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Full Length Article

Androstachys johnsonii Prain aqueous leaf extract attenuates oxidative stress in hyperglycaemic-induced spermatozoa: An *in vitro* study

Murendeni Nethengwe[a], Kunle Okaiyeto[a], Chinyerum S. Opuwari[b], Oluwafemi O. Oguntibeju[a,*]

[a] *Phytomedicine and Phytochemistry Group, Department of Biomedical Sciences, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, Bellville 7535, South Africa*

[b] *Department of Medical Biosciences, University of the Western Cape, Bellville 7535, South Africa*

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ABSTRACT

Background: Oxidative stress is a key instigator in the development of hyperglycaemia-associated complications in individuals with diabetes mellitus (DM). Male infertility is one of the complications associated with oxidative stress and hyperglycaemia. *Androstachys johnsonii* Prain has been used traditionally to treat hyperglycaemic-related male infertility. Although its antioxidant capacity and phytochemical composition were previously reported, the effect of the plant extract on male parameters under oxidative stress conditions remains unclear.

Objective: This study investigated the effect of the aqueous leaf extract of *A. johnsonii* on hyperglycaemic-induced sperm cells.

Methods: A total of 25 male participants, aged 18-40, were recruited voluntarily following ethical guidelines. Sperm parameters, including reactive oxidative species (ROS), total protein content in the plasma, catalase activity, superoxide dismutase (SOD) activity, DNA fragmentation were measured after hyperglycaemia induction and treatment with (0.001 μg/mL, 0.01 μg/mL, 0.1 μg/mL, and 1 μg/mL) of plant extract, and 100 μg/mL of acarbose. Capacitation and acrosome reaction, mitochondrial membrane potential, sperm vitality and motility were also assessed.

Results: The exposure of sperm cells to hyperglycaemic conditions for 24 h resulted in a significant ($P < 0.05$) increase in the production of reactive oxidative species (ROS), DNA fragmentation, and early capacitation and acrosome reaction, and a significant ($P < 0.05$) decline in mitochondrial membrane potential (MMP), vitality, motility. After the administration of *A. johnsonii* extract, there was a significant ($P < 0.05$) concentration-dependent reduction in ROS production, DNA fragmentation, and an increase in MMP integrity and vitality. The effect of acarbose on the sperm parameters was comparable to the highest concentrations (0.1 μg/mL and 1 μg/mL) of the extract. However, no significant ($P > 0.05$) change in motility and vitality was observed after treatment with acarbose. There was a significant ($P < 0.05$) reduction of capacitated and acrosome-reacted sperm cells post-acarbose treatment.

Conclusion: These findings revealed the evident potential of *A. johnsonii* in the improvement of male sperm parameters in DM through the reduction of ROS.

1. Introduction

The production of free radicals within the body occurs in support of physiological functions such as signal transduction, immunity, and sperm cell maturation in the male reproductive system (Dutta et al., 2021). The antioxidant system functions in response to an increase in free radicals by the production and activation of non-enzymatic and enzymatic antioxidants, maintaining oxidative status (Raza et al., 2020). An imbalance in the production and removal of free radicals results in the excessive production of free radicals such as reactive oxidative species (ROS), and consequently oxidative stress (Di Meo and Venditti, 2020). Oxidative stress plays a pivotal role in the development of disease (Kowalczyk et al., 2021). The overproduced ROS reacts with biomolecules such as proteins, lipids, and DNA and alters their functions (Zhang et al., 2020). Oxidative stress is also key in the development of diabetes mellitus (DM) (Singh et al., 2022). DM is a non-communicable chronic metabolic disease defined by hyperglycaemia (Ohiagu et al., 2021). The occurrence of oxidative stress in DM is associated with prolonged hyperglycaemia (González et al., 2023).

Pathologically high glucose levels serve as excessive substrates to the mitochondrial and lead to the overproduction and excessive leak of ROS coupled with a decrease in mitochondrial membrane potential

* Corresponding author.
E-mail address: OguntibejuO@cput.ac.za (O.O. Oguntibeju).

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(Deragon et al., 2020; González et al., 2023). Hyperglycaemia causes increased interaction of glucose molecules with macromolecules in sperm cells, leading to alteration and a decline in sperm parameters such as sperm concentration, motility, volume, and morphology (Facondo et al., 2022). In addition to this, a significant decrease in sperm maturity and vitality was previously observed in diabetic individuals (Facondo et al., 2022). Although the direct implications of hyperglycaemia in male reproductive dysfunction are unclear, some complications involved in male infertility such as erectile dysfunction, disruption of spermatogenesis, and male hypogonadism arise in diabetic individuals (Huang et al., 2024). Over 500 million individuals are affected by DM and its accompanying complications (Sun et al., 2022). Oxidative stress is implicated in DM-related complications such as cardiovascular disease, eye cataracts, kidney diseases, neurological diseases, and male infertility (Aitken et al., 2022; Andreadi et al., 2022; López-Contreras et al., 2020; Vodošek Hojs et al., 2020). Amongst the aforementioned DM complications, male infertility is less commonly studied. However, in the overall percentage prevalence of DM, the prevalence of male infertility ranges from 35% to approximately 50% (Lotti and Maggi, 2023). Hyperglycaemia-induced oxidative stress in male reproductive organs leads to the oxidation alteration of essential proteins necessary for the production and maturation of spermatozoa, and fertilisation (Ayad et al., 2022). Oxidative stress has also been implicated in the decline of sperm parameters such as DNA integrity, motility, and vitality in diabetic individuals (Nowicka-Bauer and Nixon, 2020; Ribeiro et al., 2022). This reveals the role of hyperglycaemia and oxidative stress in male reproductive dysfunction. The reduction of glucose levels and free radicals is therefore part of the therapeutic strategies to treat DM and male infertility. Due to their antioxidant composition, possible hypoglycaemic effect, and less toxicity, medicinal plants have drawn scientific research focus (Mthiyane et al., 2022).

Androstachys johnsonii Prain, also known as Lebombo wood, belongs to the *Picrodendraceae* family (previously belonged to *Euphorbiaceae*) and is indigenous to the Southern part of Africa (Zimbabwe and South Africa) (Maroyi, 2013). It is a 15 m tall plant with green leaves (Gandiwa et al., 2011). *A. johnsonii* is commonly known in Limpopo, South Africa as Musimbiri, and has been widely harvested for both its medicinal use and source of wood (Gandiwa et al., 2011; Mudau et al., 2022). As a medicinal plant, the leaves of *A. johnsonii* are traditionally used in the form of concoction to treat stomach pain and for the aphrodisiac effect on men (Mfotie Njoya et al., 2024). The roots of the plant have been recorded for their antimicrobial properties (Maroyi, 2013). A previously done phytochemical analysis of *A. johnsonii* leaves has revealed the presence of antioxidant phytochemical compounds such as phenolic glycosides, quinic, gallic, and coumaric acids, catechins, flavonoids and tannins (Mfotie Njoya et al., 2024). Although the phytochemical content of *A. johnsonii* leaf extract has been previously reported, there is limited experimental work done to investigate the effect of the plant extract on cells. In addition to this, the potential therapeutic benefit of the *A. johnsonii* extract in the treatment of DM-related male infertility is less known, and the mechanism behind the effect is unclear. This study reveals the role of hyperglycaemia in the occurrence of oxidative stress in spermatozoa, and the possible antioxidant and therapeutic effect of the aqueous leaf extract of *A. johnsonii* on the sperm parameters.

2. Materials and methods

2.1. Plant collection

Fresh leaves of *A. johnsonii* (Fig. 1) were harvested at the Brackenridgea Nature Reserve in Limpopo, South Africa. Plant authentication was performed at the University of Venda by Professor Tshisikhawe, and the specimen was kept at the Herbarium of University of Venda, with a voucher number (MNU001/10/22).



Fig. 1. The fresh leaves of *Androstachys johnsonii* Prain.

2.2. Plant extract preparation

The leaves were washed in clean water and dried in the shade for five days. After drying, they were manually crushed and then blended into a powder using a blender. A total of 100 g of this powder was mixed with 1000 mL of boiled water (100 °C) in a 2000 mL flask to prepare an aqueous extract. The mixture of the plant powder and boiled water was left for 24 h at room temperature to allow extraction. The liquid extract was collected after 24 h and the residual of plant material was discarded. The extract was then filtered using filter paper, after which it was freeze-dried and kept at −20 °C. The yield was 46 g of dried extract powder. The detailed characterisation of *A. johnsonii* leaf extract, including its antioxidant properties, will be reported in a forthcoming publication (currently under review).

2.3. Ethical considerations and participant selection criteria

Ethics approval was granted the Cape Peninsula University of Technology Research Ethics Committee (CPUT-REC) (Reference number: CPUT/HWS-REC 2024/H9) and the Biomedical Research Ethics Committee (BMREC) at the University of the Western Cape (Reference number: BM22/10/24) before the commencement of the study. A total of 25 male participants, aged 18–40, were recruited voluntarily following ethical guidelines. The age range was selected to cover the common male child-bearing age while excluding aging as a possible factor for the decline in fertility. Participants were recruited from Cape Peninsula University of Technology, University of Western Cape, and the surrounding areas. Only individuals with no chronic illnesses, including DM, were selected to participate in the study. All participants selected were under no chronic medication or supplements and did not smoke or consume alcohol. Baseline sperm parameters were evaluated as part of the screening process, and only participants who met the World Health Organisation standards were included in the study (WHO, 2010). All participants abstained for 3–5 days before the collection of semen.

2.4. Hyperglycaemia induction and treatment

Fresh semen was collected by the participant in a sterile specimen container through masturbation, and immediately kept in a 37 °C incubator for 30 min to allow liquefaction. The semen was then analysed using *sperm class analyser* (version 6.4, Microptic). Semen samples were classified using the WHO (2010) criteria where semen with sperm concentration > 15×10^7 /mL, sperm motility > 40%, sperm count > 39×10^7 per ejaculate at baseline was classified as normozoospermic. Only normozoospermic semen was used in the experiment. During preparation, the sperm cells were separated from other seminal components using a gradient, washed in human tubal fluid with bovine serum albumin (HTF-BSA) through centrifugation at 300 × *g* for 10 min, and

resuspended in 2 mL–3 mL of sperm wash to achieve the desired concentration.

Induction of hyperglycaemia was done following a previously utilised protocol (Portela et al., 2015). Prepared sperm was incubated for 3 h to allow capacitation. To induce hyperglycaemia, 5 mL of induction media (containing 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM sodium lactate, 1 mM sodium pyruvate, 0.3% BSA (w/v), 1% Penicillin/Streptomycin (v/v), 5 mM D-glucose for optimal concentration, and 100 mM D-glucose for hyperglycaemic concentration) was added to the cells, reaching a final concentration of 10 $\times 10^7$ cells/mL. The samples were incubated for 24 h. After incubation, the cells were centrifuged and washed with sperm wash before being subjected to various treatments.

Both the plant extract and acarbose treatments were prepared using HTF-BSA. Four concentrations (0.001 μg/mL, 0.01 μg/mL, 0.1 μg/mL, and 1 μg/mL) were selected for the study. To determine the selected optimal plant extract concentrations for this study, we conducted a preliminary experiment using a 10-fold serial dilution. We started with an initial concentration of 1 μg/mL and performed a series of 10-fold dilution to generate a range of concentrations. Acarbose was prepared at a concentration of 100 μg/mL. After 24 h of hyperglycaemia induction, 100 μL of each sample was centrifuged, and the supernatant was removed. Then, 100 μL of the respective treatments were added, with normal and hyperglycaemic control cells receiving HTF-BSA, and the acarbose group receiving acarbose. Treated samples were incubated at 37 ˚C for 1 h then centrifuged for 10 min at 300 ×*g*, after which the different sperm parameters were measured.

2.5. Reactive oxidative species determination

The determination of reactive oxidative species (ROS) was performed using the dihydroethidine (DHE) staining assay, as described by Henkel et al. (2012). A stock solution of DHE (20 μM) was prepared in PBS. After treating the sperm cells, the cell suspension was mixed with 20 μL of DHE solution and 180 μL of PBS, then incubated at 37 ˚C for 15 min–20 min. After incubation, 10 μL of the sample was transferred to a clean frosted slide, covered with a coverslip, and analyzed under a Nikon Eclipse 50i fluorescence microscope (Zeiss, Oberkochen, Germany) with oil immersion. A total of 200 sperm cells were assessed; red-stained sperm cells indicated high levels of ROS production, while non-stained cells suggested very low or no ROS production (Shalaweh et al., 2015).

2.6. Total protein content

Bradford method was followed to calculate the total protein content in the plasma of the spermatozoa. A protein standard with a series of concentrations (0.25 mg/mL, 0.5 mg/mL, 1 mg/mL, 1.4 mg/mL, and 2 mg/mL) was prepared from 2 mg/mL of bovine serum albumin (BSA) protein in 1% sodium dodecyl sulfate (SDS). A volume of 5 μL of the blank, standard concentrations, and the samples was loaded in the wells of a 96-well plate, after which 250 μL of the Bradford reagent was added. The plate was gently shaken and incubated in the dark for 30 min. The plate was read at 595 nm wavelength in an Omega Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA) (Kummari et al., 2022).

2.7. Catalase activity

Catalase activity in the plasma of sperm cells was measured by determining the dissociation of H_2O_2 in the lysates. The catalase activity assay was performed following the methods of Brannan et al. (1981). The H_2O_2 solution was prepared by the addition of 50 μL of H_2O_2 into 10 mL of the phosphate-buffered saline (PBS) buffer. During plating 170 μL of PBS buffer was first plates in a 96-well UV plate followed by 10 μL of the sample. The H_2O_2 solution (75 μL) was then added into the wells. The plate was immediately inserted in an Omega Spectrum plate reader and analysis was done (Thermo Fisher Scientific, Waltham, MA, USA) at 232 nm wavelength.

2.8. Superoxide dismutase activity

The activity of superoxide dismutase (SOD) in the plasma of sperm cells was determined through the calculation of inhibition (50%) of the oxidation of 6-hydroxydopamine hydrobromide (6-OHD) by superoxide radicals. Diethylene triamine pentaacetic acid (DETAPAC) was used to limit free radical production to only superoxide radicals. A 6-OHD solution was made from 4 mg of 6-OHD, 50 μL of perchloric acid, and 10 ml of distilled water. To prepare DETAPAC solution, 2 mg of DETAPAC was added into 50 ml of 100 mM phosphate buffered saline (PBS) buffer. Each sample (10 μL) was plated into the wells of a 96-well plate in triplicates after which 15 μL of 6-OHD was added. DETAPAC solution (170 μL) was then added to all wells. The plate was read immediately at 490 nm using an Omega Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA) (Brannan et al., 1981).

2.9. DNA fragmentation

DNA fragmentation was determined with the use of GoldCyto DNA kit protocol (Goldcyto Biotech Corp, Guangzhou, China). After exposing sperm cells to various treatments, the suspensions were resuspended in HTF-BSA to a concentration of 1 $\times 10^8$ cells/mL. A 30 μL aliquot of the sample was added to pre-heated agarose in an Eppendorf tube (heated at 90 ˚C–100 ˚C for 5 min, followed by 37 ˚C for 5 min). Then, 20 μL of the sample in agarose was placed on a clean slide and covered with a coverslip. The slide was refrigerated at 4 ˚C for 5 min, after which the coverslip was removed. The slide was then incubated horizontally in a series of solutions at room temperature: acid denaturation solution for 7 min, lysis solution for 25 min, distilled water for 5 min, followed by 70% ethanol for 2 min, and 90% ethanol for 2 min. The slide was left to air dry and stored in the dark until staining.

For staining, the slide was immersed in solution A for 1 min, followed by solution B for 2 min, then gently rinsed with tap water and left to dry. The slide was examined under a fluorescent microscope (Zeiss, Oberkochen, Germany) using 488 nm excitation and 510 nm–530 nm emission filters, with a 400 × oil-immersion objective. A total of 200 sperm cells were analyzed. Sperm cells with no halo or with a halo smaller than one-third of the minor diameter of the core were classified as fragmented, while those with halos larger than one-third of the core were considered non-fragmented.

2.10. Capacitation and acrosome reaction

The assessment of acrosome reaction and capacitation was conducted using the chlorotetracycline (CTC) fluorescence assay, as described by Shalaweh et al. (2015). After treatment, sperm cells were resuspended in HTF-BSA, and 1 μL of Hoechst 32258 dye was introduced. The samples were then incubated with the dye for 2 min, after which 400 μL of 2% polyvinylpyrrolidone (PVP40) was added. The mixture was centrifuged for 5 min at 900 × *g* and 37 ˚C. Once the supernatant was discarded, 45 μL of a 750 μM CTC solution (which contained 130 mM NaCl, 5 mM cysteine, and 20 mM Tris–HCl at pH 7.8) and 8 μL of a Tris–HCl solution (12.5% paraformaldehyde (w/v) in 0.5 M Tris–HCl at pH 7.4) were incorporated into the cell suspension. A 10 μL aliquot of this mixture was placed on a frosted slide and covered with a coverslip for analysis under a Nikon Eclipse 50i fluorescence microscope (Zeiss, Oberkochen, Germany). A minimum of 200 sperm cells were evaluated and categorized into three patterns:

Non-capacitated, acrosome-intact sperm (F-pattern) exhibited fluorescence throughout the entire head, while capacitated, acrosome-intact sperm (B-pattern) displayed fluorescence over the head, excluding the post-acrosomal region. In contrast, capacitated, acrosome-reacted sperm

(AR-pattern) cells were identified by the absence of fluorescence over the head (Shalaweh et al., 2015).

2.11. Mitochondrial membrane potential

A mitochondrial staining kit was utilized to evaluate the mitochondrial membrane potential according to the mitochondria staining kit protocol (CS0390) (Sigma-Aldrich Inc., St Louis, MO, USA). Following treatment, 100 μL of the cell suspension was stained using the MMP staining solution, which consisted of 80 μL of distilled water, 20 μL of JC-5 buffer, and 0.5 μL of frozen 200 × MMP stock solution in a 1:1 ratio, and incubated in the dark at 37 ˚C for 20 min. Following incubation, the samples were centrifuged at 500 ×*g* and 5 ˚C–7 ˚C for 5 min. The cell pellet remaining after discarding the supernatant was resuspended in 100 μL of chilled JC-1 buffer, prepared with 80 μL of distilled water and 20 μL of JC-5 buffer, and centrifuged again under the same conditions. After discarding the supernatant, the cells were resuspended in another 100 μL of JC-1 buffer. A 5 μL–10 μL aliquot of the suspension was placed on a frosted slide and covered with a coverslip. The slide was analysed in the dark using a Nikon Eclipse 50i fluorescence microscope (Zeiss, Oberkochen, Germany). At least 200 sperm cells were assessed, and the percentage of sperm cells with intact mitochondria was recorded. Green-stained sperm cells were classified as having non-intact mitochondria, while red-stained sperm cells indicated intact mitochondria.

2.12. Vitality and motility

Sperm vitality was assessed following the eosin-nigrosin staining method. A 1:1 ratio of sperm sample and eosin-nigrosin dye (Sigma Aldrich, St Louis, MO) was prepared and incubated at 37 ˚C for 15 min. A smear of the sample mixture was made on a slide and left to air dry. The slide was examined under a light microscope (Zeiss, Oberkochen, Germany) using a 100 × oil-immersion objective. A minimum of 200 sperm cells were analyzed, where live cells appeared white and dead cells were stained pink (Shalaweh et al., 2015). To assess total sperm motility percentage of the different sperm cell treatments, 3 μL of the sample was loaded in a Leja slide pre-warmed at 37 ˚C. The slide was analysed using a sperm class analyser (SCA, Version 6.4, Microptic) and 200 sperms were assessed.

2.13. Data analysis

Data obtained from this study was analysed by a paired *t*-test (and nonparametric tests) using GraphPad Prism version 5. The mean points were plotted in a column mean graph. Significance was determined using *P*-value, with a *P*-value < 0.05 representing significant differences between compared groups.

3. Results

3.1. Reactive oxidative species production in hyperglycaemic cells

The production of ROS was measured using a DHE stain which stains ROS-positive sperm cells in red colour. The total percentage of sperm cells with ROS production was determined and recorded as shown in Fig. 2A. The sperm cell group with hyperglycaemia (HC) showed a significant increase ($P < 0.05$) in ROS production compared to the normal control group (NC). A concentration-dependent reducing effect of *A. johnsonii* leaf extract in the production of ROS was observed. There was a significant reduction ($P < 0.05$) in ROS after treatment of the hyperglycaemic sperm cells with the plant extract. The highest concentration (1 μg/mL) compared to the lower concentrations of the plant extract caused an enhanced reduction in ROS . Although the administration of acarbose also resulted in a significant reduction ($P < 0.05$) in ROS production, its ROS-reducing capacity was lower compared to the highest concentration (1 μg/mL) of *A. johnsonii*.

3.2. Effect of A. johnsonii on total sperm motility and vitality

The total percentage motility of the sperm cells was determined to investigate the effect of hyperglycaemia on sperm motility, and the ameliorative effect of *A. johnsonii*. The results are depicted in Fig. 2B. High glucose conditions in sperm cells led to a significant decrease ($P < 0.05$) in total sperm motility. However, the treatment of hyperglycaemic cells with the aqueous leaf extract of *A. johnsonii* led to a slight non-significant increase in motility ($P > 0.05$) compared to the normal control. There was no significant difference ($P > 0.05$) in motility between the sperm cells treated with the different concentrations of the plant extract and the untreated hyperglycaemic cells. There was also no significant difference ($P > 0.05$) between the sperm cells treated with acarbose compared to the untreated hyperglycaemic cells and the sperm cells treated with the plant extract.

The treatment of sperm cells with high levels of glucose led to a significant decrease in the number of live cells (Fig. 2C). After the hyperglycaemic sperm cells were treated with *A. johnsonii* leaf extract, the number of live cells significantly ($P < 0.05$) increased dependent on the concentration of the plant extract. The highest concentration (1 μg/mL) of the plant extract led to a significant ($P < 0.05$) increase in live cells. No significant difference ($P > 0.05$) in sperm cell vitality was observed after the treatment of the hyperglycaemic sperm cells with acarbose, compared to that of the hyperglycaemic sperm cells without treatment.

3.3. Effect of A. johnsonii on DNA integrity and mitochondrial membrane potential

The percentage of sperm cells with DNA fragmentation was determined and represented in Fig. 2D. A significant increase ($P < 0.05$) in DNA fragmentation was evident in sperm cells under high glucose conditions. Exposure of the hyperglycaemic sperm cells to the aqueous extract of *A. johnsonii* leaves led to a significant decrease ($P < 0.05$) in sperm cells with fragmented DNA, with the highest tested concentration (1 μg/mL) of the plant extract exhibiting the highest effect. Treatment with acarbose showed a comparable effect on DNA fragmentation compared to the highest concentration of the plant extract.

The integrity of sperm mitochondria was determined with respect to MMP and the results are shown in Fig. 2E. A significantly ($P < 0.05$) lower number of hyperglycaemic sperm cells had intact MMP compared to the normal control cells. Exposure of the hyperglycaemic sperm cells to 0.001 μg/mL and 0.01 μg/mL of the plant extract did not show a significant difference ($P > 0.05$) in MMP compared to the untreated hyperglycaemic sperm cells. However, a significant increase in sperm cells ($P < 0.05$) with intact MMP was observed after treatment with 0.1 μg/mL and 1 μg/mL concentrations. Treatment of the hyperglycaemic cells with acarbose resulted in a significant ($P < 0.05$) increase in the number of cells with intact MMP. The effect of acarbose on the MMP of hyperglycaemic cells was comparable to that of 0.1 μg/mL and 1μg/mL concentrations of the plant extract.

3.4. Antioxidant enzyme activity

Fig. 3 illustrates the results obtained after the determination of antioxidant enzyme activities of catalase and SOD after the induction of hyperglycaemia and treatment with *A. johnsonii* aqueous leaf extract. The activity of catalase remained the same after hyperglycaemia induction compared to the normal control plasma. Similarly, SOD activity in hyperglycaemic plasma was not different from the normal control plasma. There was no significant change in both SOD and catalase activity in the plasma after the treatment of hyperglycaemic sperm cells with *A. johnsonii*. There was also no significant difference in the total protein content amongst the study groups.

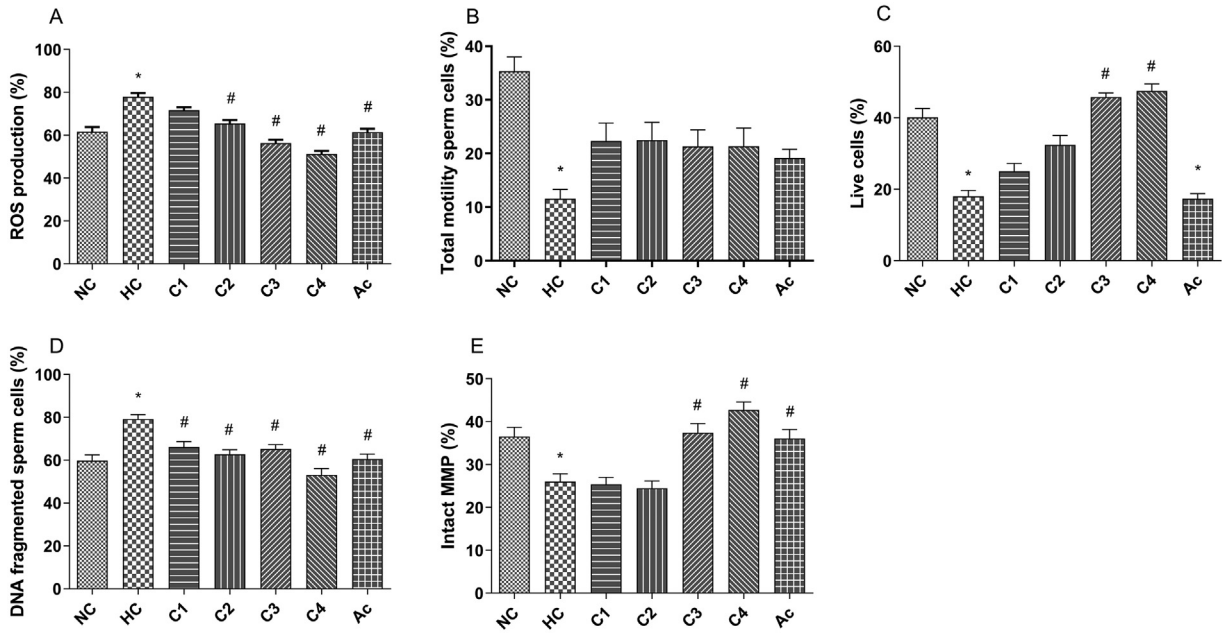


Fig. 2. The ameliorative effect of aqueous leaf extract of *A. johnsonii* on A). reactive oxidative species production, B). Total sperm motility, C). Sperm cell vitality, D). DNA fragmentation, E). Mitochondrial membrane potential in hyperglycaemic sperm cells. Data is represented as mean ± SEM of 200 sperm cells. Statistical difference is shown using $^{*}P < 0.05$ as compared to normal control and $^{\#}P < 0.05$ as compared to hyperglycaemic control. NC: normal control; HC: hyperglycaemic control, Ac: acarbose, C1: 0.001 µg/mL, C2: 0.01 µg/mL, C3: 0.1 µg/mL, C4: 1 µg/mL.

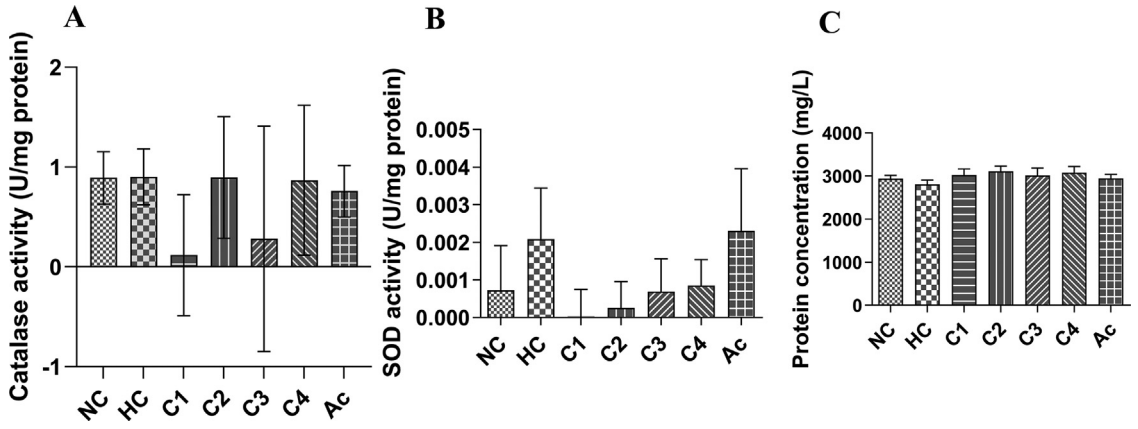


Fig. 3. The effect of aqueous leaf extract of A. *johnsonii* on A). catalase activity, B). SOD activity, C). total protein content, in the plasma of hyperglycaemic sperm cells. Data is represented as mean ± SEM. Statistical difference is shown using $^{*}P < 0.05$ as compared to normal control and $^{\#}P < 0.05$ as compared to hyperglycaemic control. NC: normal control; HC: hyperglycaemic control, Ac: acarbose, C1: 0.001 µg/mL, C2: 0.01 µg/mL, C3: 0.1 µg/mL, C4: 1 µg/mL.

3.5. Sperm capacitation and acrosome reaction

The difference in capacitation and acrosome reaction amongst the sperm cell treatment groups is depicted in Fig. 4. Exposure of sperm cells to high glucose conditions resulted in significant ($P < 0.05$) increase in capacitated, acrosome-reacted sperm cells (AR-pattern), a decrease ($P < 0.05$), in non-capacitated, acrosome-intact sperm cells a (F-pattern), and a decrease ($P < 0.05$) in capacitated, acrosome-intact sperm cells (B-pattern). The majority of the hyperglycaemic sperm cells were capacitated and contained reacted acrosomes. There was no significant difference ($P > 0.05$) in sperm cell capacitation and acrosome reaction after treatment with the aqueous leaf extract of *A. johnsonii*. Treatment with acarbose led to significant ($P < 0.05$) decrease in the AR-pattern cells and a significant increase ($P < 0.05$) in F-pattern sperm cells compared to the untreated hyperglycaemic sperm cells.

4. Discussion

DM is one of the causative factors of male infertility (He et al., 2021). The pathological pathways involved in the metabolism of excessive glucose molecules lead to the overproduction of ROS *in vivo* (Yaribeygi et al., 2020). The *in vitro* hyperglycaemic study model can be established using different cell types to study the effect of hyperglycaemia and possible treatment (Portou et al., 2020; Sharma et al., 2020). Although hyperglycaemia induction is not common in sperm cells, few studies have been conducted to investigate the implications of high glucose conditions on sperm cells (Hernández-Avilés et al., 2020; Moraes et al., 2021).

In this present study, the hyperglycaemic model was established in sperm cells using high concentration of glucose. Excessive production of ROS was observed in the hyperglycaemic cells. In the cells, an excessive substrate supply of glucose leads to the shift of the metabolic

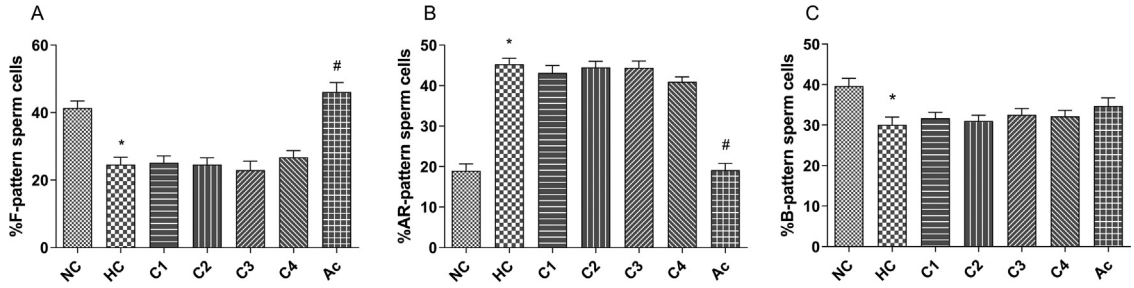


Fig. 4. The ameliorative effect of aqueous leaf extract of A. *johnsonii* on A). percentage non-capacitated with intact acrosomes (F-pattern B). percentage capacitated, acrosome-intact (B-pattern), and C). percentage capacitated and acrosome reacted (AR-pattern) in hyperglycaemic sperm cells. Data is represented as ±SEM of 200 sperm cells. $^{*}P < 0.05$ as compared to normal control and $^{\#}P < 0.05$ as compared to hyperglycaemic control. NC: normal control; HC: hyperglycaemic control, Ac: acarbose, C1: 0.001 μg/mL, C2: 0.01 μg/mL, C3: 0.1 μg/mL, C4: 1 μg/mL.

pathway to the polyol pathway, an alternative source of free radical (Garg and Gupta, 2022; Thorne et al., 2024). In the mitochondria, excessive ATP production through oxidative phosphorylation caused by hyperglycaemia leads to the overproduction of ROS from impartial reduction of oxygen (Prasun, 2020). An overload of free radicals in the mitochondrial leads to reduced mitochondrial membrane potential and a leak of excessive ROS (Tabassum et al., 2020). Due to hyperglycaemia, the non-enzymatic formation of advanced glycation products occurs rapidly and leads to increased production of ROS (Mori et al., 2024). In the current study, the excessive production of ROS in hyperglycaemic sperm cells can be associated with induced hyperglycaemia. The lower amount of ROS in the normal control group possibly represents the normal amount of ROS necessary for sperm maturation and fertilisation (Baskaran et al., 2021).

Flavonoids are a major abundant group of antioxidants in plants (Roy et al., 2022). Previous studies have reported the antioxidant capacity of flavonoids and their evident effect in the reduction of ROS (Dias et al., 2021). Flavonoids are the most abundant phenolic compounds in A. *johnsonii* aqueous leaf extract (Mfotie Njoya et al., 2024). In the present study, treatment of hyperglycaemic sperm cells with the aqueous leaf extract of A. *johnsonii* led to a significant concentration-dependent decrease in ROS which supports the previous finding that the plant extract has high antioxidant capacity. Higher concentrations of the plant extract contain a higher concentration of the phenolic compounds; hence the highest antioxidant capacity is observed at the highest tested plant extract concentration (1 μg/mL). Antioxidant enzymes such as catalase and SOD exhibit protective effects against cell and tissue damage during oxidative stress (Demirci-Çekiç et al., 2022). Previous studies have reported the reduction of antioxidant enzymes during oxidative stress (Birari et al., 2020; Yildirim et al., 2021).

In our present study, although the production of ROS was high after hyperglycaemia induction, the activity of antioxidant enzymes (catalase and SOD) remained constant. This shows that there was no compensation by the antioxidant enzyme system to reduce ROS. Most medicinal plants with antioxidant capacity activate the antioxidant system by increasing the antioxidant activity of enzymes (Mostafa et al., 2021; Soliman et al., 2020). However, this study suggests that the *A. johsonii* leaf extract may not exert its antioxidant effects through the activation of antioxidant enzymes, but through direct reaction of phenolic compounds with free radicals.

It is also evident that the accumulation of ROS is linked to the disruption of the mitochondrial membrane, i.e., the MMP (Chianese and Pierantoni, 2021; Zhao et al., 2021). The decrease in mitochondrial function also contributes to increased ROS (Prasun, 2020). In the present study, cells with higher levels of ROS (hyperglycaemic cells) also showed a decrease in mitochondrial membrane potential. The excessive supply of glucose into the sperm cell mitochondria results in the overactivate oxidative phosphorylation pathway, which releases excessive ROS and overloads the mitochondria (Chianese and Pierantoni, 2021). The treatment of hyperglycaemic sperm cells with the extract leads to a concentration-dependent increase in MMP intact sperm cells. Besides its well-known hypoglycaemic effect, acarbose exhibits antioxidant properties (AL-Zuwayni and Khudair, 2022; Cardullo et al., 2021).

In this current study, acarbose reduced ROS levels in hyperglycaemic sperm cells, and increased MMP. However, the effect of acarbose in ROS reduction is lower compared to the plant extract. In a previous study, the induction of hyperglycaemia in rats led to a decline in sperm cell motility (Pacheco-Castillo et al., 2024). In another study, the occurrence of oxidative stress also interfered with sperm cell motility, leading to decreased sperm cell motility (Kurkowska et al., 2020; Moichela et al., 2021).

In our current study, both the induction of hyperglycaemia and oxidative stress can be linked to the observed significant decrease in total sperm cell motility. A decreased sperm motility is detrimental to reproductive function and can lead to compromised male fertility. Although both acarbose and *A. johnsonii* plant extract reduced ROS levels and increased MMP in hyperglycaemic sperm cells, total sperm motility remained the same after both treatments. These findings show that the effect of the A. *johnsonii* leaf extract on hyperglycaemic sperm cells targeted the damage caused by oxidative stress. Mitochondrial function is crucial to produce ATP for the movement of the sperm cells (Nesci et al., 2020). However, the repair of MMP in this study did not lead to increased sperm cell motility. Oxidative stress leads to the oxidation and destruction of biomolecules such as lipids, proteins, and DNA (Zhang et al., 2020). Sperm cell DNA integrity is important during fertilisation for the exchange of genetic material between males and females (Nisa and Waqas Abduallah, 2023). A decreased DNA fragmentation can interfere with fertilisation and can lead to male infertility (Nisa and Waqas Abduallah, 2023).

In the current study, oxidative stress resulted in a decrease in DNA integrity. In corroboration to previous studies, ROS led to increased fragmentation of the sperm cell DNA. It is evident from this study that the reduction of ROS production leads to a lower number of fragmented DNA, as the treatment of cells with A. *johnsonii* leaf extract increased DNA integrity. Destruction of biomolecules in the sperm cells can ultimately lead to apoptosis (Asadi et al., 2021; Takalani et al., 2021). The damage of sperm cells caused by ROS in hyperglycaemic group led to decreased vitality. Exposure of the cells to the plant extract treatment led to an increase in vitality possibly caused by preventing the oxidation of the sperm biomolecules. However, the observed decrease in sperm cell vitality after acarbose treatment could suggest a possible toxicity of the standard drug on sperm cells.

Successful fertilisation also depends on the when the sperm cells undergo capacitation and acrosome reaction (Dahan and Breitbart, 2022). Capacitation involves physical and chemical alteration of the sperm cell and initiates the acrosome reaction (Chakraborty and Saha, 2022). Although fertilisation is initiated by capacitation, early/premature capacitation is detrimental (Dahan and Breitbart, 2022). The normal amount

of ROS produced in the plasma contributes to normal sperm cell capacitation (Nowicka-Bauer and Nixon, 2020). However, oxidative stress and hyperglycaemia have been reported as contributors of early capacitation and reduction of acrosome-intact sperm cells (Benko et al., 2022; O'Flaherty and Scarlata, 2022). Normally, capacitation starts immediately after ejaculation in the female reproductive tract (Pitnick et al., 2020). However, in *in vitro* studies, capacitation occurs in selected conditions with the availability of inducers (Fuentes et al., 2024).

In our present study, no capacitation inducers were added and the aim was to detect premature capacitation through the determination of the percentage of sperm cells that capacitated and contained reacted acrosomes in the absence of inducers. An increase in capacitated and acrosome-reacted sperm cells in hyperglycaemic sperm cells was observed. This can be linked to the increase in ROS. There was no significant effect of the plant extract on the capacitation of the sperm cells regardless of antioxidant capacity. Previous studies revealed that early capacitation can also possibly occur due to HTF media (Ruiz-Díaz et al., 2020). However, exposure of the cells to acarbose led to an increase in acrosome-intact and non-capacitated sperm cells.

5. Conclusion

Hyperglycaemia and resulting oxidative stress are crucial instigators of the decline in male reproductive parameters and should be a point of focus for diabetic males with male infertility. Although the use of acarbose as a standard drug is effective in the amelioration of male reproductive dysfunction, the findings of this study show that *A. johnsonii* aqueous leaf extract shows a higher therapeutic effect in the amelioration of the studied male parameters. This suggests the importance of further studies of more efficacious and less toxic alternative treatment of diabetic-related male infertility, and other complications involving oxidative stress. However, acarbose is highly effective in the improvement of other sperm parameters compared to the plant extract. The combination of medicinal plant extracts with standard drugs could mask the toxicity of the standard drug and synergistically improve the effect of the treatment. The choice of appropriate extract preparation, concentrations and dosage is crucial in determining high efficacy and lower toxicity.

Ethical approval

All procedures performed in this study were in compliance with the guidelines established by the Cape Peninsula University of Technology Research Ethics Committee (CPUT-REC) (Reference number: CPUT/HWS-REC 2024/H9) and the Biomedical Research Ethics Committee (BMREC) at the University of the Western Cape (Reference number: BM22/10/24), and both institutions have approved them.

Data availability

Data is available upon request.

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ORCID

Oluwafemi Omoniyi Oguntibeju, https://orcid.org/000-0002-7299-1128.

Declaration of competing interest

The authors declare that they have no known competing interests.

CRediT authorship contribution statement

Murendeni Nethengwe: Conceptualization, Methodology, Investigation, Data analysis and Curation, Writing-original draft. **Kunle Okaiyeto**: Supervision, Conceptualization, Writing- review and editing, Validation. **Chinyerum S. Opuwari**: Supervision, Writing- review and editing, Validation. **Oluwafemi O. Oguntibeju**: Supervision, Conceptualization, Resources, Secured funding for the study, Writing review and editing, Validation.

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