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ORIGINAL ARTICLE

Aqueous leaf extract of *Moringa oleifera* reduced intracellular ROS production, DNA fragmentation and acrosome reaction in Human spermatozoa in vitro

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Abstract

The effects of aqueous leaf extract of *Moringa oleifera* (MO) on human sperm functions and integrity was studied in vitro. Semen was obtained by masturbation after 3–5 days' abstinence from 34 healthy donors in Western Cape, South Africa. Liquefied semen was washed in human tubular fluid supplemented with 1% bovine serum albumin (HTF-BSA;1:5) with 10 min centrifugation at 300 *g*. Sperm suspensions were subsequently incubated with MO extract (0.625, 6.25, 62.5 and 625 µg/ml) for 1 hr, where HTF-BSA served as control. Sperm motility, vitality, DNA fragmentation, reactive oxygen species production, mitochondrial membrane potential, capacitation and acrosome reaction were assessed. Sperm motility, vitality, mitochondrial membrane potential and capacitation remained unchanged ($p > .05$). A dose-dependent decrease in sperm reactive oxygen species production ($p < .0001$), DNA fragmentation ($p < .0001$) and acrosome reaction ($p < .001$) was observed. An increase in the percentage of non-capacitated sperm ($p < .01$) was noted at 625 µg/ml. The antioxidant properties of MO actively maintained basic sperm functions, inhibited excess sperm free superoxide production and preserved acrosome reaction and DNA integrity. Further studies are needed to confirm the effect of aqueous MO leaf extract on fertility potential.

KEYWORDS
antioxidants, infertility, *Moringa oleifera*, reactive oxygen species, spermatozoa

1 | INTRODUCTION

Despite the common misconception that men are immune to infertility, 7% of males are diagnosed with some form of infertility in their lifetime(Schwetz et al., 2013), which accounts for about 30 million men worldwide (Agarwal et al., 2015). Males are solely responsible for nearly 20%–30% of the identifiable causes of infertility (Agarwal et al., 2015). Deficiencies in semen quality and quantity have been identified as the most common cause of male infertility, with about 90% of cases attributed to suboptimal spermatogenesis and counts (Kumar & Singh, 2015). A decline in sperm count has been reported worldwide (Virtanen et al., 2017). About 40% of normozoospermic men still fail to establish a clinical pregnancy with proven fertile females (Cho & Agarwal, 2018).Oxidative stress is mentioned as being central and independent in male infertility (Agarwal et al., 2019). High production of endogenous reactive oxygen species (ROS) is negatively correlated with sperm functional integrity (Tatone et al., 2010). Poorsperm motility and viability decreased mitochondrial membrane potential (Dobrakowski et al., 2017), and high levels of DNA fragmentation (Iommiello et al., 2015), which reduces the

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chances of successful fertilisation, embryo integrity and development were found to be secondary to oxidative stress (Nishihara et al., 2018). Despite the advances made with assisted reproductive technology, access to treatments remain unequal due to disparity of income (Adageba et al., 2015; Makuch et al., 2011), this incited the quest for identification and investigation of affordable and indiscriminately accessible therapeutic alternatives of treating infertility such as plant-based medicine.

For many centuries, traditional medicines have been used for the improvement of sexual and reproductive issues through the establishment of healing systems such as Ayurveda, Chinese and African healing systems (Kotta et al., 2013). Several scientific efforts have been concerted towards describing the efficacy of plants and their derivatives in reproductive functions. Some examples of such medicinal plants include *Aloe barbadovera* (Erhabor & Idu, 2017), *Eurycoma longifolia* (Tongkat ali) (Tambi & Imran, 2010), *Kigelia africana (sausage tree)* (Azu et al., 2010), *Mondia whitei* (white ginger) (Lampiao et al., 2008) and *Rhoicissus tridentatabushmen grape (bitter grape)*(Rakuambo et al., 2006). The World Health Organisation (WHO) asserts that about 80% of the world population relies exclusively on natural resources for the provision of their health care needs (Yuan et al., 2016).

Moringa oleifera (MO) of the family *Moringaceae* is indigenous to South Asian and is now vastly naturalised in other parts of the world (Gandji et al., 2018). *Moringa oleifera*, popularly known as the ‘horseradish’ tree, has vast phytochemical and pharmacological attributes in African folklore (Bhattacharya et al., 2018; Farooq et al., 2012), which has led to the widely held conception that MO could be the *panacea* of modern-day herbal therapy (Koul & Chase, 2015). Various parts of MO are used as traditional medicine and possess variable phytochemicals, with the leaves cited as containing high levels of the medicinal fractions (Vergara-Jimenez et al., 2017). Isolated and characterised secondary metabolites include flavonoids, vitamins, alkaloids, glucosinolates (Maldini et al., 2014), tannins and saponins (Kwaghe & Ambali, 2009) phenolic acids (Rodríguez-Pérez et al., 2015), essential amino acids (Prasanth et al., 2011) minerals (Ahmed et al., 2018), simple and complex sugars (Anudeep & Radha, 2018)), proteins (Teixeira et al., 2014), sterols (Leone et al., 2016), carotenoids (Elayaraja et al., 2017) and isothiocyanates (Fahey et al., 2019). Health benefits of MO include anti-hypertensive (Acuram & Chichioco Hernandez, 2019), antimicrobial (Thilza et al., 2010) anti-nociceptive and analgesic (Sulaiman et al., 2008), anti-inflammatory (Waterman et al., 2014), anti-peroxidative and anti-oxidative (Nandave et al., 2009).

Anecdotal evidence points out that traditional healers have been prescribing MO for treatment of sexual inadequacies (Nazim et al., 2017) and fertility-related issues (MacDOnalD et al., 2016). For instance, the seeds of MO are used by traditional healers in India as an aphrodisiac (Lalas & Tsaknis, 2002). Several studies have confirmed the anti-oxidative activity of MO leaves in improving reproductive, sexual and seminal functions in rat models (Aprioku & Onyenaturuchi, 2018; Awodele et al., 2012; Cajuday & Pocsidio, 2010; Wafa et al., 2017). This study sought to investigate the effects of aqueous leaf extracts of *M. oleifera* on human sperm functions in vitro.

2 | MATERIALS AND METHODS

2.1 | Plant collection

Fresh leaves of MO were collected from the experimental farm of the University of Limpopo, South Africa. The leaves were authenticated by Dr Egan Bronwyn Ann, University of Limpopo. A voucher specimen (121,625) was deposited at the Larry Leach Herbarium of the University of Limpopo.

2.2 | Preparation of aqueous leaf extract of *Moringa oleifera*

Fresh leaves of MO were allowed to dry for 7 days at room temperature and pulverised. MO leaf powder was infused in distilled water (100 g/L), allowed to boil for 20 min, filtered using cheesecloth, and centrifuged at 800 *g* for 10 min at room temperature (Shunmugam, 2016). Thereafter, filtered using Whatman filter papers 4 and 1 respectively, and subsequently freeze-dried. The concentration for this study was calculated based on the assumption that an average healthy man weighs about 80 kg (Shalaweh et al., 2015) together with a previous study where a capsule containing 500 mg MO leaf powder was prescribed to diabetes mellitus type-2 (DM2) patients daily (Taweerutchana et al., 2017) as follows:

$$\text{Therapeutic concentration} = \frac{\text{prescribed dose (mg)}}{\text{Average weight of man (kg)}}$$

where 1 kg = 1,000 ml.

The in vitro therapeutic concentration obtained was 6.25 μg/ml. A stock solution (62.5 mg/ml) was reconstituted in HTF-BSA.

2.3 | Source and preparation of semen

The study received ethical approval from the Turfloop Research Ethics Committee (TREC) of the University of Limpopo (TREC/45/PG: 2019) and the Biomedical Research Ethics Committee (BMREC) of the University of Western Cape (BM17/7/14). Following informed consent, semen was collected in sterile vials from 34 healthy donors in the Western Cape, South Africa, by masturbation after a 3–5 days' abstinence and prepared as described by Shalaweh et al. (2015). Semen was classified normozoospermic samples according to WHO (2010) criteria (sperm concentration > 15 million/ml, sperm count > 39 million/ejaculate, sperm motility > 40%). In brief, samples were incubated at 37°C for 30 min for liquefaction. In other to obtain the baseline sperm concentration and motility, liquefied semen (3 μl) was placed on

pre-warmed Leja slides and analysed using sperm class analyser® (SCA, version 6.4: Microptic). After that, the semen sample was diluted withHTF-BSA(1:5)and centrifuged at 300 *g* for 10 min, and the pellet was re-suspended in fresh HTF-BSA. Sperm suspensions (sperm concentration: 7.5×10^6/ml) were mixed with a stock solution of aqueous leaf extract of MO(62.5 mg/ml) to final concentrations of 0.625, 6.25, 62.5 and 625 μg/ml and incubated at 37°C for 1 hr to analyse the various sperm parameters. HTF-BSA served as control. Therefore, the total number of samples for each treatment group was 34.

2.4 | Determination of sperm motility and kinematic characteristics

Following exposure of sperm to MO aqueous leaf extract, 3 μl sperm suspension was loaded on a pre-warmed Leja slide (Leja Products B.V.). Thereafter, sperm motility (total motility (%), progressive motility (%), non-progressive motility (%)) and motion kinematics (hyperactivation (%), linearity index (%), straightness(%) wobble (%), amplitude of lateral head displacement (ALH, μm), average path velocity (VAP, μm/s), beat cross frequency (BCF, Hz), curvilinear velocity (VCL, μm/s),straight line velocity (VSL, μm/s)) were analysed using sperm class analyser. A total of 200 spermatozoa were assessed according to the WHO (2010) criteria.

2.5 | Determination of sperm vitality

Sperm vitality was determined with eosin–nigrosin Y stain (Shalaweh et al., 2015; WHO, 2010). After exposure of sperm to the various concentrations of MO aqueous leaf extract, the cells were mixed with eosin–nigrosin dye (1:1). After that, 10 μl of the mixture was smeared on frosted slides and left to air-dry. Slides were viewed at 100× objective with immersion oil using a light microscope. Live and dead spermatozoa were stained white and red, respectively. Two hundred spermatozoa were counted, and results represented as a percentage of live spermatozoa.

2.6 | Determination of sperm mitochondrial membrane potential ($\Delta\psi_m$)

Sperm mitochondrial membrane potential was assessed using 5,5′,6,6′-tetrachloro-1-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide(DePsipher™, Trevigen), a lipophilic cationic dye. The protocol was applied as previously described (Henkel et al., 2012). In brief, after exposure of spermatozoa to MO leaf extract, they were washed with HTF-BSA. The recovered pellet was re-suspended in 100 μl of diluted DePsipher solution, centrifuged for 10 min at 300 *g* and incubated in 100 μl reaction buffer for 20 min at 37°C away from light. After that, the sperm suspension was centrifuged at 300 *g* for 10 min and the pellet re-suspended in 100 μl of pre-warmed reaction buffer. An aliquot of 10 μl from each sample was used to make smears and observed at 1,000× magnification using an epifluorescence microscope (Zeiss). Spermatozoa with green fluorescing mid-pieces were regarded as having disturbed mitochondrial membrane potential (MMP), while those with red fluorescence had intact MMP(Erasmus et al., 2012; Shalaweh et al., 2015). A total of 200 spermatozoa were analysed and result expressed as the percentage of sperm with intact MMP.

2.7 | Determination of spermatozoa reactive oxygen species production

Reactive oxygen species production in spermatozoa was determined as previously described by Shalaweh et al. (2015). In brief, a stock solution of 20 μM dihydroethidium (Molecular Probes) in PBS was prepared (pH of 7.4). Following the treatment of spermatozoa with various concentrations of MO aqueous leaf extract for 1 hr at 37°C, 100 μl was diluted with HTF-BSA (1:5) and centrifuged for 10 min at 300 *g*. The pellet was re-suspended in 200 μl of PBS and centrifuged. Then, diluted DHE was mixed with sperm samples (1:5) and incubated at 37°C for 20 min. Subsequently, 10 μl of each sample was placed on a slide, covered with a coverslip and viewed under oil immersion using an epifluorescent microscope with 488 nm excitation and 590 emission filters (Zeiss). Two hundred spermatozoa were analysed and result expressed as the percentage of spermatozoa with red/orange fluorescence (indicative of excess ROS production).

2.8 | Determination of sperm DNA fragmentation

Sperm DNA fragmentation was determined using the TUNEL assay (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling) (Dead End™ Fluorometric, Promega) according to Erasmus et al. (2012). Spermatozoa exposed to different concentrations of MO extract for 1 hr were centrifuged for 10 min at 300 *g* in HTF-BSA. After that, 100 μl of the sperm suspension was re-suspended in PBS and centrifuged again. Re-suspended pellets were then smeared on Starfrost slides (Knittel Gläser) and allowed to air-dry. Subsequently, slides were fixed with 4% methanol-free formaldehyde (KIMIX Chemicals) in PBS for 25 min at 4°C, washed with PBS for 5 min and allowed to dry at room temperature. The sperm plasma membrane was permeabilised with 0.2% Triton X-100 (Sigma-Aldrich) in PBS for 5 min, rinsed twice with PBS, and 100 μl of equilibration buffer was added for 10 min. After that, 50 μl TdT reagent buffer was added, a coverslip placed on slides and incubated for 1 hr in a dark humidified chamber. Then, slides were submerged in 2× SSC buffer (Promega) for 15 min, washed three times with PBS for 5 min each and analysed at 1,000× using an epifluorescence microscope with excitation and emission filters at 488 and 530 nm, respectively. DNA intact sperm exhibited a bit of a background staining (TUNEL-negative), whereas DNA-fragmented sperm

emitted a bright green fluorescence (TUNEL-positive). Two hundred spermatozoa were counted, and the result expressed as a percentage of TUNEL-positive spermatozoa.

2.9 | Determination of sperm capacitation and acrosome reaction

The dual staining method of Hoechst 32258/CTC for assessment of capacitation and acrosome status of human spermatozoa was adopted for this study, according to Green et al. (1996). Chlortetracycline (CTC; 750 μM) solution (containing 130 mM NaCl, 5 mM cysteine and 20 mM Tris-HCl, pH 7.8) was prepared on the day of use. Following the 1 hr incubation of spermatozoa with MO aqueous leaf extracts, Hoechst 32258 was mixed with the sperm suspension in HTF-BSA (1:100), and incubated for 2 min at room temperature and centrifuged with 2% polyvinylpyrrolidone (PVP40; 40 ml) in HTFat 900 *g* for 5 min. CTC solution (45 μl) and 12.5% w/v paraformaldehyde in 0.5 Tris-HCl (pH 7.4) (8 μl) was mixed with the Hoechst-stained sperm suspension (45 μl). After that, 10 μl of the mixture was placed on Starfrost slide and viewed with a 100× objective using an epifluorescence microscope. From each slide, 200 live spermatozoa (Hoechst-negative) were identified and assessed for their CTC fluorescence patterns as follows: a uniform fluorescence over the entire sperm head was classified as non-capacitated and acrosome intact spermatozoa (F pattern); fluorescence over the sperm head except the post-acrosomal region was classified as capacitated and acrosome intact spermatozoa (B pattern); and a lack of fluorescent over the entire head with/without a fluorescent post-acrosomal region was regarded as capacitated and acrosome-reacted spermatozoa (AR pattern).

2.10 | Data analysis

Data were analysed using GraphPad Prism 6.0.1 for Windows (GraphPad Software). Test for normality was conducted using the D'Agostino-Pearson Omnibus test. One-way analysis of variance (ANOVA) and Tukey's test were used for normally distributed data, while Kruskal–Wallis test and Dunnets' multiple test were employed for non-parametric analysis for comparisons of group means. Results were regarded as statistically significant when $p < .05$.

3 | RESULTS

Baseline analysis of samples from the donors demonstrated that semen volume (Mean: 2.8 ± 0.2; Min: 1; Max: 5 ml), sperm concentration (Mean: 82.7 ± 6.5; Min: 18.5; Max: 180.5 million/ml) and percentage of sperm motility (Mean: 63.9 ± 2.7; Min: 40.1; Max: 96.3%) were in the range of values defined by WHO (2010).

3.1 | Sperm motility parameters

Table 1 shows that aqueous leaf extract of MO did not affect any of the sperm motility parameters or motion kinematics ($p > .05$).

3.2 | Sperm vitality and mitochondrial membrane potential

Figures 1a,b demonstrate that the various concentrations of MO aqueous leaf extract had no effect on the human sperm vitality ($p > .05$) and mitochondrial membrane potential($p > .05$) following a 1-hr incubation.

3.3 | Sperm reactive oxygen species production

Aqueous leaf extract of MO caused significant inhibition of sperm intracellular ROS production at 62.5 μg/ml ($p < .01$) and 625 μg/ml ($p < .0001$)compared to the control (Figure 1c).Repeated measure ANOVA trend analysis revealed a significant dose-dependent decrease ($p < .0001$) in the production of ROS with increasing concentrations of the plant extract. Also, a significant decrease in percentage of intracellular ROS production was observed between treatment groups: 0.625 and 625 μg/ml ($p < .0001$), 0.625 and 62.5 μg/ml ($p < .01$) and 6.25 and 625 μg/ml ($p < .01$).

3.4 | Sperm DNA fragmentation

A significant reduction in the percentage of sperm with DNA fragmentation was observed at 625 μg/ml ($p < .0001$) of MO leaf extract (Figure 1d) compared to the control. In addition, a significant reduction in DNA fragmentation was observed between treatment groups, that is, 0.625 and 62.5 μg/ml ($p < .0001$), 0.625 and 625 μg/ml ($p < .0001$) and 6.25 and 625 μg/ml ($p < .0001$). Furthermore, repeated measure ANOVA trend analysis revealed a dose-dependent decrease in sperm DNA fragmentation with increasing concentrations of the plant extract ($p < .0001$).

3.5 | Sperm capacitation and acrosome reaction

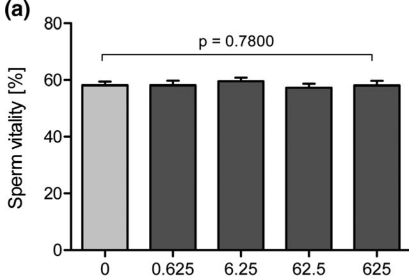
In other to assess capacitation and acrosome reaction, different patterns of CTC staining during the capacitation process were analysed. The highest concentration of MO aqueous leaf extract significantly increased the percentage of uncapacitated and acrosome intact spermatozoa (F pattern; $p < .01$; Figure 2a). Consequently, a significant decrease in the percentage of uncapacitated and acrosome intact spermatozoa was observed between the lowest and highest dose of the extract, that is, between 0.625 and 625 μg/ml ($p < .05$), 6.25 and 625 μg/ml ($p < .01$), and 62.5 and 625 μg/ml ($p < .05$).No significant

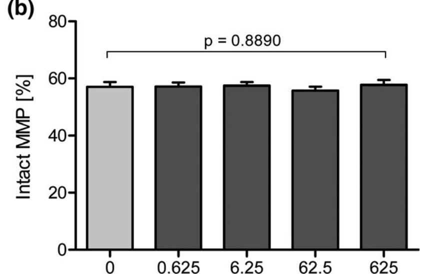
TABLE 1 Sperm motility parameters after 1-hr incubation with various concentrations ofaqueous leaf extract of *Moringa oleifera*

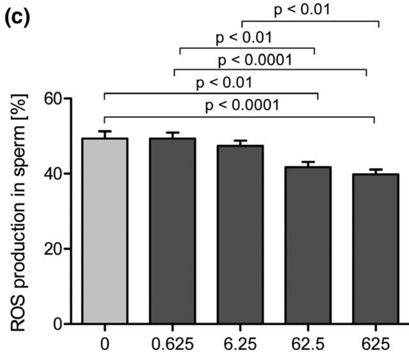
Sperm motility parameters	*Moringa oleifera* aqueous leaf extract (μg/ml) 0	0.625	6.25	62.5	625	*p*-value
Total motility (%)	24.97 ± 2.17	23.42 ± 2.59	24.68 ± 2.23	24.15 ± 3.13	24.50 ± 2.86	0.9188
Progressive motility (%)	9.58 ± 1.52	8.14 ± 1.28	7.72 ± 1.18	6.65 ± 1.14	7.43 ± 1.40	0.6594
Non-progressive motility (%)	12.46 ± 1.24	12.83 ± 1.51	14.04 ± 1.37	13.75 ± 1.92	14.88 ± 1.90	0.9206
Hyperactivation (%)	4.74 ± 1.55	6.98 ± 1.99	4.80 ± 1.18	3.38 ± 1.08	5.45 ± 1.72	0.8197
LIN (%)	39.19 ± 1.90	37.31 ± 1.87	38.15 ± 1.93	36.11 ± 2.20	37.87 ± 2.25	0.6833
STR (%)	65.87 ± 1.76	63.68 ± 1.73	64.16 ± 2.01	62.36 ± 2.26	60.21 ± 2.34	0.3661
WOB (%)	55.14 ± 1.49	54.33 ± 1.52	55.22 ± 1.55	53.99 ± 1.63	54.33 ± 1.91	0.9041
VCL (μm/s)	71.64 ± 3.28	71.11 ± 3.95	67.67 ± 3.19	67.46 ± 2.86	66.59 ± 3.29	0.7873
VAP (μm/s)	37.95 ± 1.47	37.00 ± 1.74	35.79 ± 1.47	35.17 ± 1.29	34.82 ± 1.32	0.5302
VSL (μm/s)	26.96 ± 1.30	25.18 ± 1.32	24.95 ± 1.26	23.63 ± 1.38	22.44 ± 1.29	0.1541
ALH (μm)	2.44 ± 0.09	2.44 ± 0.10	2.35 ± 0.08	2.36 ± 0.09	2.31 ± 0.11	0.9807
BCF (Hz)	15.69 ± 0.98	14.57 ± 1.03	14.42 ± 0.94	15.78 ± 1.42	13.23 ± 0.91	0.4147

Note: Data were represented as mean ± *SEM* of 200 spermatozoa from 34 healthy donors.

Abbreviations: ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN, linearity; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; WOB, wobble.







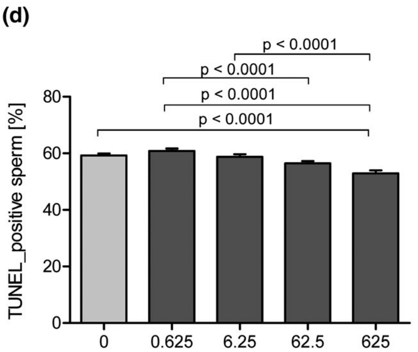
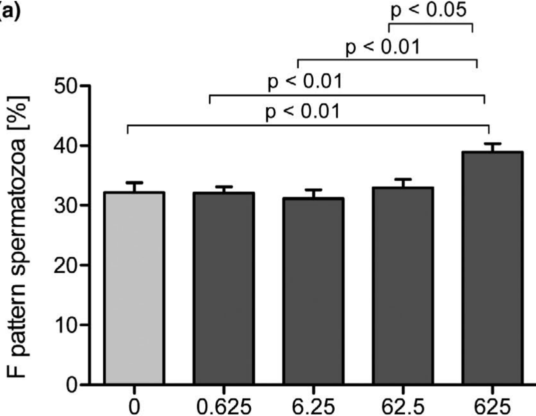


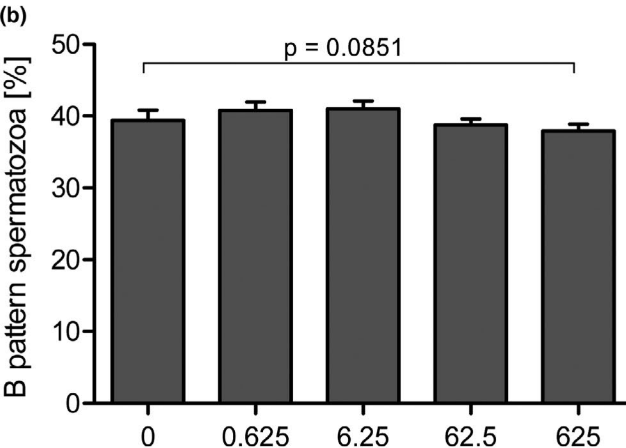
FIGURE 1 Effects of aqueous leaf extracts*Moringa oleifera*on (a) sperm vitality and (b) sperm mitochondrial membrane potential (MMP) (c) intracellular reactive oxygen species (ROS) production in sperm and (d) DNA fragmentation in sperm in vitro. Data were represented as mean ± *SEM*of 200 spermatozoa from 34 healthy donors

changes were observed in the percentage of capacitated spermatozoa (B pattern)in all groups treated with MO leaf extracts when compared to the control ($p > .05$; Figure 2b). Furthermore, no significant change in the percentage of acrosome-reacted spermatozoa was observed by the various concentrations of MO aqueous leaf extract (Figure 2c). However, Mann–Whitney test showed a significant difference at 625 μg/ml of the extract when compared to the control ($p < .05$). Besides, a significant decrease in the percentage of acrosome-reacted spermatozoa was observed between treatment groups, that is 6.25 and 625 μg/ml ($p < .05$), and 62.5 and 625 μg/ml ($p < .05$).

4 | DISCUSSION

Pharmacological properties of *M. oleifera* have been studied at length using in vivo and in vitro models for efficacy assessments in nearly all body systems (Igado & Olopade, 2016), and biological activities were ascribed to its antioxidant activities(Vergara-Jimenez et al., 2017). However, most reports on the effects of MO on male reproductive, sexual and seminal functions were conducted using animal models (Prabsattroo et al., 2012).A previous in vivo study demonstrated the fertility properties of MO by the increased FSH and LH levels, the





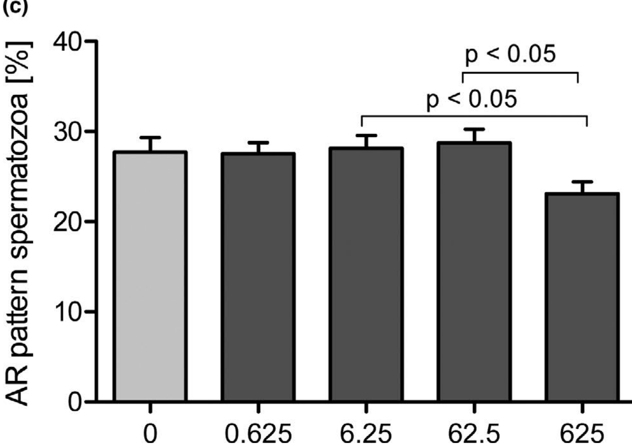


FIGURE 2 Effects of aqueous leaf extracts of*Moringa oleifera*on capacitation and acrosome reaction in vitro. (a) F pattern corresponded to non-capacitated and acrosome intact spermatozoa (b) B pattern corresponded to capacitate and acrosome intact spermatozoa and (c) AR pattern corresponded to acrosome-reacted spermatozoa. Data were represented as mean ± *SEM*of 200 spermatozoa from 34 healthy donors

number of Leydig and Sertoli cells, testicular weight, caudal epididymal sperm count and serum testosterone levels, with a resultant decrease in prolactin, in male albino rats (Orji et al., 2016). *Moringa* saponins, flavonoids and alkaloids have been implicated in some of the observed changes due to their ability to affect androgen synthesis and spermatogenesis (Zade et al., 2013). In other in vivo studies, however, sperm qualitative characteristics in male rats remained unchanged following treatment with MO (Awodele et al., 2012; El-Sheikh et al., 2016). Sperm functional capacity in mammals depends strictly on their morphology, progressive motility, intact plasma and acrosome membranes (Boshoff et al., 2018). To the best of our knowledge, this is the first scientific report to evaluate the effects of aqueous MO leaf extract on the physiological functions of human spermatozoa in vitro.

Our study revealed that MO aqueous leaf extract did not induce any significant changes in percentages of sperm motility parameters, vitality, and capacitation. A possible explanation for this is that the level of sperm ROS produced did not elicit any deleterious effects on sperm membrane integrity, viability and motility (Afolabi et al., 2013). Higher levels of ROS in semen and low levels of antioxidants are observed in infertile men compared to their fertile counterpart (Agarwal et al., 2016; Roychoudhury et al., 2016). Inclusion of semen samples from infertile men (e.g. asthenozoospermia or teratozoospermia) may have demonstrated the anti-oxidative effects of *M. oleifera* leaf on sperm motility and viability.

Furthermore, this study revealed that the level of MMP in sperm remained unchanged following the treatment with MO aqueous leaf extract. MMP is an indicator of the energetic state of mitochondria and determines functional intact mitochondria that have been related mainly to sperm motility (Marchetti et al., 2002). MMP provides insightful information on the sperm fertilising capacity in comparison to the sperm motility, as spermatozoa with high MMP are shown to undergo acrosome reaction (Gallon et al., 2006; Slowińska et al., 2018).

Nutritional antioxidants have been prescribed for pathologies in which oxidative stress was implicated as a cause, including male infertility (Balercia et al., 2005; De Young et al., 2004). *Moringa oleifera* had been shown to possess a high amount of antioxidant with increasing concentration (Luqman et al., 2012), for which quercetin and kaempferol are amongst the abundant flavonoids in MO leaves (Verma et al., 2009). In the present study, the aqueous leaf extract of MO showed a significant decrease in intracellular ROS production in sperm in a dose-dependent manner, specifically at 62.5 and 625 μg/ml, which could be due to scavenging activities of the antioxidants present in the extracts. Addition of quercetin to sperm suspension significantly improved sperm viability, morphology, motility and antioxidants and inhibited lipid peroxidation (Ben Abdallah et al., 2011). Also, alpha-lipoic acid, an organo-sulphur compound derived from octanoic acid, is regarded as a universal antioxidant as it acts as a cofactor for many enzymes and is shown to restore endogenous antioxidants such as oxidised vitamin Cand E, thereby protecting organelles, cells and tissue from the damaging effects of excessive ROS production(Taherian et al., 2019). *Moringa oleifera* leaves are also rich in vitamin C and E (Ajantha et al., 2018), which have been indicated as major chain-breaking antioxidants with strong reducing potential that attenuate free radical generating reactions (Brash & Havre, 2002). Seminal intracellular enzyme complexes and

their involvement in male fertility regulation are highly dependent on cofactors such as Zn, Se, Mg, Mn, $Fe^{3+}$and Cu (Dobrakowski et al., 2018) whose presence in MO leaves has been validated (Dilawar et al., 2018). Centrifugation during the processing of semen increases the generation of ROS results in a loss in sperm motility and viability and induces DNA damage in spermatozoa (Henkel et al., 2012; Taherian et al., 2019). Semen samples obtained from normozoospermic men, washed and centrifuged with sperm washing media, in the presence of alpha-lipoic acid (0.02 mM) maintained sperm viability, and motility through the reduction of ROS production as well as prevention DNA damage (Taherian et al., 2019). Therefore, the addition of aqueous leaf extract of MO in the sperm wash media before centrifugation may improve sperm motility and viability. To support this, the addition of crude extracts of MO in bovine semen extender significantly increased Bull's sperm motility(Sokunbi et al., 2015).Hence, the reduced ROS production, as well as the maintained motility and viability seen in this study, may be attributed to the antioxidant activities of the leaf extract of MO.

An inverse relationship exists between oxidative stress and sperm DNA integrity (Dorostghoal et al., 2017).Excessive ROS augments DNA instability through direct modification of nitrogenous bases (Koh et al., 2016), disruption of a maturation stage in spermatogenesis where spermatozoal histones are protaminated (chromatin condensation) (Hamilton et al., 2018), and occurrence of dysregulated abortive apoptosis (Sakkas et al., 2003). Lipid peroxidation and increased membrane fluidity due to polyunsaturated fatty acid (PUFA) oxidation (Tvrdá et al.,), phosphatidyl-serine translocation (Barroso et al., 2000) and caspase and endonuclease activation (Jeng et al., 2015; Wang et al., 2003) are the hallmarks of sperm ROS-induced apoptotic DNA damage (Aitken & Koppers, 2011). Our study revealed a significant reduction in the percentage of TUNEL-positive sperm (DNA fragmentation) following a 1-hr incubation with aqueous leaf extract of MO. Since superoxide availability precedes free radical production in sperm microenvironment (Gosalvez et al., 2017), it is possible that the absence or inhibition of superoxide (as scavenged or inhibited by MO leaf extracts) resulted in subsequent inhibition on the production of peroxidative derivatives such as H_2O_2 and nitric oxide that initiate and facilitative DNA damage (Verma et al., 2009), hence the significant reduction. Therefore, to the best of our knowledge, this study is first to demonstrate the protective ability of MO leaf extracts on human sperm nuclear DNA and oxidative stress in vitro.

Low levels of ROS are prerequisites for induction of processes crucial for fertilisation such as sperm hyperactivation and capacitation, acrosome reaction, gamete recognition and binding as well asgamete fusion (Aitken et al., 1995; Glenn et al., 2007; de Lamirande & Gagnon, 1993, 1995; Sánchez et al., 2010). Premature acrosome reaction in spermatozoa renders them incapable of fertilisation (Glenn et al., 2007). Aqueous leaf extract of MO significantly increased the percentage of non-capacitated and acrosome intact spermatozoa (F pattern) at 625 μg/ml but had no significant effect on the percentage of acrosome-reacted spermatozoa, which may be associated with the decreased level of ROS observed in this study. Also, the number of capacitated and acrosome intact spermatozoa (B pattern) remained unchanged at all concentrations of the plant extract. Since MO leaf extracts did not affect sperm hyperactivation, a possible explanation for the unaltered percentage of capacitated spermatozoa could be associated to the fact that the onset of capacitation coincides with hyperactivation.

Moringa, which is a rich source of vitamin E, is accessible, affordable and a readily available source of major essential nutrients and nutraceuticals (Kunyanga et al., 2013). The estimated cost for MO leaf powder is about US$0.05/g, MO capsule (450 mg dried leaf) is US $0.07/capsule and compared to vitamin E capsule (1,000 IU, 671 mg) is US$ 0.21/capsule. Dried MO leaves retain their nutrients and can be stored for up to 1 year provided they are stored in airtight containers, away from light and humidity, and kept below 24°C (Doerr & Cameroon, 2005; Gopalakrishnan et al., 2016). Drying of leaves with an oven at 50°C for 16 hr was shown to maintain most of the nutrients and phytochemicals in the leaves except for vitamin C compared to the freeze-dried leaves. Thus, the mild heating and drying processes of the leaves could be achieved using household appliances such as stove (Yang et al., 2006), making the plant readily available for use in enhancing sperm functions.

To conclude, the aqueous leaf extract of *M. oleifera* maintained human sperm function by the scavenging ability of its antioxidant compounds. Concentration-dependent effects of MO proved that the extract confers significant protection against excess ROS production and DNA fragmentation in sperm population and inhibited premature capacitation and acrosome reaction. The preservation of sperm acrosome integrity and inhibition of spontaneous AR in this study are indicative of the fertility potential of MO extract. MO might be valuable in infertility therapies, and more research is required, particularly in vivo studies, to understand the fertilising capacity of the plant extract and to establish whether moringa products have clinical value. For future work, a study on the antioxidant capacity of MO leaf extract in human sperm should be conducted with standardised compounds such as vitamin E, butylated hydroxytoluene or melatonin for evaluating the efficacy of the plant.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

AUTHORS' CONTRIBUTIONS

FTM conducted the experiments, performed the statistical analysis and wrote the first draft of the manuscript. GAA and RRH revised

the manuscript. CSO conceptualised the study and revised the manuscript. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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