recorded after oral administration of *Moringa oleifera* aqueous seed extract in any of the groups of the Wistar rats as presented in (Table 4).

There was significant increase in live body weight of Wistar rats treated with different dose of *Moringa oleifera* at third week and second week of group C and D compared to the negative control group A as shown in (Figure 1).

The results obtained for sexual behaviour among groups B, C and D treated *M. oleifera* aqueous seed extract at the dose rate of 100 mg, 200 mg and 300 mg respectively. While group A and E were treated with distilled water and sildenafil citrate (Embragra-100®) at the dose rate of 1 ml/kg, and 5 mg/kg respectively as shown in (Table 5). Group B, C and D showed significant increase on mounting frequency (MF) intromission Frequency (IF) and Ejaculatory frequency (EF) compared to control group A and E as presented in (Table 5).

Table 3: First Phase of Acute Toxicity Evaluation of *Moringa oleifera* Aqueous Seed Extract

Extract	Doses (mg/kg) per os	Mortality per group
	10	0/3
Moringa oleifera aqueous	100	0/3
seed extract	1000	0/3

Table 4: Second Phase of Acute Toxicity Evaluation of *Moringa oleifera* Aqueous Seed Extract

Extract	Doses (mg/kg) per os	Mortality per group
	1600	0/1
Moringa oleifera aqueous	2900	0/1
seed extract	5000	0/1

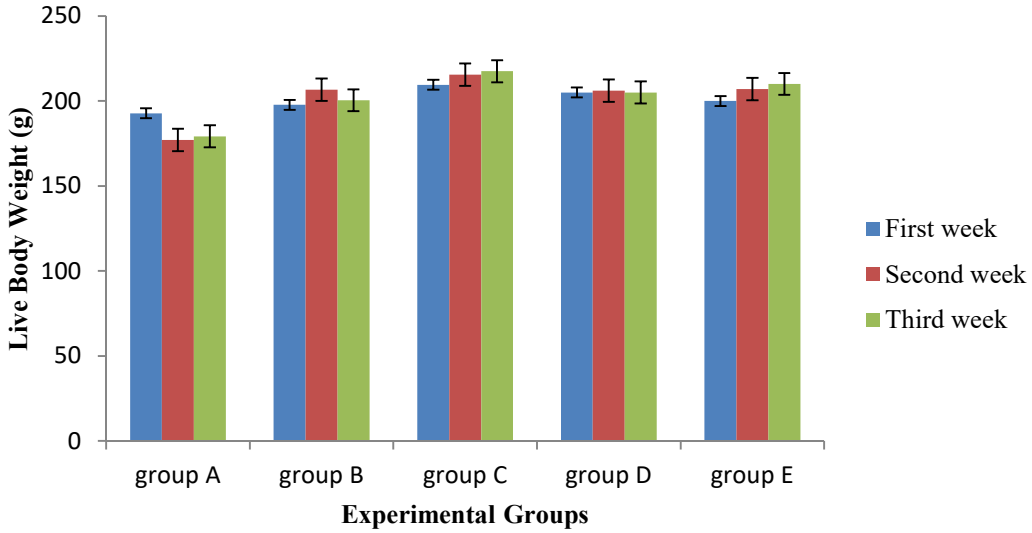

Figure 1: Changes in Live Body Weight of Wistar Rats Fed *M. oleifera* Aqueous Seed Extract

Table 5: Evaluation of Aphrodisiac Effects of *Moringa oleifera* on Wistar Rats

Treatment Groups	Dose mg/kg/bw	Mounting Frequency	Intromission Frequency	Ejaculation Frequency
A	NC H_20(1 ml)	6.2 ± 2.4^a	5.2 ± 1.5^a	0.0 ± 0.0^a
B	MOASE (100 mg)	9.0 ± 2.5^b	8.0 ± 1.0^b	2.0 ± 0.0^b
C	MOASE (200 mg)	12.6 ± 2.7^b	10.21 ± 3.7^b	3.2 ± 2.1^b
D	MOASE (300 mg)	24.5 ± 3.6^b	16.2 ± 3.2^b	9.5 ± 2.5^b
E	PC SC (5 mg)	11.6 ± 3.7^b	13.8 ± 3.3^b	5.3 ± 1.4^b

Mean ± SE with different superscript [a, b] within columns are significantly different at $P < 0.05$ in comparison to the negative control group.
Key: NC= Negative control group; MOASE= *Moringa oleifera* aqueous seed extract; Positive control group (PC); SC= sildenafil citrate.

Gonadal and extragonadal sperm reserves were evaluated for all the groups. The treated groups B, C and D, showed significant differences at $P < 0.05$. Thus, the increase in semen volume, mass sperm motility and sperm concentration were compared to the control group A and E as presented on (Table 6). Individual sperm motility was observed in group A, B, C, D and E. Sperm motility in group C, D and E are very active compared to group A and B as presented in (Table 7).

Table 6: Evaluation of Semen Characteristics of Wistar Rats Fed *Moringa oleifera*

Groups/ Parameters	Dose mg/kg/bw	Semen Volume (ml)	Sperm Motility (%)	Sperm Concentration ($\times 10^6$)
A	NC H_20(1 ml)	0.2 ± 0.1^a	80.2 ± 0.3^a	19.5 ± 1.7^a
B	MOASE (100 mg)	0.2 ± 0.1^a	85.3 ± 1.5^b	19.0 ± 2.1^a
C	MOASE (200 mg)	0.3 ± 0.0^b	95.2 ± 2.1^b	20.5 ± 1.4^b
D	MOASE (300 mg)	0.3 ± 1.1^b	95.6 ± 1.3^b	20.0 ± 1.5^a
E	PC SC (5 mg)	0.4 ± 0.0^b	85.7 ± 1.2^b	19.0 ± 1.3^a

Mean ± SE with different superscript [a, b] within columns are significantly different at $P < 0.05$ in comparison to the negative control group.
Key: - NC= Negative control group; MOASE= *Moringa oleifera* aqueous seed extract; Positive control group (PC); SC= sildenafil citrate

Table 7: Evaluation of Individual Sperm Motility of Wistar Rats Fed *Moringa oleifera*

Groups/ Parameters	Dose mg/kg/bw	Sperm Motility
A	NC H_20(1 ml)	++
B	MOASE (100 mg)	+++
C	MOASE (200 mg)	++++
D	MOASE (300 mg)	++++
E	PC SC (5 mg)	++++

Keys: - ++++ (very active) +++ (active) and ++ (less active), (MOSE) *M. oleifera* aqueous seed extract, (H_20) water, Positive control group (PC); SC= sildenafil citrate. (NC) negative control

DISCUSSION

The seed of *M. oleifera* has been used extensively in folklore (Mishra *et al*., 2011; Kumbhare *et al*., 2012). Phytochemical screening and quantitative analysis revealed the secondary metabolites carbohydrate, alkaloids, flavonoids, saponins, steroids and tannin which are in disagreement with the findings reported by Patel *et al*. (2014). *Moringa oleifera* seed extract is safe to be used up to 5000 mg/kg and this agrees with the findings reported by Varsha *et al*. (2013).

The secondary metabolites found in the present study have been reported to serve as potential antioxidant agents (Elzein *et al*., 2018). Phytochemical constituents of the *Moringa oleifera* seed extract are important bioactive agents used in the synthesis of some useful pharmacological compounds used as fertility enhancing drugs (Ma *et al*., 2019). It has been reported that steroid and saponin present in many plants possess fertility potentiating properties, and they are useful in the treatment of impotence that occurred due to reproductive hormonal imbalance (Shukla and Khanuja, 2004). Saponins found primarily in the leaf *Tribulis terrestris* L. have been used as an aphrodisiac agent in rats (Goel and Maurya, 2020). It is also known to stimulate testicular androgenesis and is essential for testicular differentiation, integrity, and steroidogenic functions (Salem *et al*., 2001; Iliyasu *et al*., 2014b) in goat and rams respectively. The present study also agrees with the findings reported by Mukhallad *et al* (2009), who studied the effects of *Nigella sativa* on spermatogenesis and fertility activities of male albino rats.

Following oral administration of aqueous seed extract of *M. oleifera* to Wistar rats at graded doses, progressive increase in the live body weight were discerned. This agrees with the findings reported by Yusuf *et al*. (2018) in rams and Varsha *et al*. (2013) in rats, which might be attributed to the high nutritional quality of *M. oleifera* seed as confirmed from the proximate analysis of the seed extract reported by (Varsha *et al*. 2013). The increase in the live body weight is in agreement with the findings reported by (Yusuf *et al*., 2018) this might be linked to the ability of the seed extract to reduce pathogenic microbial activities in the stomach, thereby improving digestibility and enhance the assimilation of vital nutrients required for muscle building. The treatment groups were orally supplemented with *Moringa oleifera* aqueous seed extract whereas the control group was not supplanted with the extract which could largely be attributable to the decrease in live body weight of the control group relative to the treatment groups.

The present finding shows that the aqueous seed extract of *Moringa oleifera* at 300 mg/kg used increased the libido of male Wistar rats. This agreed with the findings reported by Wattanathorn *et al*. (2012). who reported aphrodisiac effects cause by *Allium tuberosum* seeds extract, on male rats treated at the dose rate of 500 mg/kg for 21 days, which significantly resulted in an increased mounting frequency MF, intromission frequency IF and ejaculation frequency EF.

The increase in IF and EF among all the treated group is in agreement with the findings recorded by Varsha *et al*. (2013). The significant increase in EF suggests that the extracts and standard drug prolonged the interest for coitus, which is an indicator of increase in sexual enthusiasm as reported in rats Wattanathorn *et al*. (2012). Presence of steroids and other antioxidant in the seed extract might be the causes of increase in these parameters. This could be regarded as influence of *M. oleifera* seed extract on male reproductive system of rats (Varsha *et al*., 2013). Since androgenic effect is attributed to the pharmacological properties of *M. oleifera* seed extract as reported by Wattanathorn *et al*. (2012). Hence the increase in the spermatogenesis activities is a reflection of pharmacological properties of *M. oleifera* seed extract. It is likely that the *M. oleifera* seed extract may have a role in stimulating testosterone secretion which allowed availability of hormone along the pituitary gonadal axis. In addition to the intensity of orgasm and ejaculations which agree with the conclusion recorded by Watcho *et al* (2005), while working on hexane extract of *Mondia whitei* on the reproductive performance of male rats. Similar finding was observed on rats treated with *M. oleifera* seed extract in the present study.

The present results confirm that *M. oleifera* aqueous seed extract ingestion has the potential to increase semen quality, live body weight and libido in male rats. It also supports the claims by herbalist that *M. oleifera* seed has sexual stimulatory properties. Thus, *M. oleifera* seed extract proved to be an effective and safer alternative to boost libido.

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Author Contribution

D. Iliyasu., J. S. Rwuaan., A. I. Nwannenna and C. O. Njoku designed the research work. D. Sani assisted and guided D. Iliyasu on plant extraction and statistics analysis under supervision of J. S. Rwuaan and C. O. Njoku. The drafted copy was reviewed by A. R. Mustapha, I. D. Peter, J. Stephen., A. M. Abdullahi., A. Abba and U. M. Bamanga.

Conflict of interest

The authors declare that they do not have any conflict of interest.

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