



COMPARATIVE EFFECTS OF FRESH AND FERMENTED FRUIT JUICE OF *Morinda citrifolia* (RUBIACEAE) ON REPRODUCTIVE PARAMETERS IN ADULT MALE RATS

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ABSTRACT

Fresh and fermented fruit juices of *Morinda citrifolia* are used in ethnomedicine to improve male fertility. The comparative effects of the fresh and fermented fruit juice of *M. citrifolia* on semen parameters and hormonal profile in adult male rats were evaluated in this study. Thirty-five matured male rats (185-220 g) were divided into seven groups of five animals each. Group 1 served as the normal control. Groups 2 - 7 were further subdivided into two sub-groups 2-4 and 5-7. Animals in groups 2 - 4 were treated orally with 2.5, 5 and 10 ml/kg of the fresh juice of *M. citrifolia* respectively while animals in groups 5- 7 received 2.5, 5 and 10 ml/kg of the fermented fruit juice respectively for 60 days. Normal control animals (group 1) received 10 ml/kg of distilled water. Serum samples obtained from animals were used to assess for levels of testosterone, progesterone, luteinizing and follicle-stimulating hormones. Sperm count, morphology and motility were equally analysed. Histological evaluation of the testis was also carried out. The Fresh juice significantly ($p < 0.05$) increased serum levels of testosterone and follicle-stimulating hormone, while the fermented juice produced a significant increase in serum level of testosterone only. The serum levels of luteinizing hormone and progesterone were unaffected by both juices. The sampled juices produced varied effects on other measured parameters. The ability to enhance the production of testosterone and follicle-stimulating hormone production was exhibited by both juices. However, the fresh juice showed a higher possibility of increasing hormone production.

Keywords: *Morinda citrifolia*; Reproductive hormones; Rubiaceae; Semen quality.

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INTRODUCTION

The inability of a couple to achieve conception after one year of unprotected sexual intercourse benchmarks infertility. As a condition, it is associated with grave psychological, emotional, social, and financial consequences on the affected individuals and their extended families (Anokye *et al.*, 2017). It is estimated that over 50 million couples are infertile globally, with a prevalence rate of 5 – 30% in different countries and regions of the world. In sub-Saharan Africa, infertility rates are estimated to be higher than 15% (Vayena *et al.*, 2001). In 30 – 40% of all infertile cases, male factor infertility (MFI) is the sole causative factor and, in combination with other factors, contributes an additional 30 – 40% to the diagnosis of all infertility cases (De Jonge and Barrat, 2019). Clinically, MFI is defined by the presence of abnormal semen parameters of count, motility, and morphology.

The spermogram of the infertile male is almost always characterized by and/or a combination of low sperm count, abnormal sperm morphology, motility, and viability (WHO, 2010). Allied to sub-normal spermogram, is the strong correlation between low sperm count, abnormal sperm morphology, motility and viability and malignancies, hypertension, diabetes, metabolic syndrome disorder, endocrine disruption, general poor health, and mortality in infertile males (Copogrosso *et al.*, 2018). Of note is the reported decline in sperm count globally, with a 1% drop reported each year in the past three decades; increasing to 2.6% each year in the last five years (Levine *et al.*, 2017). Between the years 1980 – 2015, Africa's diminution in sperm concentration is estimated at 72%, suggesting a decrease in male fertility (Sengupta *et al.*, 2017). The effort to reverse the observed decline in sperm quality and attendant male fertility has led to the increased investigation of medicinal plants with the claimed ability to improve fertility by boosting sperm quality. One such plant is *Morinda citrifolia*.

Morinda citrifolia, Linn, popularly commonly called Indian Noni is an evergreen tree belonging to the Rubiaceae family. Various parts of the plant are used in folkloric medicine in the treatment of cough, cold, pain, liver diseases, hypertension, tuberculosis, malaria, diabetes, urinary tract infection, menstrual disorder, cancer, and male-related fertility disorders (Nagalingam *et al.*, 2012). Ethnomedicinal claims credit the fermented juice with a strong potential to improve male fertility by enhancing sperm quality. Hence, this study aimed at comparatively evaluating the effects of the fresh and fermented fruit juices on sperm qualities of count, motility, and morphology. The effects of both juices on serum levels of testosterone, progesterone, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) as well as the histo-architecture of the testes in male rats, were also evaluated.

MATERIALS AND METHOD

Collection and Identification of Plant

The ripe fruits of the plant were sourced around the Ugbowo area of Benin City, Edo State, Nigeria in the month of December. The plant was identified and authenticated by Dr Henry Akinnibosun of the herbarium unit, Department of Plant Biology and Biotechnology, University of Benin. The voucher number UBHM427 was assigned, and a herbarium specimen was deposited for future reference.

Preparation of Fresh and Fermented Juice

The fermented juice (Fmt) was prepared according to the method of Konsue *et al.* (2018), with a slight modification. Fresh ripe fruits (980.20 g) were cut into chunks and placed in a stainless-steel strainer with a glass bowl collector underneath it. The top of the strainer was covered with a plastic film and a lid. The setup was kept at room temperature ($28^{\circ}\text{C}\pm 3^{\circ}\text{C}$), away from light for 28 days. The ensuing dark brown liquid (37.70 ml) was filtered through Whatman

filter paper (Number 1), pasteurized by heating at 70°C for 30 secs and kept in the refrigerator at 4°C till needed for use.

The fresh juice (Frs) was obtained by blending fresh ripe fruits (606.50 g) in an electric blender and straining the marc sequentially through a muslin cloth, and filter paper. The cream-coloured juice (70.52 ml) was pasteurized and preserved as stated for the fermented juice, till needed.

Animal care

Thirty-five, 16-week-old male adult Sprague Dawley rats (185-220 g) were obtained from and maintained at the Animal house of the Department of Pharmacology and Toxicology, Faculty of Pharmacy University of Benin. Animals were housed in clean well-ventilated non-toxic polyethene cages, with wood shaving as beddings. They were fed pelletized animal chow (Life feed, Ibadan) and allowed access to water ad libitum. Room temperature of 25°C±3°C, relative humidity of 68.00±3% and natural day/night cycles were observed. The study was approved by the Ethics Committee of the Faculty of Pharmacy, University of Benin vide reference EC/FP/019/10.

Experimental Design

The method by Mensah (2019), with a slight modification was adopted. Animals were randomly assigned into seven groups of five rats each as detailed below:

Group 1 served as the normal control and received distilled water – 10 ml/kg.

Group 2: 5 mL/kg of the fresh juice

Group 3: 5 mL/kg of the fresh juice

Group 4: 10 mL/kg of the fresh juice

Group 5: 2.5 mL/kg of the fermented juice

Group 6: 5 mL/kg of the fermented juice

Group 7: 10 mL/kg of the fermented juice

All administrations were par oral and lasted for 60 days. At the end of the treatment period, rats were fasted overnight, anaesthetized in a chloroform chamber and blood was collected by cardiac puncture into plain bottles, centrifuged at 3000 rpm for 10 minutes and the serum obtained was used for hormonal analysis. Testes were excised, cleaned, weighed, and preserved in Bouin's solution for histological examination. Spermatozoa from the epididymis were surgically removed and used for sperm analysis.

Assessment of gonadosomatic index (GSI)

The body weight of animals in each group was determined as well as the weight of both testes. The gonadosomatic index was calculated from the formula below.

$$\text{Gonadosomatic Index (\%)} = \frac{\text{Testes weight}}{\text{body weight}} \times 100$$

Hormone Assay

Serum from blood collected as specified was analysed for testosterone, progesterone, (FSH) and (LH) using commercial enzyme-linked immune-sorbent assay (ELISA)® kits, according to the manufacturer's instruction.

Assessment of sperm count

The WHO (2010) method was adopted for this evaluation. Sperm suspension (0.1 mL) was added to 0.9 mL of normal saline, thoroughly mixed, transferred (0.5 µL) to a hemocytometer and viewed under the microscope. Spermatozoa within five of the red blood cell squares including those lying across the outermost lines, at the top and right sides were counted, while those at the bottom and left-hand sides were left out. Sperm count was obtained from the equation; Sperm count = $\frac{\text{Number of spermatozoa} \times \text{dilution factor} \times \text{depth factor}}{\text{Number of areas counted}}$

Assessment of sperm motility

This was carried out according to the procedure outlined by the WHO (2010), with a slight modification. The sperm suspension (0.1 mL) was dispensed onto a glass slide and viewed under a microscope (X400). Sperm motility was scored in ten separate fields, first non-motile and then motile sperm and classified as progressively motile, non-progressively motile and immotile sperm.

Assessment of sperm morphology

The sperm morphology was assessed according to the WHO, method (2010). The sperm suspension (0.5 µL) was dispensed on a glass slide from which a smear was made. The slide was allowed to dry in air for 10 minutes, flooded with the improved Eosin and Leishman stain for 15 minutes, rinsed with normal saline and left to air dry. The slide was viewed under a microscope at X400, scored at 10 different fields and normal and abnormal sperm cells were identified and scored as a percentage of the total sperm counted.

Histo-anatomical assessment of the testes

The testes were dissected out, cleaned dry of excess blood and fluid, weighed, and fixed in Bouin's solution. Tissues were dehydrated with upgraded ethanol, cleared with xylene, and embedded in paraffin. Sectioning was done by use of a microtome (5 µm) and counter-stained with haematoxylin in eosin. Sections obtained were examined under a light microscope and photographed.

STATISTICAL ANALYSIS

Results are expressed as Mean ± Standard Error of Mean (S.E.M) and, analysed with one-way analysis of variance (ANOVA), followed by Dunnett's test. Statistical analysis test was performed using SPSS (version 17.0; SPSS Inc., Chicago, IL, USA). Data were considered significant at $p \leq 0.05$.

RESULTS

Effect of fresh and fermented *M. citrifolia* fruit juice on Gonadosomatic Index (GSI)

Compared with the fermented juice, the fresh juice produced a dose-dependent and significant increase ($p < 0.05$) in the GSI of treated rats, while a decrease was observed in rats treated with the fermented juice (Figure 1). Effect of

Fresh and Fermented *M. citrifolia* Fruit juice on the hormonal profile of male rats

The fresh and fermented juice respectively effected a non-significant dose-dependent increase in serum concentration of LH in rats in all the treatment groups compared to the control animals. Equally, the fresh and fermented juice

evoked significant ($p < 0.05$ and $p < 0.01$) dose-dependent increments in serum concentration of testosterone. The increment by the fresh juice was higher than that produced by the fermented juice. Also, the fresh juice evoked a significant increase ($P < 0.05$) in serum concentration of FSH at 10 mL/kg compared to the fermented juice. The fresh juice effected a dose-dependent but non-significant increase in the serum concentration of progesterone. The increase in progesterone produced by the fermented juice was neither significant nor dose-dependent.

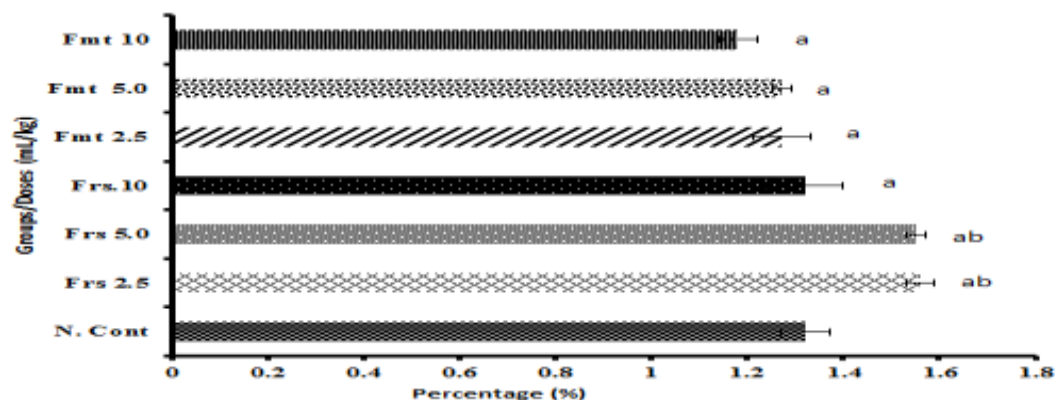


Figure 1: Effect of fresh and fermented juice of *M. citrifolia* on the Gonadosomatic index of rats
Bars with different alphabet indicate significance – $p < 0.05$.

Table 1: Effect of fresh and fermented fruit juice of *M. citrifolia* on male reproductive hormones

Treatment/Hormone (mL/kg)	LH (IU/L)	TEST (ng/mL)	FSH (mIU/mL)	PROG (ng/mL)
N. Control	2.10±0.05	3.60±0.11	3.70±0.41	3.40±0.16
Frs 2.5	2.50±0.03	5.10±0.21 ^{ab}	4.30±0.37	3.30±0.18
Frs 5.0	2.60±0.06	5.90±0.15 ^{ab}	4.70±0.22	3.60±0.31
Frs 10.0	2.90±0.09	6.40±0.32 ^{bc}	5.20±0.25 ^{ab}	3.85±0.22
Fmt 2.5	2.40±0.10	4.50±0.55	3.90±0.32	3.70±0.15
Fmt 5.0	2.45±0.08	4.80±0.23	4.10±0.28	4.15±0.33
Fmt 10.0	2.50±0.05	5.05±0.28 ^{ab}	4.30±0.19	4.10±0.27

Results are presented as mean± S.E.M; LH = Luteinizing hormone; TEST= Testosterone; FSH = follicle-stimulating hormone; PROG = Progesterone; Frs= Fresh Juice; Fmt =Fermented juice. Figures with different alphabet indicate significant differences-^{ab} ($p < 0.05$); ^{ac} ($p < 0.01$)

Effect of *M. citrifolia* Fresh and Fermented fruit juice on the sperm count

The fresh juice of *M. citrifolia* fruit produced a non-linear, but significant ($p < 0.05$) increase in sperm count with the increase observed, at the lowest dose (2.5 mL/kg) higher than that produced by the 10 mL/kg dose. The fermented juice of *M. citrifolia* fruit evoked a decrease in sperm count at all doses compared to observations with the fresh juice treated and control animals. The decrease in sperm count produced by the 10 mL/kg dose of the fermented juice was significant ($p < 0.01$) compared to the negative control (Figure 2).

Effect of *M. citrifolia* Fresh and Fermented fruit juice on sperm motility

The fresh juice produced a dose-dependent increase in the percentage of progressive motile (PM) sperm of treated animals, compared to the fermented juice which evoked a dose-dependent decrease in the percentage of PM sperm. The fresh juice evoked a dose-dependent decrease of the non-progressive motile (NPM) sperm, which was significant ($p < 0.05$) at the highest dose. Comparably, the decrease evoked by the fermented juice on NPM was neither dose-dependent nor significant. The fresh juice decreased the proportion of immotile sperm (IM) significantly, while the fermented juice dose-dependently and significantly increased it (Table 2).

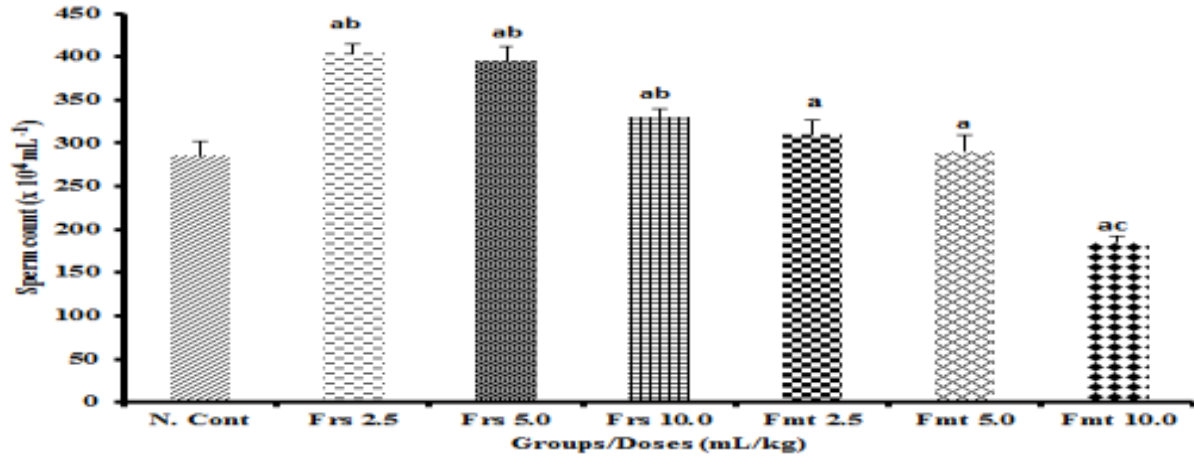


Figure 2: Effect of *M. citrifolia* fresh and fermented juice on the sperm count (Bars with different alphabets indicate significance; ^{ab} = $p < 0.05$; ^{ac} = $p < 0.01$)

Table 2: Effect of *M. citrifolia* Fresh and Fermented fruit juice on sperm motility

Group/Parameter	PM (%)	NPM (%)	IM (%)
N. Cont.	80.50±5.03	12.50±0.50	7.00±0.35
Frs 2.5 mL/kg	84.10±3.67	10.30±0.42	5.60 ±0.25
Frs 5.0 mL/kg	84.30±4.60	12.20±0.51	3.50±0.12 ^{ac}
Frs 10.0 mL/kg	90.10±4.21	5.60±0.25 ^{ab}	4.30±0.23 ^{ab}
Fmt 2.5.0 mL/kg	78.70±3.45	12.30±0.30	9.90±0.18
Fmt 5.0 mL/kg	74.20±2.70	10.20±0.20	15.60±0.64 ^{ab}
Fmt 10.0 ml/kg	70.10±3.30	10.50±0.28	19.40±0.45 ^{ab}

Results are presented as mean± S.E.M. Frs= Fresh Juice; Fmt =Fermented juice. Figures with different alphabet indicate significant difference -^{ab} = $p < 0.05$; ^{ac} = $p < 0.01$

Effect of *M. citrifolia* Fresh and Fermented Juice on sperm morphology

A decrease in the percentage of sperm with normal morphology was produced by the fresh and fermented juice respectively. The effect produced by the fermented juice was marginally higher and significant. Similarly, the fresh juice observably evoked varied effects on the percentage of sperm with abnormal morphology. Comparatively, the fermented juice dose-dependently and significantly increased the percentage of abnormal sperm cells (Figure 3).

Effect of fresh and fermented juice of *M. citrifolia* on histo-architecture of rat testes

Section of the testicular tissue from the control animals (I) indicated normal histology of the testis with the presence of mature sperm cells (A); engorged blood vessels (B); and seminiferous tubules and Leydig cells in the interstitial space (C). Testicular tissue from rats treated with 2.5 mL/kg of fresh juice (II-IV) showed different degrees of sequential sperm maturation in the seminiferous tubules (A); and mild vascular engorgement (B) engorgement of blood vessels. Section of testicular tissue from rats administered the fermented juice (V – VII) indicated the presence of sperm cells with complete sequential maturation (A). moderate vascular dilatation (B) and active interstitial engorgement (C) (Plate 1).

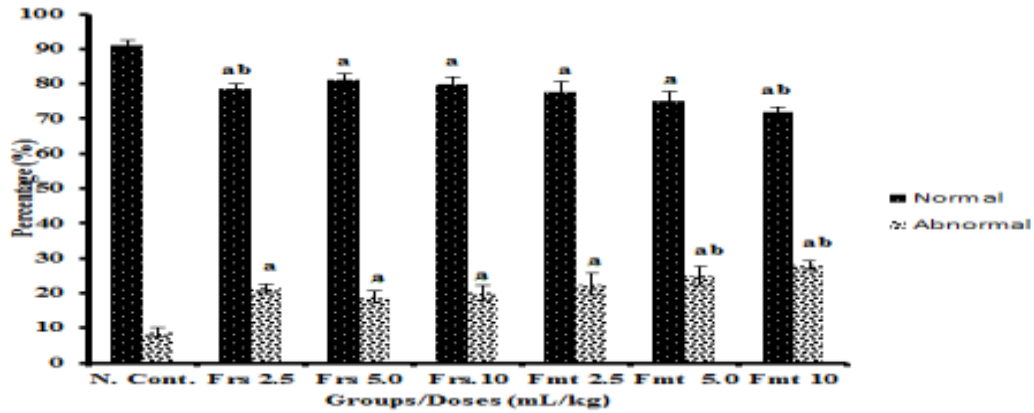


Figure 3: Effect of fresh and fermented juice of *M. citrifolia* juice on sperm morphology.

(Bars with different alphabets indicate significance; ^{ab} = p<0.05; ^{ac} = p<0.01)

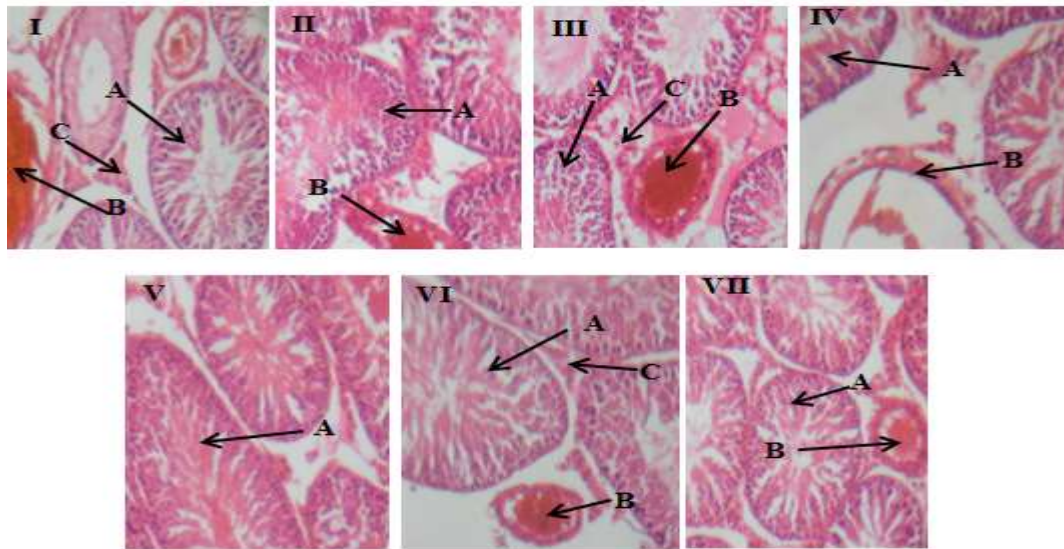


Plate 1: Photomicrographs of testes of male rats in groups I – VII

(A- sperm cell in normal maturation sequence; B- engorged blood vessel within the testes; C- interstitial spaces)

DISCUSSION

This study aimed at evaluating comparatively the effects of the fresh and fermented fruit juice of *M. citrifolia* on semen parameters and the hormonal profile of male rats treated for sixty days. The treatment duration was adopted to obtain a clear picture of the effect of the fresh and fermented juice on the spermatogenetic cycle. According to Marchetti *et al.* (2018), the time duration for complete spermatogenesis in rats range from 50 – 56 days. Hence, the effects of exogenous and endogenous substances on the spermatogenetic process are better assessed when animals are exposed for this length of time, as seen in the present study (Babaei *et al.*, 2021).

The GSI is an important indicator of gonadal development and reproductive output. Its increase is closely associated with the proliferation and activity of the Sertoli cells of the testes (Babaei *et al.*, 2021). In the present study, the fresh fruit juice caused a significant increase in GSI at 2.5 and 5 mL/kg doses, while there was a decrease in the GSI in rats treated with the fermented juice. This suggests the ability of fresh juice to promote gonadal development and reproductive output more effectively compared to fermented juice. These findings are consistent with reports from similar studies on the ethanol leaf extract of *Morinda lucida* and the fresh juice of *M. citrifolia* respectively (Raji *et al.*, 2005; Mensah, 2019).

The production and secretion of LH, FSH and testosterone are controlled by the hypothalamic-pituitary-gonadal (HPG) axis in males. Testosterone, the principal male reproductive hormone is responsible for the development of the male gonads and sexual behaviour (Clavijo and Hsiao, 2018). In the present study, the fresh and fermented fruit juices of *M. citrifolia* increased the serum concentration of LH, FSH and testosterone, indicating that they may play a role in supporting the functions of the HPG axis as it relates to the release and activity of these hormones. The findings with the fresh fruit juice of *M. citrifolia* in this study are contrary to those cited by Mensah (2019) where a decrease in serum concentration of LH and FSH was indicated, but an increase in levels of testosterone was noted. The differences observed could be due to the differences in the duration of treatment and rat breed used in the two studies. The duration of treatment in the present study was 60 days as against 28 reported by the earlier study. Previous reports on the activity of the fermented juice on LH, FSH and testosterone could not be retrieved at this time.

Although the role of progesterone in male reproduction is shrouded in controversy, some reports cite its requirement in spermatogenesis and testosterone biosynthesis in the Leydig cells (Anderson and Tufik, 2006). The fresh and fermented juices of *M. citrifolia* were observed to increase the serum concentration of progesterone in this study, pointing to a possible positive influence in increased spermatogenetic activity and testosterone secretion. Results from sperm analysis of count, motility and morphology have been used as a qualitative biomarker of idiopathic male infertility and a proxy of good health (Omu, 2013). In the present study, rats treated with the fresh and fermented juice of *M. citrifolia* were observed to have increased sperm count, a higher percentage of progressive motile and normal sperm cells, and a decreased percentage of immotile and abnormal sperm cells compared to the control rats. This suggests that the fresh and fermented juice may play a role in supporting spermatogenesis and by extension male fertility. A similar study reported that the fresh juice of *M. citrifolia* caused an increase in the sperm count and motility of rats treated consecutively for 28 days (Mensah, 2019). Some medicinal plants are reported to cause a decrease in sperm count, viability, motility, and reproductive hormones by disrupting the spermatogenetic pathways (Ikpeme *et al.*, 2007; Mohammed *et al.*, 2014). This was contrary to our present findings, which may indicate that the fresh and fermented juices of *M. citrifolia* positively impact the spermatogenetic process.

Histo-anatomical evaluation of the testes of treated rats revealed an increase in spermatogenesis at all the stages of the spermatogenesis cycle: including the secondary spermatocyte which is usually transient. A mild to prominent vascular engorgement was also observed, suggestive of improved blood supply to the testes and support of spermatogenesis. Rats treated with fermented juice presented evidence of a dose-dependent decrease in spermatogenetic activity.

CONCLUSION

Taken together, the effects of the fresh and fermented fruit juice of *M. citrifolia* on the parameters evaluated demonstrate their pro-male fertility properties in rats, with the fresh juice exhibiting a greater potential. Although this study provides scientific evidence for the claimed pro-male fertility-enhancing effects of the fresh and fermented juice of *M. citrifolia* as regards semen quality and reproductive hormone, it should be noted that this may not translate to the same effects in humans.

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CONFLICT OF INTEREST

The authors declare nil conflict of interest.

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