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Received: 22 September 2021 | Revised: 31 March 2022 | Accepted: 18 April 2022

DOI: 10.1111/and.14452

ORIGINAL ARTICLE

First International Journal of Andrology ANDROLOGIA WILEY

In vitro effects of aqueous extract of unfermented rooibos on human spermatozoa

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Funding information
The National Research Foundation of South Africa (TTK170426228930) funded this study.

Abstract

The inability to conceive is a baleful experience for thousands of couples worldwide. Among different well-known reproductive techniques, medicinal plants have been utilized to treat male infertility. Medicinal plants, provide a therapeutic alternative, which is available and affordable for infertile couples. We investigated the direct effect of unfermented rooibos aqueous extract on human spermatozoa. Semen samples ($n = 50$) collected from donors and patients consulting for fertility were reassigned as normal ($n = 22$) and abnormal ($n = 28$) samples based on the outcome of the baseline semen analysis, using the World Health Organization (WHO) cut off value. Semen samples were allowed to liquefy and subsequently washed with human tubular fluid in bovine serum albumin medium. The samples were then treated with aqueous extracts of unfermented rooibos (0, 0.15, 1.5, 15, 150 μg/ml) at 37°C for 1 h and assessed thereafter. Sperm motility, vitality, DNA fragmentation, intracellular reactive oxygen species and mitochondrial membrane potential in both groups remained unchanged ($p > 0.05$). However, aqueous extract of unfermented rooibos (only at 1.5 μg/ml) significantly increased capacitation and acrosome reaction in the abnormal sample group ($p > 0.05$). Unfermented rooibos aqueous extract had no deleterious impact on human spermatozoa's function and might be attributed to its antioxidant properties.

KEYWORDS
antioxidant, infertility, reactive oxygen species, spermatozoa, unfermented rooibos

1 | INTRODUCTION

Millions of couples worldwide have experienced infertility leading to social segregation, with the female being considered the main culprit (Stentz et al., 2019). The inability to conceive after 1 year of regular unprotected sexual intercourse is regarded as infertility (Vander Borght & Wyns, 2018). About 15% of couples of reproductive age experience infertility, of which 50% is reported to be caused by a male factor (Agarwal et al., 2019). Nearly 580 million individuals (5%–8% of couples) experience the ill effects of infertility at some stage in their reproductive lives, in which, about 372 million people (186 million couples) living in developed and underdeveloped countries are affected (Kumar & Singh, 2015). Approximately 20%–50% couples of reproductive age experience infertility in Sub-Saharan Africa, in which 30% of these are diagnosed with infertility.

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The high prevalence of sexually transmitted infections (STI's) in Africa is attributed as the major cause of increased incidence of infertility (Dyer et al., 2002). Other causes of infertility include erectile dysfunction, sperm DNA damage and disruption in delicate processes such as spermatogenesis, that are linked with excessive production of reactive oxygen species (ROS) and oxidative stress (OS) (Direkvand-Moghadam et al., 2015). OS occurs due to excessive ROS production or inefficient antioxidant scavenging mechanism (Direkvand-Moghadam et al., 2015). ROS production must be at optimal level to maintain normal cell functioning or process (Agarwal et al., 2019), especially considering their harmful impacts on human spermatozoa.

A large percentage of the population (60%–80%) depends on medicinal plants for treatment of medical conditions due to its accessibility, affordability and effortlessness compared with modernized healthcare services (Agbor & Naidoo, 2016). Plant parts are commonly used in the management of diverse aspects of male infertility such as erectile dysfunction, sperm abnormalities, endocrine disorders, ejaculatory and relaxation dysfunction and the absence or loss of orgasm (Prasad et al., 2014). However, in Africa, proof of the effectiveness and safety of traditional medicines remains uncertain and can only be verified by the knowledge of traditional practitioners (Chatfield, 2018).

Nevertheless, antioxidants in plants is beneficial in the management of male infertility, as plant phenolic and flavonoids that are mostly found in fruits, vegetables, and tea beverages possess antioxidant and chelating properties (Adewoyin et al., 2017). Their protective effects include ability to prevent lipid peroxidation, chelate redox-active metals as well as inhibiting other processes involving ROS. In high physiological sums ROS cause oxidative damage to nucleic acids, lipids and cellular proteins (Jat & Nahar, 2017). Despite, their antioxidant benefits, they could exhibit mutagenic and or pro-oxidant effects (Galati & O'brien, 2004). The pro-oxidant activity of the plant extracts should be monitored before being considered for therapeutic purposes (Joubert et al., 2005).

Aspalathus linearis (commonly referred to as rooibos) is a leguminous shrub indigenous to the Cederberg region of the Western Cape of South Africa, and usually produced as unfermented, semi-unfermented and fermented rooibos (Abuaniza, 2013). Rooibos, a caffeine-free drink with low concentrations of tannin is beneficial for pregnant women, infants and people who are caffeine sensitive (Joubert et al., 2016). The increase in rooibos preference is due to its taste, affordability and health benefits (Joubert et al., 2016). Polyphenols, flavonoids and phenolic acids scavenge free radicals, which then hinders oxidative cellular damage (Jat & Nahar, 2017). *Aspalathus linearis* contain compounds such as cyclic dihydrochalcone (aspalalinin), flavonols (e.g., quercetin), monomeric flavan-3-ol (+)-catechin, dihydrochalcone (e.g., aspalathin), oligomeric flavan-3-ol (procyanidin B3), flavones and flavanones (Canda et al., 2014; van der Merwe et al., 2015). Aspalathin (C-linked dihydrochalcone glucoside) and aspalalinin (cyclic dihydrochalcone) are monomeric flavonoid that are uniquely present in rooibos (Ajuwon et al., 2015). Compared with fermented rooibos, unfermented rooibos possesses a higher level of aspalathin (Canda et al., 2014; Kwak et al., 2015). *Aspalathus linearis* reduced testosterone production in TM3 Leydig cells (Opuwari & Monsees, 2015) and diabetic male rats (Omolaoye et al., 2021). In other studies, *A. linearis* improved male reproductive functions in rat models (Awoniyi et al., 2012; Opuwari & Monsees, 2014). In human sperm in vitro, fermented rooibos had no effect on sperm motility, vitality, intracellular ROS and acrosome reaction, while reducing intact mitochondrial membrane potential (MMP) and DNA fragmentation (Takalani et al., 2021). We sought to explore the direct effect of aqueous extract of unfermented rooibos on human spermatozoa.

2 | MATERIAL AND METHODS

2.1 | Preparation of unfermented rooibos aqueous extract

Dried leaves of unfermented rooibos (Five Roses™) were purchased from a retail shop. The leaves (20 g) were permeated in freshly boiled distilled water (1 L) for 5 min, and respectively filtered through cheese cloth and Whatman filter papers (no. 4 and 1) (Whatman, Madestone, England). Thereafter the filtrate was freeze-dried, with an average yield of 1.34 g (6.7%). Unfermented rooibos aqueous extract was reconstituted in human tubular fluid (Quinn et al., 1985) supplemented with 1% bovine serum albumin (HTF-BSA; sperm wash media) to final concentrations of 0, 0.15, 1.5, 15, 150 μg/ml, with the therapeutic concentration calculated to be 1.5 μg/ml.

2.2 | Collection and preparation of semen samples

Semen samples were voluntarily obtained with informed consent following 3–5 days of sexual abstinence by masturbation and allowed to liquefy. Donors included healthy men (that is of unproven fertility status) at the University of the Western Cape ($n = 25$) and patients ($n = 25$) attending the Reproductive Biology Unit, University of Stellenbosch, in Tygerberg Academic Hospital, South Africa for fertility. Baseline analysis of semen samples was conducted and reassigned as normal (normozoospermic; $n = 22$) and abnormal (non-normozoospermic; $n = 28$) samples using the World Health Organization criteria (WHO, 2010). Semen samples with semen volume >1.5 ml; sperm concentration $>15 \times 10^6$ spermatozoa/ml; total sperm count $>39 \times 10^6$ spermatozoa/ejaculate), progressive motility >32% and total motility >40% were classified as normal, whereas samples with atleast one abnormal sperm parameter was regarded as abnormal (Espinoza et al., 2009). Samples were diluted with sperm wash media (1:5), and centrifuged (10 min; 300 *g*). The pellets were re-suspended in fresh sperm wash media and incubated with the plant extract (0.15, 1.5, 15, and 150 μg/ml) for 1 h at 37°C. Thereafter, various sperm parameters (capacitation and acrosome reaction, DNA fragmentation, MMP, motility, ROS, and vitality) were analysed. The study was approved by Turfloop Research Ethics Committee of the University of Limpopo (TREC/46/2019:PG) and Biomedical Research Ethics Committee of the University of the Western Cape (BM18/3/17), South Africa.

2.3 | Assessment of sperm motility parameters

Following the treatment of human spermatozoa, sperm suspension (10 μl) was positioned on a Leja slide (Nieuw Vernep, The Netherlands). The kinematics parameters (total motility [%], progressive motility [%], beat cross-frequency [BCF; Hz], linearity [LIN; %], straightness [STR; %], average path velocity [VAP; μm/s], curvilinear velocity [VCL; μm/s], straight line velocity [VSL; μm/s] and hyperactivation [%]) of at least 200 spermatozoa were analysed according to WHO criteria using Sperm Class Analyser 5.0 (SCA® Evolution, Microptic, Barcelona, Spain) with a Nikon Microscope (Nikon Instruments Inc., Tokyo, Japan) at 100×.

2.4 | Assessment of sperm vitality

Vitality was determined using the eosin-nigrosin dye (Sigma Aldrich, St Louis, MO) exclusion staining technique according to WHO (2010). Eosin-nigrosin (50 μl) was mixed with treated samples (50 μl), and thereafter, a smear was prepared on a slide. The slides were then air-dried and viewed with an oil-immersion objective (100×) in the bright field using a light microscope (Zeiss, Oberkochen, Germany). A total of 200 spermatozoa were counted, with live and dead sperm, respectively appearing white and pink. The results were expressed as percentage live sperm.

2.5 | Determination of mitochondrial membrane potential in human spermatozoa

Mitochondrial membrane potential in spermatozoa were determined using a lipophilic cationic dye (DePsipher™, Trevigen, Minneapolis, USA) according to the manufacturer's instructions. Phosphate buffered saline (100 μl; Oxoid, Basingstoke, Hampshire, UK) was added to each treated sample (100 μl) and centrifuged (10 min; 300 *g*), with the pellets re-suspended in prepared DePsipher solution (50 μl) and incubated (37°C; 20 min), away from light. Afterwards, the samples were centrifuged (10 min; 300 *g*) and re-suspended in reaction buffer. At least 200 spermatozoa were analysed using fluorescence microscope with a 488 nm excitation filter (Zeiss, Oberkochen, Germany) at 400× magnification. Spermatozoa that fluoresce intense red/orange in the midpiece were classified to have intact MMP, while those that fluoresced green as disrupted MMP. The results were denoted as percentage of spermatozoa with intact MMP.

2.6 | Determination of intracellular reactive oxygen species in human spermatozoa

The level of ROS in spermatozoa was determined according to Henkel et al. (2003). Firstly, a stock solution of dihydroethidine (DHE; 20 μl) (Molecular Probes, Eugene, OR) was prepared in PBS (pH 7.4). Treated samples (100 μl) were centrifuged (10 min; 300 *g*), re-suspended in PBS (200 μl) and DHE (20 μl), and then incubated (20 min; 37°C). Afterwards, each sample (10 μl) was placed on a slide and evaluated with oil immersion objective using an epifluorescence microscope (Zeiss, Oberkochen, Germany) with 488 nm excitation and 590 emission filters. Spermatozoa with excessive intracellular ROS production appeared red or orange (ROS+), while those without ROS where non-fluorescing (ROS−). A total of 200 spermatozoa was counted and expressed as the percentage of ROS positive spermatozoa.

2.7 | Assessment of sperm DNA fragmentation

The terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) assay was used in determining DNA fragmentation in spermatozoa, according to Henkel (2005). The treated samples (100 μl) were added to PBS (100 μl; Oxoid), centrifuged (300 *g*; 10 min), re-suspended and smeared on a StarFrost™ slide (Knittel Gläser, Braunschweig, Germany), allowed to air dry at RT, and then fixed in 4% formaldehyde in PBS (pH 7.4; 4°C; 25 min). Afterwards, samples were washed in PBS (RT; 5 min) and permeabilized in Triton™ X-100 (0.2%; Sigma-Aldrich) in PBS (RT; 5 min). Equilibration buffer was added (100 μl) to each slide (10 min), after rinsing the slides twice in PBS (RT; 5 min). TUNEL

TABLE 1 Summary statistics of sperm motility parameters for normal and abnormal semen sample groups, following an hour incubation with aqueous extract of unfermented rooibos

		Aqueous extract of unfermented rooibos (μg/ml)					
Motility	**Group**	**0**	**0.15**	**1.5**	**15**	**150**	***p*-value**
Total motility (%)	Normal	23.5 ± 16.5	28.2 ± 19.7	26.7 ± 25.4	27.6 ± 22.4	23.7 ± 18.8	0.9307
	Abnormal	27.9 ± 20.7	22.9 ± 17.0	23.5 ± 15.4	22.7 ± 16.2	23.5 ± 16.0	0.8878
Progressive motility (%)	Normal	12.8 ± 13.0	17.4 ± 18.0	19.4 ± 23.9	18.4 ± 21.4	14.2 ± 14.9	0.9207
	Abnormal	17.7 ± 14.5	15.2 ± 14.9	15.3 ± 13.0	15.2 ± 14.2	14.9 ± 14.3	0.7572
Non progressive motility (%)	Normal	10.7 ± 9.8	10.0 ± 6.2	7.8 ± 4.6	9.1 ± 6.2	9.3 ± 8.2	0.8333
	Abnormal	11.4 ± 8.7	8.3 ± 5.8	8.7 ± 5.4	8.0 ± 4.7	8.5 ± 6.2	0.5977

Note: Values represented are the mean ± SD of analysis of spermatozoa from normal ($n = 22$) and abnormal ($n = 28$) semen samples following exposure to the various concentrations of unfermented rooibos.

reagent (20 μl) was added to each slide, covered with a cover slip, and incubated (37°C) in a humidified chamber, away from light (60 min). The reaction was stopped by immersing the slides in 2× SSC for 15 min and washed in PBS thrice. At least 200 spermatozoa were immediately analysed with an oil immersion objective (400×) using a fluorescence microscope (Zeiss, Oberkochen, Germany) with a 488 nm excitation filter and a 510–530 nm emission filter. DNA fragmented spermatozoa fluoresced bright green (TUNEL+), while spermatozoa with intact DNA only showed a slight background stain (TUNEL−). The results were represented as the percentage of TUNEL-positive spermatozoa.

2.8 | Assessment of sperm capacitation and acrosome reaction

Capacitation and acrosome reaction was assessed with the chlorotetracyclin (CTC; Sigma-Aldrich) fluorescence assay (Green et al., 1996). Stock solution of Hoechst 33258 (100 mg/ml; Sigma-Aldrich), mixed with HTF (1:1000) was added to the treated samples in sperm wash media (1:100). They were then incubated (2 min; RT) and centrifuged (900 *g*; 5 min) with 2% polyvinylpyrrolidone (PVP40; 4 ml) in HTF. The CTC solution (pH 7.8) containing CTC (750 μM) in a buffer of NaCl (130 mM), cysteine (5 mM) and Tris–HCl (20 mM) (Merck, Sandton, Gauteng, South Africa) was freshly prepared and kept at 4°C away from light. Hoechst-treated spermatozoa (45 μl) were mixed with CTC solution (45 μl) and 12.5% w/v paraformaldehyde (8 μl; Sigma-Aldrich) in 0.5 M Tris–HCl (pH 7.4) and subsequently was placed on a slide. To prevent fading, a drop of 1.4 diazabicyclo (2.2.2) octane (DABCO, 0.22 M, Merck) dissolved in glycerol and PBS (9:1) was added onto each slide and viewed with an oil immersion objective lens (100×) using a fluorescence microscope (Zeiss, Oberkochen, Germany). At least 200 spermatozoa were analysed using the following patters. Spermatozoon with a uniform fluorescence over its entire head was classified as non-capacitated, acrosome-intact (F-pattern). Spermatozoon with a fluorescence-free band in the post-acrosomal region was classified as capacitated, and acrosome-intact (B-pattern), while those with no fluorescence over the sperm head was classified as capacitated and acrosome-reacted (AR pattern).

2.9 | Statistical analysis

Data obtained was analysed using Graph Pad Prism version. 5.01 (Graph Pad Software Inc. San Diego, CA). One-way

TABLE 2 Summary statistics of sperm kinematic motility parameters for normal and abnormal semen samples, following an hour incubation with aqueous extract of unfermented rooibos

		Aqueous extract of unfermented rooibos (μg/ml)					
Sperm kinematic parameter	Group	0	0.15	1.5	15	150	*p* value
VAP (μm/s)	Normal	18.7 ± 12.0	23.5 ± 17.2	26.1 ± 20.5	22.3 ± 13.6	24.5 ± 14.5	0.7887
	Abnormal	19.4 ± 6.6	19.8 ± 6.0	19.5 ± 7.7	17.9 ± 6.6	20.2 ± 7.6	0.9047
VCL (μm/s)	Normal	50.3 ± 21.9	53.0 ± 22.0	57.3 ± 26.0	52.7 ± 19.0	52.3 ± 20.7	0.9514
	Abnormal	47.7 ± 14.7	48.3 ± 13.3	46.6 ± 18.4	46.5 ± 14.9	48.7 ± 13.7	0.8587
VSL (μm/s)	Normal	27.3 ± 13.6	30.8 ± 17.2	30.9 ± 16.5	28.7 ± 13.5	30.7 ± 14.6	0.9096
	Abnormal	27.4 ± 8.2	27.9 ± 7.0	28.8 ± 14.3	26.7 ± 8.8	29.1 ± 9.1	0.9035
ALH (μm)	Normal	2.2 ± 0.6	2.2 ± 0.5	2.3 ± 0.5	2.2 ± 0.4	2.1 ± 0.5	0.7087
	Abnormal	2.2 ± 0.5	2.2 ± 0.5	2.2 ± 0.5	2.2 ± 0.6	2.3 ± 0.5	0.8794
Hyperactivation (%)	Normal	0.5 ± 1.3	0.4 ± 0.6	0.6 ± 1.0	0.3 ± 0.7	0.2 ± 0.5	0.7526
	Abnormal	0.4 ± 1.0	0.3 ± 0.7	0.2 ± 0.4	0.3 ± 0.6	0.3 ± 0.8	0.8908
LIN (%)	Normal	58.1 ± 11.6	63.2 ± 13.4	63.4 ± 13.4	60.9 ± 14.2	64.3 ± 15.0	0.5235
	Abnormal	63.0 ± 7.7	62.6 ± 7.9	64.2 ± 10.4	62.0 ± 11.9	63.2 ± 10.7	0.9378
STR (%)	Normal	34.0 ± 10.2	39.8 ± 14.4	37.7 ± 13.3	37.7 ± 14.0	39.9 ± 12.2	0.5448
	Abnormal	37.8 ± 8.8	37.0 ± 7.4	38.1 ± 10.4	36.6 ± 11.3	38.4 ± 9.2	0.9218
WOB (%)	Normal	52.8 ± 7.3	56.7 ± 11.4	54.2 ± 10.6	55.1 ± 10.2	57.2 ± 9.4	0.5746
	Abnormal	55.8 ± 7.6	54.8 ± 5.6	54.7 ± 9.2	54.3 ± 8.7	56.1 ± 8.0	0.9776
BCF (Hz)	Normal	7.0 ± 5.8	7.5 ± 5.3	7.3 ± 4.6	8.2 ± 6.0	8.4 ± 6.0	0.7687
	Abnormal	7.4 ± 5.6	7.1 ± 4.5	6.9 ± 4.9	6.7 ± 4.4	6.8 ± 3.6	0.9363

Note: Values represented are the mean ± SD of spermatozoa from normal ($n = 22$) and abnormal ($n = 28$) semen samples exposed to the various concentrations of unfermented rooibos.

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.

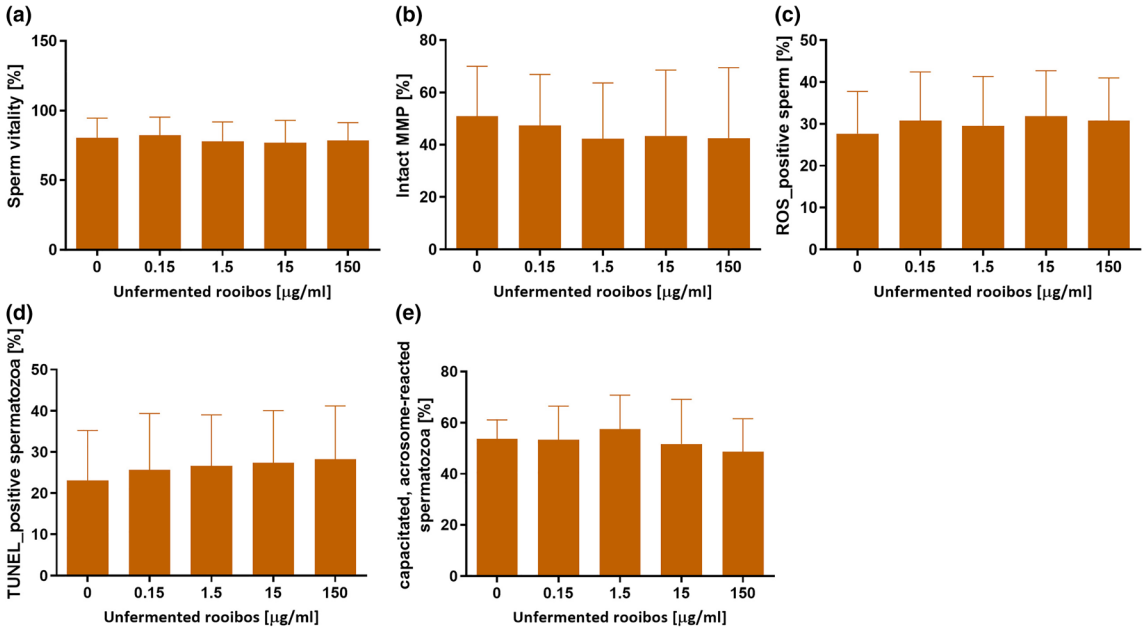

FIGURE 1 Effects of aqueous extracts unfermented rooibos on (a) sperm vitality, (b) mitochondrial membrane potential (intact), (c) intracellular reactive oxygen species production, (d) DNA fragmentation (TUNEL), and (e) acrosome reacted spermatozoa from normal semen samples ($n = 22$). Values represented are the mean ± SEM of human spermatozoa

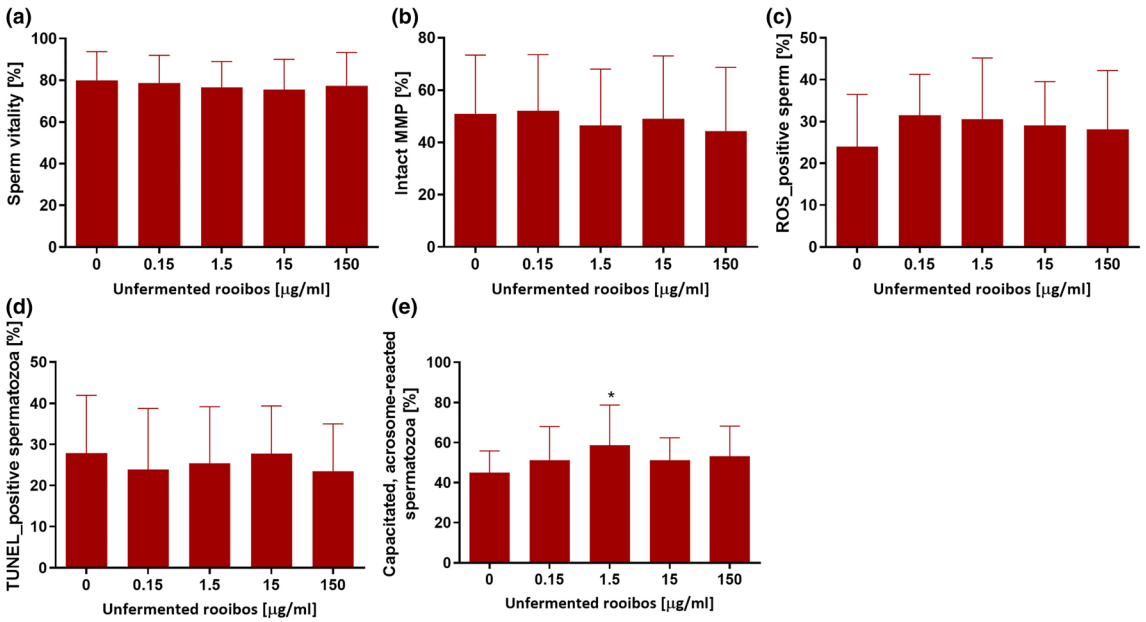

FIGURE 2 Effects of aqueous extracts unfermented rooibos on (a) sperm vitality, (b) mitochondrial membrane potential (intact), (c) intracellular reactive oxygen species production, (d) DNA fragmentation (TUNEL), and (e) acrosome reacted spermatozoa from abnormal semen samples ($n = 28$). Values represented are the mean ± SEM of human spermatozoa

analysis of variance (ANOVA) and Tukey's post-test were used for normally distributed data. For data not normally distributed Kruskal–Wallis test and Dunnetts' multiple post-test was employed. Two-way analysis of variance was used to compare the respective parameters between the unproven fertile s and infertile donors. A p-value less than 0.05 was set to be statistically significant.

3 | RESULTS

3.1 | Baseline analysis

Baseline values of semen samples of the normal and abnormal groups are respectively as follows: semen volume (Mean: 2.5 ± 0.8 ml) and (Mean: 2.0 ± 0.9 ml), sperm concentration (Mean: 62.8 ± 27.9 × 10^6/ml)

and (Mean: 54.8 ± 27.2 × 10^6/ml), total motility (Mean: 71.0 ± 14.9%) and (Mean: 40.6 ± 20.7%), progressive motility (Mean: 52.7 ± 12.6%) and (Mean: 22.7 ± 15.5%). Semen volume and concentration were comparable between the normal and abnormal sample groups while total motility ($p < 0.001$) and progressive motility ($p < 0.001$) was significantly higher in the normal group compared to the abnormal group.

3.2 | Sperm motility

Table 1 demonstrated that total motility ($p > 0.05$), progressive motility ($p > 0.05$), non-progressive motility ($p > 0.05$) remained unchanged in the normal sample group. Similarly, total motility ($p > 0.05$), progressive motility ($p > 0.05$) and non-progressive motility ($p > 0.05$) was unaltered in the abnormal sample group (Table 1). Total and progressive motility appeared to be higher in the abnormal samples compared to the normal samples, however, two-way ANOVA revealed no statistical difference between the group ($p > 0.05$). Furthermore, sperm kinematic parameters remained unchanged in both the normal and abnormal sample groups (Table 2; $p > 0.05$) after exposure to the aqueous extract of unfermented rooibos.

3.3 | Sperm vitality

The increasing concentrations of aqueous extracts of unfermented rooibos had no effect on live spermatozoa in the normal sample groups ($p > 0.05$; Figure 1a) compared to the control (mean ± SD 0 μg/ml; 80.4 ± 14.2; 0.15 μg/ml; 82.3 ± 13.0; 1.5 μg/ml; 77.7 ± 14.1; 15 μg/ml; 77.0 ± 16.0; 150 μg/ml; 78.3 ± 13.1). Similarly, the percentage of vital spermatozoa remained unchanged in the abnormal sample group following the period of treatment with the extract ($p > 0.05$; Figure 2a) (mean ± SD: 0 μg/ml; 80.0 ± 13.7; 0.15 μg/ml; 78.7 ± 13.3; 1.5 μg/ml; 76.7 ± 12.2; 15 μg/ml; 75.6 ± 14.4; 150 μg/ml; 77.3 ± 15.9).

3.4 | Sperm mitochondrial membrane potential (intact)

The percentage of spermatozoa with intact MMP remained unchanged in the normal sample (mean ± SD: 0 μg/ml; 50.9 ± 19.0; 0.15 μg/ml; 47.3 ± 19.5; 1.5 μg/ml; 42.4 ± 21.2; 15 μg/ml; 43.3 ± 25.2; 150 μg/ml; 42.6 ± 26.9) group ($p > 0.05$; Figure 1b) compared to the control as well as in the abnormal sample (mean ± SD: 0 μg/ml; 51.0 ± 22.4; 0.15 μg/ml; 52.1 ± 21.4; 1.5 μg/ml; 46.5 ± 21.5; 15 μg/ml; 49.1 ± 23.9; 150 μg/ml; 44.4 ± 24.3) group ($p > 0.005$; Figure 2b).Two way ANOVA analysis revealed a comparable result between both groups ($p > 0.05$).

3.5 | ROS-positive sperm

The amount of ROS positive sperm remained unaltered in the normal sample (mean ± SD: 0 μg/ml; 27.7 ± 10.1; 0.15 μg/ml; 30.8 ± 11.6; 1.5 μg/ml; 29.6 ± 11.7; 15 μg/ml; 31.9 ± 10.8; 150 μg/ml; 30.8 ± 10.2) group ($p > 0.05$; Figure 1c) as well as in the abnormal sample (mean ± SD: 0 μg/ml; 24.0 ± 12.5; 0.15 μg/ml; 31.5 ± 9.7; 1.5 μg/ml; 30.6 ± 14.6; 15 μg/ml; 29.1 ± 10.4; 150 μg/ml; 28.2 ± 14.0) group ($p > 0.05$; Figure 2c) compared to their controls.

3.6 | Sperm DNA fragmentation

Increasing concentrations of aqueous extracts of unfermented rooibos had no significant effects on the proportion of TUNEL-positive sperm in the normal sample (mean ± SD: 0 μg/ml; 23.1 ± 19.2; 0.15 μg/ml; 25.7 ± 13.7; 1.5 μg/ml; 26.7 ± 12.4; 15 μg/ml; 27.5 ± 12.6; 150 μg/ml; 28.2 ± 13.0) group compared to the control ($p > 0.05$; Figure 1d). Likewise, the percentage of TUNEL-positive spermatozoa remained unchanged following the treatment of spermatozoa from the abnormal sample group (mean ± SD 0 μg/ml; 27.9 ± 14.1; 0.15 μg/ml; 23.9 ± 14.9; 1.5 μg/ml; 25.5 ± 13.7; 15 μg/ml; 27.3 ± 11.7; 150 μg/ml; 23.5 ± 11.5) with the aqueous extract of unfermented rooibos ($p > 0.05$; Figure 2d).

3.7 | Capacitation and acrosome reaction

Capacitated and acrosome reacted spermatozoa remained unchanged in the normal sample (mean ± SD: 0 μg/ml; 53.8 ± 7.4; 0.15 μg/ml; 53.4 ± 13.2; 1.5 μg/ml; 57.6 ± 13.2; 15 μg/ml; 51.7 ± 17.5; 150 μg/ml; 48.7 ± 12.9) group ($p > 0.05$; Figure 1e), while in the abnormal sample (mean ± SD: 0 μg/ml; 45.1 ± 10.8; 0.15 μg/ml; 51.3 ± 16.8; 1.5 μg/ml; 58.8 ± 20.0; 15 μg/ml; 51.1 ± 11.4; 150 μg/ml; 53.1 ± 15.1) group a significant increase was observed only at 1.5 μg/ml compared to the control ($p < 0.05$; Figure 2e).

4 | DISCUSSION

Overall, the baseline assessment of the semen samples revealed a comparable result in the semen volume as well as sperm concentration between the normal and abnormal samples, although sperm motility parameters were of better quality in the normal sample compared to the abnormal sample group. Furthermore, the current study demonstrated that unfermented rooibos had no substantial effect on motility parameters. Although, total motility and progressive motility appeared to be higher in the abnormal group compared to the normal sample group, the difference is not statistically significant. A study reported that sperm motility and viability decrease over time after ejaculation (Singer et al., 1980).Our study revealed a decline in the percentage of total and progressive motility following the incubation period, with however, a great percentage of viable spermatozoa. A direct relationship exist between sperm velocity parameters and sperm fertilizing capacity (Boryshpolets et al., 2013; Collodel et al., 2007). In addition, sperm VCL and BCF have been reported to be important markers of sperm vitality, while LIN, STR, VAP, and VSL

are indicators of spermatozoa movement, with LIN and STR being associated with the control of swimming arrays (Fair & Romero-Aguirregomezcorta, 2019; King, 2018). The non-significant effect of the plant extract on VCL and BCF might explain the resultant no significant effect of unfermented rooibos on sperm vitality.

Spermatozoa are susceptible to oxidative damage by lipid peroxidation, due to the lack of cytoplasmic enzymes, antioxidant defence mechanism and DNA repair system, as well as high content of polyunsaturated fatty acid (PUFA), particularly when they are exposed to high levels of ROS (Bisht et al., 2017; Sabeti et al., 2016; Tremellen, 2008). Further to this, physiological level of ROS is a prerequisite for sustaining redox-sensitive biochemical processes such as capacitation, acrosome reaction and hyperactivation, of which, failure of this impairs fertilization (Durairajanayagam, 2019; Ickowicz et al., 2012). Potential sources of ROS in human semen may be endogenous (mainly from immature spermatozoa and leukocytes) (Du Plessis et al., 2015; Kothari et al., 2010) or exogenous (including exposure to visible light, composition of culture media, pH and temperature, oxygen concentration, ART technique involving handling of gamete/embryo and cryopreservation technique, and centrifugation during spermatozoa preparation (see review Agarwal et al., 2014). In addition, the length of centrifugation time as opposed to the centrifugal force induces ROS generation, and exposes the spermatozoa to higher temperature, that is detrimental to the spermatozoa parameters (Henkel & Schill, 2003). Addition of antioxidants during spermatozoa preparation prior to centrifugation is shown to reduce centrifugation induced ROS formation and damage to processed spermatozoa (Lampiao et al., 2010). ROS generation can also be increased by genetics and external lifestyle factors (such as cigarettes smoking and alcohol intake) that contribute significantly to OS (Agarwal et al., 2014). Nonetheless, spermatozoa are covered in a seminal milieu dominated by enzymatic and non-enzymatic antixidants (Tan et al., 2018). However, a balance between ROS and antioxidants is required since both reductive and OS are detrimental to spermatozoa. Thus, an individual's OS levels should be accurately determined before prescribing supplementary antioxidants (Opuwari & Henkel, 2016). The present study demonstrated that the level of ROS production in sperm remained unchanged in both the abnormal and normal sample groups, which may be attributed to the antioxidant property of the unfermented rooibos extract.

Spermatozoa must undergo the process of capacitation in order to fertilize an egg. The capacitated spermatozoon subsequently acquires the capacity to display hyper-activated motility and to undergo acrosome reaction (Morales et al., 1989). Although it can occur spontaneously under suitable conditions in vitro, its progress is regulated in vivo by the female reproductive tract (Francou et al., 2017). The present study demonstrated that the amount of AR pattern spermatozoa remained unchanged in the normal sample and abnormal samples (except at 1.5 μg/ml, where an increase was observed) groups, and may be associated with the ROS production A previous study indicated that consumption of *A. linearis* may result in early acrosome reaction of the male rat's spermatozoa (Opuwari & Monsees, 2014), and might possibly impair fertility. On the contrary, Isotani et al. (2017) demonstrated that acrosin-disrupted rats spermatozoa had a comparable ability to infiltrate the zona pellucida and were shown to be fertile with reduced litter size, even though, the dispersal of cumulus oophorus cells were slower. Premature (spontaneous) AR in spermatozoa renders the sperm cells incapable of fertilization (Francou et al., 2017). To establish the effect of unfermented rooibos on acrosome reaction and outcome of fertilization, further study is necessitated to better understand the mechanism of action.

Mitochondrial membrane damage is a crucial initiator of apoptosis in germ cells in the human testis (Wang & Youle, 2009). Furthermore, MMP is an accurate marker for the assessment of spermatozoa's integrity (Marchetti et al., 2002). An increased permeability of mitochondrial membranes to H^+ results in a dissipation of H^+ gradient and acts as the driving force for ATP production (Espinoza et al., 2009; Fisher & Henkel, 2020). MMP remained unchanged in the normal as well as the abnormal sample. It is well known that excessive ROS damages both the internal and external mitochondrial membranes, resulting in a discharge of cytochrome c, a protein known to activate caspases while inducing cell death (Zorov et al., 2014). Unfermented rooibos extract had no effect on ROS production, and could be a possible explanation for the maintenance of MMP in this study. Further to this, sperm vitality and motility also remained unchanged, and maybe associated with the unaltered level of MMP. Usually, a significant loss of MMP occurs before the loss of integrity of the spermatozoa's plasma membrane and is an irrevocable stage of cell death (Espinoza et al., 2009). In addition, a positive correlation was found between the percentage of MMP in sperm cells and sperm concentration and motility (Marchetti et al., 2002).

Sperm DNA as another crucial indicator of fertility is an important genomic material, and is highly condensed and compacted within the sperm cell to prevent fragmentation (Aitken & Koppers, 2011; Esteves et al., 2017). Exposure of human spermatozoa to increasing concentrations of aqueous extracts of unfermented rooibos revealed no change on the amount of DNA fragmented spermatozoa in both groups. Banihani et al. (2012) suggested that supplementation of semen samples with antioxidants does not affect sperm DNA integrity in vitro and our results are in line with this observation, which demonstrated that unfermented rooibos did not have a significant effect in reducing DNA oxidative damage in spermatozoa. Sperm DNA fragmentation occurs due to poor disulphide cross-links within mature spermatozoa usually as a result of modification in the chromatin packaging and may also be due to high level of ROS (Esteves et al., 2015). ROS cause damage to DNA through modification of all bases, synthesis of base free sites, frame shifts, elimination of DNA cross-links and chromosomal aberrations (Esteves et al., 2015). The high prevalence of single strand and double strand DNA breaks is also linked to OS. ROS have been reported to trigger gene mutations that include polymorphism and point mutation, resulting in low semen quality (Di Meo et al., 2016). However, some evidence show that DNA fragmentation and ROS may not always have a relationship. This suggests that DNA fragmentation may solely be responsible for cell death, and that ROS may sometimes directly damage the

nuclear DNA within the spermatozoa (Schulte et al., 2010). Other mechanisms such as DNA base-pair oxidation and denaturation may be involved. However, when DNA damage is minimal, spermatozoa undergo self-repair.

In conclusion, aqueous extract unfermented rooibos had no effect on sperm functions including motility, viability, DNA fragmentation, intracellular ROS, and MMP except in the abnormal samples where a significant increase was observed at the therapeutic concentration. This study may have been limited due to the use of unproven fertile donors as well as the lack of detailed information on the cause of infertility of the patients.

ACKNOWLEDGEMENTS

The authors thank the Department of Medical Biosciences, University of the Western Cape for providing us with training, laboratory space and equipment to successfully carry out this study.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Takalani, N. B., Adefolaju, G. A., Henkel, R. R., & Opuwari, C. S. (2022). In vitro effects of aqueous extract of unfermented rooibos on human spermatozoa. *Andrologia*, e14452. https://doi.org/10.1111/and.14452