



Evaluation of antioxidant potential, anti-inflammatory effect and the mechanisms of action of phenolic compounds from South African indigenous plants used in the management of diabetes-related male infertility

by

MURENDENI NETHENGWE

Student number: 219477221

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Faculty of Health and Wellness Sciences

Cape Peninsula University of Technology

Supervisor: Prof. O.O. Oguntibeju

Co-supervisors: Dr C.S. Opuwari

: Dr K. Okaiyeto

Bellville

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DECLARATION

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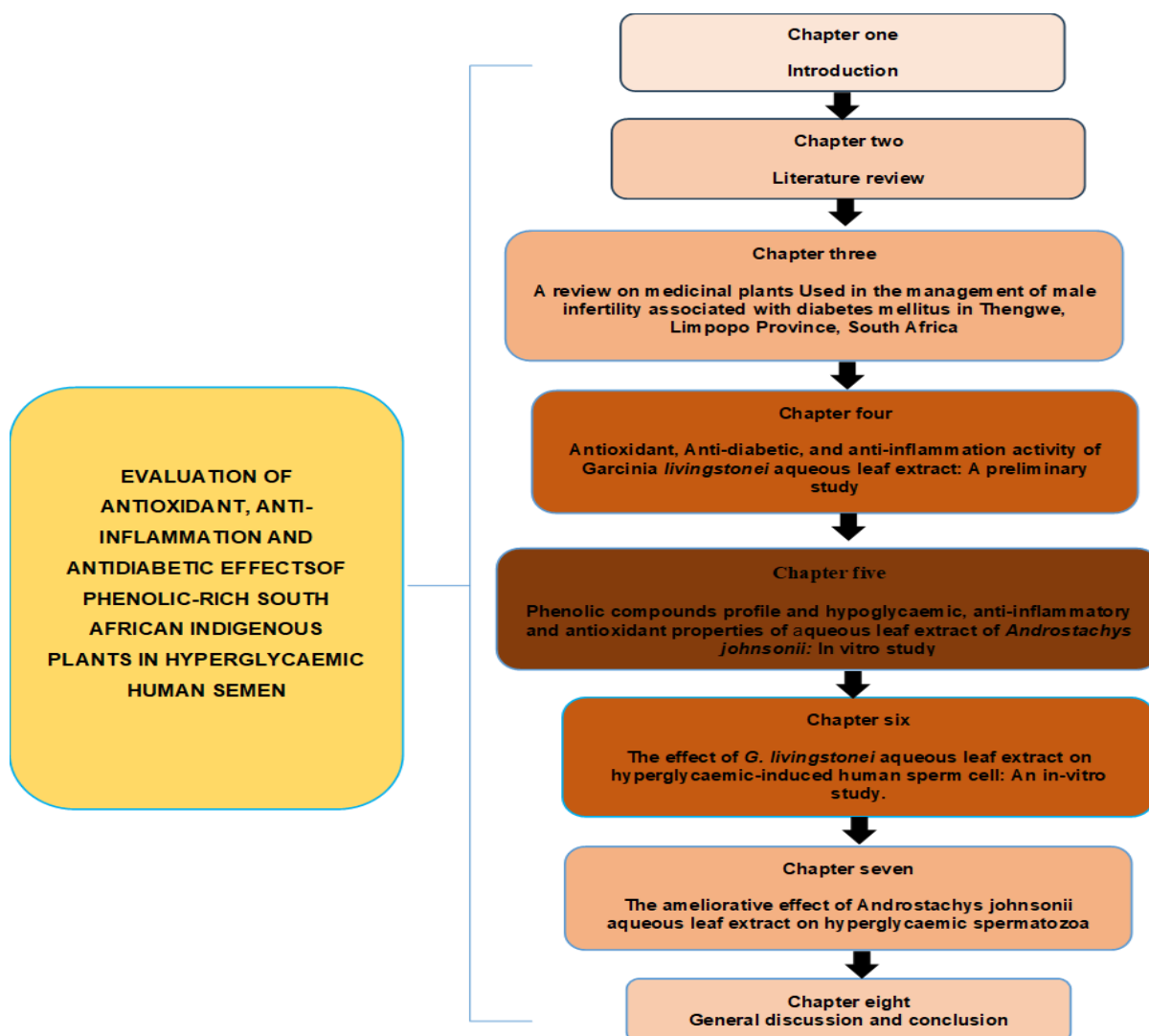
General abstract

Diabetes mellitus (DM) is a chronic metabolic disease characterised by hyperglycaemia. The rise in mortality associated with DM is attributed to the complications driven by prolonged hyperglycaemia which leads to excessive production of free radicals. The imbalance in the level of free radicals causes oxidative stress and inflammation. Hyperglycaemic-induced oxidative stress and inflammation have previously been shown to cause damage and a significant impact on male sub-/infertility by reducing sperm parameters such as sperm motility, sperm DNA and mitochondrial membrane integrity, and sperm capacitation. Over the years, these diabetic complications have been treated with conventional drugs, however, the adverse effects associated with their long usage have necessitated searching for safe and effective treatment that targets the pathological pathways leading to diabetic-related male infertility. Therefore, the present study identified potential medicinal plants (*Androstachys johnsonii* and *Garcinia livingstonei*) used in traditional medicine for the treatment of DM-related male infertility and investigated their effects in the amelioration of reproductive dysfunction linked to hyperglycaemia. The study is premised on the hypothesis that phenolic-rich South African medicinal plants could exhibit some antidiabetic effects, and attenuate oxidative stress and inflammation in the semen of diabetic individuals with male infertility. The medicinal plants were identified in the first phase of the study, and the plant extracts were prepared. Preliminary studies were conducted to determine the hypoglycaemic, anti-inflammatory, and antioxidant properties of the plant extract. The plant extracts were tested on sperm cells induced with hyperglycaemia in vitro. Normal sperm cells were collected from 25 male individuals. Each sample was divided into a normal group sample, hyperglycaemia-induced sample, and a sample induced with hyperglycaemia and treated with the plant extract. Phenolic compounds that could be responsible for the biological activities were identified from the plant extracts and among which, flavonoids were majorly abundant in both plant extracts. The results demonstrated high antioxidant capacity in both plant extracts through the determination of ferric reducing antioxidant power (FRAP), Trolox equivalent antioxidant capacity (TEAC), and

2,2-diphenyl-1-picrylhydrazyl (DPPH). Also, the extracts exhibited significant ($P < 0.05$) α -glucosidase inhibitory activity higher than acarbose. Both extracts displayed anti-inflammatory effects significantly ($P < 0.05$), depending on the concentration of the plant extract. While no cell toxicity on C3A hepatocarcinoma cells was observed with all test concentrations of *G. livingstonei*, the highest concentration (250 $\mu\text{g/mL}$) of the *A. johnsonii* extract showed cell toxicity. Hyperglycaemia induction led to a significant ($P < 0.05$) increase in the production of ROS, which was significantly reduced after exposure to both plant extracts. Both catalase and superoxide dismutase (SOD) activities remained unchanged after hyperglycaemia induction and treatment with both plant extracts. Induction of hyperglycaemia led to the decline of sperm parameters: motility, vitality, DNA integrity, mitochondrial membrane potential, capacitation, and acrosome reaction. Treatment with *G. livingstonei* led to increasing in all tested sperm parameters, with the lowest concentration exhibiting maximum protective effects. The administration of the *A. johnsonii* extract on hyperglycaemic cells also led to an increase in all, with the highest concentration (1 $\mu\text{g/mL}$) leading to the highest protective effects. The findings of this study show that both medicinal plants have potential therapeutic effects to ameliorate oxidative stress-causing hyperglycemia in human semen. The results of this study revealed that both *A. johnsonii* and *G. livingstonei* can potentially reduce glucose levels, and exhibit protective effects on the sperm cells of males with DM. However, further studies *in vitro* and *in vivo* are necessary to understand the safe and effective optimum concentrations, and other mechanisms of action through which the plant extracts exhibit their effects in the treatment of diabetic complications.

PREFACE

This is an article-based thesis containing eight chapters. The chapters of this thesis are written following the author guidelines of the journal of interest for publication.



Chapter 1 is a brief introduction to the concept of DM, male infertility as a complication, its prevalence, and the significance of the search for effective and safe medication, and the research aims and objectives. Chapter 2 contains the literature review, which outlines the link between hyperglycaemia, oxidative stress, and inflammation in the development of male infertility in diabetic individuals. Chapter 3 is an ethnobotanical study published as a

proceeding by the International Conference on “Chemical, Biological and Environmental Engineering”. Chapters 4-7 are original articles reporting the experimental findings in the current study. Chapter 4 has been published in the “International Journal of Molecular Sciences”. Chapters 5, 6, and 7 will be sent to journals of choice for consideration. Chapter 8 contains the general discussion and conclusion of the study.

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Dedication

This thesis is primarily dedicated to God and secondarily to my parents, Johannes Khubana, and Makhado Khubana, my pillars and great support system.

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CHAPTER ONE

INTRODUCTION

1.1. Background

Diabetes mellitus (DM) is a chronic disease that arises from complications in the metabolism of glucose, which leads to hyperglycaemia (Yang et al., 2021). Globally, statistics have shown an increase in the prevalence of DM from 108 million affected in 1980 to 463 million in 2019 (Grundlingh et al., 2022), and 536.6 million in 2021 (Sun *et al.*, 2022). South Africa is one of the countries with a high DM prevalence, rising from 4.5% in 2010 to 12.7% in 2019 (Grundlingh et al., 2022). The South African government's initiative to tax sweetened beverages according to the sugar content in 2018 to lower sugar intake, highlights the significant health concern (Grundlingh et al., 2022).

Due to hyperglycaemia, carbonyl groups from the glucose molecules react with proteins in a non-enzymatic reaction, thereby modifying them (Shigeta et al., 2021; Wang et al., 2021). The products formed during the reaction of glucose molecules with amino acid groups forms advanced glycation end products (AGEs) (Wang et al., 2021; Yang et al., 2021). AGEs accumulate in tissues leading to tissue damage, production of excessive reactive oxygen species (ROS), inflammation, and development of complications (Wang et al., 2021). The body produces a normal amount of ROS during glucose metabolism in the mitochondria, through the electron transport chain, to support some physiological functions such as signal transduction (Alkahtani et al., 2021). Prolonged hyperglycaemia increases the production of ROS above normal through the electron transport chain and the AGEs pathway (Oguntibeju *et al.*, 2019). As the production of free radicals exceeds normal levels, antioxidants are activated and increased in the area susceptible to damage to neutralise the damaging effect (Khorramabadi *et al.*, 2018). Antioxidants can also be obtained from food products and plant material to boost the body's antioxidant system. When the rise in free radicals is rapid and prolonged, the antioxidant system is compromised, leading to oxidative stress (Ostovan *et al.*,

2017). Oxidative stress causes damage to the biomolecules such as lipids, DNA, and proteins, leading to the progression of hyperglycemic-induced complications (Khorramabadi *et al.*, 2018).

The production of AGEs has also been associated with an inflammatory response causing an excessive production of pro-inflammatory cytokines in tissues (Wang *et al.*, 2021). Inflammation is the body's response to tissue damage by secretion of necessary cytokines to activate the immune system (Dutta *et al.*, 2021). Receptors of AGEs (RAGEs) in cells are expressed with the increase of AGEs in the occurrence of hyperglycaemia, and the binding of the increased AGEs to these receptors initiates downstream cascades that lead to the production of cytokines such as IL-1B, IL-6, IL-8, and TNF- α (Wang *et al.*, 2021). The increase in AGEs and RAGEs complexes causes endoplasmic reticulum stress which consequently triggers the production and release of more inflammatory cytokines (Li *et al.*, 2021). Inflammatory markers have been detected in hyperglycaemic models in different tissues (Wang *et al.*, 2021). The production of inflammatory cytokines is also mediated by the excessive production of ROS (Fraczek & Kurpisz, 2015). Tissue damage caused by oxidative stress leads to an inflammatory response (Oguntibeju, 2019). The interaction between the production of ROS and the secretion of inflammatory cytokines occurs in a positive feedback mechanism where both affect each other, i.e. the inflammatory response caused by oxidative stress also leads to more production of ROS (Oguntibeju, 2019).

Both inflammation and oxidative stress occurring in the male reproductive organs can be detrimental to the male reproductive cells and can potentially lead to male infertility (Dutta *et al.*, 2021; Tian *et al.*, 2020). Male infertility is another complication of concern that is coupled with hyperglycaemia. Male infertility occurs when a male is unable to fertilise an ovum after 12 months of regular, unprotected sexual intercourse (Silva *et al.*, 2020). Hyperglycaemia is a causative factor of testicular tissue damage that leads to male reproductive dysfunction (Tian *et al.*, 2020). Approximately half (40% to 50%) of male infertility cases are associated with oxidative stress (Dutta *et al.*, 2021). A controlled amount of ROS in the cytoplasm of

spermatozoa is produced to aid in the process of spermatogenesis and decreases as the spermatozoa mature and their cytoplasm is decreased in size (Agarwal et al., 2018). However, due to the impairment of the process of spermatogenesis caused by hyperglycaemia, the spermatozoa cytoplasm retains its size leading to the continuous production of ROS in high amounts (Agarwal et al., 2018). Khorramabadi *et al.* (2018) reported high levels of testicular and epididymis ROS in 25– 40% of men with infertility issues. Previous studies have reported that hyperglycaemic complications were accompanied by downregulation of the antioxidant system, including upstream factors (Nrf2/ARE) and inactivation of antioxidant enzymes such as catalase, superoxide dismutase, and glutathione peroxidase leading to oxidative stress (Ostovan *et al.*, 2017; Nna *et al.* 2019). Testicular tissue damage caused by oxidative stress leads to an inflammatory response following the production of cytokines in the testes and the spermatozoa. A controlled concentration of cytokines in the testes is responsible for the maintenance of the cells, and an increase in these cytokines above normal leads to chronic inflammation (Agarwal et al., 2018). Inflammation is one of the factors that lead to the progression of male infertility (Dutta et al., 2021). In the semen of infertile individuals, inflammatory cytokines such as IL-6, IL-8, and TNF- α have been found as biomarkers of inflammation (Fraczek & Kurpisz, 2015). These cytokines have been associated with sperm DNA fragmentation, sperm apoptosis, and reduction in sperm motility (Fraczek & Kurpisz, 2015). The increase in inflammatory cytokines has also been reported to cause a reduction in the mobilisation of cholesterol in Leydig cells, thereby hampering spermatogenesis (Dutta et al., 2021).

The use of synthetic drugs such as metformin, glibenclamide, insulin, acarbose, miglitol, and voglibose to treat hyperglycaemia has been documented and used over the years (Hussain *et al.*, 2021; Lv & Guo, 2020). The three later drugs work by blocking the enzymes that break down carbohydrate chains into glucose, which lowers the amount of glucose produced and absorbed into the blood. The side effects of metformin and glibenclamide have been well reported in previous studies (Liu *et al.*, 2018; Roxo *et al.*, 2019). Synthetic drugs are still

employed because they have a therapeutic effect on patients with hyperglycaemia. However, adverse reactions including severe hypoglycaemia have been documented and remain a concern (Khaki *et al.*, 2014). Consequently, there is a dire need for a continuous search for safe and effective drugs for the treatment of hyperglycaemia.

To treat hyperglycaemia and its complications with agents that demonstrate fewer adverse effects, natural therapies derived from medicinal plants have also been explored following the practice of traditional medicine since ancient times (André Patrick *et al.*, 2020). Traditional medicine has drawn the interest of researchers over the years (André Patrick *et al.*, 2020). The discovery of the therapeutic potential of several plants has led to research into plant composition and their mechanisms of action in treating diseases, including DM, characterised by hyperglycaemia. In a study conducted by Tchicailat-Landou and colleagues (2018), about 70 plants were found to be used by traditional healers in Congo, to treat diseases that are associated with oxidative stress such as asthma, hypertension, DM, and infertility. The interest in the investigation of traditional plants is on the antioxidant composition of these plants (Georginah *et al.*, 2012). Several studies have reported different plants as possible therapeutic agents of DM due to their evident effect in the amelioration of complications accompanying DM (Oguntibeju *et al.*, 2020; Oyenihni *et al.*, 2020).

However, there are numerous plants currently used traditionally for the treatment of DM that have not been reported in the literature. *Garcinia livingstonei* (also known as African mangosteen) is an evergreen plant found in the tropical areas of Africa (Joseph *et al.*, 2017). It is about 2-10 m in height and can grow from a small size to medium. *Garcinia livingstonei* has been studied due to its use in traditional medicine and has been found to have benefits such as antioxidant, anti-inflammatory, and antibacterial effects in the treatment of abdominal pains (Joseph *et al.*, 2017). Compounds such as Morelloflavone, morelloflavone-7-sulphate, guttiferone A, sargaol (Mulholland *et al.*, 2013), Amentoflavone, 4'-momomethoxy amentoflavone (Kaikabo & Eloff, 2011), Biflavonoids, and phenols (flavonoids and alkaloids) (Joseph *et al.*, 2017) have been isolated from the different parts of *Garcinia livingstonei*. These

compounds have treated different ailments such as watery eyes, melanoma (Mulholland et al., 2013), and bacterial infections (Kaikabo & Eloff, 2011), and may potentially be therapeutic in other diseases.

Androstachys johnsonii, also known as Lebombo ironwood, is a tall (15 m) evergreen plant found in areas of Africa with very high temperatures and low rainfall (Gandiwa et al., 2011). A concoction made out of leaves of *A. johnsonii* has been recorded to have an aphrodisiac effect on men (Maroyi, 2013).

Ethnobotanical studies have shown that *G. livingstonei* and *A. johnsonii* are some of the plants that are used in Limpopo Province for the treatment of DM and related complications (Yang et al., 2010). However, the literature does not show how the above-mentioned plants are potent in the treatment of male infertility as a diabetic complication. Therefore, it is highly imperative to conduct a more comprehensive scientific study on these plants and their potential antidiabetic, antioxidant activities and the possibility of exploring their commercial application in the treatment of hyperglycaemia and its complications. Although *G. livingstonei* and *A. johnsonii* have been continuously used in Limpopo province, South Africa, to reduce hyperglycaemia and to treat male infertility, only ethnobotanical information about the plants is recorded. This study is the first to investigate the mechanisms of action in the treatment of male infertility, by finding out their antidiabetic, anti-inflammatory, and antioxidant capacity. Hence, the results of this study could contribute highly to validating the use of these plants.

1.2. Rationale of the study

DM is a chronic disease of global public health concern due to the rise in mortality and prevalence (Maresch et al., 2019). There is a strong relationship between hyperglycaemia and male infertility (Dutta et al., 2021; Tian et al., 2020). Over 35% of males with DM are infertile (Lotti & Maggi, 2023). This has led to an increased urge for effective medication. The reduction of blood glucose levels has been the main target in the treatment of diabetic complications

over the years (Demir et al., 2021). However, the current implications of oxidative stress and inflammation provide alternative insight into the development of therapeutic options. Current synthetic drugs such as metformin, glibenclamide, insulin, acarbose, miglitol, and voglibose that are used to treat hyperglycaemia have shown a significant concern due to the adverse effects (Grammatiki et al., 2021; Liu et al., 2018). Besides the side effects, the use of synthetic drugs is limited to affordability and accessibility. These concerns have led to a large number of untreated diabetic patients, with an increase in the prevalence of male infertility. It is evident that the treatment of hyperglycaemia and male infertility with lesser adverse effects is of need, hence the need to explore medicinal plant/natural products in the treatment of DM and its related male reproductive dysfunction. Although several medicinal plants have been identified, a comprehensive study on the effective dosage, phytochemical content, and mechanism of action both at cellular and physiological levels is paramount.

1.3. Aim

This study aims to investigate the antioxidant, anti-inflammatory, and antidiabetic effects of selected traditional medicinal plants (*Garcinia livingstonei* and *Androstachys johnsonii*) found in Limpopo Province, South Africa in the treatment of male infertility in diabetic individuals.

1.4. Objectives of the research

- To collect and identify selected plants used in the treatments of diabetes and its complications such as male infertility, through ethnobotanical surveys.
- To carry out plant extraction and identify all phenolic compounds and antioxidant potentials of the crude extracts using HPLC-MS.
- To determine the cytotoxic effect of the plant extracts on L6 myocytes and C3A hepatocytes using MTT assay.

- To determine the antidiabetic potential (α -glucosidase inhibition, glucose intake and glucose utilisation) of the crude extracts in-vitro by using L6 myocytes and C3A hepatocytes.
- To assess the anti-inflammatory effect of the plant extracts through the measurement of nitrite formation in macrophages.
- To determine the ameliorative effect of oxidative stress in normal and hyperglycaemic-induced semen through the measurement of antioxidant enzyme activities of catalase, superoxide dismutase through spectrophotometry, and the production of ROS through fluorometric assays.
- To assess the effect of the plant extracts compared to acarbose on sperm parameters by the measurement of sperm vitality, sperm motility, capacitation, and acrosome in normal and hyperglycaemic-induced semen.
- To determine the effect of the plant extracts compared to acarbose on molecular parameters such as DNA fragmentation and mitochondrial membrane potential in normal and hyperglycaemic-induced semen.

1.5. Research questions

- Does hyperglycaemia induction in spermatozoa lead to excessive production of ROS and impairment of the sperm parameters?
- Do *A. johnsonii* and *G. livingstonei* contain any phenolic compounds, and what potential effects do they have on glucose uptake and utilisation, α -glucosidase inhibition, inflammation, and oxidative stress

- What are the potential protective effects of *A. johnsonii* and *G. livingstonei* against on sperm cells with induced with hyperglycaemia, compared to acarbose?

1.6. Hypothesis

The study is premised on the hypothesis that phenolic-rich South African medicinal plants could exhibit some antidiabetic effects, and attenuate oxidative stress and inflammation in the semen of diabetic individuals with male infertility.

1.7. Ethical consideration

1.7.1. Ethics approval

The experimental protocols of this study were approved by the Biomedical Research Ethics Committee (BMREC) of the University of Western Cape (Reference number: BM22/10/24). Approval from the Human Ethics Committee of the Faculty of Health and Wellness Sciences of the Cape Peninsula University of Technology (Reference number: CPUT/HWS-REC 2024/H9) was sought and obtained before the commencement of the study. A letter of approval for the collection and investigation of the plants was obtained from the Chief of Tshidongololwe village.

1.7.2. Protection from psychological harm

The participants were apparently healthy individuals with no record of DM or any other chronic illness. The participants reported to the laboratory following an agreement to participate in the study. This was sequel to the explanation of the protocol of the study to the participants and their willingness to participate in the study. This research carried minimal risks of possible psychological harm from the outcomes of the basic semen analysis. All research procedures were explained to the participants, and an information sheet was provided to the donors,

explaining the details of the research. In cases where the participant was psychologically affected by the outcomes, it was reported firstly to the Comparative Sperm Laboratory manager, and the participants were immediately referred to a relevant professional for counseling at the UWC Campus Health and Wellness Centre or the campus health care and counseling services at the Cape Peninsula University of Technology (for those who are CPUT students), which offer counseling services to students and employees at a lower cost depending on where the participant studies. In case of low or abnormal semen samples, participants were informed and advised to see their doctor.

1.7.3. Health and safety, and disposal of waste

All laboratory safety procedures were followed; all members of the laboratory wore a protective clothing (laboratory coat) before entering the laboratory, by rule. The protective clothing was removed and hung in a designated area before leaving the laboratory to protect the public from possible contamination. Visitors were not allowed access to the laboratory. Emergency contact numbers in case of any incident in the laboratory were always pasted in visibly in the laboratory. All samples left over after the conduction of the experiment were treated as biohazardous waste and were discarded into red bins. Waste was then stored in boxes and collected by the waste management company under the University of the Western Cape.

1.7.4. Data handling

To achieve anonymity, participants in this study were identified using codes instead of names. These codes were chosen by the participants to assure confidentiality. All participant identifiers such as date of birth, names and surnames, and age were excluded in this study. Only researchers involved in this study have access to data. Data is stored and kept in password-protected computers where only the researchers have access to it. No other group

has access to the identifiable data. Consent forms are stored in a protected cupboard in the manager's office at UWC.

1.7.5. POPIA ACT

POPIA ACT was followed. For example, confidential or personal information obtained from the participants was kept confidential and not made available to a third party without the consent of the participants. All precautionary measures were followed to ensure that the information given by the participant was used for this study as agreed on in the consent form.

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Chapter 2

The role of hyperglycaemia, oxidative stress, and inflammation in diabetes-related male infertility: Therapeutic properties of medicinal plants

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, South Africa.

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Abstract

The prevalence of diabetes *mellitus* (DM) continues to rise at an alarming rate. The increase in DM cases has led to a decline in male reproductive function. Hyperglycaemia, a hallmark of DM, is an instigator of both oxidative stress and inflammation in the male reproductive system and ultimately leads to male infertility. The presence of excessive reactive oxygen species (ROS) and inflammatory markers such as cytokines in the semen results in a decline in sperm parameters. Despite ongoing advancements in the use of conventional drugs to treat complications related to DM, concerns about treatment costs and side effects remain. Scientific research focus has therefore shifted to investigating naturally occurring safer, cheaper, and more effective treatments. This review outlines the link between hyperglycaemia and diabetic complications and the role oxidative stress and inflammation serve in the development of male infertility. We also review the effects of phytochemicals in medicinal plants in treating DM-related male infertility. This review concluded that oxidative stress and inflammation are instigators of the decline in sperm parameters in diabetic conditions. The

administration of medicinal plant extracts with hypoglycaemic, anti-diabetic, antioxidative, and anti-inflammatory properties can potentially restore diabetic-related male reproductive dysfunction.

Keywords: Diabetes mellitus, inflammation, male infertility, medicinal plants, oxidative stress, phytochemical compounds.

1. Introduction

Diabetes *mellitus* (DM) is a chronic metabolic disease characterised by hyperglycaemia (Yedjou et al., 2023). Several complications, such as nephropathy, cardiovascular diseases, neurodegenerative diseases, and reproductive dysfunction, are known to accompany the manifestation of DM due to hyperglycaemia (Lima et al., 2022). DM is a social and economic burden globally and is one of the leading causes of mortality (Hu et al., 2023). Approximately 463 million people worldwide are affected by DM (Guerra & Gastaldelli, 2020). DM incidences continue to rise at an alarming rate, with the global prevalence estimated to reach 700 million people that will be affected by DM by 2045 (Khalid et al., 2022; Lima et al., 2022). Amongst the aforementioned complications accompanying DM, male infertility continues to rise due to the decline in sperm quality caused by hyperglycaemia-induced oxidative stress and inflammation (S. Kumar et al., 2021). The rise in male infertility is associated with increased distress, depression, and psychological issues, which have led to a decrease in the overall quality of life (Opheelia et al., 2023). It is, therefore, crucial to treat DM and its underlying complications that lead to male reproductive dysfunction. The increased interest in drug therapy search for the treatment of diabetes complications is due to the flaws of the present conventional drugs, which have been reported to have adverse effects and cost limitations (Razavi-Nematollahi & Ismail-Beigi, 2019). Research has shifted the search for an efficacious, inexpensive, and safe treatment for DM towards medicinal plants (Yedjou et al., 2023).

Medicinal plants are plants containing compounds suitable for the treatment of different diseases (Mustafa et al., 2017). The use of medicinal plants originates from the ingestion of

plants as a source of nutrients (W. Sun & Shahrajabian, 2023). Presently, the literature reveals a record of many medicinal plants related to the treatment of many ailments (Emre et al., 2021; Huang et al., 2018; Mudau et al., 2022). From previous studies, different phytochemical compounds were identified and isolated to investigate the possible mechanisms through which these plants are useful in treating diseases (Pintatum et al., 2020). Among many, polyphenols (mainly flavonoids) have been recorded as potent free radical scavenging agents in ameliorating oxidative stress (Jubaidi et al., 2021). Other acids, such as hydroxycinnamic and coumaric acids, are well known for their anti-inflammatory activities (Ambika et al., 2013). Both oxidative stress and inflammation are manifestations of DM, and their amelioration can reduce DM complications such as male infertility (S. Kumar et al., 2021).

Medicinal plants such as *Turnera diffusa*, (S. Kumar et al., 2021) *Retama raetam* (Alshehri et al., 2024), *Garcinia livingstonei* (Abdul-Rahman et al., 2023), and red onion (Zahir and Ghaffar, n.d.), and phytochemicals such as flavonoids, flavones, and phenolic acids have been reported for their potential benefits in the treatment of DM complications (Ramírez-Alarcón et al., 2021). Although many medicinal plants have been identified, reported, and documented traditional medicine and their anti-diabetic effects [13], in-depth knowledge of their mechanisms of action is vital to maximising efficacy and effectiveness. This current study reviews the course of DM development from hyperglycaemia to tissue damage and male reproductive dysfunction. This review explains the link between hyperglycaemia, oxidative stress, inflammation, and the decline in sperm quality. We further review the phytochemical composition of a few medicinal plants and the possible treatment of DM-related male infertility with medicinal plants.

2. Carbohydrate/glucose metabolism

Glucose is the primary source of energy for all mammals and is used by cells to produce energy in the form of adenosine triphosphate (ATP) (Tang, 2020; Jiang et al., 2020). During carbohydrate metabolism, starch is broken down into glucose by small intestine's enzymes,

such as α -glucosidase and α -amylase (Khan et al., 2024). The postprandial breakdown of carbohydrates and proteins leads to a rise in blood glucose concentration by 40-50 mg/dl more, compared to the normal fasting glucose level of 80-90 mg/dl (Norton et al., 2022). Blood glucose is gradually taken in by cells over 2- 3 hours postprandial, reducing its level back to normal (Norton et al., 2022). Glucose concentration in the blood is regulated by the secretion and action of insulin and glucagon in the cells (Aronoff et al., 2004; Jiang et al., 2020). While insulin reduces the blood glucose level, glucagon increases the blood glucose level when in need, thus maintaining a homeostatic balance. Excess postprandial glucose is stored in the muscles and liver as glycogen for future use in fasting stages (Dimitriadis et al., 2021). The compensative increase of blood glucose level during fasting is initiated by the formation of glucose molecules from amino acids (gluconeogenesis) and the breakdown of glycogen (glycogenolysis) released into the plasma from the liver catalysed by glucose-6-phosphatase (Aronoff et al., 2004; Jiang et al., 2020). Furthermore, glucagon suppresses insulin secretion and action, which leads to the acceleration of gastric emptying and increases blood glucose concentration (Aronoff et al., 2004).

Insulin, a major regulator of blood glucose concentration, is produced and secreted by the beta cells of the pancreas (Campbell & Newgard, 2021). The rise in blood glucose is sensed by the beta cells of the pancreas, initiating a feedback reaction that leads to insulin secretion into the blood through exocytosis (Norton et al., 2022). Insulin initiates a cascade of events by binding onto its receptors, leading to glucose uptake augmentation as represented in Figure 1 (Jiang et al., 2020). Insulin receptors are glycoproteins with extracellular alpha and β -intracellular subunits (Huang et al., 2018). The binding of insulin to the extracellular subunits causes phosphorylation of the intracellular subunits and subsequent activation of the phosphoinositide 3-kinase (PI3K) protein through the tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) (Chen et al., 2022). The activation of PI3K leads to the production of 3,4,5 triphosphate (PIP₃) and the activation of protein kinase B (Akt), an activator of glucose transporter-4 (GLUT-4) vesicles translocation from the cytoplasm to the plasma membrane

(Huang et al., 2018). Although GLUT-4 is the most abundant glucose transporter in muscles and adipose tissue, glucose transporter-1 (GLUT-1) is the basal glucose transporter that functions without insulin dependency (Norton et al., 2022). Glucose transporter-2 (GLUT-2) also augments the uptake of glucose in the liver and beta cells of the pancreas insensitive to insulin, causing the release of insulin into the plasma(Sun et al., 2023).

Phosphorylation of glucose molecules occurs in the cell after glucose uptake, producing glucose-6-phosphate (G-6-P) in a reaction catalysed by hexokinase in all cells and glucokinase in the liver (Ramírez-Pérez et al., 2020). In the liver, the appropriate high amount of plasma insulin 1) activates glucokinase, which subsequently activates glycogenesis by activating glycogen synthase, and 2) inhibits glycogenolysis through the inactivation of glycogen phosphorylase (Jiang et al., 2020). Glucose-6-phosphate is a substrate for ATP production through glycolysis reaction, which produces 2 pyruvate molecules (Feng et al., 2020). Pyruvate is converted into coenzyme A, which enters the mitochondrial cytoplasm, where more ATP is produced (Norton et al., 2022). During the conversion of pyruvate into coenzyme A, hydrogen molecules are released into the mitochondria, assisting in the production of ATP through oxidative phosphorylation (Norton et al., 2022). Oxidative phosphorylation occurs through the mitochondria transport chain, a chain of complexes through which electrons are passed to maintain a gradient for ATPase to produce ATP (Norton et al., 2022). In the mitochondria transport chain, a normal physiological level of free radicals is also produced for normal signal transduction, immune reaction, and other development processes such as spermatogenesis (Papachristoforou et al., 2020; Simas et al., 2021). When the process of glucose metabolism is disrupted, glycaemic balance is lost, consequently leading to the development of metabolic disease.

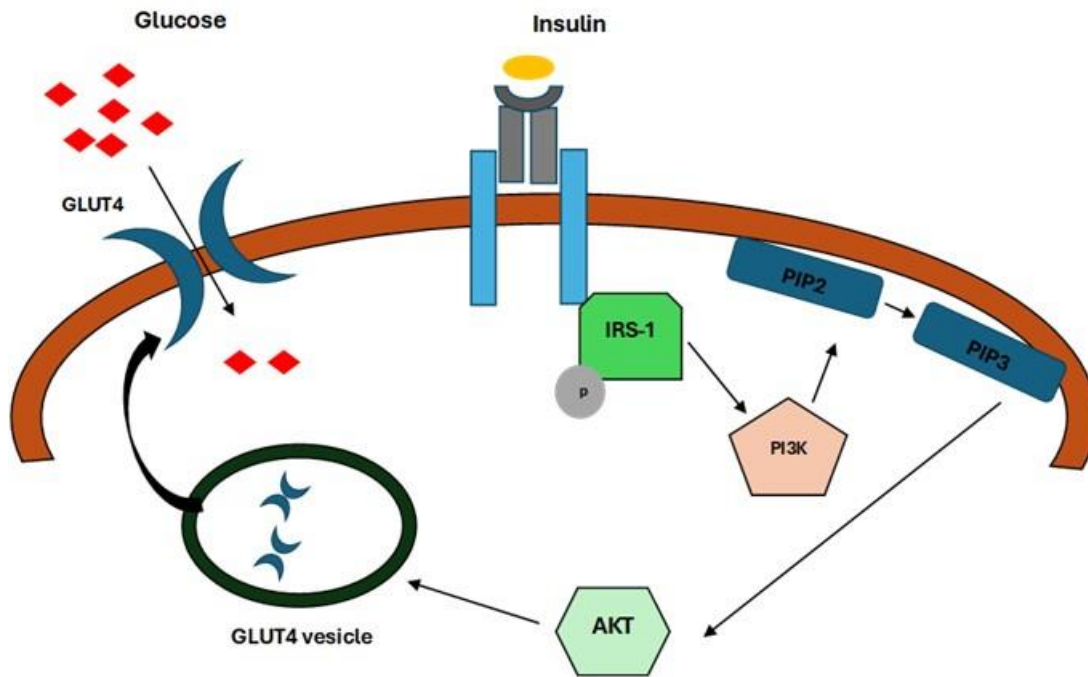


Figure 1: Insulin signalling pathway. Insulin binds to the extracellular units of the insulin receptor and causes autophosphorylation of IRS-1 which activates PI3K. PI3K causes the conversion of PIP2 to PIP3 which activates AKT. AKT causes translocation of the GLUT4 vesicle to the surface of the membrane where it releases GLUT4 and leads to the transportation of glucose molecules into the cell.

3. Pathophysiology (DM)

The regulation of blood glucose by insulin and glucagon maintains a homeostatic balance of blood glucose (Lewis et al., 2021). A disruption in insulin production, secretion, or action leads to an abnormal and prolonged increase in blood glucose level (hyperglycaemia) and an inappropriate increase in glucagon (Jiang et al., 2020). The impairment of the glucose metabolism homeostatic balance and an abnormal rise in blood glucose level is a characteristic of the development of DM (R. Kumar et al., n.d.). DM is a metabolic disease associated with complications arising from hyperglycaemia and consequent macromolecular disruptions (Jiang et al., 2020). Both major types of DM (type 1 diabetes *mellitus* (T1DM) and type 2 diabetes *mellitus* (T2DM)) are characterised by hyperglycaemia and impaired glucose and lipid metabolism (Kupriyanova et al., 2021). T1DM is distinguished by its pathology arising

from the deficiency of insulin in the plasma, which is mostly caused by the autoinflammation of the pancreas (Lima et al., 2022). T2DM is the most common type of DM (90% of diabetic cases), characterised by insulin resistance (Guerra & Gastaldelli, 2020). Although sufficient insulin is present in the plasma, insulin action is impaired due to the insensitivity of insulin receptors (Jiang et al., 2020). Excessive plasma insulin leads to further worsening of insulin resistance and dysregulation of glucose metabolism (M. Li et al., 2022).

Among several causative factors associated with the development of DM, such as genetic, lifestyle, and environmental factors, obesity remains an important role player in DM morbidity (Tsalamandris et al., 2019; H. Wu & Ballantyne, 2020). Obesity occurs when excessive energy and fats accumulate due to high-calorie intake, physical inactivity, or genetic factors (Silveira et al., 2022). At the onset of DM, the accumulation of excess adipocytes around the pancreas leads to the destruction of the beta cells of Langerhans and reduces the level of insulin produced and secreted (Zatterale et al., 2020). In both the onset and the course of DM development, an uncontrolled increase in calorie intake increases the accumulation of triglycerides and hypertrophy of fat cells and increases the risk of insulin resistance, subsequently leading to uncontrolled hyperglycaemia (Lima et al., 2022). The accumulation of fat around muscles and the liver also causes insulin insensitivity (Ahmed et al., 2021).

Fat accumulation in obese individuals leads to the secretion of inflammatory markers by adipocytes, which leads to the inflammation of the pancreas and other organs, leading to insulin resistance and the reduction of insulin secretion, hence the resulting hyperglycaemia (Tsalamandris et al., 2019). Complications such as male infertility, accompanying DM, and metabolic syndrome are also alluded to obesity (Torres-Arce et al., 2021). While obesity leads to insulin insensitivity and hyperglycaemia, the accumulation of fats in males around the testes increases temperature and is detrimental to spermatogenesis (Torres-Arce et al., 2021). In correlation to these findings, it was deduced in a similar study that physical activity improves semen parameters and ameliorates male reproductive dysfunction (Gaderpour et al., 2021). Over the past years, the prevalence of obesity and DM has been managed by a decrease in

calorie intake and increased physical activity, which maintains the glycaemic index by improving beta cell function and precluding insulin resistance (Lima et al., 2022).

The liver serves a crucial role in storing and releasing glucose in regulating carbohydrate metabolism (Guerra & Gastaldelli, 2020). Due to the activity of glucose-6-phosphatase, which reverses the phosphorylation of glucose, the liver contributes to the increase in blood glucose levels (Aronoff et al., 2004). In diabetic conditions, the absence or impairment of insulin action on hepatic cells causes an increase in the release of glucose into the blood through the initiation of gluconeogenesis (Jiang et al., 2020). The inactivation of glucokinase and reduction in glucose uptake during postprandial blood glucose increase has been noted in diabetic models, and it plays a causative role in the initiation of hyperglycaemia (Nakamura et al., 2021). In most diabetic patients, less glycogen storage and impaired hepatic glucose production have been observed (Guerra & Gastaldelli, 2020). The activation of glucose-6-phosphatase causes the exacerbation of hyperglycaemia in DM, and the inhibition of glucokinase and glycogen synthase, consequently leading to the efflux of glucose from the liver into the blood (Jiang et al., 2020). Due to prolonged hyperglycaemia in diabetic patients, excessive glucose molecules form reactions and interactions that lead to the development of DM complications such as cardiovascular diseases, neurological degeneration, nephropathy, retinopathy, and male infertility (H.-Q. Wu et al., 2021). The implications of hyperglycaemia in DM are discussed in the subsections below.

4. Implications of hyperglycaemia

The hallmark of DM, hyperglycaemia, is the basis of subsequent complications prominent in diabetic patients. Several reactions, such as advanced glycation end-products (AGEs) formation, oxidative stress (OS), and inflammation, dependently arise excessively in the occurrence of hyperglycaemia (Khalid et al., 2022). Although the reactions mentioned above exacerbate each other, leading to continuous damage, each reaction majorly contributes to the progression of DM by instigating macromolecular and tissue damage (Figure 2) (Han et

al., 2019). This section outlines the implications of hyperglycaemia, linking DM to its consequent complications.

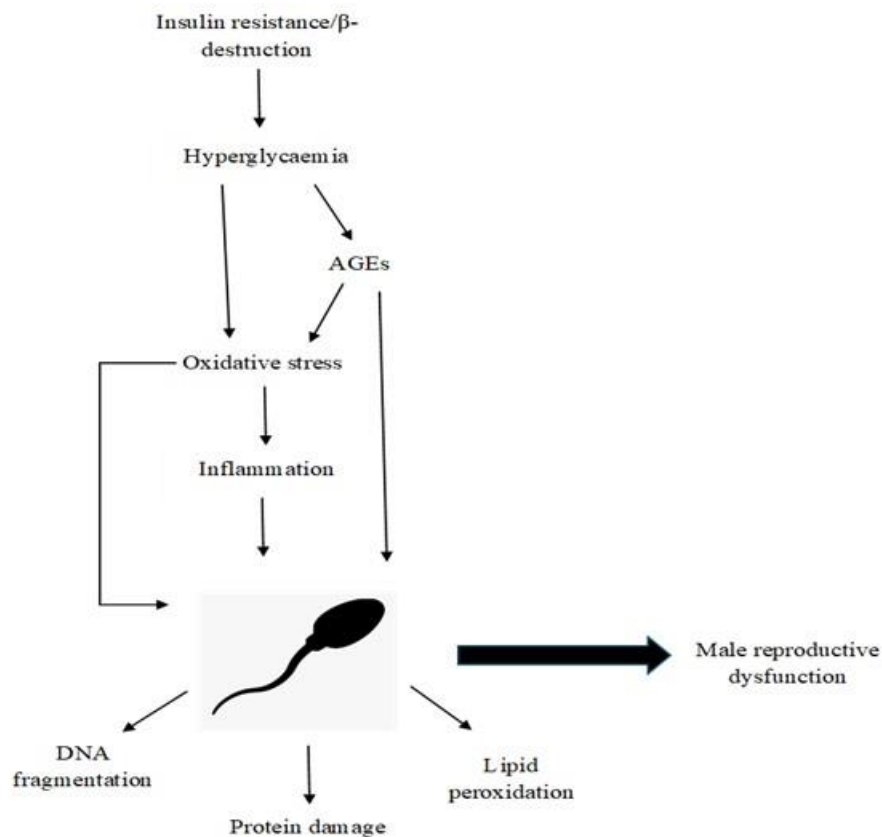


Figure 2: The link between hyperglycaemia, advanced glycation end-products (AGEs), oxidative stress, and inflammation in developing male reproductive dysfunction.

4.1. Oxidative stress (OS)

4.1.1. Overview of the oxidative balance

Oxidative stress occurs due to the dysregulation of free radicals, particularly reactive oxidative species (ROS), caused by either increased generation of free radicals, reduced capacity of the free radical-scavenging system, or both (Ritchie & Ko, 2021). Free radicals are highly reactive molecules due to their instability caused by a free electron in their outermost orbital, which reacts with adjacent macromolecules (Papachristoforou et al., 2020). Amongst different free radicals, such as reactive nitrogen species (RNS) and reactive sulfur species, ROS are

the main instigator of OS (Papachristoforou et al., 2020). Therefore, this review will refer to free radicals as ROS. Although oxygen is a fundamental physiological element, it is the primary substrate of the generation of ROS (Hernansanz-Agustín & Enríquez, 2021). The excessive generation of ROS, such as superoxide (O_2^-), hydrogen dioxide, hypochlorous acid (HOCL), lipid peroxides, and hydrogen peroxide (H_2O_2) are associated with macromolecular damage caused by the resulting OS (Torres-Arce et al., 2021).

The presence of the typically regulated ROS is crucial in some physiological functions, such as the defence against pathogens and signal transduction under normal conditions (Sies & Jones, 2020). The regulation of ROS is maintained by the balance between the generation and the removal of the ROS (Lima et al., 2022). The antioxidant system consists of protein and non-protein molecules that neutralise the oxidation of other biological molecules by free radicals (Sarker & Oba, 2020). The increase in free radicals activates the antioxidant system to release endogenous antioxidants to either scavenge or neutralise free radicals by binding them and blocking them from oxidising other molecules (Mu et al., 2021). However, antioxidants can also be obtained from diet, such as fruits and vegetables (exogenous antioxidants) (Rahaman et al., 2023).

Some endogenous antioxidants, such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (Gpx), are enzymatic and catalyse reactions that lead to the elimination of free radicals (Nna et al., 2019). Non-enzymatic antioxidants include glutathione, vitamins C and E, carotenoids, and flavonoids (Papachristoforou et al., 2020). The perturbation of the antioxidant system caused by the reduction/deactivation of enzymatic activity or the decrease in the production of antioxidants caused by upstream factors such as the nuclear factor erythroid 2-related factor 2 (NRF2), can lead to OS (Papachristoforou et al., 2020). The reaction of ROS with lipids, proteins, and DNA leads to cell death and tissue damage (Baskaran et al., 2021). Depending on where oxidative damage occurs, different complications arise.

4.1.2. The role of hyperglycaemia in oxidative stress

Although other causative factors of OS, such as smoking, excessive ultraviolet light, environmental pollutants, hyperglycaemia, are a driving force in the generation of ROS and the development of OS (Papachristoforou et al., 2020). Hyperglycaemic-induced OS plays a role in the development of diabetic complications (Iacobini et al., 2021). Besides the direct molecular damage caused by ROS in diabetic patients, OS also exacerbates insulin resistance and increases chronic inflammation (Charlton et al., 2020). Excessive generation of free radicals from hyperglycaemia occurs through the following pathways: 1) the leaking of electrons from the mitochondria, 2) AGEs formation, and 3) the polyol pathway (Lima et al., 2022).

ROS are mainly produced in the mitochondria's electron transport chain (ETC) (Hernansanz-Agustín & Enríquez, 2021). During a critical stage of ATP formation in the ETC (oxidative phosphorylation), ROS are generated mainly from the partial reduction of oxygen caused by the electrons leaking out of the ETC (Kaludercic & Di Lisa, 2020). Leaked electrons can also be transported to extracellular oxygen through the NADPH oxidases that the donation of an electron from NADPH to FAD to form FADH₂, which donates the electron to Fe³⁺ to form Fe²⁺, subsequently transferring the electron through the membrane to reduce extracellular oxygen and create free radicals (Papachristoforou et al., 2020). Due to excess glucose, glucotoxicity arises and leads to the impairment of the mitochondrial function by increasing mitochondrial membrane potential and ROS generation (Lima et al., 2022).

AGEs are products of the non-enzymatic Maillard reaction between reducing sugars (glucose) and the amino groups of proteins (Shen et al., 2020). In the early stages of the Maillard reaction, a reducing sugar such as glucose reacts with a protein to form Schiff bases (Peng et al., 2024). In the intermediate stage, further rearrangement of Amadori adducts leads to the formation of amadori products (Khalid et al., 2022). The major amadori products, such as methylglyoxal (MGO) and 3-deoxyglucosone (DG), are important hall-markers of AGEs production (Khalid et al., 2022). Further oxidation of the Amadori products leads to the final

formation of AGEs (Liu et al., 2023). Besides the Maillard pathway, AGEs are formed through the polyol pathway, a bypass pathway initiated by converting glucose to sorbitol in hyperglycaemic conditions instead of channelling to the glycolysis pathway (Khalid et al., 2022), as depicted in Figure 3. The accumulation of intermediate products and AGEs is the instigator of vascular damage in DM.

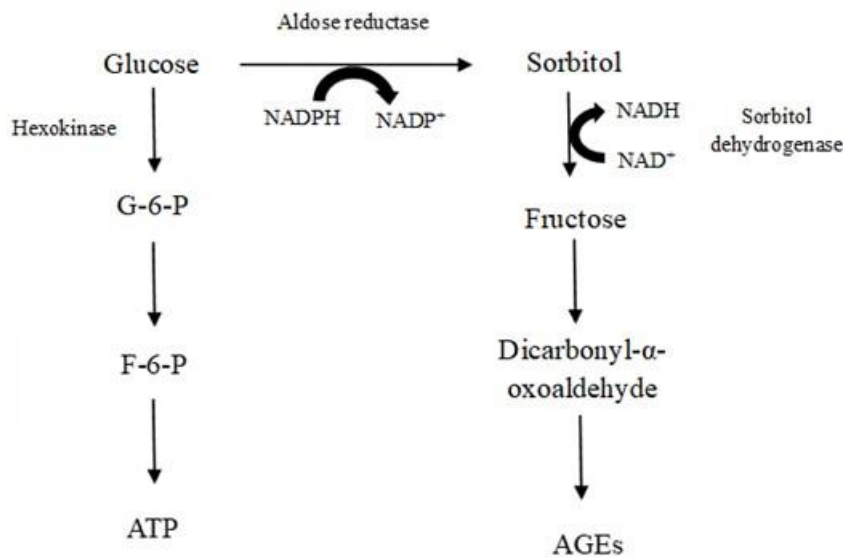


Figure 3: The polyol pathway. Basal glucose is converted into glucose 6-phosphate (G-6-P) by hexokinase, which is further converted to fructose 6-phosphate (F-6-P) to produce adenosine triphosphate (ATP). Excess glucose in hyperglycaemia is converted to sorbitol catalysed by aldose reductase. Sorbitol dehydrogenase converts sorbitol to fructose with NADH as an end-product. The polyol pathway leads to the production of advanced glycation end-products (AGEs).

In normal blood glucose conditions, the production of AGEs is regulated by the production of anti-stressors (Yue et al., 2023). However, in hyperglycaemic conditions, the production of anti-stressors is exceeded by the excessive production of AGEs (Khalid et al., 2022). The interaction of AGEs with proteins, lipids, and DNA is a major contributor to macrovascular and macromolecular damage in diabetic patients (Perrone et al., 2020). Due to excess AGEs, cross-linkages between neighbouring AGEs are formed, and they interact and deactivate crucial proteins (Kamml et al., 2023). Proteins affected by AGEs become dysfunctional and resist degradation, becoming agents of free radical formation and consequently leading to

oxidative stress (Salazar et al., 2021). The deleterious effect of AGEs is also associated with the binding of AGEs to receptors of advanced glycation end-products (RAGE), eliciting downstream signal transduction, which subsequently leads to inflammation and oxidative stress (Adeshara et al., 2022). The binding of AGEs to RAGEs downstream activates c-Jun N-terminal kinase (JNK), which activates IRS-1 and causes a false insulin signalling cascade and consequent insulin resistance (Khalid et al., 2022). AGEs exert detrimental effects through the increase in ROS initiated by binding to RAGEs (Jiao et al., 2020).

During hyperglycaemia, excess glucose is transferred to a bypass pathway called the polyol/sorbitol pathway to physiologically compensate for the increase in glucose concentration, as depicted in Figure 3 (Lima et al., 2022). In the polyol pathway, aldose reductase uses NADPH (producing NADP⁺) to convert glucose to sorbitol, which is further converted to fructose, catalysed by sorbitol dehydrogenase with the consumption of NAD⁺ and the production of NADH (Garg & Duggal, 2022). The sorbitol pathway leads to the excessive increase in NADH, which overloads the mitochondria and leads to increased ROS generation (Yan, 2021). Furthermore, the reduction in NADPH reduces the amount of GSH formed, leading to a compromised antioxidant system. Both the production of NADH and the consumption of NADPH contribute to the pathological increase of ROS and OS (Black, 2022).

4.2. Inflammation

4.2.1. Inflammation as an immune response

Pathogens and foreign molecules elicit an immune response that subsequently leads to pro-inflammatory cytokine secretion in a normal physiological reaction called inflammation (Oronsky et al., 2022). Chronic inflammation occurs due to the prolonged activation of immune cells and the excessive secretion of inflammatory markers after severe and prolonged damage (Rohm et al., 2022). In both stages of inflammation, immune cells such as natural killer cells, T-lymphocytes, B-lymphocytes, and macrophages are activated and mobilized to the area of

damage where they secrete pro-inflammatory markers (Yaribeygi et al., 2020). The availability of inflammatory markers such as TNF- α , Interleukin-1 (IL-1 β), and Interleukin-6 (IL-6) also recruits more immune cells to the area of damage (Yaribeygi et al., 2020). Nitric oxide (NO) is also an important mediator of inflammation, and its elevation is associated with tissue damage (Matsunaga et al., 2021). The accumulation of immune cells in the injured or infected area (immune cell infiltration) leads to tissue damage (Apostolova et al., 2020). Inflammation is initiated by NF- κ B, a transcription factor that leads to the transcription of genes that code for mediators of inflammation (C. Li et al., 2020). The promotion of NF- κ B activity during tissue damage or invasion by foreign material is the initial cause of inflammation, followed by the downstream expression of pro-inflammatory cytokines (Ilchovska & Barrow, 2021). Although inflammation is a normal physiological process to get rid of pathogens, it has been implicated in the pathogenesis of several health complications, including DM (Apostolova et al., 2020). Previous research focussing on the amelioration of chronic inflammation has therefore targeted the augmentation of NF- κ B activity to reduce the level of inflammatory cytokines (Nna et al., 2019).

4.2.2. The role of Hyperglycaemia in inflammation

The development of DM complications is associated with chronic inflammation (Forrester et al., 2020). Hyperglycaemia leads to the activation of several inflammatory pathways and the secretion of excessive levels of cytokines (Ilchovska & Barrow, 2021). In addition, it has been deduced that the overall upregulation of inflammation in diabetic patients has alluded to the coupled and individual actions of obesity, hyperglycaemia, and oxidative stress (Lima et al., 2022). In DM-associated obesity, hypertrophic adipocytes secrete excessive cytokines (Al-Mansoori et al., 2022). The recruitment of more cytokines from macrophages is augmented by the release of fatty acids in obese diabetic individuals (Lima et al., 2022).

As previously discussed, hyperglycaemia upregulates inflammation through the formation of AGEs. The binding of AGEs to RAGE leads to the downstream activation of I κ B kinase (IKK β),

which inhibits I κ B. This protein binds to the main regulating transcription factor of inflammation (Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B)) (Khalid et al., 2022). Inhibition of I κ B releases NF- κ B, which freely exerts its effect in the nucleus, producing pro-inflammatory markers (Khan et al., 2024). The high concentration of AGEs leads to exaggerated activation of NF- κ B and excessive production of inflammatory markers such as IL-1 β , IL-6, and TNF α (Khalid et al., 2022). In the prediabetic stage, hyperglycaemia-induced OS activates the excessive production of cytokines from adipose tissue (Papachristoforou et al., 2020). In a previous study conducted by (Han et al., 2019), the elevation of TNF-caused by hyperglycaemia was observed and associated with the possible subsequent upregulation of NF-Kb, followed by further immune cell recruitment.

5. The effect of hyperglycaemia on male reproduction

The increase in infertility cases has become a global public concern, with approximately 15% of couples affected (Wagner et al., 2023). Couples affected by infertility are mostly stigmatized and suffer from financial stress and depression, which ultimately leads to mental issues and overall reduced quality of life (Opheelia et al., 2023). Amongst the overall prevalence of infertility, over 40% of the cases are alluded to male reproductive dysfunction (Khojasteh Rad et al., 2021; Nowicka-Bauer & Nixon, 2020). Approximately 7% of male individuals are affected by male infertility globally (Nowicka-Bauer & Nixon, 2020). The causative factors of male infertility are not limited to obesity (Ameratunga et al., 2023), age (Opheelia et al., 2023), and physical and environmental factors (Torres-Arce et al., 2021). In obese individuals, excessive adipose tissue accumulation leads to a decrease in the production of testosterone and a decline in sperm parameters (Barbagallo et al., 2021). Previous studies have also reported the age-related alteration and damage of testicular mitochondria which leads to reduced ATP production and reduced motility of sperm cells (Wang et al., 2022). The decline in male reproductive parameters has also been associated with environmental toxins from metals, food additives, and pollutants, which cause reactions with sperm cells and lead to apoptosis

(Kamiński et al., 2020). Amongst the aforementioned causes of male reproductive dysfunction, DM has been listed as a causative factor in the decline in sperm quality and an increase in male infertility (Han et al., 2019; Salazar et al., 2021). Figure 2 shows the link between hyperglycaemia, oxidative stress, inflammation, and male infertility.

5.1. Hyperglycaemia-induced oxidative stress in male infertility

The male reproductive organs generate free radicals balanced by the available antioxidants during normal conditions (Simas et al., 2021). In normal conditions, a controlled level of free radicals produced in the plasma membrane assists in spermatogenesis processes such as sperm capacitation (Gaderpour et al., 2021). During oxidative stress, free radicals are produced in the spermatozoa mainly through mitochondrial leakage of electrons, which ultimately react with oxygen (Torres-Arce et al., 2021). Besides the spermatozoa, immature cells and round cells such as leucocytes also contribute to the production of free radicals in the semen (Nowicka-Bauer & Nixon, 2020).

In hyperglycaemic conditions, excessive generation of free radicals is promoted in the testicular tissue and germ cells through the leaking of electrons of the mitochondria (Simas et al., 2021). DM and obesity are collectively factors leading to the excessive production of free radicals and the cause of oxidative stress in male reproductive organs (Torres-Arce et al., 2021). The overload of free radicals in the reproductive organs compromises the antioxidant system and reduces antioxidant enzyme activities, leading to oxidative stress (Simas et al., 2021). In a previous study, the increase in free radicals associated with hyperglycaemia negatively correlated with the activities of antioxidants such as SOD and catalase in the semen (Aksu et al., 2021). Oxidative stress contributes to approximately 35% of male infertility cases globally (Nowicka-Bauer & Nixon, 2020). The manifestation of oxidative stress in diabetic models is a major cause of apoptosis of testicular cells (Barkabi-Zanjani et al., 2020). Accumulated free radicals lead to both lipid peroxidation of sperm cell membranes and the

oxidation of sperm organelles, leading to the fragmentation of DNA, thereby reducing sperm viability and motility (Uribe et al., 2022).

Sperm DNA damage is a crucial concept in male reproduction and is the leading cause of infertility in diabetic complications (Barkabi-Zanjani et al., 2020). Excessive levels of free radicals in the testes and epididymis have been reported to lead to sperm DNA damage in diabetic-related male infertility cases (Barkabi-Zanjani et al., 2020). Sperm cells are most vulnerable to lipid peroxidation due to the abundance of polyunsaturated fatty acids as part of their membranes (Simas et al., 2021). Although polyunsaturated acids in the sperm cell serve a crucial role in the fluidity of the plasma membranes, their reaction with free radicals leads to the formation of toxic end-products such as malondialdehyde (MDA), which leads to compromised cell motility and cell death (Nowicka-Bauer & Nixon, 2020). The presence of elevated MDA levels in diabetic semen has revealed the role of hyperglycaemia in the lipid peroxidation of sperm cells and the consequent male reproductive dysfunction (Gaderpour et al., 2021). Sperm parameters, such as acrosomal reaction, mitochondrial membrane potential, and DNA integrity, are compromised due to oxidative stress, thus the consequent apoptosis of the sperm cells (Simas et al., 2021; Torres-Arce et al., 2021).

5.2. Hyperglycaemia-induced inflammation in male infertility

Hyperglycaemia-associated inflammation contributes to male reproductive dysfunction (Barkabi-Zanjani et al., 2020; Han et al., 2019). Inflammation in hyperglycaemic conditions is primarily initiated by the activation of NF- κ B by oxidative stress (Jiao et al., 2020). Reports have shown the accumulation of pro-inflammatory cytokines in male reproductive cells and tissue of individuals with male infertility (Lu et al., 2017). Although cytokines detected in different pathologies are not specific to diseases, some cytokines are predominantly found in accompanying certain complications and are used as predictors of specific diseases (Yaribeygi et al., 2020)). Cytokines such as TNF- α , IL-6, and IL8 have been primarily found in

the reproductive parameters of diabetic patients (Lu et al., 2017). In a previous study, pro-inflammatory markers IL-17 and IL-18, which are positively correlated with the above-mentioned main male reproductive cytokines, were elevated in diabetic semen samples and associated with the resulting decrease in sperm parameters such as sperm DNA integrity, sperm motility, and vitality (Lu et al., 2017). A similar study by (Samie et al., 2018) investigated the effect of diabetes induction in the manifestation of inflammation and the development of spermatogenetic damage. In their study, an increase in TNF- α and IL-17 in the sperm and testicular tissue of rats revealed the involvement of inflammation in diabetes-related male infertility (Samie et al., 2018). Besides causing organ damage, TNF- α is also involved in the recruitment of other inflammatory markers, such as NO, which both cause a reduction in sperm parameters and prolong inflammation by promoting immune cell infiltration and oxidative stress (Barkabi-Zanjani et al., 2020; Han et al., 2019). Synergistic to the effect of oxidative stress, the elevation of inflammatory cytokines in diabetic conditions also causes lipid peroxidation of sperm cells, consequently leading to reduced sperm quality and possible male infertility (Lu et al., 2017).

6. The role of phytochemicals in medicinal plants

The use of medicinal plants is tied to their chemical composition (Saparbekova et al., 2023). Scientific focus in the treatment of diseases currently depends on the presence of phenolic compounds in medicinal plants (Sun & Shahrajabian, 2023). Following carbohydrates, phenolic compounds are the second most abundant compounds in most medicinal plants (Nurzyńska-Wierdak, 2023). Phenolic compounds in plants include but are not limited to, flavonoids (flavonols and flavanols), flavones, alkaloids, and phenolic acids (hydroxycinnamic acids, hydroxybenzoic acids). Amongst phenolic compounds, flavonoids are mostly reported as potent antioxidants (Sun & Shahrajabian, 2023). The structural composition of phenolic compounds (one aromatic ring attached to hydroxyl groups) contributes to their health benefits (Nurzyńska-Wierdak, 2023). Phenolic compounds are well-known for their ameliorative health

benefits in the treatment of diseases associated with inflammation, oxidative stress, and microbial infection (Rajashekar, 2023).

Certain phenolic compounds in medicinal plants are responsible for the beneficial effect in the treatment of DM-related male infertility (Alshehri et al., 2024). In our previous ethnobotanical study, we identified several medicinal plants used to treat DM and male infertility in traditional medical practice (Nethengwe et al., 2022). A similar study conducted by (Mudau et al., 2022) revealed a vast availability of medicinal plants used in the treatment of DM complications. However, fewer studies have focussed on the phytochemical screening of these plants. Many other medicinal plants are recorded in the literature for their potential benefit in treating DM-related male infertility worldwide, as represented in Table 1. Countries including Nigeria, South Africa, Cameroon, China, India, and Europe appear in the literature for their scientific studies on potentially effective medicinal plants and their role in the treatment of DM-related male infertility (Fadahunsi et al., 2021; Ghosh et al., 2019; Heidari et al., 2021; Moichela et al., 2021; Oridupa et al., 2020). However, the discovery and research of more medicinal plants are paramount in discovering, discovering more effective bioactive compounds that are different in different plants. Additionally, the accessibility of medicinal plants and their status in biodiversity are important factors in choosing suitable medicinal plants. Therefore, creating a large pool of beneficial medicinal plants is necessary. More DM and male infertility studies have tested the efficacy of medicinal plants in in vivo animal models, while fewer studies focussed on in vivo and in vitro human studies (Adeleye et al., 2020; Kouassi et al., 2022; Oji et al., 2022).

In a previous study, the antioxidant and anti-inflammatory effect of *Cleome rutidosperma*, a plant used for treating male infertility in diabetic men, was unfolded using male Wistar rats (Oridupa et al., 2020). In their study, the beneficial effect of *Cleome rutidosperma* was linked to bioactive compounds such as tannins, flavonoids, and phenols (Oridupa et al., 2020). In a similar animal study conducted in Europe, the effect of *Alpinia officinarum* in increasing sperm quality of diabetic rats was discovered (Heidari et al., 2021). This effect was linked to

glycosides, flavonoids, diarylheptanoids, and flavonol, which scavenge ROS and reduce the secretion of inflammatory cytokines in male reproductive organs (Heidari et al., 2021). The frequent appearance of phenolic compounds in different medicinal plants used in treating DM-related male reproductive dysfunction, as observed in Table 1, suggests the important role of these bioactive compounds in treating diabetic complications and the improvement of male reproductive parameters. In these previous studies, it is deduced that phenolic compounds increase antioxidant capacity and antioxidant enzyme activity, thereby reducing oxidative damage in reproductive organs and cells (Mohlala et al., 2023; Zhang et al., 2024). The presence of a bioactive compound, berberine, is linked to the reduction of inflammatory cytokines through the inhibition of NF- κ B in an animal model (Song et al., 2020). Besides the antioxidant and anti-inflammatory effect of phenolic compounds in different DM medicinal plants, some studies have deduced that different compounds such as alkaloids, flavonoids, alkaloids, coumarin, tannins, and gallic acid also led to the increase in insulin sensitivity and a reduction in blood glucose levels (Kouassi et al., 2022; Sarkar et al., 2022; Song et al., 2020). In corroboration to this, phenolic compound-rich *Coptis chinensis* found in China was found to improve glucose metabolism in diabetic mice through the increase in the stimulation of beta cells to produce and secrete insulin (Zhang et al., 2024). *Moringa oleifera* is also a common medicinal plant originally from Middle Eastern Africa, India, and Southern Asia and used in different parts of the world to treat skin complications (Bhalla et al., 2021). The leaves of *M. oleifera* are used in these parts of the world for the treatment of DM complications, including male infertility (Mohlala et al., 2023). Phytochemical profiling of *M. oleifera* leaves has shown the presence of bioactive compounds such as 2-Isopropoxyethyl propionate, propanamide, carbonic acid, and citramalic acid, which exhibit anti-inflammatory and antioxidant activities (Bhalla et al., 2021). *M. oleifera* also reduces lipid peroxidation by eradicating ROS in human spermatozoa and increases sperm quality (Moichela et al., 2021).

Table 1: Medicinal plants used in different parts of the world for the treatment of DM-related male infertility and other ailments.

Medicinal plant	Family	Country of use	Pharmacologically active compounds	Study mode	Effect in DM-related male infertility	Other medicinal use	Reference
<i>Cleome rutidosperma</i>	Cleomaceae	Southern Nigeria	Tannins, flavonoids, phenols	In vivo (male Wistar rats)	Reduces inflammation and oxidative stress	Relieves pain and fever, diuretic	(Ghosh et al., 2019; Oridupa et al., 2020)
<i>Moringa oleifera</i>	Moringaceae	Middle East Africa, Southern Asia	2-Isopropoxyethyl propionate, Propanamide, carbonic acid, citramalic acid	In vitro (Human sperm)	Antioxidative effect	Prevents skin damage	(Bhalla et al., 2021; Mohlala et al., 2023; Moichela et al., 2021)
<i>Alpinia officinarum</i>	Zingiberaceae	Europe	Glycosides, flavonoids, diarylheptanoids, flavonols	In vivo (male rats)	Increase in sperm quality	Anti-cancer reduces inflammation, antimicrobial, anti-ulcers, relieves pain	(Heidari et al., 2021)

<i>Anchomanes difformis</i>	Araceae	Nigeria	Tannins, flavonoids, phenols	In vivo (Male Wistar rats)	Hypoglycemia	Hepatoprotective	(Alabi et al., 2020; Kouassi et al., 2022)
<i>Psidium Guajava</i>	Myrtaceae	Around the world	Quercetin	In vivo (Male Wistar rats)	Hypoglycemic reduces oxidative stress	Antimicrobial effect: treats allergic reactions, relieves coughs, hepatoprotective	(Adeleye et al., 2020)
Carica Papaya	Caricaceae	Mexico and Central America	Alkaloids, glycosides, flavonoids, and tannins	In vivo (Male Wistar rats)	Increases sperm motility	Colic, fever, Malaria, asthma	(Oji et al., 2022)
Curcuma amada	Zingiberaceae	India	Alkaloids and flavonoids	In vivo (Albin rats)	Hypoglycemic,	Antibacterial and anti-fungal effect	(Sarkar et al., 2022)

Coptis chinensis	Ranunculaceae	China	Berberine, alkaloids, coumarin, tannins	In vivo (Sprague-Dawley rats)	Hypoglycaemic reduces inflammation, hypolipidaemic, antioxidative	Bowel disease, arthritis	(M. Li et al., 2023; Song et al., 2020; Yang et al., 2021)
Hunteria umbellata	Apocynaceae	Cameroon, Ghana, Senegal, Congo	Quercetin, apigenin, alkaloids, gallic acid, triterpenoids	Ethnobotanical study	Afrodisiac, anti-inflammatory, hypoglycaemic	Wound healing in leprosy, stomach ache, menstrual complications	(Fadahunsi et al., 2021)
Ficus carica	Moraceae	Malaysia, Turkey	Anthocyanins, flavonoids, kaempferol, myricetin, quercetin	In vivo (Sprague-Dawley rats)	Hypoglycaemic improves sperm quality	Cardiovascular diseases	(Abu Bakar et al., 2020; Gündeşli et al., 2021)

In a previous study, the antioxidant effects of a medicinal plant, *Retama raetam*, in the treatment of male infertility in diabetic rats were linked to the presence of flavonoids such as kaempferol, apigenin, and quercetin (Alshehri et al., 2024). In a similar study, the effect of red

onion powder in the improvement of male reproductive function in diabetic rats was linked to profiled phytochemicals such as flavonoids, anthocyanins, quercetin glucosides, S-methyl cysteine sulfoxide (Zahir and Ghaffar, n.d.). *Turnera diffusa* showed a protective effect in the testes of diabetic-induced rats by restoring sperm vitality, motility, and DNA integrity (Kumar et al., 2023). The protective effect of *T. diffusa* was linked to the anti-inflammatory effect (downregulation of NF-Kb) and the antioxidant effect (increase in antioxidant enzyme activities) potentially exhibited by phytochemicals such as p-coumaric acid, kaempferol, and protocatechuic acid (Kumar et al., 2023). *Garcinia livingstonei* has also been reported for its hypoglycaemic effect in the treatment of diabetic complications through the inhibition of α -glucosidase (Abdul-Rahman et al., 2023). The composition of *G. livingstonei* has also been reported to contain flavonoids and benzophenones, which can be anti-inflammatory and antioxidative (Muriithi et al., 2016).

Phytochemical compounds have been identified and isolated from medicinal plants and investigated to understand the pathways involved in the beneficial effects of medicinal plants. In a study conducted (Simas et al., 2021), a bioactive compound called resveratrol was also reported to exhibit anti-apoptotic effects in diabetic rat sperm cells by amelioration oxidative stress, thereby reducing DNA fragmentation and mitochondrial damage. The health benefit of flavonoids in medicinal plants was confirmed in a previous study by the isolation of a biflavonoid compound, troxerutin, for the treatment of DM-related male reproductive dysfunction (Shokri et al., 2023). In their study, troxerutin exhibited antioxidant effects and improved sperm quality (viability, chromatin integrity, and motility (Shokri et al., 2023). The ameliorative effect of hesperidin, a flavonone, on oxidative stress was reported in a previous study (Aksu et al., 2021). The free radical-scavenging activity of some plants through direct neutralisation and upregulation of antioxidant enzyme activity is associated with hydroxycinnamic acids (Ambika et al., 2013). In addition, a previous study has revealed the effect of hydroxycinnamic acids in inhibiting macrophage infiltration, thereby reducing inflammation (Alam et al., 2016).

7. Conclusion

This review gives an overall overview of how prolonged hyperglycaemia in diabetic conditions leads to glucotoxicity in male reproductive tissue through the generation of excessive ROS and pro-inflammatory cytokines. The mechanisms through which oxidative stress and inflammation lead to the detrimental damage of testicular tissue, the disruption of spermatogenesis, and the decline in sperm parameters are highlighted. Although the main instigator of tissue damage and cell death in DM is hyperglycaemia, the treatment target cannot be limited to the reduction of glucose but to the decrease of the elevated ROS and inflammatory markers. The effectiveness of medicinal plants in restoring spermatogenic damage in diabetic conditions is linked to the antioxidant and anti-inflammatory phytochemicals in the plants. Many medicinal plants have been identified in previous ethnobotanical studies. However, phytochemical screening of these identified plants is paramount in discovering more compounds that can be isolated and used to treat diabetic complications.

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CHAPTER THREE

A review on medicinal plants Used in the management of male infertility associated with diabetes mellitus in Thengwe, Limpopo Province, South Africa

Murendeni Nethengwe ^a, Nasifu Kerebba ^b, Kunle Okaiyeto ^a, Chinyerum S. Opuwari ^c and 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 Chemistry, Makerere University, Kampala 7062, Uganda.

^c Department of Medical Biosciences, University of the Western Cape, Bellville, 7535, South Africa.

* Correspondence: oguntibejuo@cput.ac.za; bejufemi@yahoo.co.uk

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Abstract— Diabetes mellitus (DM) is a metabolic chronic disease characterized by persistent hyperglycemia resulting in oxidative stress. Excessive production of reactive oxidative species (ROS) through the mitochondrial electron transport chain and other pathways destroys the macromolecules such as proteins, lipids, and DNA thereby leading to an inflammatory response involving the excessive production of cytokines, which leads to the augmentation of the production of ROS and tissue damage. One of the complications of DM is male infertility which results from reproductive tissue damage. Oxidative stress and chronic inflammation in

the male reproductive tissue pose a great risk in the development of male infertility. Although synthetic drugs are conventionally used in the treatment of diabetes and its complications, the cost implications and adverse effects associated with the use of the drugs pose a concern. Hence, the quest for cost-effective alternatives with fewer side effects such as natural products is important. Therefore, the present review aimed at providing available ethnobotanical information (such as methods of extraction, dosage, and observed effectiveness) about medicinal plants used in Thengwe, Limpopo Province in the management of diabetes and its complications such as male infertility. The findings of this study revealed ten medicinal plants (*Androstachys johnsonii*, *Annona senegalensis*, *Brackenridgea zanguebarica*, *Garcinia livingstonei*, *Peltophorum africanum*, *Rhoicissus tridentate*, *Securidaca longepedunculata*, *Senna petersiana*, *Terminalia servicei*, *Ximenia caffra*) used in the treatment of DM and male infertility.

Keywords—Diabetes mellitus-related male infertility, inflammation, oxidative stress, medicinal plants.

1. Introduction

Diabetes mellitus (DM) is a non-communicable metabolic disease defined by different complications that are caused by hyperglycaemia (Wang *et al.*, 2021). Hyperglycaemia is the hallmark of DM and a consequence of the alterations in the secretion and/or action of insulin (insulin resistance) (Mudau *et al.*, 2022). Major predisposing factors, including genetic predisposition, obesity, poor food, and other lifestyle selections, are linked to insulin resistance and pancreatic beta cell degeneration (Kumar *et al.*, 2021). Hyperglycaemia is implicated in complications such as cardiovascular disease, nephropathy, neurodegenerative diseases, and male infertility (Abou Zeid *et al.*, 2021; Johnson *et al.*, 2019; Nanti *et al.*, 2019). The increased blood glucose molecules react with macromolecules in the body, altering and damaging them (Roxo *et al.*, 2019). The rise in DM prevalence contributes to the rise in male

infertility and is a great financial and public health concern (Temidayo & Stefan, 2017). The link between DM and male infertility is mitigated by the excessive production of ROS and inflammatory cytokines, led by hyperglycaemia (Oguntibeju, 2019).

The exposure of cells to high glucose levels causes the mitochondria to produce excess ROS and leads to oxidative stress (Nolfi-Donagan *et al.*, 2020), an instigator of the progression of DM-related complications (Olofinisan *et al.*, 2022). Furthermore, the interaction of glucose molecules with other macromolecules also increases ROS (Fishman *et al.*, 2018). ROS are highly reactive and unstable and can easily oxidise other molecules such as proteins, lipids, and DNA thereby damaging them (Hosseini *et al.*, 2019). Oxidative stress occurs when the production of ROS exceeds their elimination by antioxidants (Kurutas, 2016). Immune cells release inflammatory cytokines in response to tissue damage (Nna *et al.*, 2019). The damage caused by both high levels of glucose and oxidative stress leads to the excessive release of inflammatory cytokines, causing more damage (Nna *et al.*, 2019). The high levels of inflammatory cytokines, and oxidative stress in the male reproductive organs are detrimental to the production and maturation of spermatozoa, and can lead to male infertility (Agarwal *et al.*, 2018).

The prevalence of DM has risen more in developing countries compared to developed countries, with DM being the second leading cause of death in South Africa. A large number of people (415 million) worldwide are affected by DM, and it is estimated that double the amount will be affected by 2040 (Ibrahim *et al.*, 2019). It is also estimated that approximately 5% of the world's population may be diabetic by the year 2030 (Abou Zeid *et al.*, 2021). A rapid rise in the prevalence of DM from 4.5% to 12.7% in South Africa was recorded between the years 2010 to 2019 (Grundlingh *et al.*, 2022). Besides the rise in mortality, DM reduces the quality of life and increases the number of males affected by infertility issues (Johnson *et al.*, 2019). It has been recorded that 50% of infertility cases involve male-related causative factors (Abdillahi & Van Staden, 2012). There is currently no known cure for DM, however,

drugs such as metformin, glibenclamide, insulin, acarbose, and miglitol are prescribed to diabetic patients for the management of DM complications (Liu *et al.*, 2018; Lv & Guo, 2020). These drugs are reported to decrease blood glucose levels, ameliorate oxidative stress and inflammation, and inhibit enzymes responsible for the breakdown of carbohydrates into glucose molecules (Ibrahim *et al.*, 2019). Currently prescribed drugs are accompanied by side effects and high production costs (Innalegwu *et al.*, 2022). The disadvantages behind the use of synthetic drugs in the treatment of DM complications show a need for a more cost-effective and efficacious method of treatment (Ibrahim *et al.*, 2019). The pathway link between DM and male infertility has led to further investigation and findings of an alternative therapeutic way of treating male infertility in diabetic individuals.

The use of medicinal plants dates back to the existence of humankind (Petrovska, 2012). Over 80% of the African population uses medicinal plants as a form of treatment (Nanti *et al.*, 2019). Although the mechanisms behind the effectiveness of these plants was not yet investigated, ancient people continued to utilise them as food and to treat ailments (Aremu, 2022). Presently, more information about medicinal plants obtained from ethnobotanical surveys and biomedical studies has been published. Through available publications, it is evident that medicinal plants contain nutrients and phytochemicals such as polyphenols, alkaloids, and flavonoids which are key to the effectiveness of these plants (Alabi *et al.*, 2019; Joseph *et al.*, 2017; Moichela *et al.*, 2021).

In Thengwe community of Limpopo Province, South Africa, traditional healers traditionally treat DM and male infertility using medicinal herbs. Phytochemicals in these medicinal plants can ameliorate oxidative stress and inflammation, thereby treating DM and male infertility (Nna *et al.*, 2019). The different plants used by these Vhavenda people for the treatment of DM have been published, however, the potential of the same plants in the treatment of male infertility in diabetic patients is not well established. Additionally, there are more medicinal plants known and used by traditional healers regardless of their appearance in the literature.

The documentation of more plants in the treatment of DM, and their potential benefits in specifically treating male infertility could lead to further investigation of the mechanisms of action of these plants, and an increase in their use to lower the prevalence of male infertility. More studies on the different plants used in traditional medicine could pave the way to more discovery of suitable dosage, preparation, and method of administration, for a more efficacious way of treatment. Improvement and more documentation of the use of medicinal plants could create a safer (less adverse effects), more accessible, and more effective way of treating DM and related male infertility.

2. Materials and methods

2.1. Study area

The ethnobotanical survey was carried out in Thengwe village in Limpopo, South Africa. Thengwe village lies in the far North of Limpopo province between coordinates 22° 24' 0.0"-23° 36' 0.0" S and 29° 12' 0.0" and 31° 12' 0.0" E. Figure 1 represents the location of Thengwe village.

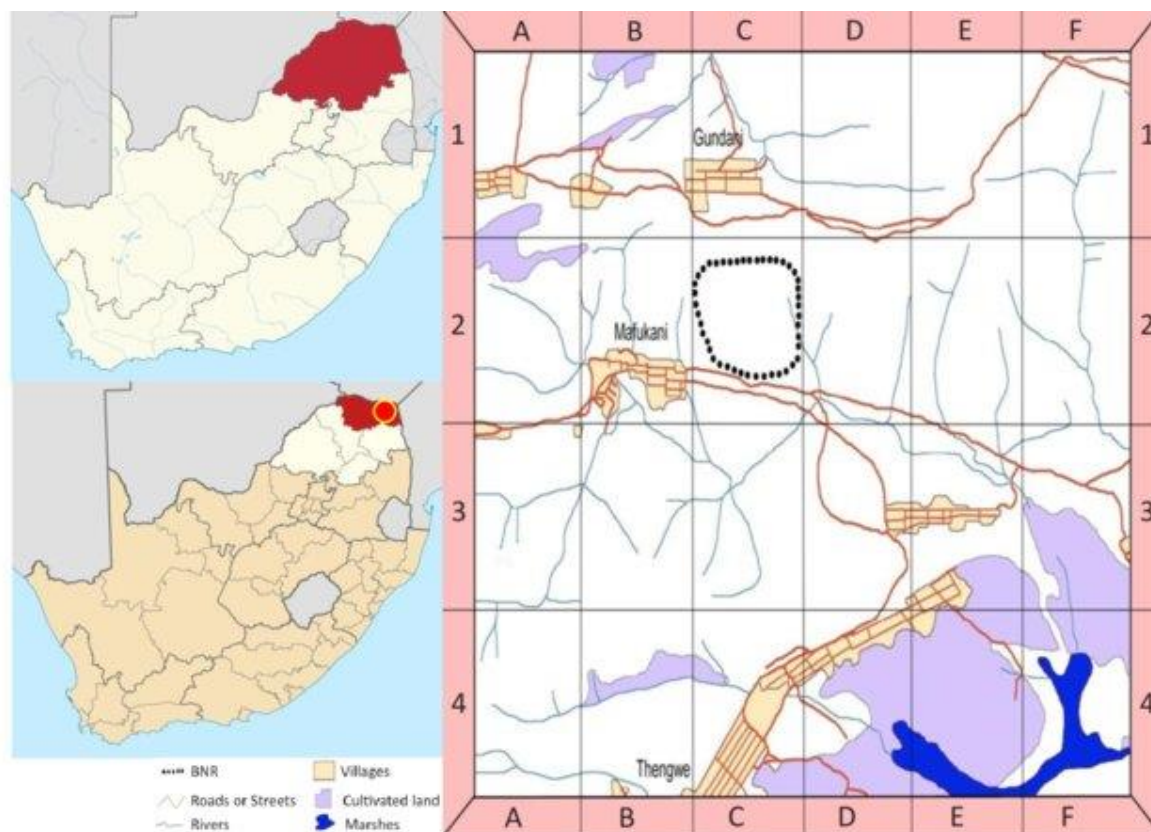


Figure 1: The location of Thengwe village in Limpopo province, South Africa, with the indication of the Brackenridge Nature reserve in Mafukani (Tiawoun *et al.*, 2019).

2.2. Data collection

An ethnobotanical survey was carried out at Thengwe village in Limpopo, South Africa, through a structured one-on-one interview with traditional healers about the medicinal plants used in the treatment of DM-related male infertility, together with their local names. Approval to get this information was obtained from the Chief of Tshidongololwe village. Information about medicinal plants used in Limpopo for the treatment of DM-related male infertility was obtained from publications found in different databases such as PubMed, Scopus, Science Direct, Wiley, and Springer, by searching for keywords such as diabetes mellitus, male infertility, medicinal plants, and Limpopo.

3. Results and discussion

Amongst others, the following plants were found in Thengwe village, Limpopo, South Africa. This study recorded 10 taxa used in the treatment of both DM and the resulting treatment of male infertility. The method of preparation for these medicinal plants was found to be majorly concoction and administration was done orally. These plants were also identified in the literature and their benefits in DM and male infertility were linked. The information on the selected plants is also represented in Table 1.

Table 1: Ethnobotanical information for plants used for the treatment of DM-related male infertility in Thengwe village, Limpopo Province, South Africa.

Name of plant	Local name	Part used for DM-related male infertility	Dosage	Method of preparation and administration	Other known benefits
<i>Androstachys johnsonii</i>	Musimbiri	Leaves	250 ml per day for 2 weeks	Orally (water decoction)	Sexually transmitted diseases
<i>Annona senegalensis</i>	Muembe	Leaves Roots	1 Tablespoon 3 times a day	Orally (water infusion)	Erectile dysfunction, malaria, and intestinal worms.
<i>Brackenridgea zanguebarica</i>	Mutavhatsindi	Roots	250 ml per day for 2 weeks	Orally (water decoction)	Healing of wounds

<i>Garcinia livingstonei</i>	Mupimbi	Leaves, roots, and stem	2 shots 3 times a day for 7 days	Orally (ethanol infusion)	Stomach cramps, bacterial infections
<i>Peltoporum africanum</i>	Musese	Leaves Roots and bark	250 ml per day for 2 weeks	Orally (water decoction)	Viral infections
<i>Rhoicissus tridentate</i>	Murumbulambudzana	Stem	Half a cup per day for 2 weeks	Orally (water infusion)	unknown
<i>Securidaca longepedunculata</i>	Mpesu	Stem bark	Half a cup per week for 4 weeks	Orally (water decoction)	Sexually transmitted diseases

<i>Senna petersiana</i>	Munembenembe	Leaves	Half a cup per week for 2 weeks	Orally (ethanol infusion)	Stomach cramps
		Seeds			
<i>Terminalia sericea</i>	Mususu	Roots	250 ml per day for 2 weeks	Orally (water decoction)	Bacterial infections
		Bark			
		Leaves			
<i>Ximenia caffra</i>	Mutshili	Roots	One cup per day for 2 weeks	Orally (water decoction)	unknown
		Leaves			
		Fruits			

Traditional healers (informants of the present study) were mainly male, although randomly selected. They reported that their way of knowing and locating these plants for any treatment was through divine communication with the ancestors. The most used parts of medicinal plants for the treatment of DM and male infertility were the roots and leaves, followed by the stem bark, fruits, and seeds. The vast availability of medicinal plants in Thengwe village supports the medical route popularly taken by the citizens of the area. Traditional healers in Limpopo have reported the use of *Androstachys johnsonii* (Musimbiri in Tshivenda), also known as Lebombo ironwood, as a treatment for DM and male infertility. *Androstachys johnsonii* is a tall (15 m) evergreen plant found in areas of Africa with very dry soil and low rainfall (Gandiwa *et al.*, 2011). In addition to its effect on male infertility treatment, the leaves of *Androstachys johnsonii* have been recorded to have an aphrodisiac effect on men (Maroyi, 2013). Besides the report from traditional healers, the treatment of DM-related complications by *Androstachys johnsonii* extract is not well investigated. *Androstachys johnsonii* has also been used to treat bacterial infections (Georginah *et al.*, 2012).



Figure 2: *Androstachys johnsonii* (Musimbiri).

Some medicinal plants have been recorded as food or food additives. *Annona senegalensis* (Muembe in Tshivenda), also known as “Wild custard apple” is a shrub (2-11 m tall) with smooth green leaves that have brownish hair on the dorsal side, a greyish-brownish stem, and green fruits that turn yellow and orange when ripe (Okhale *et al.*, 2016). These plants are found in hot climates next to river banks in forests (Okhale *et al.*, 2016). The fruits of *A. senegalensis* are edible, with the flowers used as a seasoning in foods (Donhouedé *et al.*, 2022). Supporting the evidence that this plant has been used in the treatment of DM and its complications, it was reported that the leaf extracts of *A. senegalensis* are beneficial in the reduction of blood glucose (Ibrahim *et al.*, 2019; Nanti *et al.*, 2019). Furthermore, the effect of *A. senegalensis* in the treatment of DM is linked to its antioxidant and anti-inflammatory capacity (Ibrahim *et al.*, 2019).

The compounds in *A. senegalensis* such as hexadecanoic acid, methyl ester, 1,3-octadecenal, and bis (2-methylpropyl) ester are associated with the inhibitory effect of the leaf extract of the plant on α -amylase and α -glucosidase, leading to the amelioration of hyperglycaemia (Ibrahim *et al.*, 2019). Previous studies have shown that phytochemicals such as flavonoids, saponosides, triterpenes, and tannins are constituents of *A. senegalensis*, corroborating the findings that this plant has antioxidant effects (Nanti *et al.*, 2019; Okhale *et al.*, 2016). The roots of the plant are prepared by the traditional healers in Limpopo to treat male infertility (Mahwasane *et al.*, 2013). Similar to this report, infusions of the roots of the plant are prepared in Nigeria for the treatment of erectile dysfunction, another male reproduction complication accompanying DM (Okhale *et al.*, 2016). It was also reported that the concoction prepared with *A. senegalensis* treats malaria and intestinal worms (Donhouedé *et al.*, 2022).



Figure 3: *Annona senegalensis* (Muembe).

Brackenridgea zanguebarica is known as Mutavhatsindi by the Vhavenda people. It has green leaves, and a yellow-pigmented stem and grows up to 10 m tall in tropical areas (Möller *et al.*, 2010; Rasethe, 2022). The *B. zanguebarica* plants are fenced at the Brackenridgea Nature Reserve in Thengwe, Limpopo where their harvest is controlled, due to the species being endangered (Rasethe, 2022). Several superstitions and witchcraft reports follow the use of *B. zanguebarica* which has been known to be a way to protect the plants from being overused (Rasethe, 2022). Traditional healers also used the roots of *B. zanguebarica* for the healing of wounds, and later switched to the leaves of the plant for the same purpose, due to raised conservation strategies. Isolation of phenolic compounds from *B. zanguebarica* has revealed the presence of different flavonoids in the crude root extract (Möller *et al.*, 2010).



Figure 4: *Brackenridgea zanguebarica* (Mutavhatsindi).

Garcinia livingstonei (Mupimbi in Tshivenda), also known as “African mangosteen” is a small evergreen plant with very small leaves found in high-temperature areas. *Garcinia livingstonei* is known for its antioxidant and anti-inflammatory effects in the treatment of DM complications (Joseph *et al.*, 2017). Compounds such as morelloflavone, morelloflavone-7-sulphate, guttiferone A, sargaol (Mulholland *et al.*, 2013), amentoflavone, 4’momomethoxy amrentoflavone (Kaikabo & Eloff, 2011), and phenols (flavonoids and alkaloids) (Joseph *et al.*, 2017) have been isolated from the leaves, roots, and stem of *Garcinia livingstonei*. The identification of the different compounds in the plant supports the benefits of the plant in treating DM-related male infertility. The leaves of this plant are also used to relieve stomach cramps.



Figure 5: *Garcinia livingstonei* (Mupimbi).

Peltophorum africanum is well known as Musese by the Vhavenda people. Phytochemical screening of the leaves of *P. africanum* revealed the availability of phytochemical compounds such as tannins, flavonoids, and saponins, contributing to the antioxidant effect of the plant (Abou Zeid *et al.*, 2021). In addition to these compounds, *P. africanum* also contains catechin, and bergenin, a C-galloylglycoside, adding to the antioxidant capacity of the plant (Theo *et al.*, 2009). The antioxidant capacity of this plant could explain its therapeutic effect in the amelioration of hyperglycaemia and the treatment of male infertility. Although the method of extraction differs in some studies compared to the method used (water extraction) by the selected traditional healers in Limpopo, findings show the antidiabetic effect of this plant through the inhibition of α -amylase (Abou Zeid *et al.*, 2021). A previous ethnobotanical study has recorded the use of the roots and bark of *P. africanum* in the treatment of male infertility (Abdillahi & Van Staden, 2012; Theo *et al.*, 2009). *P. africanum* is also beneficial in the

treatment of viral infections due to its anti-inflammatory effect (Adebayo et al., 2017). The reduction of proinflammatory cytokines caused by *P. africanum* could contribute to its benefit in the treatment of DM-related male infertility.



Figure 6: *Peltophorum africanum* (Musese).

Rhoicissus tridentate (Murumbulambudzana in Tshivenda), also known as “Wild grape” is a shrub with wedged green to black leaves and greenish-yellow flowers, known for its benefit in the treatment of DM (Aremu, 2022; Mukundi et al., 2015). Traditional healers have reported that the plant is mostly used for the treatment of sexually transmitted diseases (Nazer et al., 2019). The identification of phenols, flavonoids, saponins, and tannins in the stems of *R. tridentate* suggests the antioxidant and anti-inflammatory effect of the plant, which explains the amelioration of the DM complications, including male infertility (Mukundi et al., 2015). Very few publications report the benefits of this plant in DM complications.



Figure 7: *Rhoicissus tridentata* (Murumbulambudzana).

Securidaca longepedunculata (Mpesu in Tshivenda) is popularly known as the “Violet tree”, grows up to 6 m tall and produces purplish-green fruits (Abubakar *et al.*, 2022). The plant is mostly used for the treatment of erectile dysfunction and for aphrodisiac effects. Its roots and stem bark are known by traditional healers for the treatment of both DM and male infertility (Innalegwu *et al.*, 2022; Musa *et al.*, 2022). This plant is known to contain useful phytochemicals such as flavonoids, xanthones, terpenes, and steroids with a high capacity to ameliorate oxidative stress (Innalegwu *et al.*, 2022). A previous study also identified antioxidants such as saponins, flavonoids, and terpenoids in the stem bark of the plant (Adefolaju *et al.*, 2019). Root-bark extracts of *S. longepedunculata* affect the testicular parameters of rabbits and improve fertility (Chika *et al.*, 2017), which may be attributed to the bioactive compound content of this plant. The treatment with *S. longepedunculata* on rats has shown the effect of this plant in the improvement of both sperm concentration and sperm motility and could reverse DM effects in the spermatozoa (Chika *et al.*, 2017).



Figure 8: *Securidaca longepedunculata* (Mpesu)

Senna petersiana (known as Munembenembe in Tshivenda) has been recorded for its benefit in the treatment of DM complications through the inhibition of α -amylase and α -glucosidase (Mudau *et al.*, 2022; Olofinsan *et al.*, 2022). The hypoglycaemic effect of this plant is also linked to the pancreatic lipase inhibitory capacity of its leaf extracts (Olofinsan *et al.*, 2022). Leaves contain phenolic compounds such as flavonoids that boost the plant's antioxidant capacity (Olofinsan *et al.*, 2022). The leaves of *S. petersiana* possess an antioxidant effect through the activation of antioxidant enzymes thereby ameliorating oxidative stress (Olofinsan *et al.*, 2022). Activation of these enzymes in the testes could be a possible mechanism behind the treatment of male infertility. The use of the seeds *S. petersiana* for the treatment of male infertility in traditional medicine is recorded (Rajkovic *et al.*, 2022).



Figure 9: *Senna petersiana* (Munembenembe).

Terminalia sericea (Mususu in Tshivenda) is one of the popular medicinal plants in Africa (Anokwuru *et al.*, 2020). It is a small plant that grows up to 8 m tall well known as the “Silver cluster leaf” (Anokwuru *et al.*, 2020). It is used as an antibiotic due to its microbial effect (Nel *et al.*, 2020). The stem of *T. sericea* is used in the treatment of DM complications due to its effect on inhibiting carbohydrate hydrolysis enzymes such as α -amylase and α -glucosidase (Nkobole *et al.*, 2011). The reduction of blood glucose could contribute to the reduction of macromolecule damage in the male reproductive organs, thereby reducing male infertility. The bark of this plant also possesses high antioxidant capacity suggested to be caused by the availability of phytochemicals such as catechin, epicatechin, gallic acid, β -sitosterol, β -sitosterol-3-acetate, and lupeol in the stem bark (Nkobole *et al.*, 2011). Flavonoids (rutinoside and quercetin galloyl-glucoside) were isolated from the leaves of *T. sericea* in support of the antioxidant content of the plant (Sobeh *et al.*, 2019).



Figure 10: *Terminalia sericea* (Mususu).

Ximenia caffra (Mutshili in Tshivenda), also known as sour plum, is a shrub that grows in tropical areas in African countries such as Tanzania, Namibia, Limpopo and Botswana (Sobeh *et al.*, 2017). The poor and dry soil in the Thengwe farms in Limpopo favour the existence of these plants (Jacob *et al.*, 2021). *X. caffra* has a stem with grey to black barks, and green to creamy white flowers that can sometimes be reddish-pink (Jacob *et al.*, 2021). *X. caffra* is known for its multiple benefits and its commercial use, due to the use of its oils for cosmetics, and the consumption of its fruits and nuts (Maroyi, 2016). The leaves and roots of *X. caffra* are used for the treatment of male infertility (Sobeh *et al.*, 2017). Phytochemical analysis of the roots of this plant revealed the identification of compounds such as tannins and other flavonoids such as catechins, epicatechins and quercetin explaining the antioxidant capacity of the plant (Nkosi *et al.*, 2022; Sobeh *et al.*, 2017). In corroboration of this finding, the antioxidant capacity of the leaves of *X. caffra* was measured and found to be high and of

potential in the treatment of DM complications (Jacob *et al.*, 2021). The association of the treatment of DM and male infertility by this plant is linked to the amelioration of hyperglycaemia and the increased levels of insulin (Sobeh *et al.*, 2017). A study conducted by Nkosi and colleagues also supported the evident hypoglycaemic effect of the plant deduced from the α -amylase and α -glucosidase inhibitory effect of the fruits it produces (Nkosi *et al.*, 2022).



Figure 11: *Ximenia caffra* (Mutshili).

4. Conclusion

Male infertility is a home-breaker in many countries of the world. It is important to seek solutions to male infertility in all ways possible including an herbal approach. The present study reviewed 10 plants used in Thengwe village, Limpopo in the treatment of DM-related male infertility. This shows the vast possibility of the discovery and improvement of more ways to treat male infertility associated with DM. Findings from the present study revealed there is limited studies documented on the reviewed plants. The indigenous knowledge of medicinal plants is a valuable resource for health management. Knowledge of traditional medicine use

needs to be protected through proper documentation of recipe enumerations. Traditional knowledge of the use of medicinal plants must be recorded before it is lost to future generations. The paucity of scientific literature information on the pharmacological activities of some plant species demonstrates the need for more ethnobotanical survey studies to capture and document the folk medicinal use of plants in South Africa and around the world. This study will provide a foundation for future phytochemical and pharmacological investigations into the beneficial medicinal properties of such plants. Rather than relying exclusively on trial and error resulting from random screening procedures, adequately documented traditional knowledge may address challenges in identifying plants with medicinal uses that could find new applications for the benefit of all humans.

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CHAPTER FOUR

Antioxidant, Anti-diabetic, and anti-inflammation activity of *Garcinia livingstonei* aqueous leaf extract: A preliminary study

Murendeni Nethengwe ^a, Nasifu Kerebba ^b, Kunle Okaiyeto ^a, Chinyerum S. Opuwari ^c
and 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 Chemistry, Makerere University, Kampala 7062, Uganda.

^c Department of Medical Biosciences, University of the Western Cape, Bellville, 7535, South Africa.

* Correspondence: oguntibejuo@cput.ac.za; bejufemi@yahoo.co.uk

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Abstract: Diabetes mellitus (DM) is the second leading cause of mortality globally. The increased concern for DM is due to the underlying complications accompanying hyperglycaemia, associated with oxidative stress and consequent inflammation. The investigation of safe and effective treatment for DM is necessary. In the present study, cytotoxicity, phytochemical analysis, antioxidant capacity, anti-inflammatory and antidiabetic effects in aqueous extract of *Garcinia, livingstonei* leaves were assessed. All tested extract concentrations showed no toxicity against C3A hepatocytes. Several phenolic compounds were identified using Ultra-High Performance Liquid Chromatography Mass Spectrometry (UHPLC-MS). The total polyphenol content was 100.9741 mg GAE/g, 16.7712 mg CE/g

flavanols, and 2.3548 mg QE/g flavonols. The antioxidant capacity values were 253.4268 mg AAE/g, 192.232 mg TE/g, and 167.8724 mg TE/g for ferric reducing antioxidant power (FRAP), Trolox equivalent antioxidant capacity (TEAC), and 2,2-diphenyl-1-picrylhydrazyl (DPPH), respectively. The plant extract significantly ($P<0.05$) demonstrated anti-inflammatory and hypoglycaemic effects in a dose-dependent manner, with α -glucosidase inhibition of the extract higher ($P<0.05$) than the standard conventional drug (acarbose). The findings of this study revealed the potential of the constituents of *G. livingstonei* aqueous leaf extract in DM treatment. Further studies on the preparation and mechanisms of action of the plant in DM treatment are recommended.

Keywords: Antioxidants; diabetes mellitus; *Garcinia livingstonei*; hyperglycaemia; inflammation; oxidative stress; phenolic compounds

1. Introduction

Diabetes mellitus (DM) is a chronic non-communicable metabolic disease distinguished by the emergence of hyperglycaemia [1]. Alteration in the production or secretion, and the signal transduction of insulin in DM patients is the main cause of hyperglycaemia [2]. DM is categorized into two major types namely, type 1 DM, caused by β -cell destruction, and type 2 DM characterized by insulin resistance [3]. The pathogenesis of DM is associated with oxidative stress and inflammation, and both are implicated in hyperglycaemia [4]. Excess glucose molecules lead to oxidative stress through mitochondrial dysfunction and glycation of biomolecules subsequently leading to chronic inflammation [5], [6]. Oxidative stress occurs due to the dysregulation of free radicals caused by the imbalance between free radical formation and elimination by the antioxidant system [4]. Excessive free radicals interact with biomolecules and consequently lead to tissue damage and chronic inflammation [7]. Chronic inflammation occurs when the activation of immune cells and secretion of inflammatory cytokines exceeds normal [8]. The infiltration of immune cells and direct accumulation of inflammatory cytokines in organs leads to damage and further pathogenesis of DM [9]. The implications of hyperglycaemia explain the accompanying complications of DM such as

cardiovascular diseases, neurological and neurogenerative diseases, nephrological damage, and male infertility [10], [11], [12].

DM is a global public health problem with a prevalence of over 546.6 million people diagnosed in 2021 [13]. It is estimated that approximately 35.1 billion US dollars will be spent globally by 2030 towards the treatment plan, hence, the need for effective and affordable treatment [14], [15]. Reduction of hyperglycaemia and alleviation of oxidative stress and inflammation is crucial in the treatment of DM complications [16]. The inhibition of carbohydrate metabolism enzymes (α -glucosidase and α -amylase) reduces hyperglycaemia [10]. In addition, several studies have uncovered the role of lipid breakdown and absorption in the manifestation of obesity and have discovered the effect of lipase inhibitors in the treatment of obesity and DM [17]. Conventional treatment of DM includes metformin [2], as a first line of treatment, and other drugs such as acarbose, voglibose, and glibenclamide [18]. However, these synthetic drugs exhibit adverse effects and are highly expensive in production and unaffordable in most developing countries such as South Africa [2]. It is therefore paramount to investigate potential effective, safe, and affordable therapy for DM. Literature reveals some medicinal plants, used for the treatment of DM with interest in their phytochemical content such as antioxidants and anti-inflammatory compounds [1], [19]. However, many of the studies have only identified the plants (ethnobotanical studies), and limited studies have been done on the phytochemical profiling and evaluation of the antidiabetic effects that these plants possess [20].

Garcinia livingstonei is an evergreen plant indigenously found in Africa and popularly known in South Africa for its beneficial use [21]. In South Africa, the plant is found in some areas in Limpopo where it is known as “Mupimbi” in the Tshivenda language. *G. livingstonei* bears green leathery-like leaves and yellowish orange round edible fruits [22]. In different countries in Africa, *G. livingstonei* is identified by traditional healers and herbalists and used for the treatment of ailments such as bacterial infections, infertility, DM, and some respiratory complications through the preparation of either its leaves, stem, or roots [22]. Although only a few studies have been conducted on the leaves of the plant, review studies have revealed the

possibility of the leaves of the plant having therapeutic effects in the treatment of DM due to the constituents [23]. Several phytochemicals of interest such as benzophenones, phenolic acids, and flavonoids have been profiled, leading to reports of the antioxidant, antibacterial, and antiviral properties of the plant [21], 24]. However, information and data revealing the antidiabetic effect of the leaves of *G. livingstonei* is insufficient. From the currently limited information on literature, our study focused on the possible effect of the leaves of *G. livingstonei* in the treatment of DM by investigating the effect of the aqueous extract on the major carbohydrate enzymes, identifying possible responsible phytochemicals in the extract, and determining antioxidant and anti-inflammatory capacity of the extract.

2. Results and discussion

2.1. Cytotoxicity effect of aqueous *G. livingstonei*

The toxicity effect of the aqueous extract of *G. livingstonei* was evaluated on C3A hepatocytes to determine the safe concentrations of the extract to use for treatment in subsequent experiments. The cell viability of C3A hepatocytes treated with all different concentrations (15.625, 31.25, 62.5, 125, 200, and 250 µg/mL) of the plant extract was significantly ($P < 0.05$) higher compared to the negative control cells treated with melphalan (include the concentration used). There was an observed significant ($P < 0.05$) increase in cell viability in the first four lower concentrations of the aqueous extract (15.625, 31.25, 62.5, and 125 µg/mL) of *G. livingstonei* compared to the negative control (untreated C3A hepatocytes) suggesting the effect of the lower concentrations of the extract in promoting cell viability. The two highest concentrations of the extract (200 and 250 µg/mL) exhibited significantly ($P < 0.05$) lower cell viability compared to the lower concentrations, although there is no significant ($P > 0.05$) difference between the positive control and these two highest concentrations. Higher concentrations than the ones tested in the present study could exhibit minimal toxicity effects, considering the decrease in cell viability as the concentration of the extract is increased. There was no significant ($P > 0.05$) decrease in cell viability of the C3A hepatocytes treated with all

concentrations of the extract tested, compared to the untreated control C3A hepatocytes. These results reveal the safety of the use of this plant extract and the necessity in studying higher concentrations for possible higher efficacy.

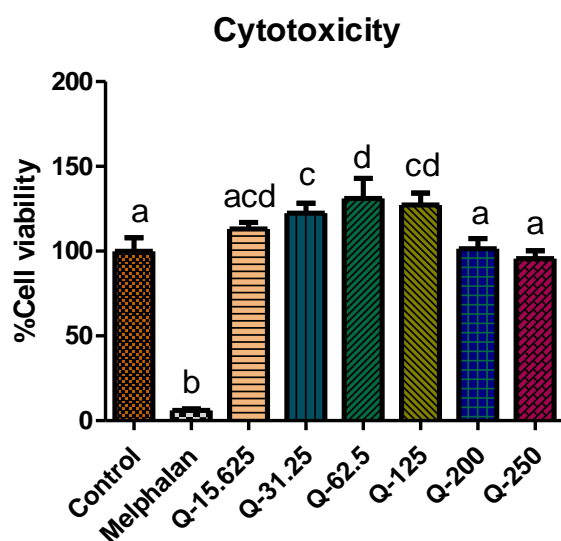


Figure 1. Cytotoxicity of the different concentrations of *G. livingstonei* leaf extract in $\mu\text{g/mL}$ against C3A cells. Cells were treated for 48 hours. Error bars indicate standard deviation of quadruplicate values done as a single experiment. Melphalan ($10 \mu\text{M}$) was used as a positive control. Bar graphs represent the mean of quadruplicate values. Error bars represent the standard deviation of the mean. Statistics was done using One-way ANOVA and Tukey post-test. Letters on bars show the significant difference where bars with different letters are significantly different ($P < 0.05$).

2.2. Quantification of specific phenolic compounds

Quantification of the present bioactive compounds of the aqueous leaf extract of *G. livingstonei* revealed the composition of bioactive chemicals (polyphenols) previously reported to be agents of therapy in the treatment of diabetic complications [23]. In previous studies, isolated polyphenols were used in drug therapy, to produce less-toxic drugs to treat diabetic complications. The current study shows a high level of total polyphenols, comparable to other medicinal plants previously studied and found to have a free radical scavenging effect.

Polyphenols serve a regulatory role in the balance of the level of inflammatory cytokines in DM by reducing the expression of mRNAs coding for chemokines and cytokines [25]. Amongst the specific phenolic compounds quantified in the study, flavanols (16.7712 mg CE/g) appear to be the highest of the total polyphenols (100.9741 mg GAE/g) in the aqueous extract of *G. livingstonei*, supporting the appearance as the vastest category of flavonoids [25]. No alkaloids were detected. The findings reveal the content of flavonoids as a possible contribution to the effects of the extract in the treatment of DM. The presence and predominance of flavonoids are possibly the cause of the high antioxidant capacity of the extract as high levels of flavonoids have been linked to high amounts of TEAC in previous studies [26]. Flavonoids have also been reported for their hypoglycaemic effect which can be linked with the inhibitory effect of the extract on carbohydrate metabolic enzymes [27].

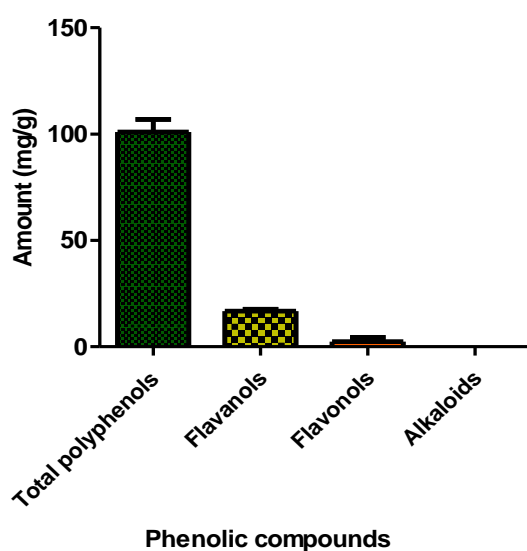


Figure 2. Quantification of phytochemical compounds in *G. livingstonei* leaf extract. Error bars indicate standard deviation of the mean of the quadruplicate values.

2.3. Total antioxidant capacity

Hyperglycaemia-induced overproduction of ROS leads to the oxidation of biomolecules, subsequently leading to DM complications [7]. The excessive production of free radicals effectuates the reduction of antioxidant capacity and subsequently exacerbates oxidative

stress damage in DM [28]. The amelioration of antioxidant capacity is therefore a therapeutic target in the treatment of DM complications facilitated by oxidative stress. In this current study, the assessment of the antioxidant capacity *G. livingstonei* aqueous leaf extract was carried out to evaluate its potential in the reduction of oxidative stress through the augmentation of the antioxidant system.

Assessment of antioxidant capacity of extracts is also an alternative way of determining the appearance of antioxidant compounds. From the phenolic content findings, it is evident that the extract possibly has high reducing power and scavenging activity. These findings are well supported by the high antioxidant capacity (DPPH = 167.8724 mg TE/g, TEAC = 192.232 mg TE/g, FRAP = 253.4268 mg AAE/g) exhibited by the plant in this study, with FRAP showing the highest value (253.4268 mg AAE/g). The ferric-reducing power of the extract, shown by the FRAP value, reveals the ability of the plant in the reduction of free radicals by the donation of an electron leading to the neutralisation of the free radical effect. Since the complications of DM are associated with the oxidation of tissues by ROS, the observed antioxidant capacity values of *G. livingstonei* reveal its antioxidant and subsequent antidiabetic potential. Alleviation of oxidative stress in DM can relieve organ damage and increase the quality of life in diabetic patients. An increase in oxidative stress consequently leads to chronic inflammation [6]. The potential of *G. livingstonei* in reducing oxidative stress leads to downstream reduction of diabetic complications caused by inflammatory damage of physiological systems. The rising interest in the use of medicinal plants is the presence of phenolic compounds linked to the increase in antioxidant capacity [19]. Phenolic compounds such as hydroxycinnamic acids have been found to reduce free radicals such as superoxide anions through the increase in antioxidant enzyme activity, ultimately leading to the increase in antioxidant capacity values as observed in the present study [29]. Treatment with the leaf extract of *G. livingstonei* can augment the antioxidant system in DM patients with compromised antioxidant enzymes and reduced non-enzymatic antioxidant proteins by directly reducing/neutralising free radicals. The antioxidant effect of *G. livingstonei* can potentially prevent further development of the

pathogenesis of DM by halting the manifestation of oxidative stress-induced organ damage. In the pathogenesis of DM, oxidative stress is an instigator of insulin resistance caused by the impairment of the insulin signal cascade, and β -cell destruction [30]. The antioxidant effect of *G. livingstonei* leaf extract can contribute to the reduction of hyperglycaemia by increasing insulin sensitivity and relieving β -cell damage through the elimination of free radicals.

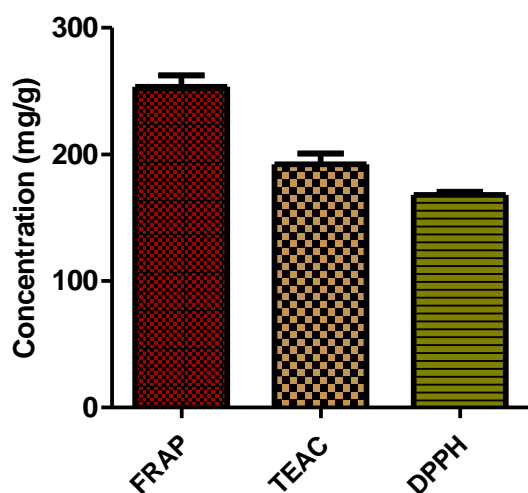


Figure 3. Antioxidant capacity of *G. livingstonei* leaf extract. Values of ferric reducing antioxidant power (FRAP), trolox equivalent antioxidant capacity (TEAC), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were determined. Error bars indicate standard deviation of the mean of the quadruplicate values.

2.4. UHPLC-MS identification of phenolic compounds

Further phytochemical analysis by the performance of UHPLC-MS identified the presence of a total of 37 phytochemical compounds found in the aqueous leaf extract of *G. livingstonei*. The phytochemical analysis in the current study demonstrates that the aqueous leaf extract of *G. livingstonei* contains flavonoids such as flavan-3-nols (catechin, epicatechin, gallic catechin), flavones (apigenin-C-pentoside-C-hexoside) and benzophenones, highly active and can react with free radicals due to their free hydroxyl and carbonyl groups which

agrees with previous similar studies [31], [32]. This section lists and explains the findings after the HPLC analysis of the extract.

2.4.1. Identification of flavan-3-ols, flavonols and flavone

As a supplement of the flavonoids detected in the quantification method, flavan-3-ols, flavonols, and flavone were detected using the UHPLC-MS analysis. Flavan-3-ols in oligomeric/ polymerised forms (peaks 18-21) were identified as catechin and epicatechin. The monomers: catechin, epicatechin, galocatechin, and gallo(epi)catechin gave various lengths of polymers called proanthocyanidins (Pas). Flavanols are a group of flavonoids with 3 joined rings (pyron ring, catechol, and resorcinol) with hydroxyl groups mainly responsible for the capacity of flavanols to eliminate free radicals [33]. The structural form of flavanols (epicatechin and catechin) determines the therapeutic role (antioxidative) they serve in the treatment of DM depending on their polymerization and the position of hydroxyl groups [33]. Using the elution order and conjugation with deoxyhexose and hexose sugars, peaks 11, 20 and 21 could be tentatively identified. Additionally, in conjugation with galloyl (152 Da), peak 23 could be tentatively identified as (-)-epicatechin-3-Ogallate. The methylated sulphate derivatives peaks 30 and 31 could also be identified with corresponding stereoisomers. Oligomeric forms were identified according to previous literature in peaks; 13, 24, and 25 [34].

Peaks 12, 14, 22, 28, and 32 were identified as flavonol peaks after the conjugation of aglycones with various sugars (hexose, 162 Da and pentose, 146 Da). The aglycone, isorhamnetin could appear in a free form at peak 28. The flavone, apigenin-C-pentoside-C-hexoside was identified in line with previous reports with the aid of accurate mass match m/z 563.2272. The hypoglycaemic effect of flavonoids is linked to the modulation of the insulin signaling pathway during glucose metabolism through the activation of Protein kinase B (Akt) and insulin receptor-1 (IRS-1) enzymes to increase glucose uptake by cells [35]. In DM, the oxidative stress-induced apoptosis of cardiomyocytes can be alleviated by the elimination of

free radicals [36]. Flavones have been previously documented to exhibit cardio-protective effects in DM by reducing both free radicals through the increase of antioxidant enzyme activities and inflammatory cytokines [37]. Therefore, the presence of flavonoids (flavanols, flavones, and flavonols) in the aqueous leaf of *G. livingstonei* contributes to the antidiabetic potential of the plant as it can possibly ameliorate hyperglycaemia, oxidative stress, and inflammation.

2.4.2. Identification of benzophenones

Peaks 7 and 8 were identified to belong to benzophenones with the aglycone ion at $[M - H]^-$; m/z 272. The fragmentation of the compound in peak 8 is similar to the one found in literature $3'$ - β -Glucosyloxy-4,4'-dihydroxy-2,6-dimethoxy-benzophenone [38]. Benzophenones are organic phenolic compounds with 2 interconnected C-rings as a basic structure. Various benzophenones compounds, artificial or naturally occurring, are derived from benzophenones to elicit therapeutic effects depending on the moiety and azines in the compound [18], 2024). The benzophenone identified in this study ($3'$ - β -Glucosyloxy-4,4'-dihydroxy-2,6-dimethoxy-benzophenone) was previously detected by in a study that specifically profiled benzophenone derivatives in *G. livingstonei* [38]. Glycoside derivatives of benzophenones were previously reported to exhibit α -glucosidase inhibitory activity [18]. The appearance of $3'$ - β -Glucosyloxy-4,4'-dihydroxy-2,6-dimethoxy-benzophenone as a constituent of *G. livingstonei* leaf extract suggests possible antidiabetic effects through the delay in a starch breakdown, which subsequently reduces blood glucose concentration. Most of the benzophenones reported from the genus *Garcinia* are polyisoprenylated benzophenones, derived from maclurin, a phenolic compound with ROS scavenging capacity [39]. Literature has also reported the effect of benzophenones in the management of obesity, as a preventative measure against the development of DM. Benzophenones improve dyslipidaemia which leads to the reduction of body fat and body weight[40]. It was also reported that benzophenones promote fuel utilisation

through carbohydrate metabolism by promoting glucose oxidation [40]. The shift of fuel utilisation to carbohydrates can ultimately lead to the reduction of blood glucose concentration i.e., the amelioration of hyperglycaemia. Besides the hypoglycaemic effect, the anti-inflammatory effect of benzophenones was revealed through the reduction of major cytokines such as TNF- α and IL-1 β observed with the treatment of cells with benzophenones [41].

2.4.3. Identification of hydroxycinnamic acids

The UHPLC-MS analysis also reveals the presence of hydroxycinnamic acids (dimethoxycinnamic acid, vanillic acid hexoside, p-coumaric acid and 3-O-malonyl-5-O- ϵ -caffeoylquinic acid). Peaks 1, 3, 4, 15, and 16 could be identified as those of hydroxycinnamic acids. Peak 3 was tentatively identified as p-Coumaric acid ethyl ester due to the presence of m/z 119 which would represent the decarboxylated coumaroyl. A free dimethoxycinnamic acid was also assigned at peak 1. Vanillic acid hexoside could be assigned on peak 4 due to the vanillic acid ion $[M - H]^-$; m/z 167 which indicated a loss of hexoside from the precursor ion m/z 329.0814. Acylation with malonyl (86 Da) on caffeoyl quinic acid led to compound in peak 16 tentatively identified being identified as 3-O-malonyl-5-O-(*E*)-caffeoylquinic acid and its derivative at peak 15. 3-O-malonyl-5-O-(*E*)-caffeoylquinic acid was first identified from fruits of wild eggplant [42]. P-coumaric acid is a natural phenolic compound derived from cinnamic acid after a shift in the position of the hydroxyl group. The antioxidant effect of *G. livingstonei* can be associated with the presence of p-coumaric acid, previously reported to increase DPPH and TEAC values, and reduce superoxide and hydrogen peroxide (free radicals) [43]. In a previous study, 3-O-malonyl-5-O- ϵ -caffeoylquinic acid was isolated from *Solanum incanum* and found to increase antioxidant capacity through the increase in DPPH value [44]. Dimethoxycinnamic acid alleviates lipid peroxidation, inflammation and hyperlipidaemia i.e., it also contributes to the therapeutic effects of *G. livingstonei* [45]. Gallic acid too, has previously been found to exhibit antioxidant potential. Hydroxycinnamic acids are well-documented for their anti-inflammatory effect, hence the anti-inflammatory effect observed [29]. The anti-

inflammatory activity of hydroxycinnamic acids is achieved by the reduction of pro-inflammatory cytokines and the blockage of macrophage infiltration [46]. These compounds also promote the increase in adiponectin secretion in reaction against inflammation [46]. It has also been recorded that hydroxycinnamic acids are free radical scavengers targeting the eradication of superoxide and reducing lipid peroxidation and increasing antioxidant capacity by increasing antioxidant enzyme activity [29].

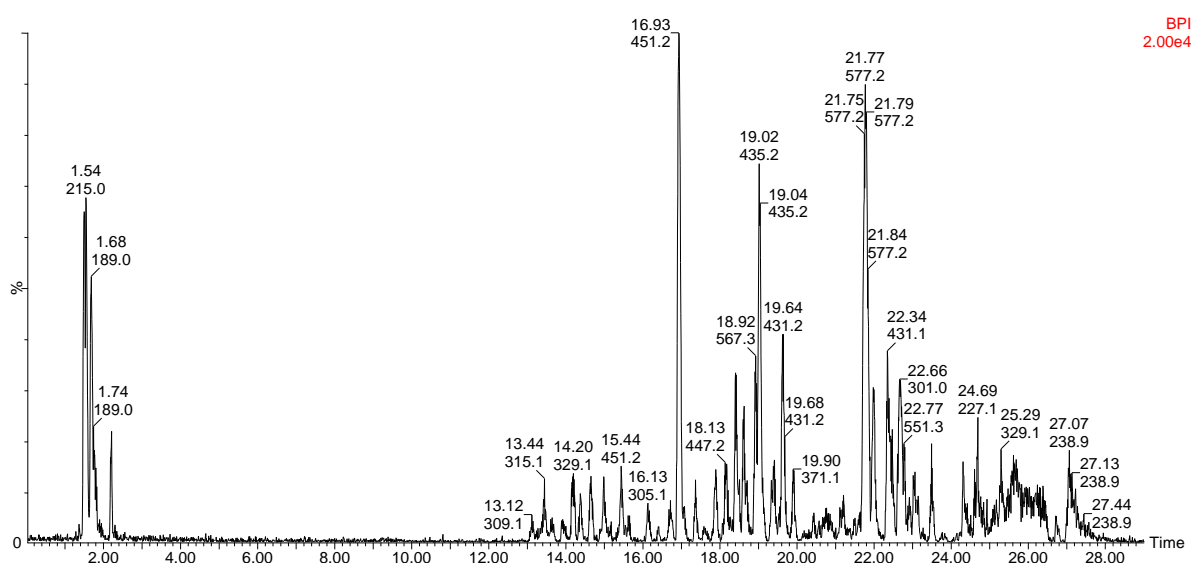


Figure 4. UHPLC-ESI-MS base peak chromatogram for the extract of *Garcinia livingstonei* analysed in the ESI negative mode.

Table 1. Phytochemicals screened from extract of *Garcinia livingstonei* using UHP-LCMS.

N o	t _R (mi n)	UV λ _{max} (nm)	m/z [M-H] ⁻	MS/MS	Tentative name	Reference s
1	1.5 4		215.022 4/ 225.052 0	179, 165, 133	Dimethoxycinnamic acid monohydrate	[47]
2	1.6 8		188.993 9	127	2,6-Diaminopimelic acid	[48]
3	2.2 1	262, 290	191.010 1	179, 119, 149	p-Coumaric acid ethyl ester	[49]
4	14. 20	254, 290	329.081 4	167, 279	Vanilic acid hexoside	[50]
5	14. 38	286, 310	423.086 2	315, 291	Unidentified	-
6	14. 65	278, 311	469.154 5	423, 391, 343, 203	Unidentified	-
7	14. 98	256, 301	361.070 6	272, 193	2,4,6-Trihydroxy-2',5'- dimethoxybenzophenone derivative	[38]

8	15.	236,	451.210	351, 272,	3'-β-Glucosyloxy-4,4'-dihydroxy-2,6-	[38]
	44	314	1	361	dimethoxy-benzophenone	
9	16.	271	305.061	236, 162	(-/+)-Galocatechin	[50]
	13		5			
1	16.	285sh	583.259	451, 293	3-hydroxyphloretin 2'-O-	[51]
0	72		5		xylosylglucoside	
1	16.	281	451.214	387, 441,	Epicatechin-3-O- hexoside	[52]
1	93		0	113, 45		
1	17.	264	417.099	159	Kaempferol-3-O-pentoside	[53]
2	36		3			
1	17.	280	577.138	449	Procyanidin B2	[54]
3	89		4			
1	18.	280	447.183	407, 437,	Kaempferol-3-O-hexoside	[55]
4	17		4	261, 327		
1	18.	300	473.123	439, 215	3-O-malonyl-5-O-(E)-caffeoylquinic acid	[42]
5	42		6		derivative	
1	18.	281	439.174	239	3-O-malonyl-5-O-(E)-caffeoylquinic acid	[42]
6	51		0			
1	18.	252,	431.188	289, 421	Epicatechin-3-O- xylosyl derivative	-
7	63	289	4			
1	18.	279	289.065	271, 215	Epicatechin	[56]
8	71		2			
1	18.	280	567.267	431, 327,	Epicatechin -3-O- (4 ¹ -xylosyl)	New
9	92		3	521	deohexoside	
2	19.	281	435.220	289, 425	Catechin -3- rhamnoside	[57]
0	02		4			
2	19.	267,	435.219	289, 431,	Epicatechin -O- rhamnoside	[57]
1	42	285,	9	421		
		337				
2	19.	285	431.186	385	Hexoside of kaempferol-3-O-(deoxy)	[52], [58]
2	64		2			
2	19.	281	371.090	247, 309,	(-)-Epicatechin-3-Ogallate	[59]
3	90		9/	157		
			441.189			
			0			
2	21.	270,	577.156	Un	Procyanidin B1 dimer	[60]
4	77	344	9	fragmented		
2	21.	268,	577.158	413, 293,	Procyanidin B11 dimer	[61]
5	84	338	2	311		
2	22.	268,	431.091	Un	Epicatechin-3-O- xylosyl derivative	-
6	00	335	2	fragmented		
2	22.	270,	431.089	289, 317	Epicatechin-3-O- xylosyl derivative	-
7	34	335	8			
2	22.	270,	316.989	237, 207,	Isorhamnetin	[62]
8	47	334	7	283		
2	22.	292	300.994	221, 157	Apigenin-C-pentoside-C-hexoside	[57], [58]
9	68		3/			
			563.227			
			2			
3	22.		551.268	447, 301,	O-Methyl-galocatechin-3-O-gallate-O-	[63]
0	80		5	541, 245,	sulfates	
				505		
3	22.		551.270	301, 447,	O-Methyl-(epi)galocatechin-3-O-gallate-	[63]
1	92		7	401, 541,	O-sulfate	
				319, 169		
3	23.		553.288	419, 507	Quercetin-3-O-acetyl-hexoside	[58]
2	49		8 ^{[M-2Na]-}			
3	24.	279	187.086	169, 127	Gallic acid	[56], [64]
3	31		7			

3	24.		227.117		Not identified	
4	69		6			
3	25.		329.059	150, 145	Fagomine derivative	[65]
5	29		9			
3	25.	302	149.984		Fagomine	[65], [66]
6	62		2			
3	27.		238.882		Unidentified	
7	07		0			

2.5. Anti-inflammatory activity

The advancement of DM is linked to the excessive release of pro-inflammatory markers in reaction to oxidative stress damage [67]. Over-activation of the inflammatory response leads to more damage to organs, more production of free radicals, and the development of DM complications [68]. Therefore, anti-inflammatory agents are of great importance in the treatment of DM. To complement the findings that the aqueous extract of *G. livingstonei* contains anti-inflammatory compounds, anti-inflammatory activity was determined and indicated by the decrease in nitrite concentration in response to LPS activation of RAW macrophages with no effect on cell viability, as seen with the AG control-treated cells. Although treatment with *G. livingstonei* aqueous extract showed a significant ($P < 0.05$) decrease in cell viability (as depicted in Fig. 5B), all tested concentrations (50, 100, and 200 $\mu\text{g}/\text{mL}$) of the extract, maintained cell viability over 100% which validates the observation of nitrite production in the determination of anti-inflammatory activity. A significant ($P < 0.05$) decrease in nitrite concentration in a dose-dependent manner is observed when compared to the lipopolysaccharide (LPS) activated cells, with the highest dose (200 $\mu\text{g}/\text{mL}$) showing a significantly ($P < 0.05$) lower concentration of nitrite compared to the other concentrations of the extract (Fig. 5A). Although the treatment of the cells with aminoguanidine (AG), as a positive control, showed a higher level of anti-inflammatory activity than the plant extract, significant ($P < 0.05$) and commendable anti-inflammatory activity is observed with the plant extract and can be linked with the appearance of both the hydroxycinnamic acids and benzophenones [29]. The reduction in nitrite concentration caused by the plant extract suggests the potential of the plant extract in reducing inflammatory cytokines which can be

linked with the effect of benzophenones identified [41]. Less production of nitrites can also be linked to reduced macrophage infiltration associated with the presence of hydroxycinnamic acids in the plant extract [46]. Besides the inhibitory activity of hydroxycinnamic acids in *G. livingstonei* aqueous leaf extract against macrophage infiltration, a possible mechanism behind the reduction of nitrites could be the increase in anti-inflammatory agents such as adiponectin, caused by hydroxycinnamic acids [46]. The anti-inflammatory effect observed can potentially ameliorate inflammation-induced insulin resistance and subsequently improve glucose metabolism in DM [69]. The synthesis of molecules caused by the reaction of excess glucose with macromolecules leads to the infiltration of immune cells which subsequently release cytokines that accumulate in organs such as the kidneys and cause apoptosis and tissue damage [9]. The Potential reduction of inflammatory cytokines by the leaf extract of *G. livingstonei* can potentially reduce tissue damage associated with DM by reducing apoptosis. In relation to the previously reported delayed wound healing caused by excess inflammatory cytokines, the anti-inflammatory effects of *G. livingstonei* can potentially improve wound healing as a complication in DM [8]. A similar previous study has also revealed the association of oxidative stress-induced myocardial damage with the excessive release of inflammatory cytokines in DM [70]. The collective anti-inflammatory and antioxidant effects of *G. livingstonei* observed in this current study can potentially treat cardiovascular complications in diabetic individuals.

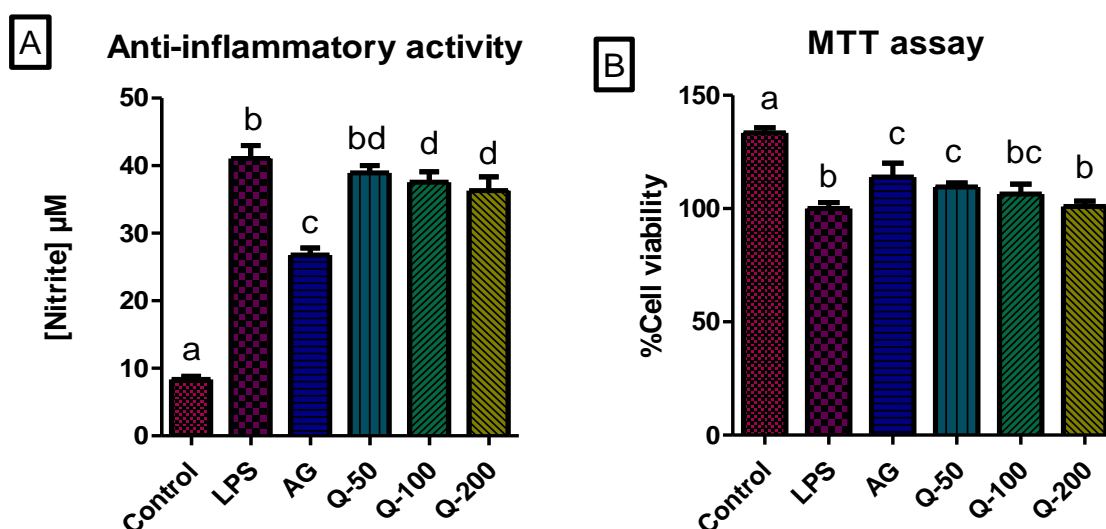
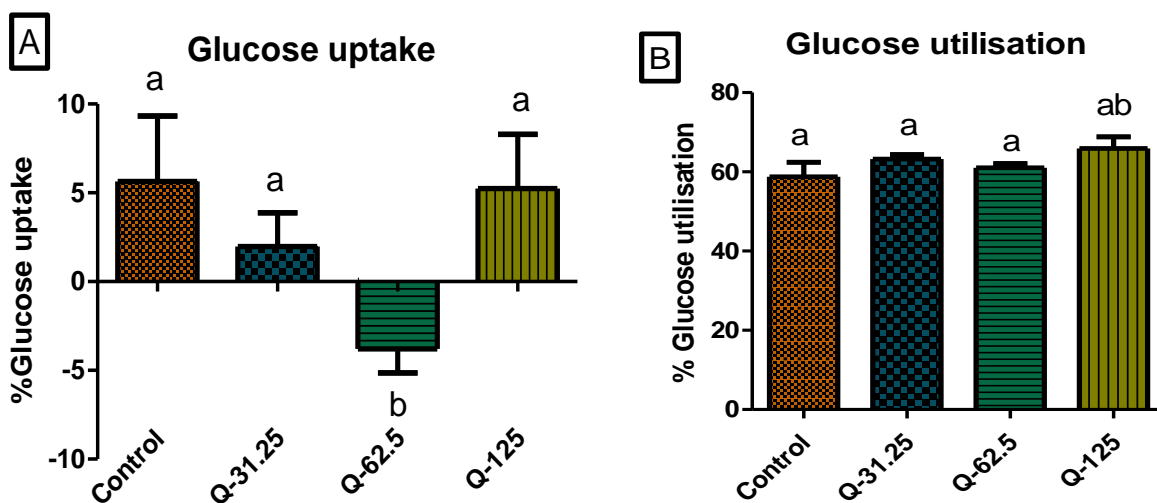


Figure 5. Nitric oxide production in LPS activated macrophages treated with samples as indicated in the Figure. Bar graph represents quadruplicate values of one experiment. Error bars represent the standard deviation of the mean. B. Cell viability (%) of LPS activated macrophages after 24 hours of exposure to treatments. Bar graph represents the mean of the quadruplicate values. Error bars represent the standard deviation of the mean. Statistics was done using One-way ANOVA and Tukey post-tests. Letters on bars show a significant difference where bars with different letters are significantly different ($P < 0.05$).

2.6. Glucose uptake and utilization

The rise in blood glucose level is highly dependent on the glucose uptake by cells, normally initiated by the binding of insulin to cell receptors, and the utilisation of the glucose taken in [71]. Besides the absorption of glucose into the blood, glucose uptake from the blood and utilisation by the cells is a contributing factor to the balance of blood sugar levels [72]. Although C3A hepatic cells treated with the concentration of $62.5 \mu\text{g/mL}$ of the plant showed a highly significant ($P < 0.05$) decrease in glucose uptake, other concentrations showed a comparable effect in glucose uptake with insulin. It was found that phenolic compounds such as hydroxycinnamic acids present in the extract promote the secretion and action of insulin in cells *in vivo* [29]. However, the present study was *in vitro*, and such effects could not possible be observed. A slight, but significant ($P < 0.05$) increase in glucose utilisation was observed

with the highest concentration of treatment (125 µg/mL). Glucose utilisation was comparable between the lower concentrations and the control, with no significant ($P>0.05$) difference observed. A previous similar study revealed the effect of benzophenones in the increase in fuel utilisation through carbohydrate metabolism by increasing glucose oxidation [40]. This present study supports the previous findings, as the link between the presence of benzophenones and the increase in glucose utilisation is observed [40]. Although the intracellular glucose concentration is not determined, given that glucose uptake through glucose transporter proteins is the rate-limiting step for cellular glucose consumption, it may be assumed that the amount of glucose remaining indirectly reflects glucose uptake. There was no significant ($P<0.05$) increase in glucose utilisation in cells treated with the plant extract. No cytotoxicity was observed with the treatment of the cells with all the plant extract concentrations. An increase in glucose utilisation can accelerate the clearance of glucose in cells which can lead to a feedback reaction increasing the production and secretion of insulin.



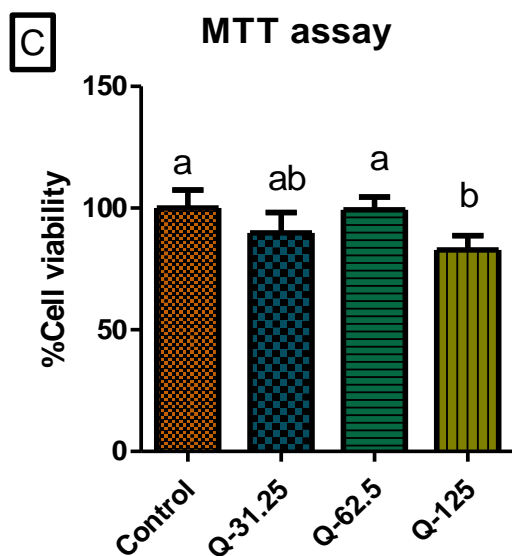


Figure 6. Glucose utilization (%) after 24 hours of treatment with different concentrations of the extract (31.25, 62.5, 125 $\mu\text{g}/\text{mL}$) in C3A cells (A) and Glucose uptake (%) after 4 hours in C3A cells, following 24-hour of the pre-treatment (B). Results were normalized to cell viability as determined using the MTT assay (C). Bar graph represents the mean of the triplicate values. Error bars represent the standard deviation of the mean. Statistics was done using One-way ANOVA and Tukey post-test. Letters on bars show the significant difference where bars with different letters are significantly different ($P < 0.05$).

2.7. α -glucosidase and lipase inhibition

Lipase inhibition was determined to evaluate the role of the aqueous leaf extract of *G. livingstonei* in the reduction of fat absorption in the intestines and the development of obesity [17]. The leaf extract showed some inhibitory activity against lipase. However, the lipase inhibitory activity was significantly ($P < 0.05$) low with all concentrations of the plant extract compared to the control. There was no significant difference in lipase inhibition amongst the plant extract concentrations. Although the lipase inhibitory activity exhibited by the plant is very low, these findings do not deduce less effectiveness of the extract in the management of obesity or dyslipidaemia but rather suggest an alternative additional pathway of the reduction of fats exhibited by the extract constituents. The AMP-activated protein kinase (AMPK) pathway activated by benzophenones identified in the extract can be a possible mechanism

in which fat reduction can be achieved through the treatment with the extract [40]. It has been concluded from a previous study that benzophenones activate the AMPK signaling pathway leading to the downregulation of downstream proteins such as the sterol regulatory element binding protein (SREBP)-1c and results in the reduction of triglycerides accumulation in the cells [41]. It is evident from the findings that *G. livingstonei* exhibits its antidiabetic effect mostly targeting the carbohydrate metabolic enzymes than triglycerides. In the process of carbohydrate metabolism (postprandial), the breakdown of carbohydrates is catalysed by carbohydrate enzymes such as α -glucosidase and α -amylase [10]. These enzymes promote the absorption of glucose from the intestines into the blood. Inhibition of α -glucosidase is of importance in glycaemic control for diabetic individuals as it slows down the rise of glucose in the blood, improving hyperglycaemia. Several previous studies have recorded comparable or lower inhibitory activity of other medicinal plants compared to acarbose [1], [2]. Interestingly, α -glucosidase inhibitory activity was found to be significantly ($P < 0.05$) higher compared to the standard drug (acarbose). The abundance of flavonoids in the leaf extract contributed to the high α -glucosidase inhibitory activity of the extract [27].

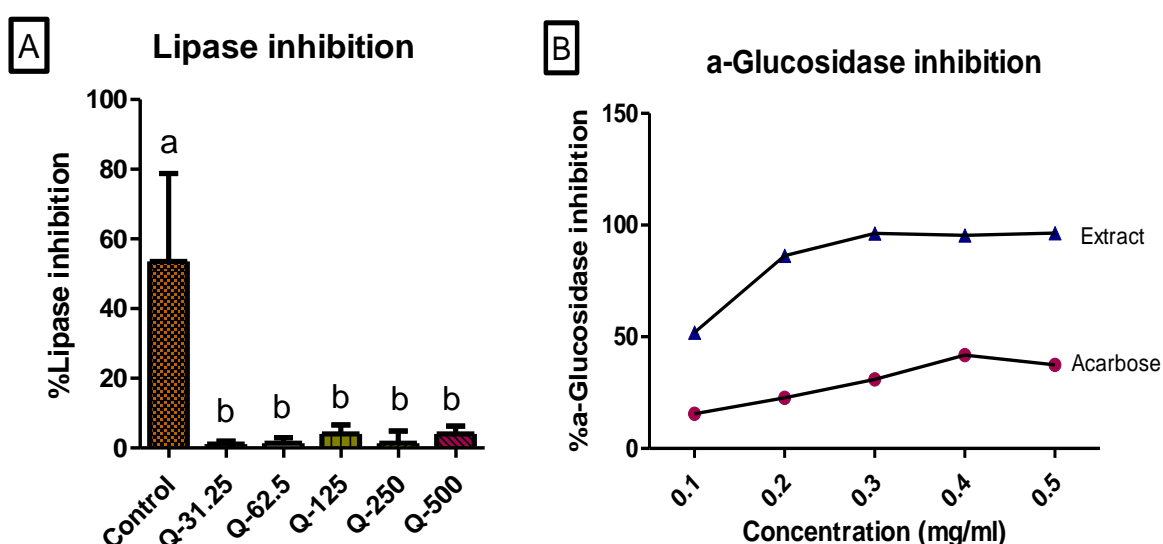


Figure 7. Pancreatic lipase inhibition and α -glucosidase inhibition. A. 100 μ M Orlistat was used as a positive control. Error bars indicate the standard deviation of the mean of 4 replicates. Bar graph represents quadruplicate values of one experiment. Error bars represent the standard deviation of the

mean. Statistics was done using One-way ANOVA and Tukey post-test. Letters on bars show the significant difference where bars with different letters are significantly different ($P < 0.05$). B. α -glucosidase inhibition was compared to acarbose as a positive control.

3. Materials and methods

3.1. Chemicals

All reagents and chemicals used in this study were purchased from Sigma-Aldrich, unless stated otherwise. The following chemicals were used; α -glucosidase (from *Sacchromyces cerevisiae*), monobasic sodium phosphate, dibasic sodium phosphate, pNPG, acarbose, MTT, dimethyl sulfoxide (DMSO), minimal essential medium (MEM) and phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} purchased from Cytiva in Marlborough, MA, USA, Foetal Bovine Serum (FBS), non-essential amino acids and penicillin/streptomycin purchased from Biowest in Nuaille, France, Lipase from porcine pancreas, Tris-HCl, *p*-nitrophenyl palmitate (pNPP), isopropanol, gum Arabic, sodium deoxycholate, Triton X-100, orlistat, RPMI-1640 Medium, RAW 264.7 mouse macrophages (purchased from Cellonex, South Africa), Sulfanilamide Solution and NED Solution (purchased from Promega), Lipopolysaccharide (LPS) and aminoguanidine, RPMI1640 culture medium (from GE Healthcare Life Sciences, Logan, UT, USA), C3A hepatocarcinoma cells (purchased from the ATCC, USA).

3.2. Plant material

The leaves of *G. livingstonei* were harvested in 2022 during the summer season (December) from the Brackenridge Nature Reserve in Thengwe, Limpopo, South Africa. The plant was authenticated by Prof. Tshisikhawe at the University of Venda (voucher number: MNU002/10/22), and a specimen of the plant was deposited at the University of Venda herbarium. The leaves of *G. livingstonei* (100g) were washed with tap water and dried for 5 days in the shade, after which they were crushed into powder and stored in an airtight container.

3.3. Plant extraction

The powdered leaves of *G. livingstonei* were weighed to up to 100 g and soaked in 1000 mL of hot (100 °C) distilled water overnight (24 hours). The liquid was extracted after 24 hours with the leaf material removed and discarded. The extract was further filtered using filter size 2 and 3 paper. The filtrate was dried overnight using a freeze-dryer. The final dried extract weight was 35.48 g and the percentage yield of 35.48%. The extract was stored at -20 °C in the freezer until use. This explained method of extraction was done following a modified version outlined by Zhao and colleagues [73].

3.4. Phytochemical analysis

3.4.1. Determination of polyphenols

Total polyphenol content was estimated following the methods used by Alabi and colleagues, [74]. A standard curve was generated from the preparation of gallic acid using different concentrations. A volume of 125 µL of Folin reagent (200 mM) was added into 25 µL of the samples (extracts) in 96-well plate wells. After 5 min, 100 µL of sodium carbonate (7.5% w/v) was added. The plate was incubated for 30 min at 37 °C and absorbances were measured using a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 765 nm. The results were expressed as mg of gallic acid equivalent to a gram dry mass of the plant material.

3.4.2. Determination of flavonol content

The concentration of flavonol was quantified using the methods followed by Alabi and colleagues, [74], and was expressed as mg of quercetin equivalent per gram of the plant (mg QE/g DM). Quercetin was used as a standard. The samples and the standard (12 µL) were added into plate wells of a 96-well plate followed by 12.5 µL of 0.1% HCl and 225 µL of 2% HCl and incubated for 30 min at room temperature. The plate was read using a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA) at a wavelength of 360 nm.

3.4.3. Determination of total alkaloid

Following the methods used by Alabi and colleagues [74], the total alkaloid content was calculated against a standard curve prepared using a series of dilutions of 0.1 mg/mL atropine in 2 M sodium phosphate buffer (pH 4.7). A mixture of 5 ml of 2 M sodium phosphate buffer (pH 4.7) with 500 mL of the sample/extract and 5 ml of BCG solution was prepared and mixed with 12 mL of chloroform. Two layers were expected to form and if so, 300 µL of the lower yellow layer was drawn and dispensed into the wells of a 96-well plate. The plate was read at 470 nm using a Multiskan spectrum plate reader (Thermo Fisher Scientific, USA).

3.4.4. Determination of flavanol content

The methods reported by Alabi and colleagues [74] were followed to determine flavanol content. Briefly, 1 mM of catechin will be dissolved in series as a standard. Twenty-five microliters (25 µL) of the samples and the standards were pipetted into the wells of a 96-well plate, and 275 µL of DMACA was added into each well. The plate was incubated for 30 min at room temperature and read at 640 nm. DMACA solution was prepared by adding DMACA in a methanol-HCl mixture at a ratio of 3:1 to a final concentration of 10 µg/mL. The concentration of flavanols was expressed as mg of catechin per gram dry mass of the plant material (mg catechin/g DM).

3.5. Ultra-High Performance Liquid Chromatography Mass Spectrometry (UHPLC-MS) analysis

Briefly, phytochemical analysis of *Garcinia livingstonei* extracts was done using a Waters Synapt G2 Quadrupole time-of-flight (QTOF) mass spectrometer (MS) connected to a Waters Acquity ultra-performance liquid chromatograph (UPLC) (Waters, Milford, MA, USA) or high-resolution analysis. Electrospray ionization was used in negative mode with a cone voltage of 15 V, a desolvation temperature of 275 °C, at 650 L/h, and the rest of the mass spectrometry

(MS) settings optimized for best resolution and sensitivity. Data was acquired by scanning from m/z 150–1500 in resolution mode as well as in MS^E mode. Two channels of MS data were captured in the MS^E mode, the first at a low collision energy (4 V), and the second at a collision energy ramp (40–100 V), which also allowed for the acquisition of fragmentation data. Leucine enkephalin was used as reference mass for accurate mass determination, and the instrument was calibrated with sodium formate. Separation was achieved on a Waters T3 HHS, 2.1x150mm, 1.7 μ m column. The mobile phase consisted of solvent A and B with the former containing 0.1% formic acid in water and the latter containing 0.1% formic acid in acetonitrile, and the injection volume was 2 μ L. A linear flow (flow rate= 0.3 mL/min) of the solvent was maintained for 22 min from a 100% gradient for solvent A (1 min) to 28% for solvent B. The gradient was then increased to 40% in solution B for 50 sec after which a wash step took over for 1.5 min at 100% solvent B. At the end of the wash step, re-equilibration occurred for 4 min. The temperature of the equipment was kept at 55 °C.

Data was processed and analysed under MS^E acquisition mode. The retention time (RT) ranged from 0.0-29.0 min with a tolerance of \pm 0.2 min. The mass accuracy tolerance was \pm 5ppm while the mass range was 40-1200 Da. Analysis was performed in negative ion mode and the adducts taken into consideration were, [M-H]⁻, [M-2H]²⁻, [M-H-H₂O]⁻, [M+Cl]⁻, [M-H-CO₂]⁻, [M+HCO₂]⁻, and [2M-H]. Data processing and acquisition were done using MassLynx™ software version 4.1 (Waters, Milford, MA, USA) and the conversion of the format from project files (.PRO) to NetCDF files (.CDF) was done using Databridge in MassLynx and MZmine. m/z tolerance was set at 0.01 or 10ppm for processing the Q-ToF data. Compounds were detected according to their RT, MS1, MS2 (m/z) and UV. During the identification of metabolites, retention times were aligned after the selection of the spectra. The unknown compounds were then detected and grouped, and their compositions were predicted, after which the list was searched, and the background compounds were marked. The N-rule and seven heuristic rules were considered during the prediction of the compounds composition to constrain all possible structures. The following N-rule and seven heuristic rules were followed: (1) restrictions for the

number of elements, (2) LEWIS and SENIOR chemical rules, (3) isotopic patterns, (4) hydrogen/carbon ratios, (5) element ratio of nitrogen, oxygen, phosphor, and sulphur versus carbon, (6) element ratio probabilities and (7) presence of trimethylsilylated compounds.

For the assignment of tentative names of the compounds, criteria were set as follows, (1) Accurate mass match: the masses were matched and linked to Metlin (<http://metlin.scripps.edu/index.php>), MassBank (<http://www.MassBank.jp/http://MassBank.normadata.eu/>), NIST (<http://chemdata.nist.gov/>), and ReSpect (<http://spectra.psc.riken.jp/>). All compounds whose accurate mass error (AME) was > 5 ppm were considered unidentified (Zubarev and Makarov, 2013). (2) mass fragmentation pattern: To identify compounds using their mass fragmentation, retention time and ionization modes, a few phenolic compounds were used as a standard of comparison and were run under the same conditions. However, not all standards were used in this experiment due to the presence of many compounds identified during UPLC-ESI-QTOF-MS analysis. Therefore, the MS¹ and MS² fragment ions of previous similar compounds were found in literature and databases. (3) Carbon atoms: In cases where there were several isotopes of compounds, the calculation of carbon atoms at the peak was used to limit false identifications.

The sensitivity of this method was previously validated [75]. Briefly, chemical markers were set according to the calculated limits of detection (LODs) and limits of quantification (LOQs). Phenolic acids were detected around the UV absorption of 300, 309, and 322 nm. Flavonols were detected at 254, 255, and 354 nm while flavanols were detected at 278 nm. Different concentrations (3.9; 7.8; 15.6; 62.5; 125.0 and 250.0 mg/L) of the standards (epicatechin and catechin) were injected and the UV absorptions were obtained to generate a calibration curve. The correlation coefficient of the linear regression model was considered ($R > 0.99$). The intra and inter-repeatability of the RT of compounds in the standard ranged from 0.14-3.14% and 1.01-2.90%, respectively.

3.6. Antioxidant capacity

3.6.1. Ferric Reducing Antioxidant Power Assay

The Ferric Reducing Antioxidant Power Assay (FRAP) method followed by Alabi and colleagues [74] was used to determine the ferric-reducing power of the plant extracts. Briefly, the sample, blank and standard (10 µl) was dispensed into the wells of a 96-well plate and 300 µl of FRAP reagent was added into the wells. The plate was incubated for 30 min at room temperature and read at 593 nm in a spectrophotometer. FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6)/ TPTZ solution, iron (III) chloride hexahydrate solution, and distilled water at a ratio of 10:1:1:2. The standard dilutions were made using ascorbic acid. The results were expressed as mg of ascorbic acid per gram of the plant extract (mg AAE/g sample). A blank sample was prepared containing all reagents except the antioxidant solution, and its absorbance value was subtracted from the samples to overcome spectral interference.

3.6.2. Trolox equivalent antioxidant capacity (TEAC) assay

The antioxidant activity of the sample was compared to the activity of Trolox as a standard in this study, following the method used by Alabi and colleagues [74]. A serial dilution was prepared from a Trolox stock solution of 1000 mg/L. A volume of 275 µl of 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) (0.4 mg/mL) was added to 25 µl of the samples in a 96-well plate. The plate was incubated at room temperature for 30 min to allow the reaction to occur. Subsequently, the plate was read at 593 nm in a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA).

3.6.3. 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The DPPH value of the plant extract was determined using trolox as a standard of comparison. Trolox standard diluted solutions were prepared with 1000 mg/L stock solution of trolox. A volume of 25 µl of the extract sample and the standard was plated in a 96-well plate, after which 275 µl of DPPH with the concentration of 0.4 mg/mL was added. The plate was left for

30 min at room temperature, then read at 593 nm in a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA) [74].

3.7. Anti-inflammatory effect determination

The mouse macrophage cell line, RAW 264.7 model was used to determine anti-inflammatory activity. RAW 264.7 cells were seeded in RPMI1640 culture medium supplemented with 10% FBS (RPMI complete medium) into 96-well plates at a density of 1×10^5 cells per well and allowed to attach overnight. The following day spent culture medium was removed and 50 μ L of sample aliquots (diluted in RPMI complete medium) were added to give final concentrations of 50, 100, and 200 μ g/mL. To assess the anti-inflammatory activity, 50 μ L of lipopolysaccharide (LPS) (final concentration of 1 μ g/mL) containing medium was added to the corresponding wells. Aminoguanidine (AG) was used as the positive control at 100 μ M. Cells were incubated for a further 24 h. To quantify NO production, nitrite was measured in a spent culture medium using the Griess reagent system based on the diazotization reaction originally described by Griess (1879). The spent culture medium was transferred (50 μ L) to a new 96-well plate and 50 μ L of sulphanilamide solution was added. Plates were incubated for 10 min at room temperature in the dark, then 50 μ L of NED solution was added followed by incubation for 10 min at room temperature in the dark. Absorbance was measured at 540 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA). A standard curve using sodium nitrite was used to determine the concentration of NO (μ M) in each sample. To confirm the absence of toxicity as a contributory factor, cell viability was assessed using MTT as described under glucose utilisation and uptake [76].

3.8. Glucose uptake and utilization

3.8.1. Cell line maintenance

Glucose uptake and utilization was tested on C3A hepatocarcinoma cells which were maintained in 10 cm culture dishes in complete medium (MEM with 1% NEAA, 10% FBS, 1% Pen-Strep) and incubated at 37 °C in a humidified atmosphere with 5% CO₂.

3.8.2. Glucose uptake

Cells were seeded in 96 well plates (2 x 10⁴ cells/well, 100 µL aliquots) and left overnight to attach. Different concentrations of the treatment (extract) were prepared in complete medium (31.25, 62.5 and 125 µg/mL) and added to cells and incubated for 24 h. Treatment was removed after 24 hours of incubation and cells were transferred to new 96 well plates after which 200 µL glucose oxidase (prepared from the mixture of 1 mU/mL *Aspergillus niger* glucose oxidase with 0.4 mM 4-aminoantipyrine, 2.5 U/mL horseradish peroxidase, 3 mM phenol, 0.25 mM EDTA and 0.5 M PBS (pH 7.0)) was added. The plate was kept at room temperature for 15 minutes and read at 510 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA). Glucose standards in the wells contained culture mediums and the incubation buffer, with no cells. The mean difference (in mM) of the concentrations of glucose remaining after the reaction, between glucose standards and the samples was used to determine glucose intake [76].

3.8.3. Glucose utilisation

Cells were seeded in 96 well plates (2 x 10⁴ cells/well, 100 µL aliquots) and left overnight to attach. Different concentrations of the treatment (extract) were prepared in complete medium (31.25, 62.5 and 125 µg/mL) and added to cells and incubated for 24 h. Treatment was removed after 24 h incubation and cells were transferred to new 96 well plates. The remaining medium was aspirated and cells were washed with 100 µL PBS and 25 µL of incubation buffer (RPMI-1640 diluted with PBS containing 0.1% BSA to a final glucose concentration of 8 mM) was added to cells followed by 4 h of incubation. A volume of 200 µL glucose oxidase reagent was added to the plates followed by incubation for 15 minutes at room temperature.

Absorbance was measured at 510 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA). Cell-free wells containing incubation buffer and complete culture mediums were used as the glucose standards. Glucose uptake was determined as a function of the concentration of glucose (mM) remaining and expressed as the difference between the mean of the standard and test sample [76].

3.9. Cytotoxicity determination-MTT assay

The MTT assay was performed on the cells tested for glucose utilisation and uptake to ensure viability of the cells during the glucose uptake and utilisation assays. After the assays, the remaining treatment medium was aspirated from all wells after 24 h, and 100 µL complete medium containing 0.5 mg/mL MTT was added to the cells. The cells were incubated for 1 hour at 37 °C. MTT was removed and 100 µL DMSO was added to each well to solubilise the formazan crystals. Absorbance was read at 540 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA) [76].

3.10. Lipase inhibition

Samples were diluted in assay buffer (100 mM Tris-HCl, pH 8.0) to concentrations of 500, 250, 125, 62.5, and 31.25 µg/mL. The pancreatic lipase inhibition assay was performed as described by Pringle and colleagues [77]. A volume of 40 µL of the lipase enzyme was added to 10 µL of the sample in a 96-well plate in quadruplicate. The plate was incubated at 37 °C for 15 min after which 170 µL substrate with reaction buffer was added. The plate was incubated again at 37 °C for 25 min. Absorbance was measured at 405 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA). No enzyme and no substrate controls were included, and the percentage lipase inhibition was calculated using as follows:

$$\% \text{ Lipase inhibition} = \frac{A_{405\text{nm of blank}} - A_{405\text{nm of test sample}}}{A_{405\text{nm of blank}}} \times 100$$

3.11. α -glucosidase inhibition

The inhibitory capacity of the extract determined, compared to that of acarbose, a standard drug. Different comparable concentrations of the plant extract and acarbose were prepared (15.625, 31.25, 62.5, 125, 200 $\mu\text{g/ml}$) using distilled water. Briefly, 100 μL of α -glucosidase was added to 50 μL of each well of the concentration of samples and the standard drug (with phosphate buffer as a negative control) in a 96-well plate and incubated at room temperature for 10 minutes. A substrate buffer solution (50 μL), prepared from 20mM phosphate buffer, pH 6.8, and 5mM of pNPG was then added into the wells to start the reaction. The 96-well plate was incubated for 30 min at 35.5 $^{\circ}\text{C}$. During the reaction, the colour of the solutions turned yellow due to the product of the breakdown of pNPG by the α -glucosidase enzyme. The %inhibition was observed by the shade of yellow, with the darker shades representing lower %inhibition. The %inhibition of each extract and acarbose concentration was calculated using the formular below [78].

$$\% \alpha\text{-glucosidase inhibition} = \frac{A_{405\text{nm of blank}} - A_{405\text{nm of test sample}}}{A_{405\text{nm of blank}}} \times 100$$

$$\% \text{inhibition} = [(A_c - A_s) / A_c] \times 100$$

A_c = Absorbance of the negative control

A_s = Absorbance of the sample

3.12. Statistical analysis

Statistical analysis was performed using GraphPad prism version 5. Analysis was done using One-way ANOVA (non-parametric) with Tukey post-test. Significance is denoted by letters on bars where bars with different letters show significant differences ($P < 0.05$), and bars with the same letter have no significant difference ($P > 0.05$).

4. Conclusions

The findings of this study revealed the therapeutic potential of *G. livingstonei* for the treatment of DM. Identification of the major antioxidant and anti-inflammatory compounds show the

potency of the plant extract in the amelioration of oxidative stress and inflammation in diabetic individuals. It is also evident that the plant can potentially improve both carbohydrate and lipid metabolism, which can promote management of obesity and hyperglycaemia. Although the preparation of the extract with a different solvent such as ethanol, and the extraction period could play a role in the determination of effects of *G. livingstonei* and the quantification of the phytochemical content, this study focused on the traditional method of preparation. Additionally, the use of water in the preparation of extracts has been proven effective and less costly. Comparison and relation to previous studies was insufficient as the studied plant has limited information in literature. Due to budget constraints, complementary experimental work such as the use of several extraction methods and the evaluation of more carbohydrate enzyme enzymes could not be conducted. However, further studies with the use of different solvents and longer extraction period could be highly useful in the findings. Further isolation of the identified compounds can also be useful in further investigations. Further *in vitro* and *in vivo* animal studies of *G. livingstonei* is needed to further validate the results from this study.

Author Contributions: Murendeni Nethengwe: conceptualisation, methodology, investigation, data analysis and curation, writing-original draft. Kunle Okaiyeto: supervision, conceptualisation. Nasifu Kerebba: data curation, writing- review and editing. Chinyerum S. Opuwari: supervision, writing- review and editing. Oluwafemi O. Oguntibeju: supervision, resources, secured funding for the study, writing-reviw and editing.

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Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER FIVE

Phenolic compounds profile and hypoglycaemic, anti-inflammatory and antioxidant properties of aqueous leaf extract of *Androstachys johnsonii*: In vitro study

Murendeni Nethengwe^a, Nasifu Kerebba^{b,d}, Kunle Okaiyeto^a, Chinyerum S. Opuwari^c, Oluwafemi O. Oguntibeju^a.

^aPhytomedicine and Phytochemistry Group, Department of Biomedical Sciences, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, Bellville 7535, South Africa.

^bDepartment of Chemistry, Makerere University, Kampala 7062, Uganda.

^cDepartment of Medical Biosciences, University of the Western Cape, Bellville, South Africa.

^dDepartment of Physical Sciences, Kampala International University, Kampala, Uganda.

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Abstract

Androstachys johnsonii has been identified through ethnobotanical studies as a medicinal plant used in traditional medicine to treat medical complications, including diabetes mellitus (DM). The development of DM complications involves hyperglycaemia and excessive production of free radicals and inflammatory cytokines. This study investigated the hypoglycaemic, antioxidant, and anti-inflammatory properties of the aqueous extract of *A. johnsonii*, to reveal the possible pathways through which the extract can treat DM complications. A total of 34 phenolic compounds (majorly flavonoids) was identified in the plant extract through the Ultra-High Performance Liquid Chromatography Mass Spectrometry (UHPLC-MS) analysis. Total polyphenols were 403.28 ± 12.75 mg gallic acid equivalent (GAE)/g with 88.35 ± 2.16 mg catechin equivalent (CE)/g of flavanols and 6.96 ± 3.10 mg

quercetin equivalent (QE)/g flavonols. For antioxidant capacity determination, ferric reducing antioxidant power (FRAP) (1342.68 ± 3.41 mg ascorbic acid equivalent (AAE)/g), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (571.57 ± 0.55 mg Trolox equivalent (TE)/g), and Trolox equivalent antioxidant capacity (TEAC) (478.88 ± 0.09 mg Trolox equivalent (TE)/g) values were obtained. The extract demonstrated a concentration-dependent anti-inflammatory activity on RAW macrophage cells. The highest ($62.5 \mu\text{g/mL}$) concentration of the extract increased glucose uptake and utilisation in C3A hepatocarcinoma cells. The extract showed a significant concentration-dependent ($P < 0.05$) increase in α -glucosidase inhibition and a significantly ($P < 0.05$) lower pancreatic lipase inhibition. These results suggest the potential therapeutic effect of *A. johnsonii* in the treatment of DM.

Keywords: Antioxidants, diabetes mellitus, hyperglycaemia, Inflammation, Phenolic compounds.

1. Introduction

Hyperglycaemia is a main characteristic of diabetes mellitus (DM) (Yao et al., 2023). DM is a global concern due to its increased prevalence and mortality (Bodke et al., 2023; Ye et al., 2023). Currently, 1 in 11 adults globally have been diagnosed with DM (commonly type 2 diabetes mellitus) (Fenta et al., 2023). It has also been estimated that 642 million people worldwide will be affected by DM in 2040 (Bodke et al., 2023). Although the pathogenesis of DM arises from reduced glycaemic control and impaired glucose metabolism, several other factors such as oxidative stress and inflammation are involved in the development of DM complications (Jubaidi et al., 2021). During the development of DM, the interaction of excessive glucose with biomolecules leads to the formation of advanced glycation end-products, and subsequent tissue damage (Khalid et al., 2022). Chronic inflammation is one of the instigators of medical complications accompanying DM (Ramesh et al., 2022; Rohm et al., 2022). Glucotoxicity leads to the upregulation of the NRF2 which causes immune cell infiltration and further damage caused by excessive production of inflammatory cytokines (Cai

et al., 2023). Hyperglycaemic complications also include the excessive production of ROS and compromised antioxidant systems leading to oxidative stress (Bhatti et al., 2022). The reaction of ROS with biomolecules causes oxidation and subsequent damage, leading to apoptosis, alteration, and deactivation of biomolecules (Liu et al., 2022). Oxidative stress is also directly linked to chronic inflammation and exacerbation of DM complications (Sahakyan et al., 2022). It is therefore evident that the direct amelioration of both oxidative stress and inflammation, and the reduction of blood glucose levels is paramount in treating DM.

The modern treatment of DM includes the use of hypoglycaemic synthetic drugs (Haq et al., 2021). As an example, the use of acarbose has also been linked with its hypoglycaemic effect through the inhibition of α -glucosidase and α -amylase (Saddique et al., 2022). Regardless of the effectiveness of synthetic drugs, traditional medicine has drawn scientific focus due to the review of the adverse effects and economic concerns that arise from the use of synthetic drugs (Blahova et al., 2021; Nguyen et al., 2022). Currently, several ethnobotanical studies have been conducted in search of medicinal plants that are effective in the treatment of DM (Amoo et al., 2022; Andrade et al., 2020; Bouyahya et al., 2021). Previous studies show that the pharmacological effect of medicinal plants is linked to the bioactive compounds constituted in the plants (Khan et al., 2022; Rahman et al., 2022; Yang et al., 2021). The isolation of individual compounds with respect to their targets and mechanisms of action is of interest in investigating therapeutic alternatives in the treatment of diseases (Ercan & Doğru, 2022). However, medicinal plants contain several phenolic compounds that can possibly act synergistically. Phenolic compounds in plants are known to have hypoglycaemic, antioxidant, and anti-inflammatory properties (Pieczykolan et al., 2021), hence, they are used in the treatment of various disease conditions including DM.

Androstachys johnsonii, also known as Lebombo ironwood, is a relatively tall (up to 15 m tall), green plant belonging to the Picrodendraceae family (Mfotie Njoya et al., 2024). *A. johnsonii* originates from Africa and Madagascar, commonly known as a source of yellowwood (Magalhães, 2021). The leaves, roots, and barks of *A. johnsonii* are used in traditional

medicine for the treatment of infections, stomach aches, DM, and as an aphrodisiac for men (Mfotie Njoya et al., 2024). Phenolic compounds such as catechins, bioflavonoids, gallic acid, tannins, quinic acid were previously identified in the leaves of *A. johnsonii* (Mfotie Njoya et al., 2024). A similar biological study revealed the antifungal effect of *A. johnsonii* roots, barks, and leaves with association to the presence of catechin as a bioactive compound (Molotja et al., 2011). Although ethnobotanical studies have identified the plant as a commonly used treatment for DM, only very scanty studies have revealed the action of the plant and its bioactive compounds possibly responsible for its effect. Therefore, this study was designed to comprehensively investigate the potential effect of *A. johnsonii* in reducing glucose levels and ameliorating oxidative stress and inflammation.

2. Materials and methods

2.1. Chemicals

The following chemicals and reagents were purchased from Sigma-Aldrich; Dimethyl sulfoxide (DMSO), α -glucosidase (from *Sacchromyces cereviciae*), Lipase from porcine pancreas, *p*-nitrophenyl palmitate (pNPP), dibasic sodium phosphate, monobasic sodium phosphate, acarbose, Tris-HCl, 4-nitrophenyl beta-D-glucoside (pNPG), triton-X100, isopropanol, sodium deoxycholate ferric reducing antioxidant power (FRAP) reagent, 2,2-diphenyl-1-1-picrylhydrazyl (DPPH) reagent, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), ascorbic acid, gallic acid, atropine, catechin. minimal essential medium (MEM) and phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} were purchased from Cytiva, in Marlborough, MA, USA. Lipopolysaccharide (LPS), aminoguanidine, and RPMI1640 culture medium were purchased from GE Healthcare Life Sciences, Logan, UT, USA. Sulphanilamide and NED Solutions were purchased from Promega. Penicillin/streptomycin, foetal bovine serum, and non-essential amino acids were purchased from Biowest in Nuaille, France. Orlistat, RPMI-1640 Medium, RAW 264.7 mouse macrophages were purchased from Cellonex, South Africa. C3A hepatocarcinoma cells were purchased from ATCC, USA.

2.2. Plant material collection and preparation

The *A.johnsonii* plant was identified at the Brackenridge Nature Reserve in Thengwe, Limpopo, South Africa. The leaves of the plant were harvested and authenticated at the University of Venda, by Prof. Tshisikhawe (voucher number: MNU001/10/22), and a specimen was kept at the herbarium at the University of Venda. An aqueous leaf extract was prepared from the leaves of *A. johnsonii*. The leaves were dried in the shade for 3 days and hand-crushed to smaller particles. The leaves were then blended into finer particles. The powder was weighed and soaked in 100 °C hot distilled water at a ratio of 1:10. The mixture was left overnight (24 hours). Extraction of the liquid in the mixture was performed using size 2 and 3 filters and the filtrate was freeze-dried overnight. The extract was kept in a -20 °C freezer.

2.3. Ultra-High Performance Liquid Chromatography Mass Spectrophotometry (UHPLC-MS) phenolic compound profiling

Phytochemical profiling of aqueous leaf extract of *A. johnsonii* was performed using a mass spectrometer, Water Synapt G2 Quadrupole time-of-flight (QTOF) coupled with a Waters Acquity ultra-performance liquid chromatograph from Waters, Milford, MA, USA. The UHPLC-MS procedure was performed following the methods followed by Idris and colleagues (Idris et al., 2024). Data processing and acquisition were performed under MS^E using MassLynx™ software version 4.1 (Waters, Milford, MA, USA), and the format was converted from project files (.PRO) to NetCDFfiles (.CDF) using Databridge in MassLynx and MZmine (Version 3). To specifically detect phytochemical compounds, ionization was in negative mode and the temperature of desolvation was set at 275 °C (650 L/h) and voltage at 15 V. The compounds were identified both in MS^E mode and in resolution mode (between the range of *m/z* 150–1500). In MS^E mode, data was captured at a low collision of 4 V and then at a higher collision energy range (40-100 V) to also obtain fragmentation data. Sodium formate was used to

calibrate the instrument and Leucine enkephalin was a reference for mass determination accuracy. A Waters T3 HHS, 2.1×150 mm, 1.7 μm column was used.

During separation, 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) were used for mobile phase with 2 μL volume initially injected. The flow was linear, and the flow rate was set at 0.3 mL/min. The flow was maintained for 22 min with solvent A flowing for 1 min at 100% gradient to 28% for solvent B which was then raised to 40% for 50 sec. A wash step was then performed for 1.5 min at 100% solvent B after-which the instrument was re-equilibrated for 4 min at 55 °C. At MS^E acquisition mode, retention time (RT) was set to start from 0.0 to 29.0 minutes and tolerance at ±0.2 min while m/z tolerance was set at 0.01 or 10ppm. Adducts in the negative ion ([M-H]⁻, [M-2H]²⁻, [M-H-H₂O]⁻, [M+Cl]⁻, [M-H-CO₂]⁻, [M+HCO₂]⁻, and [2M-H]) were considered. Mass range was 40-1200 Da and the mass accuracy tolerance was set at 5 ±5ppm. Detection of compounds was done according to UV, MS¹, MS² (*m/z*) and RT. Compounds were predicted according to the N-rule and the seven heuristic rules. The selection of the spectra was performed, and the retention times were aligned during the identification of metabolites. Unknown compounds were grouped, and their compositions were predicted. Compounds were named tentatively using 3 steps: 1) The masses of the compounds were matched and compared to existing data in Metlin (<http://metlin.scripps.edu/index.php> (accessed on 15 October 2023)), MassBank (<http://www.MassBank.jp>) (accessed on 15 October 2023), NIST (<http://chemdata.nist.gov/> (accessed on 15 October 2023)), and ReSpect (<http://spectra.psc.riken.jp/> (accessed on 15 October 2023)). 2) Compounds were identified by comparing their mass fragmentation, retention time and ionization modes to existing phenolic compounds standards run at the same conditions. Due to the presence of many compounds identified in this present study, not all standards were used. For other compounds, the MS¹ and MS² fragment ions were obtained from previous literature and databases. 3) The calculation of carbons atoms at the peak of compounds with several isotopes was done to limit false identifications.

2.3.1. Specificity and sensitivity

Phenolic compounds such as epicatechin and catechin were run at the same conditions and used as standards for the detection of phenolic compounds in the plant extract. This study did not focus on specific phenolic compounds, leading to many compounds detected. Due to too many compounds detected, not all standards were used. However, the MS¹ and MS² fragment ions of previous similar compounds were found in databases and used to determine the detected compounds.

The sensitivity of the method used in this study was validated in a previously similar study (Idris et al., 2024). In brief, the limits of detection (LODs) and limits of quantification (LOQs) were calculated and chemical markers were set. A UV and concentration standard curve for calibration was generated from epicatechin and catechin standards at 3.9; 7.8; 15.6; 62.5; 125.0 and 250,0 mg/L concentrations. The intra (0.14-3.14%) and inter (1.01-2.90%) repeatability of the RT of standards ranging from was considered. Phenolic acids were detected at 300, 309, and 322 nm, and flavonols and flavanols were detected at 254, 255, and 354, and 278 nm respectively. The correlation coefficient of linear regression was maintained at R>0.99.

2.4. Flavonol

Quercetin was used as a standard to determine the concentration of flavonols, and the quantity of flavonol was expressed as mg quercetin equivalent per gram of the extract (mg QE/g). The standard and samples were plated in a 96-well plate with 12.5 µL of 0.1% HCl and 225 µL of 2% HCl. The plate was incubated at room temperature for 30 minutes before reading at 360 nm in a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA) (Matsabisa et al., 2022).

2.5. Flavanol

Catechin (1 mM) was used as a standard to quantify the amount of flavanol in the extract. Samples and concentrations of the standard (25 µL) were plated into a 96-well plate after

which 275 μ L DMACA solution (DMACA in methanol-HCl to a concentration of 10 μ g/mL) was added. The plate was kept at room temperature for 30 minutes before reading. Absorbance was read at 640 nm. Results were expressed as mg catechin equivalent per gram of the extract (mg CE/g) (Matsabisa et al., 2022).

2.6. Alkaloid

To detect the presence alkaloids, the sample (500 mL) was mixed with 2 M sodium phosphate buffer (pH 4.7), BCG solution (5 ml), and chloroform (12 mL). The presence of alkaloids was noticed by the presence of 2 layers (yellow and blue). If so, 0.1 mg/mL Atropine (prepared in 2 M sodium phosphate buffer, pH 4.7) was used as a standard to calculate the concentration of alkaloids in the extract. The yellow layer in the extract sample/standard (300 μ L) was plated into a 96-well plate and the plate was immediately read at 470 nm in a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA). Results were expressed as mg atropine equivalent per gram of the extract (mg AE/g) (Alabi et al., 2019).

2.7. Total polyphenols

Gallic acid was used as a standard in the quantification of total polyphenols. Both the standard and the extract sample (25 μ L) were loaded in a 96-well plate. A concentration of 200 mM Folin-Ciocalteu reagent (125 μ L) was then added in the wells containing the samples/standard after which sodium carbonate was added. After an incubation of 30 minutes at 37 $^{\circ}$ C, the plate was read at 765 nm in a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA). The quantity of total polyphenols was expressed as mg of gallic acid equivalent to a gram dry mass of the extract. Results were expressed as mg gallic acid equivalent per gram of the extract (mg GAE/g) (Mndi et al., 2023).

2.8. Trolox equivalent antioxidant capacity assay

Trolox equivalent antioxidant capacity (TEAC) of the plant extract was measured following the methods by Alabi and colleagues (Alabi et al., 2019). Briefly, Trolox (1000 mg/L) was used as a standard in the assay. Both the serial dilution concentrations prepared from Trolox and the extract (25 μ L) were added into wells of a 96-well plate in triplicates. Immediately after, 275 μ L of 0.4 mg/mL 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) was added into the wells, and the plate was kept at room temperature for 30 minutes before reading. The plate was read at 593 nm using a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA). Results were expressed as mg Trolox equivalent per gram of the extract (mg TE/g).

2.9. Ferric Reducing Antioxidant Power assay

The ferric-reducing antioxidant power (FRAP) assay of the plant extract was measured following the methods by Okaiyeto and colleagues (Okaiyeto et al., 2023). 1000 μ M of vitamin C was diluted into a series of concentrations and used as a standard in the assay. A volume of 10 μ L of the standard and extract were added into a 96-well plate in triplicates. Immediately after, 300 μ L of FRAP reagent solution (300 mM acetate buffer, pH 3.6, iron (III) chloride hexahydrate solution, and TPTZ solution in distilled water at 10:1:1:2 ratio) was added into each well and the plate was kept at room temperature for 30 minutes before reading. The plate was read using a Multiskan Spectrum plate reader purchased from Thermo Fisher Scientific, Waltham, MA, USA at 593 nm. Results were expressed as mg ascorbic acid equivalent per gram of the extract (mg AAE/g).

2.10. 2,2-diphenyl-1-picrylhydrazyl assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was performed following the methods by Okaiyeto and colleagues (Okaiyeto et al., 2023). Briefly, Trolox (1000 mg/L) was used as a standard in the assay to express the results as mg of Trolox per gram of the plant. Both the

serial dilution concentrations prepared from Trolox and the extract (25 μ L) were added to a 96-well plate in triplicates. Immediately after, 275 μ L of 0.4 mg/mL DPPH was added into the wells, and the plate was kept at room temperature for 30 minutes before reading. The plate was read at 593 nm using a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA). Results were expressed as mg Trolox equivalent per gram of the extract (mg TE/g).

2.11. Anti-inflammatory effect determination

Anti-inflammatory activity was determined by quantifying the amount of nitric oxide (NO) produced by RAW 264.7 cells treated with the extract. A macrophage cell line (RAW 264.7 cells) from mice was used to determine anti-inflammatory activity. The cells were grown overnight in a 96-well plate (1×10^5 per well), in RPMI1640 culture medium supplemented with 10% FBS. After the overnight incubation, the culture media was removed and cells were treated with different concentrations of the plant extract (50, 100, and 200 μ g/mL) prepared in the culture media. The extract concentrations were chosen from a preliminary trial with consideration of the highest concentration that caused less toxicity (200 μ g/mL). Positive control cells were treated with 100 μ M aminoguanidine (AG). A volume of 50 μ L of 1 μ g/mL lipopolysaccharide (LPS) prepared in the media was then added to the cells and the plate was incubated for 24 hours. NO production was determined by measuring the concentration of nitrite in the each well using the Griess reagent. A volume of 50 μ L of the spent medium from the plate was added into the wells of a new plate after which 50 μ L of sulphanilamide solution (1% sulphanilamide in 5% phosphoric acid) was added. After a 10 min incubation in the dark at room temperature, the plate was read at 540 nm in a spectrophotometer (BioTek® PowerWave XS from Winooski, VT, USA). Sodium nitrite was used as a standard and the concentration of nitrite was measured in μ M. The MTT assay was also performed to determine the toxicity of the extract concentrations as a function of the NO production (van de Venter et al., 2008).

2.12. Glucose and lipid metabolism

2.12.1. Glucose utilisation

Glucose utilisation was measured on treated hepatocarcinoma cells (C3A). The cells were maintained in a complete medium prepared by adding 10% FBS, and 1% Pen-Strep to MEM with 1% NEAA in a 10 cm culture dish. A volume of 100 μL of the C3A hepatocarcinoma cells was then seeded in 96-well plates at a density of 2×10^4 cells/well and left overnight to attach. The cells were then treated with the extract concentrations (15.625, 31.25, and 62.5 $\mu\text{g}/\text{mL}$) prepared in the complete medium for 24 h before the cells were transferred to a new 96-well plate, and the medium was removed. The cells were washed with PBS (100 μL) and 25 μL of incubation buffer prepared by diluting RPMI-1640 with PBS in 0.1% BSA to a final glucose concentration of 8 mM and incubated for 4 h. Glucose oxidase (200 μL) was added into the cells and the 96-well plate was incubated for 15 minutes at room temperature before reading at 510 nm using a BioTek[®] PowerWave XS spectrophotometer (Winooski, VT, USA) (van de Venter et al., 2008).

2.12.2. Glucose uptake

C3A hepatocarcinoma cells were maintained in a complete medium prepared by adding 10% FBS, 1% Pen-Strep to MEM with 1% NEAA in a 10 cm culture dish and then seeded in wells of a 96-well plate (100 μL of 2×10^4 cells/well) overnight. The cells were then treated with the extract concentrations ((15.625, 31.25, and 62.5 $\mu\text{g}/\text{mL}$) prepared in the complete medium for 24 hours. The cells were then transferred to another 96-well plate where 200 μL of glucose oxidase prepared from 1 mU/mL *Aspergillus niger* glucose oxidase, 2.5 U/mL horseradish peroxidase, 0.25 mM EDTA, 3 mM phenol, 0.5 M PBS, pH 7.0, and 0.4 mM 4-aminoantipyrine, was added. The standard contained only glucose, medium, and the buffer without cells. The plate was kept at room temperature for 15 min and then read in a BioTek[®] PowerWave XS spectrophotometer (Winooski, VT, USA) at 510 nm. Glucose intake (in mM) was measured by

finding the difference between the glucose concentration in the standard and the glucose remaining in the samples after the reaction (van de Venter et al., 2008).

2.12.3. Cytotoxicity

The MTT assay was performed after the glucose uptake and utilisation assays. After both assays, the medium was removed, and the cells were treated with 100 μ L of complete medium with 0.5 mg/mL MTT followed by a 1-h incubation at 37 °C. The MTT solution was then aspirated, and DMSO (100 μ L) was added to the wells containing the cells. The plate was read using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA) at 540 nm (van de Venter et al., 2008).

2.12.4. Lipase inhibition

Lipase inhibition was determined following the methods by Pringle and colleagues (Pringle et al., 2021). Briefly, 10 μ L of extract concentrations (31.25, 62.5, 125, 250, 500 μ g/mL) prepared in 100 mM Tris-HCl, pH 8.0 were aliquoted into wells in quadruplicate of a 96-well plate. The concentrations were prepared from the highest soluble concentration to the lowest concentration that at least showed lipase inhibition activity. A volume of 40 μ L Porcine lipase enzyme was then added to the wells containing the extract solutions. The plate was kept at 37 °C for 15 minutes. A volume of 170 μ L of the 1 mg/mL *p*-nitrophenyl palmitate (pNPP) substrate (diluted in isopropanol) was added and the plate was incubated again at 37 °C for 25 min. The plate was then read in a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA) at 405 nm.

2.12.5. α -glucosidase inhibition

Following the methods of Kwon and colleagues (Kwon et al., 2006), α -glucosidase inhibition of the plant extract was measured against acarbose, as a standard. Both the standard and

concentrations (0.1, 0.2, 0.3, 0.4, 0.5 mg/mL) of the plant prepared in distilled water were plated into a 96-well plate in triplicates up to 50 μ L. The extract concentrations were chosen following the dilution series of α -glucosidase concentrations used and reported from previous studies (Telagari & Hullatti, 2015). A volume of 100 μ L of α -glucosidase was then added to each well. The plate was incubated for 10 minutes at room temperature. A volume of 50 μ L of the substrate (5 mM of 4-nitrophenyl beta-D-glucoside (pNPG) in 20 mM phosphate buffer, pH 6.8) was added to the wells and the reaction was allowed to occur for 30 min at 35.5 °C. The plate was read at 405 nm using a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA).

2.13. Statistical analysis

Data was analysed using Prism version 5 (GraphPad software). A non-parametric test was done using One-Way ANOVA and the Turkey post-test with the value of ($P < 0.05$) indicating significance. The significance in the data is indicated by letters of the alphabet, with different letters showing significance between groups and the same letters denoting no significance between groups.

3. Results

3.1. Cytotoxicity

The toxicity of the aqueous extract of *A. johnsonii* leaves was investigated against C3A hepatocarcinoma cells and the results are shown in Figure 1. Melphalan was used as a positive control and successfully showed a significant ($P < 0.05$) decrease of 93.95% in cell viability in the untreated C3A cells compared to the negative control cells (100% cell viability). Cell viability in the cells treated with the extract was concentration-dependent, with the lower concentrations (15.625, 31.25, 62.5, and 125 μ g/mL) showing significantly ($P < 0.05$) higher cell viability compared to the negative control cells. The higher concentrations (200 and 250

$\mu\text{g/mL}$) reveal the possible cytotoxicity of the plant extract as shown by a significant ($P < 0.05$) decrease in cell viability with the highest concentration showing almost 50% decrease in cell viability compared to control cells.

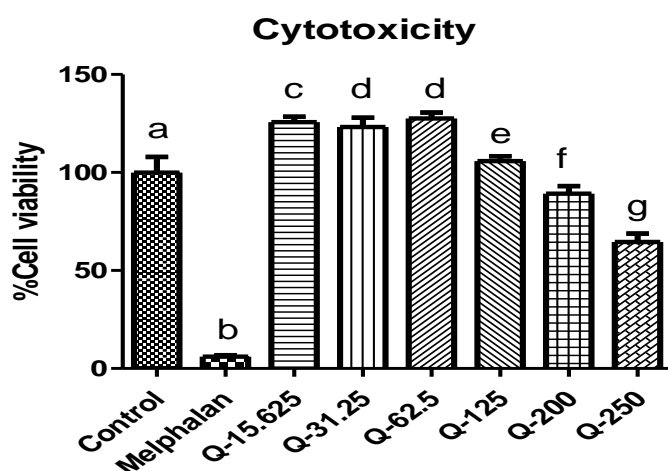


Figure 1. Toxicity of different concentrations (15.625, 15.625, 31.25, 62.5, 125, 200, and 250 $\mu\text{g/mL}$) of the *A. johnsonii* aqueous leaf extract and Melphalan (10 μM) against C3A hepatocarcinoma cells after 48 h. The bar graph is a result of the mean of values in quadruplicate with error bars representing standard deviation. Data was analysed using One-way ANOVA and Tukey post-test. The letters on top of the bars indicate significance, and significant difference is shown by different letters ($P < 0.05$).

3.2. Ultra-High Performance Liquid Chromatography Mass Spectrometry (UHPLC-MS) analysis

Phytochemical screening of the aqueous leaf extract of *A. johnsonii* identified a total of 34 phenolic compounds in the extract as depicted in Table 1. The chromatograph obtained from the phytochemical screening is shown in Figure 2. Flavonoids (flavonols, flavanols, and

flavones) were the most abundant phenolic compounds identified in the plant extract amongst other compounds such as hydroxybenzoic acids, gallotannins, quinic acid, citric acid and trihydroxy-octadecadienoic acid.

3.2.1. Identification of hydroxybenzoic acid and derivatives

In Figure 2, Peak 7 was identified as gallic acid whose m/z value is consistent with earlier studies owing to UV absorption of about 276 nm and m/z 125 [gallic acid $-H_2O-CO_2$]. Its derivatives were identified at peaks 6 and 7. Peak 6 was identified as 5-galloylquinic acid with m/z 343.0566 [M -H]⁻, and MS² fragments m/z 191.0440 [M -H -galloyl]⁻, m/z 169.0091 [M -H -191]⁻ consistent with the literature (Brescianiet al., 2015). Similarly, peak 9 was identified as 3-galloylquinic acid. The order of elution and thus the loss of the galloyl group (G) is 5-G>3-G.

3.2.2. Identification of hydroxycinnamic acid

Ferulic acid [M-H]⁻, could be detected with an adduct [M-Na]⁻, m/z 215.0231 and it characteristic MS² fragment m/z 174.0336 [M-H-H₂O]⁻, m/z 165.0247[feruloyl-CO]⁻ and these results were tabulated in Table 1.

3.2.3. Identification of flavonols

Five flavonols could be detected and identified (peaks 27, 28, 29, 30, and 31 of Figure 2). They were identified at characteristic UV maximum of 352 or 354 nm. Quercetin hexoside at peak 30 exhibited parent ion at m/z 463.0855 and MS² fragments at m/z 300.9797 signifying loss of sugar moiety (-162). Additional acylation of quercetin hexoside with a galloyl group could result in quercetin-3-O- β -D-(6¹¹-galloyl) glucopyranoside and quercetin-3-O- β -D-(6¹¹-galloyl) galactopyranoside at peaks 27 and 28, respectively identified using their order of retention on the column. Rutin was identified at peak 29 with parent ion 609.1478 [M - H]⁻ and MS² fragment m/z 300.0137 [M -H-glc - rha - H]⁻ i.e., consecutive loss of glucose (glc) (162

Da) and rhamnose (rha) (146 Da) sugars. The acetyl hexoside conjugate derivative of quercetin was identified at peak 31.

3.2.4. Identification of flavones

Two flavone peaks could be detected and identified (peaks 32 and 33 of Figure 2). They could exhibit characteristic maximum wavelength of 341 nm. These included luteolin-7-galactoside with 447.0903 [M- H]⁻ and its MS² fragments *m/z* 285.0282, [M- H -Glc]⁻ *m/z* 171.0058 and nicotiflorin, *m/z* 593.1525 [M- H]⁻, its MS² fragments *m/z* 285.0388 [M- glc- rha- H]⁻.

3.2.5. Identification of flavan-3-ols

Eleven flavan-3-ol, including oligomeric flavan-3-ols were identified and depicted in Figure 2 and Table 1. A few of them were in oligomeric/ polymerised forms (peaks 10, 11, 12, 15, 16, 17, 18, 19, 20, 24, 26) were identified from the stereoisomers; catechin and epicatechin and (Table 1). The monomers: catechin, epicatechin, galocatechin, and gallo(epi)catechin gave various lengths of polymers called proanthocyanidins (PAs). The two types of *B*-type PAs are linked by C4-C8 or C4-C6 interflavan linkages and *A*-type which have an additional C2-C5 or C2-C7 interflavan ether-linkages between the oligomers. The order of hydrophobicity for flavan-3-ol monomers in the reversed-phase liquid chromatography ie (-/+)-epicatechin > (-/+)-catechin > (-/+)-epigallocatechin > (-/+)-galocatechin also enabled identification of the monomers and the oligomers. The order of elution from the column is in the opposite direction. Thus (-/+)-galocatechin elutes earlier and epicatechin comes out last. Therefore, (-/+)-galocatechin (peak 11), and (-/+)-epicatechin (peak 17) could be identified. Catechin and epicatechin exhibit fragments *m/z* 151, 135 (due to retro Diels Alder (RDA) fragmentation) upon collision-induced dissociation (CID) of their precursor ions [M -H]⁻; *m/z* 289. In addition, fragment ions *m/z* 245 ([epicatechin -H-44]⁻; loss of CO₂), *m/z* 205 ([epicatechin -H-84]⁻; loss of flavonoid A ring) and *m/z* 179 ([M-H-110]⁻; loss of flavonoid B ring) were consistent with that of literature (Escobar-Avello et al., 2019).

Some polymers of PAs such as B-type procyanidin trimer ((Epi)catechin-4,8'-(epi)catechin-4',8''-(epi)catechin) (peak 10), (Epi)catechin-(epi)gallocatechin (peak 12), procyanidin B1 dimer (also called B-type procyanidin dimer)- (Epi)catechin-4,8'- catechin (peak 15), procyanidin B11 dimer ((Epi)catechin-4,8'-(epi)catechin (peak 16) which is an isomer among others were formed from the interflavan linkages between different monomers. The main fragment ions of parent ions of procyanidin B1 dimer (B-type procyanidin dimer) i.e. (Epi)catechin-4,8'- catechin at m/z 577.1438 were m/z 425.0825 [M - H - 152]⁻ from RDA of the heterocyclic rings, 407.0655 [M- H- 170]⁻ from RDA of the heterocyclic rings and loss of water, m/z 289.0684 [M -H- 288]⁻ from interflavanic bond cleavage, 451.1040 [M +H-126]⁻ from cleavages between C4–C5 and O–C2 of one of pyran rings.

Similarly, fragment ions of B-type procyanidin trimer with m/z 865.2118 and/ or 577.1343 included those at m/z 577.1438 [M - H -288]⁻ from interflavanic bond cleavage, m/z 695.1594 [M- H-170]⁻ from RDA of the heterocyclic rings and loss of water, m/z 425.0729 [M- H- 288 - 152]⁻ from interflavanic bond cleavage plus RDA of the heterocyclic rings, m/z 407.0655 [M- H- 288- 170]⁻ from interflavanic bond cleavage and RDA of the heterocyclic rings and loss of water, etc. In addition, another main fragment ion m/z 577.1343 [M- H- 290]⁻ might arise from interflavanic bond cleavage following the quinone-methide mechanism (Karchesy et al 1989). Procyanidin B11 dimer may be characterised with m/z 575.0995.

3.2.6. Identification of gallotannins

Gallotannins constituted another significant group of phytochemicals to be identified. They were detected at peaks (13, 14, 21, 22, 23, and 25) in Figure 2. The main HHDP galloyl glucose moiety could be detected at peak 21. They are ellagic acid derivatives in polyol form distinguished by their characteristic fragment ion in their spectra leading to sequential losses of galloyl (-152 Da), gallate (170 Da), and HHDP residues (301 Da) (Singh et al., 2016). HHDP-galloyl|glucose has a precursor ion of m/z 633.0814 [M- H]⁻ and its MS² fragments 463.0589

[M- H- galloyl- H-H₂O]⁻, 300.9778 [M- H- galloyl- H₂O- Hex]⁻. Trisgalloyl HHDP glucose could be detected at *m/z* 951.0864 [M- H]⁻, and its MS² fragment 300.9938 [M- H- galloyl- H₂O- HHDP]⁻, 475.0364, 169.0060 [M - H -galloyl -191]⁻, 125.0157[gallic acid -H₂O-CO₂]⁻. Mucic acid lactone digallate at peaks 13 and 14 could also be identified with *m/z* 495.0747 ([M -H]⁻), 343.0668 [M -H- galloyl]⁻, 169.0009 [M - H -galloyl -191]⁻, 169.0009 [gallic acid -H]⁻ and *m/z* 125.0170 [gallic acid -H₂O-CO₂]⁻, while *m/z* 325.0498 signifies possibility of lactonisation.

3.2.7. Identification of other compounds

Other compounds like quinic acid, citric acid and trihydroxy-octadecadienoic acid using accurate mass match.

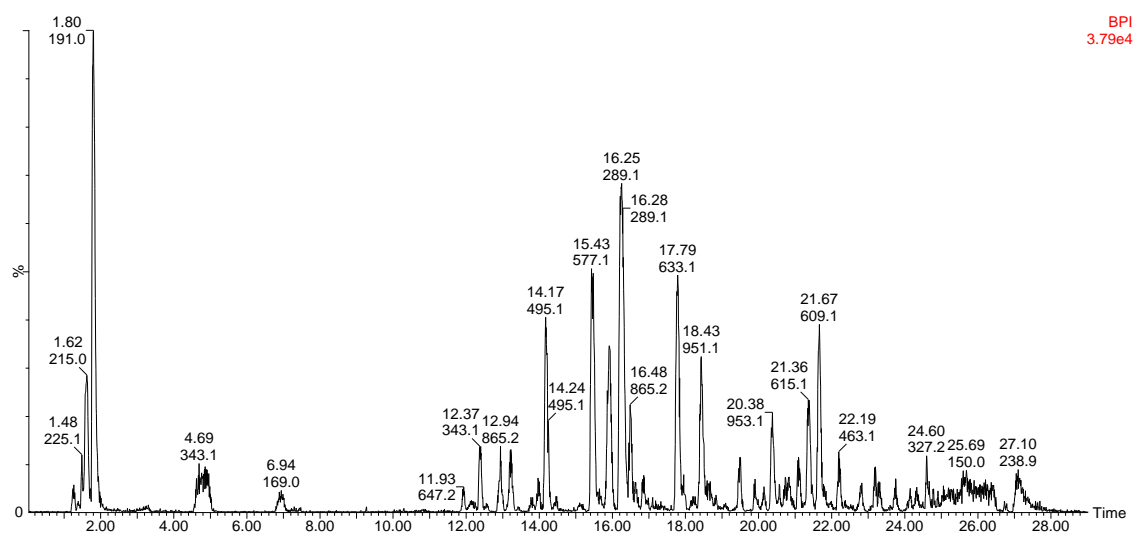


Figure 2: *Androstachys johnsonii* aqueous leaf extract UHPLC-ESI-MS base peak chromatogram obtained after analysis in the ESI negative mode.

Table 1: Compounds identified from the extract of *Androstachys johnsonii*

Pea k No	t _R (min)	UV λ _{max} (nm)	m/z [M-H] ⁻	MS/MS	Tentative name
1	1.48		225.0510	179.0437, 131.0379	unidentified
2	1.62		215.0231 ^[m- Na]	165.0247, 174.0336, 96.9604	Ferulic acid
3	1.72		191.0080	unfragmented	Quinic acid
4	1.80	225	191.0457	unfragmented	Citric acid
5	1.83	225	191.0459	85.0176	Isocitric acid
6	4.69	273	343.0566	191.0440, 169.0091	5-Galloylquinic acid
7	6.94	272	169.0052	125.0175	Gallic acid
8	11.93	273	647.2078	343.0649, 191.0471	Galloylquinic acid derivative
9	12.37	274	343.0596	169.0050, 175.9536	3-Galloylquinic acid
10	12.94	279	865.2118	323.1297, 695.1594	B-type procyanidin trimer ((Epi)catechin-4,8'- (epi)catechin-4',8''-(epi) catechin)
11	13.23	273	305.0587	261.0300	(+)-Gallocatechin
12	13.96	277	593.1240	449.1241, 433.0793	(Epi)catechin- (epi)gallocatechin
13	14.17	274	495.0747	169.0009, 125.0170,	Mucic acid lactone digallate)

				325.0498,	
				343.0668	
14	14.24	274	495.0744	345.0760,	Mucic acid lactone digallate
				203.0758	isomer
15	15.43	279	577.1438	289.0684,	Procyanidin B1 dimer (B-
				125.0148,	type procyanidin dimer)-
				425.0825,	(Epi)catechin-4,8'- catechin
				407.0655,	
				451.1040	
16	15.91	279	577.1367	289.0710,	Procyanidin B11 dimer
				425.0725,	((Epi)catechin-4,8'-
				125.0126,	(epi)catechin
				407.0942	
17	16.25	279	289.0624	245.0777	(Epi)catechin
18	16.28	279	579.1522	289.0627	Procyanidin B4
					(Catechin-4,8'-(epi)
					catechin)
19	16.48	279	865.2097	289.0622,	Procyanidin trimer
				325.0828	((Epi)catechin-4,8'-(epi)
					catechin-4',8''-(epi)catechin
					(isomer))
20	16.86	279	865.2111	291.0057,	Procyanidin trimer
				575.0995	(Epi)catechin-4,8'-(epi)
					catechin-4',8''-
					(epi)catechin (isomer))
21	17.79	268	633.0814	463.0589,	HHDP galloyl glucose
				300.9778	isomer.

22	17.95		647.0881	305.0703, 485.0419, 633.0795	Methyl galloyl-HHDP-hexose
23	18.43	277	951.0864	300.9938, 475.0364, 169.0060, 125.0157	Trisgalloyl HHDP glucose isomer
24	19.49	279	577.1343	289.0629, 425.0729	Procyanidin B5 Catechin-4,8'-(epi)catechin (isomer)
25	20.38	276	953.1051	476.0410, 541.2192 300.9952, 169.0020, 463.0730	Galloyl chebuloyl -HHDP glucose
26	20.80	278	595.1254	541.2440, 481.1049, 385.1217	(Epi)gallocatechin-(epi)catechin
27	21.10	266, 353	615.1038	300.0220, 463.1964	Quercetin 3-O- β -D-(6 ¹¹ -galloyl) glucopyranoside
28	21.36	264, 354	615.1016	537.2035, 463.0755	Quercetin 3-O- β -D-(6 ¹¹ -galloyl) galactopyranoside
29	21.67	256, 354sh	609.1478	300.0137	Rutin (Quercetin 3-O-(6-O-rhamnosyl-glucoside))
30	22.19	254sh, 352	463.0855	300.9797	Quercetin hexoside

31	22.83	268sh, 352	761.1660	549.2669, 505.2141, 485.1760, 300.9898	Quercetin O-acetyl Hexoside derivative
32	23.20	266, 341	593.1525	300.9981, 285.0388	Nicotiflorin
33	23.75	266, 341	447.0903	285.0282, 171.0058	Luteolin 7- O -glucoside
34	24.60		327.2097	215.1185, 211.1227	Trihydroxy-octadecadienoic acid

HHDP = hexahydroxydiphenic acid, ^[m-Na] = [M-Na]⁻, sh = shoulder

3.3. Phenolic compounds quantification and antioxidant capacity

It was deduced from the phytochemical profiling (Table 1) that flavonoids are majorly present in the aqueous leaf extract of *A. johnsonii*. Flavonoids (flavanol and flavonol) and alkaloids were quantified, and the results are shown in Table 2. In the total polyphenol content (403.28 ± 12.75 mg GAE/g), 88.35 ± 2.16 mg CE/g consisted of flavanols and 6.96 ± 3.10 mg QE/g flavonol. No alkaloids were identified in the extract. Antioxidant capacity was determined by measuring DPPH, FRAP, and TEAC values, and the results are depicted in Table 2. The plant extract showed an overall high antioxidant capacity. The extract showed a higher FRAP value (1342.68 ± 3.41 mg AAE/g) compared DPPH (571.57 ± 0.55 mg TE/g) and TEAC (478.88±0.09 mg TE/g).

Table 2: Antioxidant capacity values and phenolic compound quantities in *A. johnsonii* aqueous leaf extract.

Phenolic compound/antioxidant capacity	Presence (+/-)	Quantity/value
Phenolic compounds		
Flavonols (mg QE/g)	+	6.96 ± 3.10
Flavanols (mg CE/g)	+	88.35 ± 2.16
Alkaloids (mg AE/g)	-	0
Total polyphenols (mg GAE/g)	+	403.28 ± 12.75
Antioxidant capacity		
DPPH (mg TE/g)	+	571.57 ± 0.55
FRAP (mg AAE/g)	+	1342.68 ± 3.41
TEAC (mg TE/g)	+	478.88±0.09

3.4. Anti-inflammatory activity of *A. johnsonii*

Anti-inflammatory activity of the aqueous extract of *A. johnsonii* leaves was investigated on RAW macrophage cells and depicted in Figure 3. The relative excessive production of nitrite represented increased inflammation in the macrophage cells. The negative control cells with no induction of inflammation by LPS showed a low production of nitrite (approximately 10 µM). All cell groups with induced inflammation through the administration of LPS (both treated and untreated) showed a significant ($P < 0.05$) increase in the production of nitrite (Figure 3A). The administration of LPS with no extract or drug treatment caused a significant ($P < 0.05$) increase in the nitrite concentration to 40 µM. The treatment of the inflammation-induced macrophage cells with AG (a control drug) showed a significant ($P < 0.05$) reduction of nitrite concentration compared to the untreated inflammation-induced cells (a difference of approximately 15 µM). The nitrite-reducing capacity of the extract was concentration-dependent, with the lower concentrations showing a non-significant ($P > 0.05$) difference amongst each other and a

significant ($P < 0.05$) decrease in nitrite concentration (50 and 100 $\mu\text{g/mL}$) compared to the untreated induced cells. The highest concentration (200 $\mu\text{g/mL}$) of the extract investigated significantly ($P < 0.05$) reduced the nitrite concentration to a concentration comparable to the treated cells. As the production of nitrite is dependent on the cell viability, the MTT assay was conducted, and the results are represented in Figure 3B. Cells induced with LPS showed a significant ($P < 0.05$) decrease in cell viability. Although there was no significant difference in cell viability between cells treated with 100 $\mu\text{g/mL}$ and those treated with 200 $\mu\text{g/mL}$, there was a significant decrease in nitrite production with 100 $\mu\text{g/mL}$ extract treatment compared to 200 $\mu\text{g/mL}$ extract treatment.

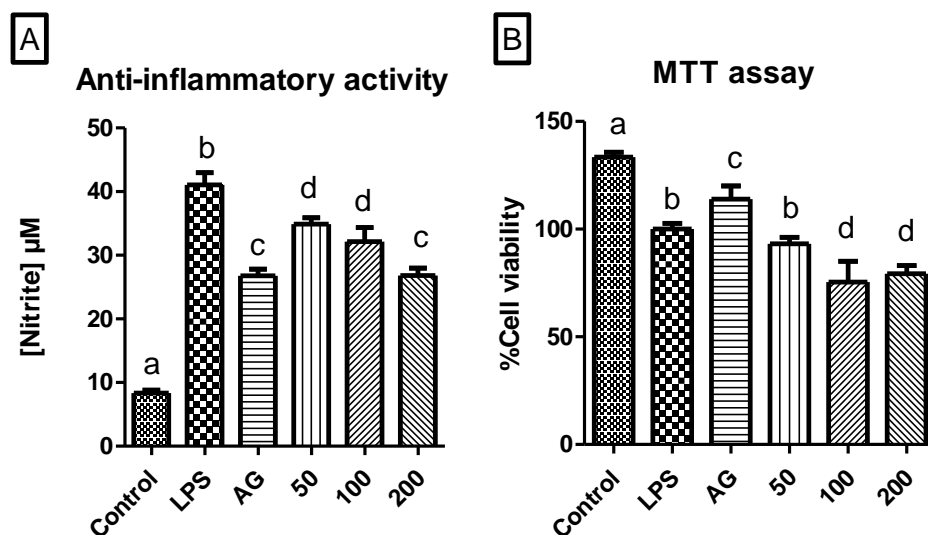


Figure 3: The effect of *A. johnsonii* on the concentration of nitrite in LPS-activated macrophages after 24 hours of exposure to treatments. Aminoguanidine (AG) was used as a control drug. B. Cell viability (%) of LPS-activated macrophages after 24 h of exposure to treatments. The bar graph is a result of the mean of values in quadruplicate with error bars representing standard deviation. Data was analysed using One-way ANOVA and Tukey post-test. The letters on top of the bars indicate significance, and significant difference is shown by different letters ($P < 0.05$).

3.5. The hypoglycaemic effect of *A. johnsonii*

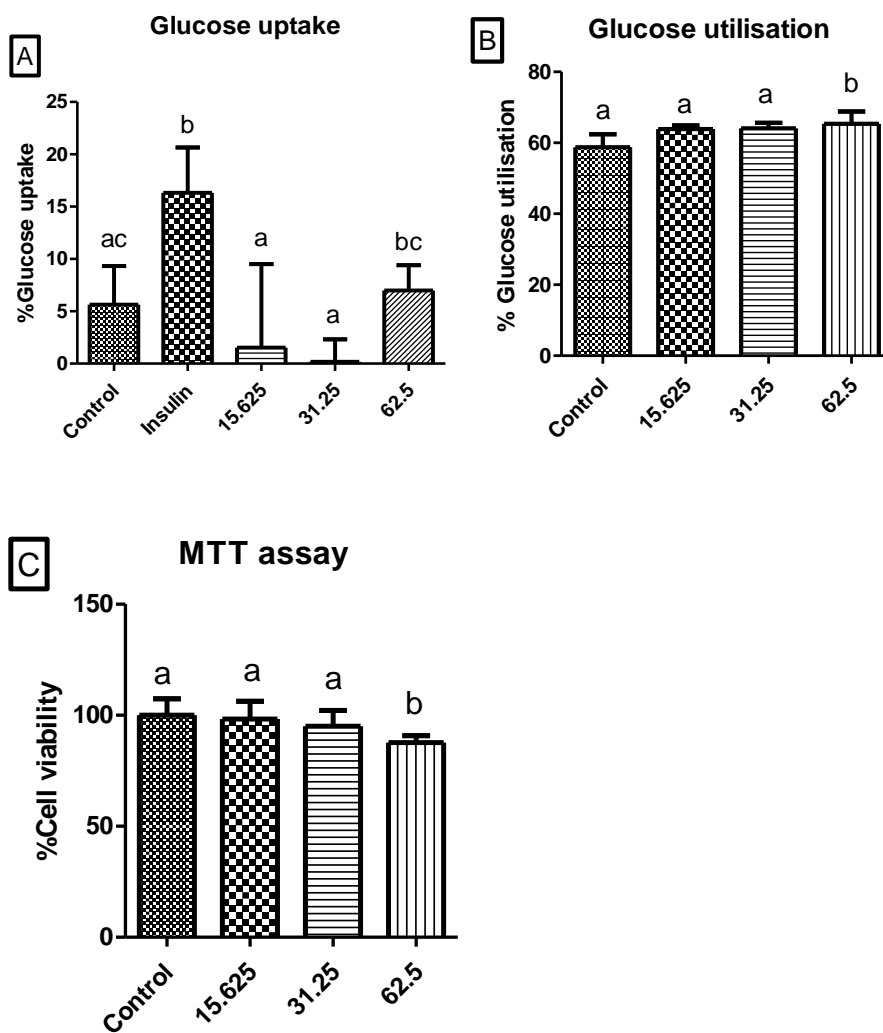
3.5.1. Glucose utilisation and intake

The effect of the *A. johnsonii* aqueous leaf extract on glucose and lipid metabolism was investigated through the determination of glucose uptake and utilisation, α -glucosidase inhibition, and lipase inhibition respectively. The results are depicted in Figure 4. Figure 4A shows the results of glucose uptake amongst the groups. There was a significant ($P < 0.05$) increase in glucose uptake following insulin treatment, compared to the control cells. However, there was a significant ($P < 0.05$) increase in glucose uptake after treatment with the highest concentration (62.5 $\mu\text{g/mL}$) of the extract compared to the lower extract concentrations (15.625 and 31.25 $\mu\text{g/mL}$), and the normal untreated cells (Figure 4A). Glucose uptake facilitated by the highest concentration of the leaf extract was comparable to insulin-induced glucose uptake. As depicted in Figure B, there was no significant ($P > 0.05$) difference observed between the lowest concentrations (15.625 and 31.25 $\mu\text{g/mL}$) of the extract investigated. However, the highest concentration (62.5 $\mu\text{g/mL}$) of the extract caused a significant ($P < 0.05$) increase in glucose utilisation compared to the control (insulin) and the lower concentrations of the plant. Although the MTT assay (Figure 4C) showed a slight significant ($P < 0.05$) decrease in cell viability in cells treated with 62.5 $\mu\text{g/mL}$ concentration of the extract, both glucose intake and glucose utilisation results were not affected by cell viability.

3.5.2. Pancreatic lipase and α -glucosidase inhibition

Pancreatic lipase inhibition results are represented in Figure 4D. The inhibitory activity of the different concentrations (31.25, 62.5, 125, 250, 500 $\mu\text{g/mL}$) of the extract was compared to that of 100 μM of orlistat. The control drug (orlistat) showed approximately 65% inhibition of pancreatic lipase activity. There was no significant ($P > 0.05$) difference in pancreatic lipase inhibition amongst the different concentrations of the extract. There was a significantly ($P < 0.05$) lower inhibitory activity against the pancreatic lipase with the extract treatment concentrations compared to the control. The inhibitory activity of the extract against α -glucosidase was compared to the standard drug, acarbose, and the results are shown in Figure 4E. There was a concentration-dependent increase in the inhibitory activity of both the

extract and acarbose. However, the inhibitory activity of the extract against α -glucosidase increased to the highest inhibition of 97.54%, while the inhibitory activity of acarbose only increased to 41.82%.



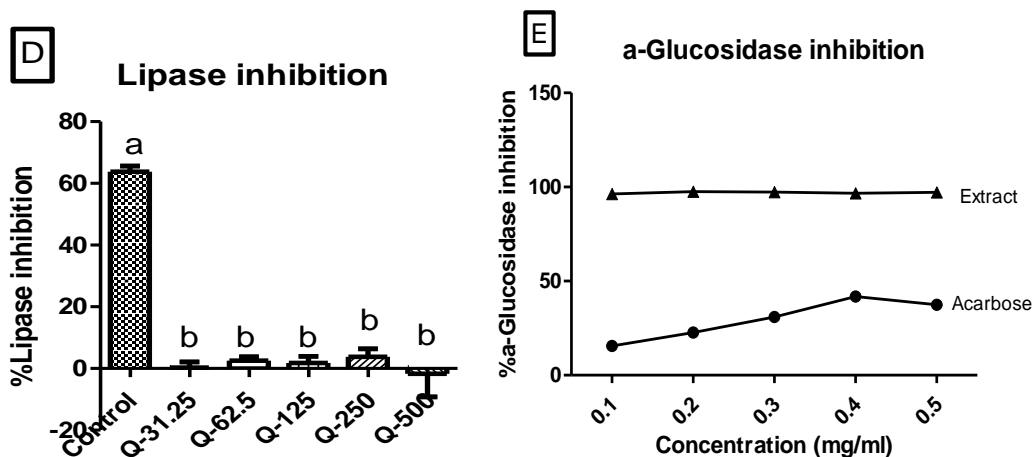


Figure 4. The effect of different concentrations of *A. johnsonii* aqueous leaf extract on glucose and lipid metabolism. A. Glucose utilization (%) compared to insulin, in C3A cells after 24 hours of treatment with different concentrations of the extract (in $\mu\text{g/mL}$ unless stated otherwise). B. Glucose uptake (%) compared to insulin after 4 h of pre-treatment, in C3A cells. C. Glucose uptake and utilisation results were normalized to cell viability as determined using the MTT assay. D. Pancreatic lipase inhibition of the extract compared to orlistat (100 μM). E. The effect of the extract on α -glucosidase inhibition was compared to acarbose as a positive control. The bar graph is a result of the mean of values in quadruplicate with error bars representing standard deviation. Data was analysed using One-way ANOVA and Tukey post-test. The letters on top of the bars indicate significance, and significant difference is shown by different letters ($P < 0.05$).

4. Discussion

Medicinal plants exert their effects in the treatment of disease through the activity of the constituent bioactive compounds (Dar et al., 2023). The resulting pharmacological effect of medicinal plants is dependent on the type of phytochemical compounds in the plant (Süntar, 2020). This study revealed and focussed on the presence of phenolic compounds in the leaves of *A. johnsonii*, and their role in potentially reducing hyperglycaemic, oxidative stress, and inflammation. Oxidative stress plays a crucial role in the development of DM complications (Unuofin & Lebelo, 2020). In previous studies, diabetic models, characterised by hyperglycaemia showed the excessive occurrence of ROS and consequent organ damage

(Abukhalil et al., 2021; Dong et al., 2022). Diabetic models have also shown the compromised antioxidant system caused by glucotoxicity (Rahmani et al., 2023). It is therefore a necessity to increase antioxidant capacity, in a hyperglycaemic state, to reduce oxidative stress. Phenolic compounds are bioactive compounds commonly known as free radical scavengers (Esmailzadeh Kenari & Razavi, 2022). The quantification of phenolic compounds in the leaves of *A. johnsonii* showed both the availability and abundance of polyphenols, specifically flavonoids.

Flavonoids are naturally occurring compounds found in fruits and vegetables and are known for their beneficial effect in ameliorating oxidative stress (Shen et al., 2022). Quantified flavonols were further confirmed by the HPLC-MS detection of quercetin, a type of flavonol. Flavonols upregulate the antioxidant system by increasing the activation of antioxidant enzymes (catalase, superoxide dismutase, glutathione peroxidase, etc.) and upregulation of the expression of other non-enzymatic antioxidants (Shen et al., 2022). The present study has revealed the presence of phenolic acids (hydroxybenzoic and hydroxycinnamic acids) such as quinic acid, ferulic acid, gallic acid, and gallonylquinic acid. The detection of hydroxycinnamic acid, and ferulic acid, revealed the antioxidant capacity of *A. johnsonii* leaves. Ferulic acids are phenolic compounds with the reported effect of reacting with DPPH and FRAP radicals, thereby reducing them (Yang et al., 2021). The observed DPPH and FRAP scavenging capacity of the plant extract can therefore be linked to the presence of ferulic acid. Although ferulic acid is abundant in daily diet, its post-prandial bioavailability is insufficient (Mancuso et al., 2021). The aqueous leaf extract of *A. johnsonii* is therefore a necessary supplement in humans, especially in those with increased free radicals. The moiety and structure of gallic acid, with three phenolic hydroxyl groups, contribute to its antioxidant effect (Yang et al., 2021). Although a previous study has concluded that quinic acid lacks antioxidant capacity due to its stable cyclohexane structure and a missing aromatic ring, it has also revealed that quinic acid reduces lipid peroxidation in small amounts (Ercan & Dođru, 2022). The antioxidant

capacity of the leaf extract of *A. johnsonii* can therefore be possibly alluded to a synergistic effect of the different phenolic acids present.

Besides oxidative stress, diabetic complications are aggravated by the excessive accumulation of proinflammatory markers (Guo et al., 2022). For instance, complications such as nephrotic failure in diabetic patients can be detected through the increase in systemic immune-inflammation index (Guo et al., 2022). This finding suggests the occurrence and contribution of inflammation in the development of DM. Therefore, the reduction of proinflammatory cytokines plays a role in the management of diabetic complications (Guan et al., 2021). The process of inflammation is initiated by the activation of NF- κ B kinases as a response to an infection or tissue damage (Bai et al., 2021). The phosphorylation of the I κ B kinases leads to the breakdown of NF- κ B inhibitory proteins, allowing NF- κ B to attach to the nucleus and initiate the expression of proinflammatory cytokines (Bai et al., 2021). Glucotoxicity caused by hyperglycaemia leads to the activation of the immune system through the increased transcription of the NF- κ B gene and downstream production and secretion of proinflammatory cytokines (Li et al., 2020). The excessive secretion of proinflammatory cytokines and inflammatory cell infiltration exacerbate insulin resistance in diabetic individuals, further increasing blood glucose levels (Zhao et al., 2024).

Besides other cytokines, the increase in the concentration of nitrite is an indicator of inflammation. In the present study, inflammation was induced by LPS, and observed by the increase in the level of nitrite as an indicator of nitric oxide production. The induction of inflammation by LPS activation caused a significant ($P < 0.05$) decrease in cell viability. However, the type and concentration of treatment played a role in cell viability. Although inflammation is one of the factors that can lead to reduced cell viability, the reduction of nitrite after extract treatment was not necessarily caused by reduced cell viability. This is observed by the significant ($P < 0.05$) change in nitrite concentration between 100 μ g/mL and 200 μ g/mL concentrations of extract regardless of the non-significant ($P > 0.05$) difference in cell viability between the two concentrations. Treatment with the *A. johnsonii* aqueous leaf extract reduced

the concentration of nitrite produced by the macrophage cells. Although the anti-inflammatory properties of *A. johnsonii* are not well-studied, individual phytochemical compounds detected in the leaf extract have been reported for their crucial role in amelioration of inflammation. Ferulic acid is associated with a significant reduction of pro-inflammatory cytokines such as IL-6 and TNF- α (Liu et al., 2022). This effect can be linked to the observed anti-inflammatory effect of the leaf extract of *A. johnsonii*. The anti-inflammatory effect of the extract can be linked to the presence of gallotannins, which were previously recorded for their anti-inflammatory activity through the upregulation of the AMPK pathway (Kim et al., 2022), and the downregulation of the NF- κ B pathway (Chicas et al., 2020) which leads to the reduction of pro-inflammatory cytokines. The HPLC-MS profile detected the presence of gallic acid. Gallic acid exerts its anti-inflammatory properties by inhibiting the phosphorylation of I κ B kinases, thereby blocking the expression of cytokines through the downregulation of NF- κ B (Bai et al., 2021).

The role of obesity in the pathogenesis of DM is crucial. Lipid accumulation leads to insulin resistance and consequent hyperglycaemia. On the other hand, DM is a metabolic disease, and its pathogenesis involves impairment in lipid metabolism, which involves the excessive release of fatty acids. Previous studies have revealed the role of lipase inhibition in the management of both obesity and DM (Liu et al., 2020). In our present study, no lipase inhibitory activity was observed with the extract. However, some phytochemicals present in the leaf extract of *A. johnsonii* (flavonol, kaempferol, and quercetin) ameliorate dyslipidaemia (Jubaidi et al., 2021), by decreasing the level of triglycerides and low-density lipoprotein cholesterol and increasing high-density lipoprotein cholesterol (Abukhalil et al., 2021). It is therefore paramount to understand that although the extract shows no lipase inhibitory activity, its benefit in the reduction of hyperlipidaemia through possible alternative mechanisms cannot be ignored. The HPLC-MS profiling revealed the presence of anthocyanidins such as procyanidin B1 and B11. Besides their antioxidant properties, anthocyanins reduce the

accumulation of lipids by decomposing cholesterol (Shen et al., 2022). This effect can reduce obesity and insulin resistance thereby alleviating hyperglycaemia.

The pathogenesis of DM arises from hyperglycaemia as a main characteristic (Galicía-García et al., 2020). Both inflammation and oxidative stress are aggravated by glucotoxicity caused by the production of AGEs (Passarelli & Machado, 2021). The reduction of blood glucose is therefore a research interest in DM management. Blood glucose levels are controlled by the production and release of glucose into the blood, and glucose uptake by the cells (Norton et al., 2022). An increase in the activity of α -glucosidase ultimately leads to the rapid breakdown of carbohydrates and a release of excessive glucose molecules into the blood (Lawal et al., 2022). Similarly, the low uptake of glucose, mainly caused by insulin resistance leads to triglyceride breakdown and hyperglycaemia (Kalra et al., 2021). The hypoglycaemic effect of *A. johnsonii* was observed through the evident inhibition of α -glucosidase.

Flavonoids improve glucose transporter expression through the activation of the insulin pathway factors such as PI3K/Akt which leads to a reduction in blood glucose (Manavi et al., 2021). Although the glucose uptake effect of the extract is comparable to that of insulin, it is arguable that the synergistic effect of the extract with insulin in vivo could result in an increased glucose uptake. It has also been reported that procyanidins function similarly to insulin in the process of glucose intake (Manavi et al., 2021). The presence of different procyanidins in the extract could have possibly played a role in both glucose intake and utilisation, leading to a similar effect caused by insulin. Therefore, *A. johnsonii* extract is a possible alternative in the amelioration of insulin resistance.

5. Conclusion

Scientific focus on medicinal plant research aims to find more effective and affordable treatments for medical complications. In this current study, the aqueous leaf extract of *A. johnsonii* was found to cause a concentration-dependent reduction in inflammation and exhibit

possible antioxidative effects as observed with antioxidant capacity values. The extract also showed very high inhibitory activity against α -glucosidase compared to the standard drug (acarbose). The hypoglycaemic, anti-inflammatory, and antioxidative effects observed in this study were linked to the phenolic compounds identified, with an abundance of flavonoids. The observed effect of the plant extract is dependent on the concentration, with very high concentrations showing cytotoxicity. It is therefore paramount to find the suitable concentration that is highly effective with lower cytotoxicity. These findings therefore serve as a contribution towards further investigation of *A. johnsonii* as a possible pharmacotherapeutic medicinal plant in the treatment of DM.

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CHAPTER SIX

The effect of *Garcinia livingstonei* aqueous leaf extract on hyperglycaemic-induced human sperm cell: An in-vitro study

Murendeni Nethengwe^a, Kunle Okaiyeto^a, Chinyerum S. Opuwari^b, Oluwafemi O. Oguntibeju^a.

^aPhytomedicine and Phytochemistry Group, Department of Biomedical Sciences, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, Bellville 7535, South Africa.

^bDepartment of Medical Biosciences, University of the Western Cape, Bellville, South Africa.

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Abstract

Hyperglycaemia is a contributing factor in the development of male infertility in men with diabetes mellitus (DM). The consequent production of excessive reactive oxidative species (ROS) and a compromised antioxidant defence system leads to testicular damage and the disturbance in spermatogenesis and maturation. Supplementation with antioxidants sourced from medicinal plants is beneficial in the treatment of DM-related male infertility. The current study investigated the effect of aqueous leaf extract of *Garcinia livingstonei* on hyperglycaemic human male sperm cell parameters. Hyperglycaemia was induced using sperm media supplemented with 100 mM D-glucose over 24 h. Hyperglycaemia induction led to a significant ($P < 0.05$) decline in sperm motility, vitality, DNA integrity, mitochondrial membrane potential (MMP), and acrosome integrity. The effect of the plant extract on spermatozoa was concentration-dependent, with the lowest concentration demonstrating the highest protective effects. A significant ($P < 0.05$) improvement in all tested sperm parameters was observed after exposure of the hyperglycaemic spermatozoa to aqueous leaf extract of *G. livingstonei*. The

effect of acarbose on all tested sperm parameters was comparable to that of the plant extract. The findings of this study suggest the potential therapeutic effect of *G. livingstonei* in the treatment of DM-related male infertility. However, the use of an appropriate dosage depending on the pathological target is paramount.

Keywords: *Garcinia livingstonei*, hyperglycaemia, male infertility, medicinal plants, oxidative stress.

1. Introduction

Male infertility is defined as when a male of child-bearing age is unable to impregnate a fertile female after 12 months of intended unprotected sexual intercourse (Agarwal et al., 2021). The World Health Organisation (WHO) has reported that between 15%-20% of couples are affected by infertility (Chhikara et al., 2023). The prevalence of male infertility has risen to approximately 50% of the total infertility cases (Liu et al., 2023). Amongst other factors such as genetics, trauma, and lifestyle choices (Okonofua et al., 2022), diabetes *mellitus* (DM) is another causative factor associated with the development of male reproductive dysfunction (Barkabi-Zanjani et al., 2020). DM is characterised by prolonged hyperglycaemia and consequent oxidative stress that leads to tissue damage and complications such as cardiovascular disease, kidney disease, and male infertility (Andreadi et al., 2022; Pang et al., 2020; Winiarska et al., 2021). The prevalence of DM was reported to be 10.5% (536.6 million people) of the global population of ages 20-79 years old and expected to rise to 12.2% by 2045 (Sun et al., 2022). Approximately 35% of DM- affected individuals are reported to be infertile (Zhu et al., 2023). Hyperglycaemia is linked to the decline in sperm parameters such as sperm motility, sperm vitality, mitochondrial membrane potential, and the fragmentation of sperm DNA, which leads to male infertility (Aksu et al., 2021; Papadopoulou et al., 2022). Male infertility is associated with socio-economic issues and depression in men (Njagi et al., 2020; Öztekin et al., 2019). The investigation of effective medication for the treatment of male infertility, with consideration of the causative factor, is necessary.

Hyperglycaemia is the main target in the treatment of diabetic complications (Taylor et al., 2021). Prolonged hyperglycaemia leads to excessive production of ROS through the mitochondria (Kaludercic & Di Lisa, 2020). Although the optimal production of ROS in the reproductive system is required in spermatogenesis and sperm maturation (Baskaran et al., 2021), excessive production of ROS in the testes is detrimental and can lead to male infertility (Takehima et al., 2021). The antioxidant system maintains the redox status by neutralising the oxidative effect of free radicals, through the synthesis of enzymatic and non-enzymatic antioxidants (Chaudhary et al., 2023). Medicinal plants are a source of antioxidant bioactive compounds (Dar et al., 2023). Traditional medicine has recorded several plants used in the treatment of DM and male infertility (Teixeira et al., 2024). However, the study of the existing medicinal plants on animal sperm cells is limited.

Garcinia livingstonei is a tall evergreen plant known in Limpopo for its benefit in the treatment of DM and its underlying complications (Ramadwa & Meddows-Taylor, 2023). The phytochemical analysis of *G. livingstonei* reveals the presence of phenolic compounds such as flavonoids, hydroxycinnamic acids, and benzophenones associated with antioxidant, anti-inflammatory, and hypoglycaemic effects linked to its benefit in the treatment of DM complications (Nethengwe et al., 2024). Although the hypoglycaemic effect of *G. livingstonei* has been reported as evidence for the potential anti-diabetic benefit of the extract, its direct effect on the hyperglycaemic sperm parameters has not been well uncovered. This study established the cellular hyperglycaemia model of the human sperm cell and investigated the effect of the aqueous leaf extract of *G. livingstonei* on sperm parameters of hyperglycaemia-induced human sperm cells.

2. Methods and materials

2.1. Plant harvest and extraction

The leaves of *G. livingstonei* were harvested from the Brackenridgea Nature Reserve at Thengwe village in Limpopo, South Africa. A sample of the plant was harvested and sent to the University of Venda for authentication. The process of authentication was carried out by Prof Tshisikhawe. The plant specimen was kept at the herbarium of the University of Venda, and a voucher was assigned (Voucher number: MNU002/10/22).

The leaves of the *G. livingstonei* were washed and left to dry in a shade for 5 days. The leaves were then hand-crushed before blending using a blender, to obtain a powder form. The powder was weighed to 100 g and mixed with 1000 mL to prepare an aqueous extract. The mixture was left overnight (24 hours) to allow extraction to occur. The liquid from the mixture was extracted and the plant material was discarded. The extract was filtered using a filter paper after which it was freeze-dried. A total yield of 35.48 g of the extract was stored at -20 °C.

2.2. Participants selection criteria

Ethical clearance for this study was obtained from Cape Peninsula University of Technology Research Ethics Committee (CPUT-REC) (Reference number: CPUT/HWS-REC 2024/H9), and the Biomedical Research Ethics Committee (BMREC) of the University of Western Cape (reference number: BM22/10/24). Male participants (n=25) between the ages of 18-40 were recruited, following ethical procedures, to voluntarily join the study. Participants with chronic diseases were excluded from the study. Baseline sperm parameters were assessed as a screening procedure, and all participants who did not meet the World Health Organisation (WHO) criteria were excluded from the study.

2.3. Collection of samples

Semen samples were collected through masturbation after 3-5 days of abstinence and incubated at 37 °C for liquefaction after the participants had agreed to join the study and had

signed the consent form. Baseline sperm parameters were then assessed using the Sperm Class Analyser to classify sperm for selection. Semen samples were classified according to the WHO criteria where normozoospermic sperm had sperm concentration >15 million/ml, sperm count >39 million/ejaculate, and sperm motility >40%. After liquefaction, 3 μ L of the sample was loaded into a Leja chamber slide, and all baseline parameters were analysed.

2.4. Hyperglycaemia induction

Sperm cells were washed and resuspended with 2-3 mL of sperm wash to achieve the desired concentration and incubated for 3 hours for capacitation. A volume of 5 mL of the induction media (0.9 mM CaCl₂, 0.5 mM MgCl₂, 10 mM sodium lactate, 1 mM sodium pyruvate, 0.3% w/v bovine serum albumin (BSA), 1% v/v penicillin/streptomycin, 5 mM D-glucose (for optimal concentration), and 100 mM D-glucose (for hyperglycaemic concentration)) was added into the cells to a final concentration of 10 mil/mL and incubated for 24 h. Samples in media were centrifuged and washed using a sperm wash before exposure to different treatments (Portela et al., 2015).

2.5. Treatment

The plant extract and acarbose were both prepared in HTF-BSA. To prepare different concentrations of the plant extract, a stock solution (1 mg/ml) was prepared. A 10-times serial dilution of the stock solution (1 mg/mL) was done to prepare 10 different concentrations that were tested on sperm cells to assess motility. Four concentrations (0.001, 0.01, 0.1, and 1 μ g/mL) of the extract were then selected for the study. Acarbose was prepared at a concentration of 100 μ g/mL). After 24 hours of hyperglycaemia induction, 100 μ L aliquots were centrifuged, and the supernatant was discarded. A volume of 100 μ L of the different treatments was added, with normal and hyperglycaemic control cells treated with HTF-BSA, and the

acarbose group treated with acarbose. Treated samples were incubated at 37 °C for 1 h, then centrifuged for 10 minutes at 300x g.

2.6. Total motility

Total motility was assessed after induction of hyperglycaemia and exposure to the different concentrations of the aqueous leaf extract of *G. livingstonei*. A volume of 3 µL of each sample was loaded into each chamber of the 8-chamber Leja slide. The slide was analysed under a microscope using computer-assisted semen analysis (CASA), with a total of 200 sperm cells analysed.

2.7. Vitality

Sperm vitality was determined using the eosin-nigrosin dye technique. The sperm sample and eosin-nigrosin dye (Sigma Aldrich, St Louis, MO) were mixed at a 1:1 ratio and incubated at 37°C for 15 min. The sample mixture was smeared on a slide and allowed to air dry. The slide was viewed under a light microscope (Zeiss, Oberkochen, Germany) using the oil immersion objective (100×). At least 200 sperm cells were analysed with white cells appearing white, and dead cells appearing pink (Shalaweh et al., 2015).

2.8. Mitochondrial membrane potential

A mitochondrial staining kit (Sigma-Aldrich Inc., St Louis, MO, USA). was used to assess mitochondrial membrane potential according to the manufacturer's instructions. After treatment, the cell suspension (100 µL) was stained by the MMP staining solution (80 µL of distilled water, 20 µL of JC-5 buffer, and 0.5 µL of frozen MMP 200x stock solution) at a 1:1 ratio and incubated at 37 °C for 20 min in the dark. After incubation, the samples were centrifuged at 500x g and 5-7 °C for 5 min and the supernatant was discarded. The cell

suspension was resuspended with 100 μL of cool JC-1 buffer prepared by 80 distilled water and 20 μL JC-5 buffer and centrifuged again at 500x g and 5-7 $^{\circ}\text{C}$ for 5 minutes. The supernatant was discarded, and the cells were resuspended with more than 100 μL of the JC-1 buffer. A volume of 5-10 μL of the suspension was placed on a frosted slide and covered by a cover slip. The slide was analysed in the dark under a Nikon Eclipse 50i fluorescence microscope (Zeiss, Oberkochen, Germany). Assessment of at least 200 sperm cells was done and a percentage of sperm cells with intact mitochondria was recorded, with green-stained sperm cells considered to have non-intact mitochondria and red-stained sperm cells with intact mitochondria.

2.9. DNA fragmentation

DNA fragmentation was assessed following the protocol of the GoldCyto DNA kit (Goldcyto Biotech Corp, Guangzhou, China). After the exposure of sperm cells to the different treatments, cell suspensions were resuspended with HTF-BSA to a final concentration of 10 mil/mL. A volume of 30 μL of the sample was added into a pre-heated (90 $^{\circ}\text{C}$ -100 $^{\circ}\text{C}$ for 5 minutes followed by 37 $^{\circ}\text{C}$ for 5 minutes) agarose Eppendorf. A volume of 20 μL of the sample in the agarose gel was placed on a clean slide and covered with a cover slip. The slide was left in the fridge at 4 $^{\circ}\text{C}$ for 5 min and the cover was gently removed. The slide was incubated horizontally in different solutions (acid denaturation solution for 7 min, lysis solution for 25 min, distilled water for 5 min, 70% ethanol for 2 min, and 90 % ethanol for 2 minutes) consecutively at room temperature. The slide was left to dry at room temperature and stored in the dark until staining. To stain, the slide was placed in solution A for 1 minute, then solution B for 2 min, then washed gently in tap water and left to dry at room temperature. The slide was viewed under a fluorescent microscope (Zeiss, Oberkochen, Germany) with 488 nm and 510–530 nm excitation and emission filters, respectively with an oil immersion objective (400 \times), scoring 200 sperm cells for analysis. Sperm cells with no halo, or with a halo similar or smaller than 1/3 of the minor diameter of the middle part of the sperm head (core) were considered fragmented.

Sperm cells with a halo higher than 1/3 of the minor diameter of the core were considered non-fragmented.

2.10. Capacitation and acrosome reaction

The chlorotetracyclin (CTC) fluorescence assay was conducted following the methods of Green et al., 1996), to assess acrosome reaction and capacitation. The sperm cells were resuspended in HTF-BSA after treatment, and 1 μ L of Hoechst 32258 was added. The samples were incubated for 2 min in Hoechst 32258 after which 400 μ L of 2 polyvinylpyrrolidone (PVP40) was added, and centrifuged for 5 minutes at 900x g and 37 °C. After the supernatant was discarded, 45 μ L of 750 μ M CTC solution (130 mM NaCl, 5 mM cysteine, 20 mM Tris-HCl, pH 7.8) and 8 μ L of Tris-HCl solution (12.5% w/v paraformaldehyde, 0.5 M Tris-HCl, pH 7.4) were added into the cell suspension. A volume of 10 μ L was placed on a frosted slide, covered with a cover slip, and analysed under a Nikon eclipse 50i fluorescence microscope (Zeiss, Oberkochen, Germany) At least 200 sperm cells were analysed and categorized into three patterns. Non-capacitated, acrosome-intact sperm (F-pattern) were represented by sperm cells with fluorescence over the entire head. Capacitated, acrosome-intact sperm (B-pattern) were represented by sperm cells with fluorescence over the sperm head excluding the post-acrosomal region. Sperm cells with no fluorescence over the head were categorized as capacitated, acrosome-reacted sperm cells (AR pattern) (Shalaweh et al., 2015).

2.11. Reactive oxidative species

ROS were determined through the dihydroethidine (DHE) staining assay following the methods of Shalaweh et al. (2015). DHE stock solution (20 μ M) was prepared in PBS. After the treatment of sperm cells, the cell suspension was resuspended with 20 μ L of DHE solution and 180 μ L of PBS and incubated for 15-20 min at 37 °C. After incubation, 10 μ L of the sample was placed on a clean frosted slide, covered with a cover slip, and analysed under a Nikon

eclipse 50i fluorescence microscope (Zeiss, Oberkochen, Germany) with oil immersion. A total of 200 sperm cells were analysed, with red-stained sperm cells indicating excessive production of ROS and non-stained sperm cells indicating very little or no ROS production (Shalaweh et al., 2015).

2.12. Superoxide dismutase activity

Superoxide dismutase (SOD) activity in the plasma of the spermatozoa was determined following the methods of Brannan and colleagues, (1981). A 6-hydroxydopamine hydrobromide (6-OHD) solution (50 µl of perchloric acid, 10 ml of distilled water, and 4 mg of 6-OHD) was freshly prepared. Diethylene triamine pentaacetic acid (DETAPAC) was prepared by adding 2 mg of DETAPAC to 50 ml of the 100 mM phosphate buffered saline (PBS) buffer. A volume of 10 µl of each sample and blank was loaded into the wells in triplicates and 15 µl of 6-OHD was added to each well. DETAPAC (170 µl) was added into the wells and the plate was read immediately at 490 nm in an Omega Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA). SOD activity was estimated by calculating the 50% inhibition of auto-oxidation 6-OHD by superoxide free radical.

2.13. Catalase antioxidant activity

Catalase activity in the plasma of sperm cells was measured using the procedure followed by Brannan and colleagues, (1981). An H₂O₂ solution was prepared by adding 50 µl of H₂O₂ to 10 ml of 100 mM phosphate buffered saline (PBS) buffer. The sample and blank were loaded into wells of a 96-well UV Greiner plate. A volume of 170 µl of PBS was added to each well, after which the H₂O₂ reagent (75 µl) was added. The plate was read immediately in an Omega Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 232nm.

2.14. Protein content determination

A modified version of the protocol followed by Kummari and colleagues, (2022) was used to determine total protein content. The Bradford reagent was used to determine protein concentration in the plasma obtained from the spermatozoa. A protein standard dilution series (0.25, 0.5, 1, 1.4, and 2 mg/ml) was prepared 1% SDS and 2 mg/ml stock solution of bovine serum albumin (BSA) protein, as the standard protein. A volume of 5 µl of the blank, standard concentrations and the samples was loaded in the wells of a 96-well plate. The Bradford reagent (250 µl) was then added to the wells. The plate was incubated in the dark for 30 min after a gentle shake. The plate was read using an Omega Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 595 nm (Kummari et al., 2022).

2.15. Statistical analysis

A two-tailed paired T-test (and nonparametric tests) was performed for statistical analysis was performed using GraphPad Prism version 5. The mean points were represented by a column mean with vertical error bars. Letters were placed on top of mean points to denote significance (CI intervals excluding zero), with different letters in different points representing significant differences between the points. Significance was determined using confidence intervals of mean differences. Confidence intervals that do not include zero represented significant differences between groups. Statistical analysis was done using GraphPad Prism version 5.

3. Results

3.1. Reactive oxidative species production

The antioxidant effect of *G. livingstonei* aqueous leaf extract on hyperglycaemia-induced human spermatozoa is depicted in Figure 1A and Table 1. A significant (MD: -16.22; CI: -24.10, -8.340) increase in ROS was observed in untreated hyperglycaemic sperm cells compared to control sperm cells. The level of ROS was significantly reduced after the treatment of the sperm cells with 0.001 µg/mL (MD: 27.62; 95% CI: 19.74, 35.50), 0.01 µg/mL

(MD: 21.72; 95% CI: 13.84, 29.60), and 0.1 µg/mL (MD: 13.30; 95% CI: 5.420, 21.18) of the extract compared to the untreated hyperglycaemic sperm cells. However, sperm cells treated with the highest concentration (1 µg/mL) of the extract showed no significant (MD: 5.50; 95% CI: -2.380, 13.38) reduction in ROS compared to the untreated hyperglycaemic sperm cells. Treatment of the hyperglycaemic sperm cells with acarbose significantly (MD: 16.38; 95% CI: 8.500, 24.26) reduced the level of ROS compared to the untreated hyperglycaemic cells. The level of ROS in sperm cells treated with acarbose was significantly (MD: -11.22; 95% CI: -19.12, -3.360) higher compared to the level of ROS in sperm cells treated with the lowest concentration of the plant extract.

3.2. Mitochondrial membrane potential

The mitochondrial membrane potential (MMP) of sperm cells before and after induction of hyperglycaemia, and after administration of the aqueous leaf extract of *G. livingstonei*, is depicted in Figure 1B and Table 1. The number of mitochondria-intact sperm cells was significantly (MD: 10.50; 95% CI: 1.778, 19.22) reduced following hyperglycaemia induction, compared to the control sperm cells. A significant increase in the number of sperm cells with intact mitochondria was observed after the treatment of the sperm cells with 0.001 (MD: -17.68; 95% CI: -26.40, -8.958), 0.01 (MD: -15.96; 95% CI: -24.68, -7.238) and 0.1 µg/mL (MD: -9.48; 95% CI: -18.20, -0.7575) concentrations of the aqueous leaf extract of *G. livingstonei*. The percentage of mitochondria-intact sperm cells treated with the highest tested concentration (1 µg/mL) of the extract was not significantly (MD: -2.32; 95% CI: -11.04, 6.402) different compared to that of untreated hyperglycaemic sperm cells. Treatment of hyperglycaemic sperm cells with acarbose also caused a significant (MD: -10.06; 95% CI: -18.78, -1.338) increase in the number of sperm cells with intact mitochondria.

Table 1: Representation of the mean differences (MD) between different treatment groups depicting the effect of hyperglycaemia on the production of reactive oxidative species (ROS) and mitochondrial membrane potential (MMP), and the effect of different concentrations 0.001, 0.01, 0.1, and 1 µg/mL of the plant, and acarbose. Negative MD values represent an increase in the parameter, and 95% confidence intervals that do not include 0 represent a significant difference. NC: normal control; DC: diabetic control, ACA: acarbose.

Groups	ROS-positive		Intact MMP	
	Mean difference	95% CI of difference	Mean difference	95% CI of difference
NC vs DC	-16.22	(-24.10, -8.340)	10.50	(1.778, 19.22)
DC vs Q-0.001	27.62	(19.74, 35.50)	-17.68	(-26.40, -8.958)
DC vs Q-0.01	21.72	(13.84, 29.60)	-15.96	(-24.68, -7.238)
DC vs Q-0.1	13.30	(5.420, 21.18)	-9.480	(-18.20, -0.7575)
DC vs Q-1	5.500	(-2.380, 13.38)	-2.320	(-11.04, 6.402)
DC vs ACA	16.38	(8.500, 24.26)	-10.06	(-18.78, -1.338)
Q-0.001 vs Q-0.01	-5.900	(-13.78, 1.980)	1.720	(-7.002, 10.44)
Q-0.001 vs Q-0.1	-14.32	(-22.20, -6.440)	8.200	(-0.5224, 16.92)
Q-0.001 vs Q-1	-22.12	(-30.00, -14.24)	15.36	(6.638, 24.08)
Q-0.001 vs ACA	-11.24	(-19.12, -3.360)	7.620	(-1.102, 16.34)
Q-0.01 vs Q-0.1	-8.420	(-16.30, -0.5403)	6.480	(-2.242, 15.20)
Q-0.01 vs Q-1	-16.22	(-24.10, -8.340)	13.64	(4.918, 22.36)
Q-0.01 vs ACA	-5.340	(-13.22, 2.540)	5.900	(-2.822, 14.62)
Q-0.1 vs Q-1	-7.800	(-15.68, 0.07972)	7.160	(-1.562, 15.88)
Q-0.1 vs ACA	3.080	(-4.800, 10.96)	-0.5800	(-9.302, 8.142)
Q-1 vs ACA	10.88	(3.000, 18.76)	-7.740	(-16.46, 0.9824)

Negative MD values represent an increase in the parameter, and 95% confidence intervals that do not include 0 represent a significant difference. NC: normal control; DC: diabetic control, ACA: acarbose.

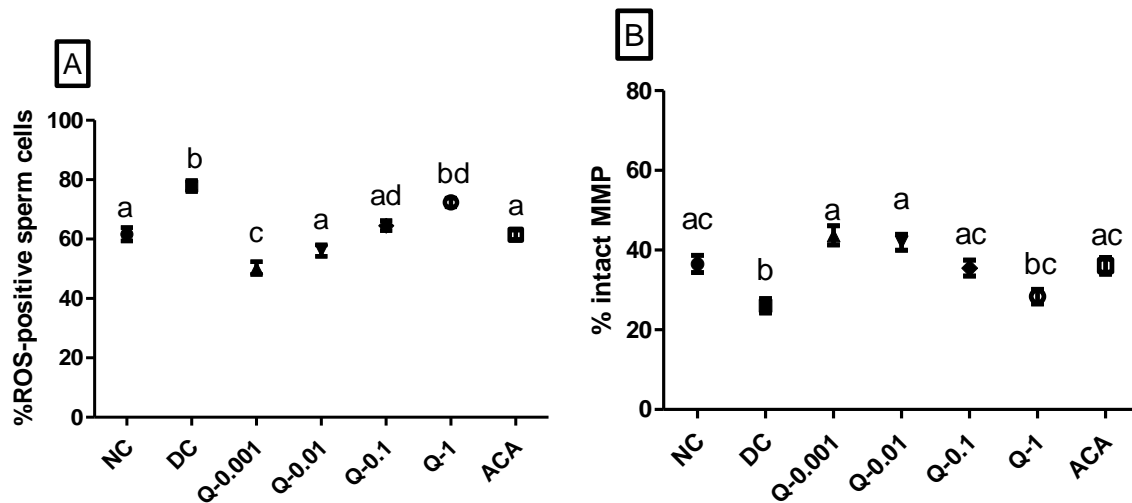


Figure 1: The effect of 0.001, 0.01, 0.1, and 1 $\mu\text{g/mL}$ concentrations of aqueous leaf extract of *G. livingstonei* on A). reactive oxidative species (ROS) production and B). mitochondrial membrane potential (MMP), in hyperglycaemic sperm cells. Data is represented as $\pm\text{SEM}$ of 200 sperm cells. Significance is denoted by the letters on each point, with letters different from each other showing significance (95% confidence interval excluding 0) while same letters show no significance (95% confidence interval including 0). NC: normal control; DC: diabetic control, ACA: acarbose.

3.3. Capacitation and acrosome reaction

The results from the chlorotetracyclin (CTC) assay are represented in Figure 2 and Table 2. There was a significant (MD: -26.32; 95% CI: -33.35, -19.29) increase in capacitated and acrosome reacted (AR-pattern) sperm cells in hyperglycaemic sperm cells compared to the normal control cells. The induction of hyperglycaemia resulted in a significant decrease in non-capacitated and contained intact acrosomes (F-pattern) (MD: 16.72; 95% CI: 7.545, 25.94) and capacitated, acrosome-intact (B-pattern) (MD: 9.58; 95% CI: 2.099, 17.06) sperm cells in

comparison to the normal control sperm cells. In addition to this, AR pattern sperm cells were dominant in the hyperglycaemic cell group, while most of the sperm cells in the normal control group showed the F-pattern. A concentration-dependent increase in a decrease in AR-pattern sperm cells compared to untreated hyperglycaemic cells was observed post-treatment with 0.001 (MD: 17.12; 95% CI: 10.09, 24.15), 0.01 (MD: 16.10; 95% CI: 9.075, 23.13), 0.1 (MD: 2.12; 95% CI: -4.905, 9.145), and 1 µg/mL (MD: 4.54; 95% CI: -2.485, 11.57) concentration of the aqueous leaf extract of *G. livingstonei*, with the lowest concentration, exhibiting more effect. Hyperglycaemic sperm cells treated with ACA showed a significant reduction in the number of AR-pattern (MD: 26.14; 95% CI: 19.11, 33.17) sperm cells and an increase in F-pattern (-21.48; 95% CI: -30.68,-12.28) sperm cells. No significant difference was observed in the number of B-pattern cells after treatment with both the plant extract and ACA as observed in Table 2.

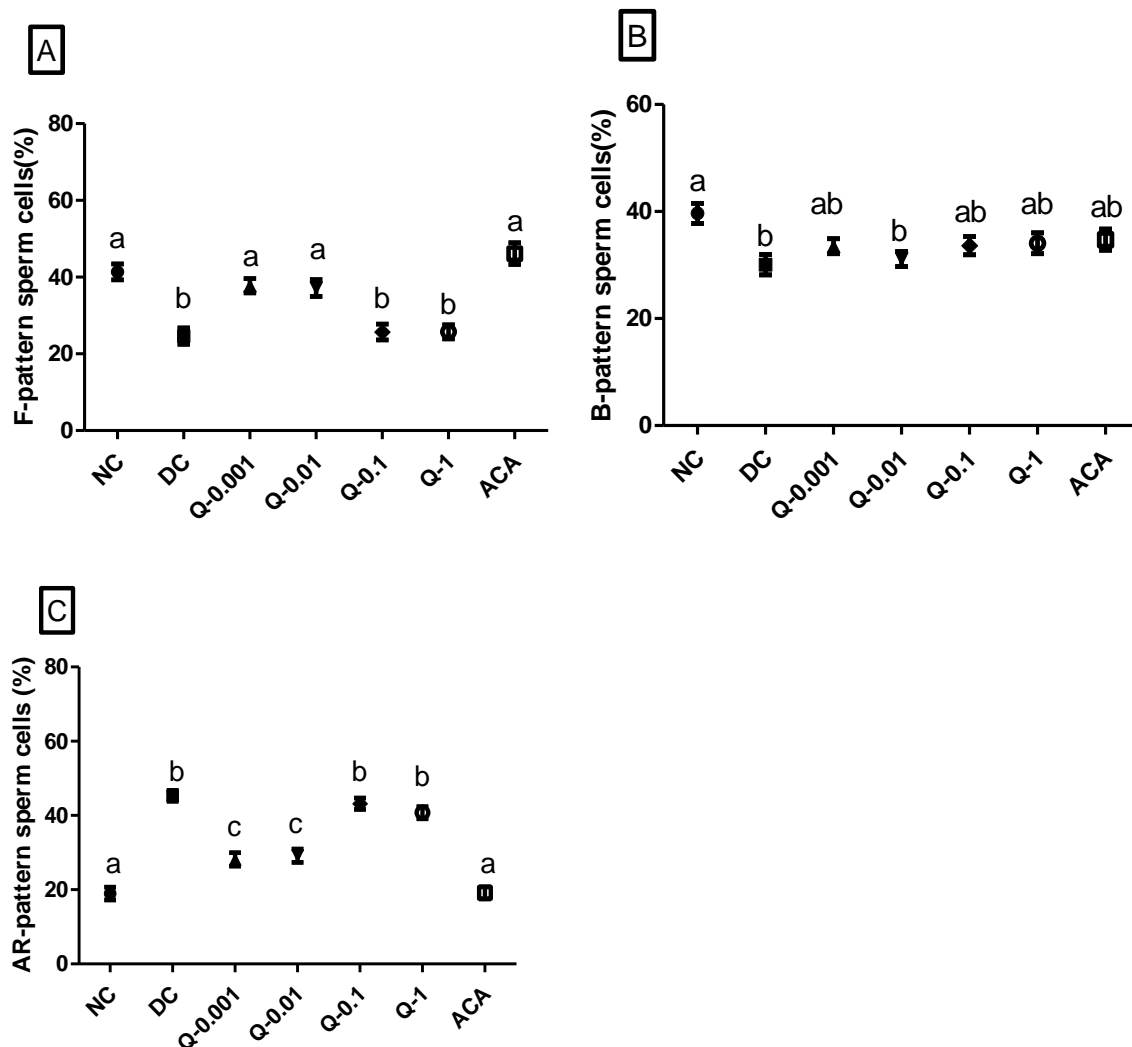


Figure 2: The effect of 0.001, 0.01, 0.1, and 1 $\mu\text{g}/\text{mL}$ concentrations of aqueous leaf extract of *G. livingstonei* on the percentage of A). non-capacitated with intact acrosomes (F-pattern), B). capacitated, acrosome-intact (B-pattern), and C). Capacitated and acrosome reacted (AR-pattern) in hyperglycaemic sperm cells. Data is represented as $\pm\text{SEM}$ of 200 sperm cells. Significance is denoted by the letters on each point, with letters different from each other showing significance (95% confidence interval excluding 0) while the same letters show no significance (95% confidence interval including 0). NC: normal control; DC: diabetic control, ACA: acarbose.

Table 2: Representation of the mean differences (MD) between different treatment groups depicting the effect of hyperglycaemia on capacitation and acrosome reaction, and the effect of different concentrations 0.001, 0.01, 0.1, and 1 µg/mL of the plant, and acarbose.

Groups	F-pattern		B-pattern		AR-pattern	
	Mean difference	95% CI of difference	Mean difference	95% CI of difference	Mean difference	95% CI of difference
NC vs DC	16.74	(7.545, 25.94)	9.580	(2.099, 17.06)	-26.32	(-33.35, -19.29)
DC vs Q-0.001	-13.12	(-22.32, -3.925)	-3.440	(-10.92, 4.041)	17.12	(10.09, 24.15)
DC vs Q-0.01	-12.54	(-21.74, -3.345)	-1.040	(-8.521, 6.441)	16.10	(9.075, 23.13)
DC vs Q-0.1	-1.080	(-10.28, 8.115)	-3.560	(-11.04, 3.921)	2.120	(-4.905, 9.145)
DC vs Q-1	-1.100	(-10.30, 8.095)	-4.000	(-11.48, 3.481)	4.540	(-2.485, 11.57)
DC vs ACA	-21.48	(-30.68, -12.28)	-4.660	(-12.14, 2.821)	26.14	(19.11, 33.17)
Q-0.001 vs Q-0.01	0.580	(-8.615, 9.775)	2.400	(-5.081, 9.881)	-1.020	(-8.045, 6.005)
Q-0.001 vs Q-0.1	12.04	(2.845, 21.24)	-0.1200	(-7.601, 7.361)	-15.00	(-22.03, -7.975)
Q-0.001 vs Q-1	12.02	(2.825, 21.22)	-0.5600	(-8.041, 6.921)	-12.58	(-19.61, -5.555)

Q-0.001 vs ACA	-8.360	(-17.56, 0.8352)	-1.220	(-8.701, 6.261)	9.020	(1.995, 16.05)
Q-0.01 vs Q-0.1	11.46	(2.265, 20.66)	-2.520	(-10.00, 4.961)	-13.98	(-21.01, -6.955)
Q-0.001 vs Q-1	11.44	(2.245, 20.64)	-2.960	(-10.44, 4.521)	-11.56	(-18.59, -4.535)
Q-0.001 vs ACA	-8.940	(-18.14, 0.2552)	-3.620	(-11.10, 3.861)	10.04	(3.015, 17.07)
Q-0.1 vs Q-1	- 0.020 00	(-9.215, 9.175)	0.2494	(-7.921, 7.041)	2.420	(-4.605, 9.445)
Q-0.1 vs ACA	-20.40	(29.60, 11.20)	- 0.6235	(-8.581, 6.381)	24.02	(16.99, 31.05)
Q-1 vs ACA	-20.38	(-29.58, 11.18)	- 0.3741	(-8.141, 6.821)	21.60	(14.57, 28.63)

Negative MD values represent an increase in the parameter, and 95% confidence intervals that do not include 0 represent a significant difference. NC: normal control; DC: diabetic control, ACA: acarbose; F-pattern: non-capacitated with intact acrosomes; B-pattern: capacitated, acrosome-intact; AR-pattern: capacitated and acrosome reacted.

3.4. DNA fragmentation

The percentage of DNA-fragmented sperm cells was recorded to determine the effect of hyperglycaemia on spermatozoa DNA integrity, and the therapeutic effect of the aqueous leaf extract of *G. livingstonei* on hyperglycaemia-induced DNA fragmentation. The results are depicted in Figure 3A and Table 3. A significantly (MD: -19.42; 95% CI: -29.03, -9.809) higher number of cells with fragmented DNA was observed with hyperglycaemia induction compared to normal control cells. However, a significantly lower number of sperm cells with DNA fragmentation was observed after treatment of the sperm cells with 0.001 (MD: 26.40; 95%

CI: 16.79, 36.01), 0.01 (MD: 15.62; 95% CI: 6.009, 25.23), 0.1 (MD: 22.76; 95% CI: 13.15, 32.37), and 1 µg/mL (MD: 11.62; 95% CI: 2.009, 21.23) concentration of the of *G. livingstonei* aqueous leaf extract. Treatment with ACA significantly (MD: 18.64; 95% CI: 9.029, 28.25) reduced DNA fragmentation in hyperglycaemic cells. As observed in Figure 3A and Table 3, the effect of acarbose on the DNA of hyperglycaemic sperm cells was comparable to the effect caused by the different concentrations of the plant extract.

3.5. Vitality

To assess the viability of sperm cells in different treatment groups, the percentage of live sperm cells in different treatment groups was compared and the results are depicted in Figure 3B and Table 3. The induction of hyperglycaemia was followed by a significant (MD: 22.10; 95% CI: 13.06, 31.14) reduction in the percentage of live sperm cells compared to the normal control sperm cells. The effect of the treatment with *G. livingstonei* aqueous leaf extract on the sperm cell viability was concentration-dependant. A significant increase in live cells was observed after treatment with 0.001 µg/mL (MD: -26.24; 95% CI: -35.28,-17.20) and 0.01 µg/mL (MD: -20.80; 95% CI: -29.84, -11.76) concentration of the plant extract compared to the untreated hyperglycaemic sperm cells. There was no significant difference in sperm cell vitality compared to the untreated hyperglycaemic sperm cells after treatment with 0.1 µg/mL (MD: -0.70; 95% CI: -9.738, 8.338) and 1 µg/mL (MD: -5.28; 95% CI; -14.32, 3.758) concentration of the plant extract. Similarly, the treatment of the hyperglycaemic cells with ACA showed no significant (MD: 0.68; 95% CI: -8.358, 9.718) difference in sperm cell vitality compared to the untreated hyperglycaemic sperm cells.

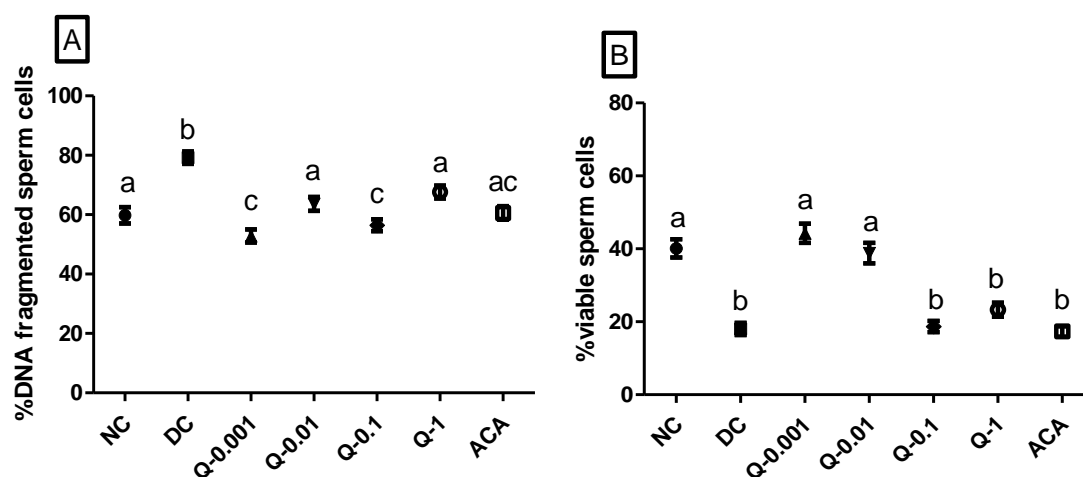


Figure 3: The effect of 0.001, 0.01, 0.1, and 1 µg/mL concentrations of aqueous leaf extract of *G. livingstonei* on A). DNA fragmentation and B). vitality in hyperglycaemic sperm cells. Data is represented as \pm SEM of 200 sperm cells. Significance is denoted by the letters on each point, with letters different from each other showing significance (95% confidence interval excluding 0) while the same letters show no significance (95% confidence interval including 0). NC: normal control; DC: diabetic control, ACA: acarbose.

Table 3: Representation of the mean differences (MD) between different treatment groups depicting the effect of hyperglycaemia on sperm cell DNA fragmentation and vitality, and the effect of different concentrations 0.001, 0.01, 0.1, and 1 µg/mL of the plant, and acarbose. Negative MD values represent an increase in the parameter, and 95% confidence intervals that do not include 0 represent a significant difference. NC: normal control; DC: diabetic control, ACA: acarbose.

Groups	%DNA fragmentation		%Vitality	
	Mean difference	95% CI of difference	Mean difference	95% CI of difference
NC vs DC	-19.42	(-29.03, -9.809)	22.10	(13.06, 31.14)
DC vs Q-0.001	26.40	(16.79, 36.01)	-26.24	-35.28 to -17.20
DC vs Q-0.01	15.62	(6.009, 25.23)	-20.80	-29.84 to -11.76
DC vs Q-0.1	22.76	(13.15, 32.37)	-0.7000	-9.738 to 8.338

DC vs Q-1	11.62	(2.009, 21.23)	-5.280	-14.32 to 3.758
DC vs ACA	18.64	(9.029, 28.25)	0.6800	-8.358 to 9.718
Q-0.001 vs Q-0.01	-10.78	(-20.39, -1.169)	5.440	-3.598 to 14.48
Q-0.001 vs Q-0.1	-3.640	(-13.25, 5.971)	25.54	16.50 to 34.58
Q-0.001 vs Q-1	-14.78	(-24.39, -5.169)	20.96	11.92 to 30.00
Q-0.001 vs ACA	-7.760	(-17.37, 1.851)	26.92	17.88 to 35.96
Q-0.01 vs Q-0.1	7.140	(-2.471, 16.75)	20.10	11.06 to 29.14
Q-0.001 vs Q-1	-4.000	(-13.61, 5.611)	15.52	6.482 to 24.56
Q-0.001 vs ACA	3.020	(-6.591, 12.63)	21.48	12.44 to 30.52
Q-0.1 vs Q-1	-11.14	(-20.75, -1.529)	-4.580	-13.62 to 4.458
Q-0.1 vs ACA	-4.120	(-13.73, 5.491)	1.380	-7.658 to 10.42
Q-1 vs ACA	7.020	(-2.591, 16.63)	5.960	-3.078 to 15.00

Negative MD values represent an increase in the parameter, and 95% confidence intervals that do not include 0 represent a significant difference. NC: normal control; DC: diabetic control, ACA: acarbose.

3.6. Motility

The percentage of motile sperm cells was assessed to determine the effect of hyperglycaemia on the motility of sperm cells, and the therapeutic effect of the aqueous leaf extract of *G. livingstonei* on the motility of the sperm cells. The total motility results were recorded and represented in Figure 4 and Table 4. A significantly (MD: 21.17; 95% CI: 9.457, 32.88) lower percentage motility was observed in untreated hyperglycaemic sperm cells compared to normal control cells. After treatment with the lowest concentration of the plant extract, the total motility of sperm cells was significantly (MD: 15.32; 95% CI: -26.76, -3.886) increased compared to the untreated hyperglycaemic cells. However, no significant difference in total motility was observed after treatment with 0.01 µg/mL (MD: -10.63; 95% CI: -22.07, 0.8107), 0.1 µg/mL (MD: -9.129; 95% CI: -20.57, 2.310), 1 µg/mL (MD: -9.395; 95% CI: -20.83, 2.044) concentrations of the plant extract and acarbose (MD: -5.350; 95% CI: -16.79, 6.089) caused

no significant difference in total motility compared to the untreated hyperglycaemic sperm cells.

Table 4: Representation of the mean differences (MD) between different treatment groups depicting the effect of hyperglycaemia on sperm cell motility and the effect of different concentrations of 0.001, 0.01, 0.1, and 1 µg/mL of the plant, and acarbose. Negative MD values represent an increase in the parameter, and 95% confidence intervals that do not include 0 represent a significant difference. NC: normal control; DC: diabetic control, ACA: acarbose.

Groups	Mean difference	95% CI of difference
NC vs DC	23.79	(12.35, 35.23)
DC vs Q-0.001	-15.32	(-26.76, -3.886)
DC vs Q-0.01	-10.63	(-22.07, 0.8107)
DC vs Q-0.1	-9.129	(-20.57, 2.310)
DC vs Q-1	-9.395	(-20.83, 2.044)
DC vs ACA	-5.350	(-16.79, 6.089)
Q-0.001 vs Q-0.01	4.697	(-6.742, 16.14)
Q-0.001 vs Q-0.1	6.196	(-5.243, 17.63)
Q-0.001 vs Q-1	5.930	(-5.509, 17.37)
Q-0.001 vs ACA	9.975	(-1.464, 21.41)
Q-0.01 vs Q-0.1	1.499	(-9.940, 12.94)
Q-0.001 vs Q-1	1.233	(-10.21, 12.67)
Q-0.001 vs ACA	5.278	(-6.160, 16.72)
Q-0.1 vs Q-1	-0.2664	(-11.71, 11.17)
Q-0.1 vs ACA	3.779	(-7.660, 15.22)
Q-1 vs ACA	4.046	(-7.393, 15.48)

Negative MD values represent an increase in the parameter, and 95% confidence intervals that do not include 0 represent a significant difference. NC: normal control; DC: diabetic control, ACA: acarbose.

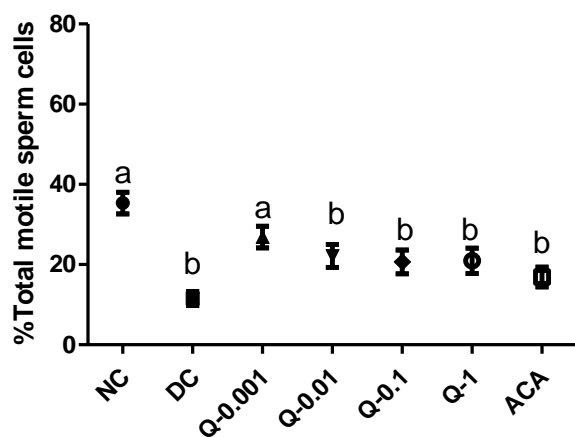


Figure 4: The effect of 0.001, 0.01, 0.1, and 1 $\mu\text{g/mL}$ concentrations of aqueous leaf extract of *G. livingstonei* on motility of hyperglycaemic sperm cells. Data is represented as $\pm\text{SEM}$ of 200 sperm cells. Significance is denoted by the letters on each point, with letters different from each other showing significance (95% confidence interval excluding 0) while the same letters show no significance (95% confidence interval including 0). NC: normal control; DC: diabetic control, ACA: acarbose.

3.7. Total protein content and antioxidant enzyme activity

The effect of the aqueous leaf extract of *G. livingstonei* on antioxidant enzymes and total protein content in the plasma obtained from the sperm cells was determined, and the results are demonstrated in Figure 5. There was no significant change in protein content of the plasma after the induction of hyperglycaemia. There was no significant difference in both catalase and SOD activity after exposure of the cells to high glucose conditions. There was no significant effect exhibited on the total protein content, catalase, and SOD activity in the plasma of

hyperglycaemic cells, after treatment with the plant extract. Also, no significant change was observed after treatment with acarbose.

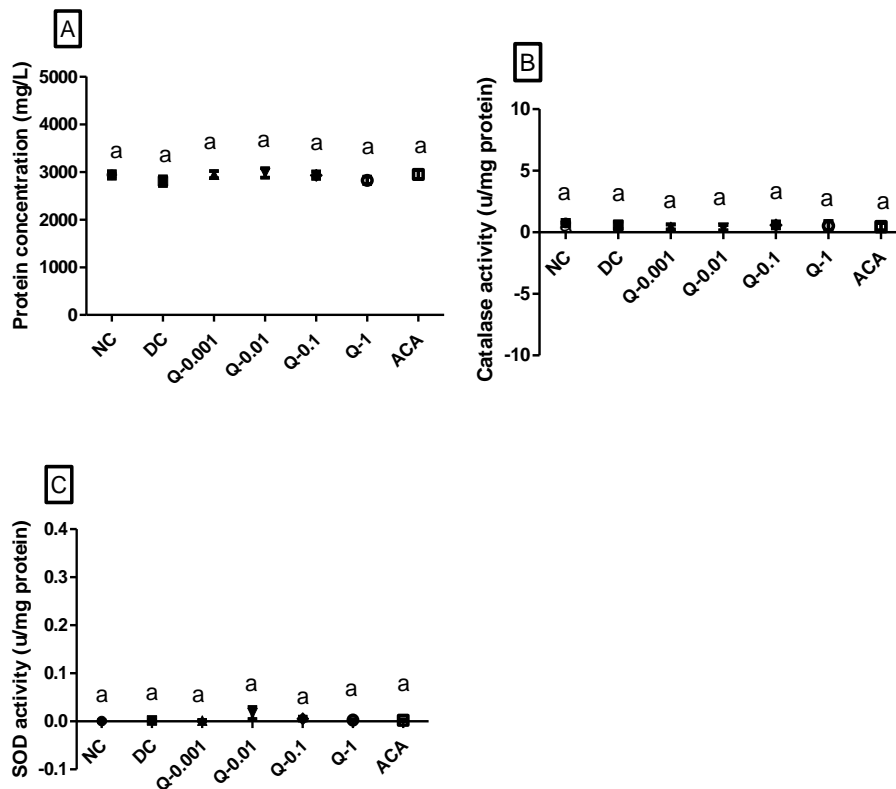


Figure 5: The effect of 0.001, 0.01, 0.1, and 1 $\mu\text{g/mL}$ concentrations of aqueous leaf extract of *G. livingstonei* on A. total protein concentration, B. catalase activity, and C. SOD activity in the plasma of hyperglycaemic sperm cells. Data is represented as $\pm\text{SEM}$. Significance is denoted by the letters on each point, with letters different from each other showing significance (95% confidence interval excluding 0) while the same letters show no significance (95% confidence interval including 0). NC: normal control; DC: diabetic control, ACA: acarbose, SOD: superoxide dismutase.

4. Discussion

Prolonged hyperglycaemia causes excessive production of ROS in the mitochondria and auto-oxidation of glucose molecules (González et al., 2023). The abnormal increase in the production of free radicals compromises the antioxidant system and leads to oxidative stress (González et al., 2023). Hyperglycaemia leads to the upregulation of a protein (Calpain-1), which reduces the activity of ATP synthase and leads to an increase in ROS (Kaludercic & Di Lisa, 2020). Free radicals accumulate in the semen due to the reaction of glucose with proteins, and the formation of AGEs (Tian et al., 2020). More than 60% of sperm cells under hyperglycaemic conditions have been found to express RAGEs and consequently contain excessive ROS (Tian et al., 2020). This present study corroborates the previous findings, as the induction of hyperglycaemia led to the excessive production of ROS compared to the normal control sperm cells.

The production of ROS in normal control cells reveals the normal production of ROS for sperm production and maturation, and the vulnerability of sperm cells to oxidative stress. The oxidation of the sperm cell lipid membrane leads to the damage and death of sperm cells via autophagy (Tian et al., 2020). In addition to this, oxidative stress leads to the formation of pores in the mitochondrial membrane and a decrease in mitochondrial membrane potential (Kaludercic & Di Lisa, 2020). The reduction in ATP in the sperm cells can lead to a reduction in sperm cell motility and apoptosis. Amongst other complications of the male reproductive function that lead to infertility, sperm cell death leads to the reduction of the chance of fertilisation (Chianese & Pierantoni, 2021). It is evident from the present study that hyperglycaemia led to a reduction in the number of viable cells. The reduction in viability of hyperglycaemic cells compared to normal control cells can be connected to the excessive production of ROS which leads to autophagy and apoptosis of sperm cells (Sadeghi et al., 2020).

It can be deduced from the reported disruptive effect of ROS on sperm cells, that the amelioration of oxidative stress in the male reproductive system could be a therapeutic target

in the treatment of hyperglycaemic-related male reproductive dysfunction. Similar to this study, *G. livingstonei* has been previously reported for its antioxidant potential associated with its high polyphenol content, and its high antioxidant capacity (Nossier et al., 2023). The treatment of hyperglycaemic sperm cells with the aqueous leaf extract of *G. livingstonei* showed a concentration-dependent oxidative stress-reducing effect. Polyphenols in medicinal plants reduce oxidative stress by neutralising and deactivation of the action of ROS. The presence of polyphenolic compounds in *G. livingstonei* could be potentially responsible for the reduction of ROS observed in this study model. However, the highest antioxidant effect of *G. livingstonei* was observed at the lowest concentration (0.001 µg/mL) of the extract. The administration of a toxic dosage of the extract possibly led to damage to the mitochondrial membrane, resulting in more production of ROS. Although the plant extract showed high antioxidant capacity as demonstrated by the significant reduction in ROS, the activity of antioxidant enzymes remained the same amongst the different study groups. The increase in the production of ROS is therefore linked to the pathways leading to the production of ROS rather than the compromised antioxidant system. It can be deduced that the antioxidant activity of the plant extract is inclined to the neutralisation of ROS than the activation of antioxidant enzymes.

The use of ACA as a standard drug is associated with its capacity to reduce ROS and increase antioxidant capacity (Arya et al., 2023). This is well depicted in this present study, as the administration of ACA on hyperglycaemic sperm cells led to the reduction of ROS production compared to the untreated hyperglycaemic cell. However, the lowest dose of the plant extract exhibits a stronger effect in the reduction of ROS compared to ACA. It can therefore be deduced that antioxidant efficacy of the plant extract can be higher than that of ACA. Although ACA exhibits an antioxidant effect, sperm cells treated with ACA showed low cell viability with a non-significant difference compared to the non-treated hyperglycaemic cells. This suggests that the death of sperm cells in hyperglycaemic conditions are not entirely caused by oxidative stress. Previous studies have reported relevant factors that could possibly lead to sperm cell death in hyperglycaemic conditions such as AGEs reaction with the sperm cells. Although the toxicity of ACA on sperm cells is not well-documented. A similar hypoglycaemic drug,

metformin, has been recorded for its toxicity on sperm cells leading to reduced viability and this effect could be exhibited by ACA (Alzain et al., 2020). Following the treatment of sperm cells with different concentrations of the aqueous leaf extract of *G. livingstonei*, sperm cell viability was increased in the lower concentrations of the extract. These findings are in correlation with the reduced ROS production in the lower-concentration extract group and show that the increase in ROS can consequently lead to a reduction in cell viability. The toxicity of *G. livingstonei* following a relative increase in dosage depending on cell type has not been concealed, hence the lower viability of sperm cells at higher concentrations (Nethengwe et al., 2024).

Sperm motility is a vital parameter of the male reproductive system in fertility. Fertilisation is achieved when sperm cells are progressively motile towards the egg cell. A drastic reduction in sperm motility reduces the chance of fertility. In the present study, prolonged hyperglycaemia led to the reduction of sperm cell motility as compared to the normal control sperm cells. These findings support the findings from previous studies that hyperglycaemia leads to reduced motility and compromises male reproductive function. Human sperm cells rely solely on mitochondrial oxidative phosphorylation for the production of ATP as fuel for sperm motility (Barbagallo et al., 2021). In normal glycaemic conditions, the sperm mitochondria utilise glucose to produce ATP for the normal motility of sperm cells. During glucose metabolism, a low number of free radicals are produced in aid of sperm maturation. However, in hyperglycaemic conditions, excessive glucose molecules undergo oxidative phosphorylation which overloads the mitochondria and produces excessive ROS (Barbagallo et al., 2021). Mitochondrial damage caused by free radical overload leads to less ATP being produced, and the reduction of percentage sperm motility. Similarly, the present study shows the reduction of MMP in hyperglycaemic sperm cells and a resultant decrease in sperm motility. As observed initially in this study, the reduction in MMP of the sperm cells can be associated with the hyperglycaemia-induced oxidative stress.

Addition of *G. livingstonei* leaf extract to the sperm cells showed a concentration-dependent effect on the sperm motility. Lower concentration of the plant extract yielded a higher percentage of sperm motility. The possible toxicity of the plant extract at higher concentrations led to the interruption of sperm cell motility. The reduction of ROS by the extract led to an increase in MMP in comparison to the non-treated hyperglycaemic cells, which increased ATP and sperm motility. Since *G. livingstonei* exhibits a hypoglycaemic effect, it reduces the amount of free radical formation in the mitochondria which reduces mitochondrial membrane damage. However, the highest concentration could have caused increase in ROS and damaged the mitochondria. The effect of ACA on MMP is comparable with that of the *G. livingstonei* leaf extract. However, due to the higher therapeutic effect of the plant extract in other sperm parameters, the general effect of ACA is lower.

Capacitation and acrosome reaction are pre-requisites for achieving fertilisation of the oocyte (Chhikara et al., 2023). Capacitation of the sperm cells begins in the female reproductive tract after ejaculation (in vivo). In an in vitro setting, different inducing factors such as calcium ionophore are used to initiate capacitation and acrosome reaction (De Villiers et al., 2022). However, slow and less capacitation occurs spontaneously due to the growth media, and incorrect handling of the sample. Although capacitation and acrosome reaction are indicators of possible fertilisation, premature capacitation, and acrosome reaction are both worrying phenomena and can lead to reduced fertility. In the present study, no inducing factors were used, hence the sperm cells suitable for fertilisation were expected to have less capacitation and contain intact acrosomes. Due to spontaneous capacitation and possible contamination in sample handling, a few cells in the normal control group were capacitated and contained reacted acrosomes. However, the comparison between normal control sperm cells and the hyperglycaemic sperm cells showed increased capacitation and a decrease in acrosome integrity in hyperglycaemic sperm cells. Although a minimum amount of ROS is required for cell capacitation and the initiation of acrosome reaction, fluctuation of the production of ROS in the sperm cells can interfere with these processes (Sanocka & Kurpisz, 2004).

Lipid peroxidation of sperm cell membranes instigated by oxidative stress interferes and blocks chemical and biophysical changes that initiate appropriate capacitation and acrosome reaction in conditions suitable for these fusion processes (Benko et al., 2022). In a previous study that investigated the effect of oxidative stress in cryopreserved sperm cells, the presence of ROS was associated with premature capacitation and acrosome reaction (Benko et al., 2022). Similarly, this present study shows premature capacitation and acrosome reaction in hyperglycaemic sperm cells, possibly caused by the excessive production of ROS. The increase in non-capacitated and acrosome-intact sperm cells after the administration of lower concentrations of the aqueous leaf extract of *G. livingstonei* on hyperglycaemic sperm cells reveals the antioxidant effect of the extract and further suggests the therapeutic effect on fertilisation fusion processes, associated with the reduction of ROS. The disruption of the sperm cell membranes and interference with the capacitation process reduce sperm motility (Balbach et al., 2023). The correlation between sperm motility and acrosome integrity is apparent in this study as sperm cells with reduced acrosome integrity showed reduced sperm motility.

The information obtained from sperm analysis parameters such as motility, concentration, and morphology is limited to conclude the fertility status (Liu et al., 2023). Sperm DNA fragmentation plays a huge role in the production, maturation, and transfer of genetic material to the egg cell, hence its in-found importance in reproduction science research (Liu et al., 2023). DNA fragmentation is common in fewer sperm cells during maturation and movement through the urethra and vaginal lining (Nielsen et al., 2023). However, increased sperm DNA fragmentation reduces sperm motility and is detrimental to male fertility (Qi et al., 2024). Oxidative stress is an instigator of sperm DNA fragmentation (Nielsen et al., 2023). The increase in sperm DNA fragmentation of hyperglycaemic sperm cells in this study reveals the contribution of high glucose levels to disrupted fertility. Hyperglycaemic-induced production of ROS is implicated in the fragmentation of sperm DNA in this study. A negative correlation between sperm DNA fragmentation and total sperm motility was observed in this study, similar

to a previous study (Qi et al., 2024). The neutralisation of free radicals in hyperglycaemic sperm cells was expected to reduce sperm DNA fragmentation. The antioxidant effect of *G. livingstonei* leaf extract was expressed, and a resultant concentration-dependent reduction in sperm DNA fragmentation was observed after treatment with the extract. The lower concentrations of the extract showed maximum antioxidant activity, hence the lower ROS and reduced sperm DNA fragmentation. The reduction of sperm DNA fragmentation in higher concentrations of the extract regardless of high ROS production suggests the alternative protective effect of the extract against sperm DNA fragmentation.

5. Conclusion and limitations

The underlying pathology of male reproductive dysfunction in DM is associated with a decline in multiple interconnected sperm parameters. Therefore, the assessment of the underlying molecular pathways that are associated with male infertility is paramount to finding a suitable treatment and dosage. For instance, this study shows that at lower concentration, the extract significantly reduced sperm DNA fragmentation compared to the higher concentration of the extract, indicating the necessity of appropriate dosage of medicinal plant extracts in the treatment of any disease. The appropriate use of *G. livingstonei* aqueous leaf extract holds therapeutic potential in the treatment of male reproductive complications related to hyperglycaemia and oxidative stress. However, this study model is an *in vitro* study and does not completely mimic DM and the male reproductive system. This study aimed at investigating the effect of the plant extract independent of any standard drug, and due to ethical concerns, the study could not include untreated diabetic individuals as a control. With these findings, future studies can focus on the coupled effect of *G. livingstonei* aqueous leaf extract and a standard drug on diabetic individuals *in-vitro* or *in vivo*.

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conceptualization. Chinyerum S. Opuwari: supervision, writing- review and editing. Oluwafemi O. Oguntibeju: supervision, resources, secured funding for the study, writing review and editing.

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CHAPTER SEVEN

Ameliorative effect of *Androstachys johnsonii* aqueous leaf extract on hyperglycaemic spermatozoa

Murendeni Nethengwe^a, Kunle Okaiyeto^a, Chinyerum S. Opuwari^b, Oluwafemi O. Oguntibeju^a.

^aPhytomedicine and Phytochemistry Group, Department of Biomedical Sciences, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, Bellville 7535, South Africa.

^bDepartment of Medical Biosciences, University of the Western Cape, Bellville, South Africa.

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Abstract

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* 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. This study investigated the effect of the aqueous leaf extract of *A. johnsonii* on hyperglycaemic-induced sperm cells of 25 male participants in Cape Town, South Africa. The exposure of sperm cells to hyperglycaemic conditions for 24 h resulted in a significant ($P<0.05$) increase in the production of ROS, DNA fragmentation, and capacitated and acrosome reacted sperm cells, 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 and 1 $\mu\text{g/mL}$) 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. 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 ROS, and

consequently oxidative stress (Di Meo & 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 (Deragon et al., 2020; González et al., 2023). 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 & 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 & 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 the amelioration of oxidative stress in diabetic individuals is therefore a therapeutic target in the treatment of DM-related 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 (*A. johnsonii*), 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 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. Methods

2.1. Plant collection

Fresh leaves of *A. johnsonii* were collected from the Brackenridgea Nature Reserve in Limpopo, South Africa. Authentication of the plant was performed at the University of Venda by Professor Tshisikhawe and kept at the Herbarium of University of Venda, with a voucher number (MNU001/10/22).

2.2. Plant extract preparation

The leaves were washed and air-dried in the shade for five days. After drying, they were manually crushed and then blended into a powder. A total of 100 g of this powder was mixed with 1000 mL of water to prepare an aqueous extract, which was left for 24 h to allow extraction. The liquid extract was separated from the plant material, which was discarded. The extract was then filtered using filter paper, freeze-dried, and stored at -20°C.

2.3. Ethical considerations and participant inclusion criteria

Ethical approval for this study was obtained from 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). A total of 25 male participants, aged 18-40, were recruited voluntarily following ethical guidelines. Individuals with chronic illnesses were excluded from participation. Baseline sperm parameters were evaluated as part of the screening process, and participants who did not meet the World Health Organisation (WHO) standards were excluded from the study.

2.4. Hyperglycaemia induction and treatment

Induction of hyperglycaemia was done following a previously utilised protocol (Portela et al., 2015). Sperm cells were washed and resuspended in 2-3 mL of sperm wash to achieve the desired concentration, then incubated for 3 h to allow capacitation. To induce hyperglycaemia, 5 mL of induction media (containing 0.9 mM CaCl₂, 0.5 mM MgCl₂, 10 mM sodium lactate, 1 mM sodium pyruvate, 0.3% w/v BSA, 1% v/v Penicillin/Streptomycin, 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 million cells/mL. The samples were incubated

for 24 hours. 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, 0.01, 0.1, and 1 µg/mL) were selected for the study, from a preliminary test with a 10-fold serial dilution from a stock solution of 1 mg/mL. 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 and then centrifuged for 10 minutes at 300 x g.

2.5. Reactive oxidative species determination

The determination of ROS was performed using the dihydroethidine (DHE) staining assay, as described by Henkel et al. (2003). 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 15-20 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 used to determine the total protein content in the plasma of the spermatozoa. A protein standard with a series of concentrations (0.25, 0.5, 1, 1.4, and 2

mg/ml) was prepared by diluting 2 mg/ml of bovine serum albumin (BSA) protein in 1% sodium dodecyl sulfate (SDS). A volume of 5 μ l of the blank, standard concentrations, and the samples was loaded in the wells of a 96-well plate, after which 250 μ l of the Bradford reagent was added. The plate was gently shaken and incubated in the dark for 30 minutes. The plate was read at 595 nm 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 H₂O₂ in the lysates. The catalase activity assay was performed following the methods of Brannan *et al.* (1981). The H₂O₂ reagent was prepared by adding 50 μ l of H₂O₂ to 10 ml of the phosphate-buffered saline (PBS) buffer. PBS buffer (170 μ l) was added to each well of the 96-well UV plate, and 10 μ l of each sample was added to each well containing the PBS buffer. The H₂O₂ reagent (75 μ l) was added to each well and the plate was read immediately in an Omega Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 232nm.

2.8. Superoxide dismutase activity

Superoxide dismutase (SOD) activity in the plasma of sperm cells was estimated by calculating 50% inhibition of auto-oxidation of 6-hydroxydopamine hydrobromide (6-OHD) by superoxide free radical. Diethylene triamine pentaacetic acid (DETAPAC) was used to inhibit any cycle formation of other free radicals. A 6-OHD solution was freshly prepared by adding 4 mg of 6-OHD in 10 ml of distilled water and 50 μ l of perchloric acid in a 15 ml plastic tube. DETAPAC was prepared by adding 2 mg of DETAPAC to 50 ml of the 100 mM phosphate buffered saline (PBS) buffer. A mixture of oxidised 6-OHD and DETAPAC forms a pink/orange colour. A volume of 10 μ l of each sample was loaded into the wells in triplicates and 15 μ l of 6-OHD was added to each well. A volume of 170 μ l of DETAPAC was then added to each well

and the plate was read at 490 nm in an Omega Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA) (Brannan *et al.*, 1981).

2.9. DNA Fragmentation

DNA fragmentation was evaluated using 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 final concentration of 10 million cells/mL. A 30 μ L aliquot of the sample was added to pre-heated agarose in an Eppendorf tube (heated at 90–100°C for 5 min, followed by 37°C for 5 min). Then, 20 μ L 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 gently 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 minutes, 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 minutes, 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 510–530 nm emission filters, with a 400 \times 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 μ L of Hoechst 32258 dye was

introduced. The samples were then incubated with the dye for 2 minutes, after which 400 μL of 2% polyvinylpyrrolidone (PVP40) was added. The mixture was centrifuged for 5 minutes at 900 x 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% w/v paraformaldehyde 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 exhibited fluorescence throughout the entire head, while capacitated, acrosome-intact sperm displayed fluorescence over the head, excluding the post-acrosomal region. In contrast, capacitated, acrosome-reacted sperm 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 200x MMP stock solution in a 1:1 ratio. The mixture was then incubated at 37°C for 20 min in the dark. After incubation, the samples were centrifuged at 500 x g and 5-7°C for 5 min, and the supernatant was removed. The cell pellet 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 5-10 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

Sperm vitality was assessed using 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 minutes. 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 100x 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).

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-values, with a P-value less than 0.05 representing significant differences between compared groups. Significance ($P < 0.05$) was denoted by alphabetical letters on top of mean points. Different points with different letters were considered significantly ($P < 0.05$) different from each other, while column means with the same letters had no significant ($P > 0.05$) difference.

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 Figure 1A. The sperm cell group with hyperglycaemia (HC) showed

a significant ($P < 0.0001$) increase 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 ($P < 0.05$) reduction in ROS after treatment of the hyperglycaemic sperm cells with the plant extract. The highest concentration (1 $\mu\text{g/mL}$) of the plant extract caused a significantly ($P < 0.001$) lower reduction in ROS compared to the lower concentrations. Although the administration of acarbose (Ac) resulted in a significant ($P < 0.0001$) reduction in ROS production, the ROS-reducing capacity was lower compared to the highest concentration (1 $\mu\text{g/mL}$) 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 Figure 1B. High glucose conditions in sperm cells led to a significant ($P < 0.001$) decrease 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 ($P > 0.05$) difference 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 ($P > 0.05$) difference 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 (Figure 1C). 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 $\mu\text{g/mL}$) of the plant extract led to a higher significant ($P < 0.0001$) increase in live cells. No significant ($P > 0.05$) difference in sperm cell vitality was observed after the treatment of the hyperglycaemic sperm cells with acarbose.

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 Figure 1D. A significant ($P < 0.0001$) increase 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 ($P < 0.05$) decrease in sperm cells with fragmented DNA, with the highest tested concentration (1 $\mu\text{g/mL}$) 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 Figure 1E. A significantly ($P < 0.001$) lower number of hyperglycaemic sperm cells had intact MMP compared to the normal control cells. Exposure of the hyperglycaemic sperm cells to lower concentrations (0.001, and 0.01 $\mu\text{g/mL}$) of the plant extract did not show a significant ($P > 0.05$) difference in MMP compared to the untreated hyperglycaemic sperm cells. However, a significant increase in sperm cells with intact MMP was observed after treatment with 0.1 ($P < 0.001$) and 1 $\mu\text{g/mL}$ ($P < 0.0001$) concentrations. Treatment of the hyperglycaemic cells with acarbose resulted in a significant ($P < 0.001$) increase in the number of cells with intact MMP. The effect of acarbose on the MMP of hyperglycaemic cells was comparable to that of the highest (0.1 and 1 $\mu\text{g/mL}$) concentrations of the plant extract.

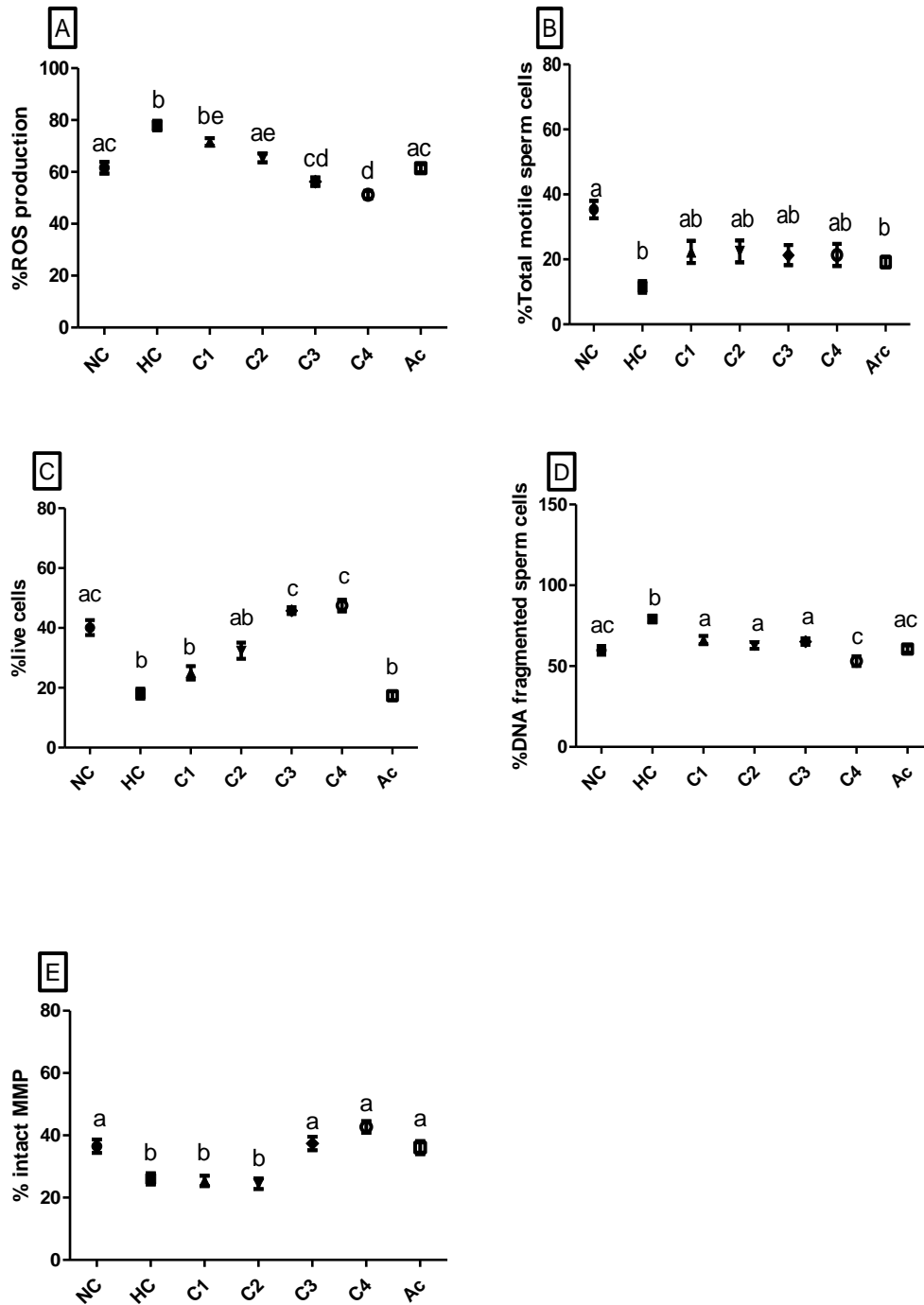


Figure 1: The ameliorative effect of aqueous leaf extract of *A. johnsonii* on A). reactive oxidative species production, B). Total sperm motility, C). Sperm cell vitality, D). DNA fragmentation, E). Mitochondrial membrane potential in hyperglycaemic sperm cells. Data is represented as \pm SEM of 200 sperm cells. Letters of the alphabet on bars represent significance, and bars with letters different from each other show a significant ($P < 0.05$) difference while the same letters show no significance ($P > 0.05$). NC: normal control; HC: hyperglycaemic control, Ac: acarbose, C1: 0.001 μ g/mL, C2: 0.01 μ g/mL, C3: 0.1 μ g/mL, C4: 1 μ g/mL.

3.4. Antioxidant enzyme activity

Figure 2 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*.

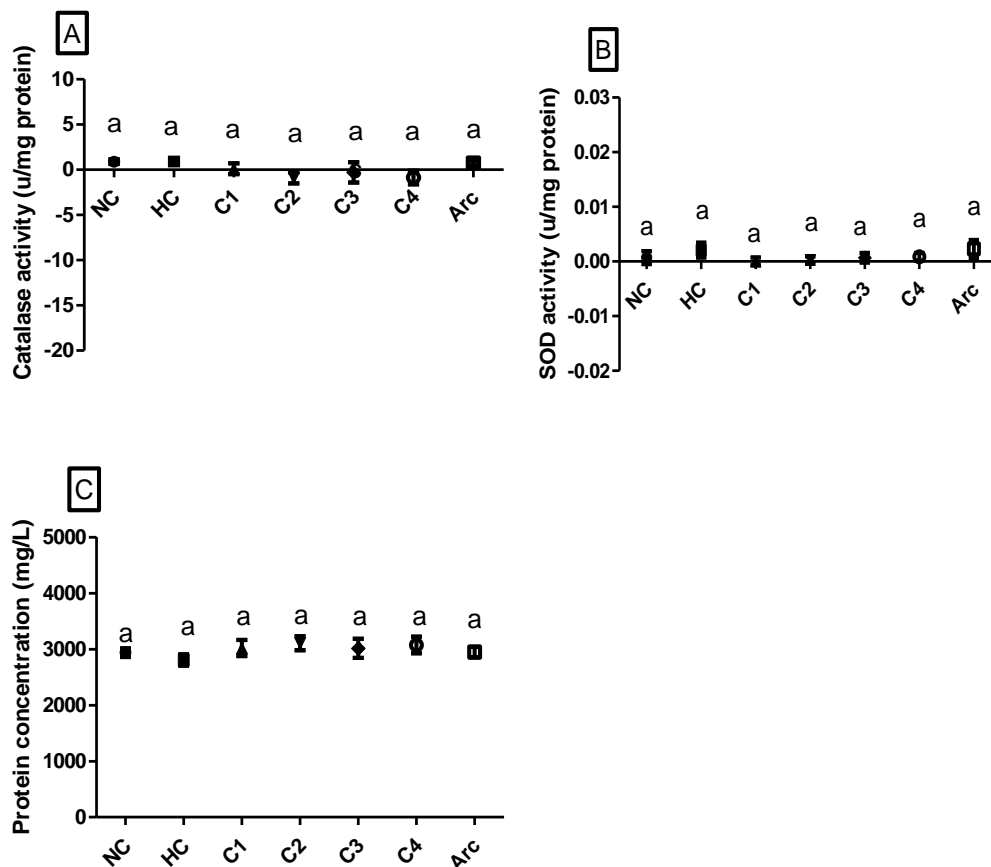
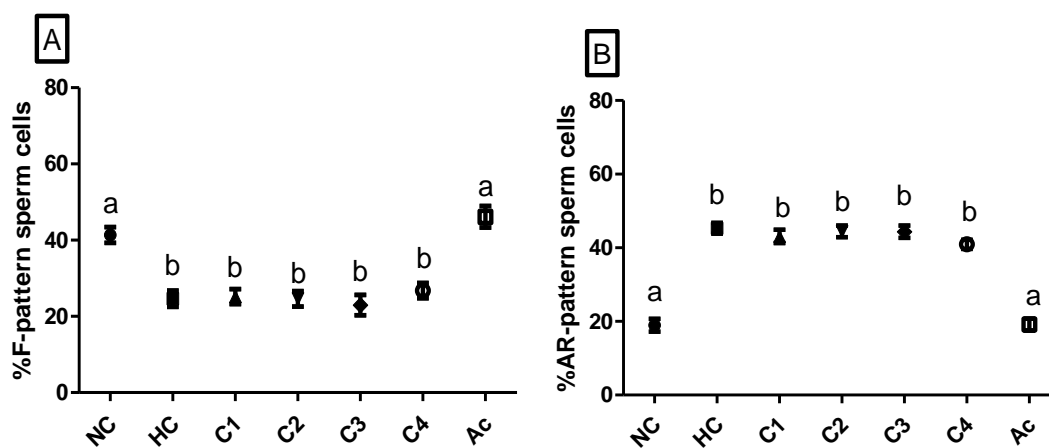


Figure 2: The effect of aqueous leaf extract of *A. johnsonii* on A). Catalase activity, B). SOD activity, C). Total protein content, in the plasma of hyperglycaemic sperm cells. Data is represented as \pm SEM. Letters of the alphabet on bars represent significance, and bars with letters

different from each other show significant ($P < 0.05$) difference while the same letters show no significance ($P > 0.05$). NC: normal control; HC: hyperglycaemic control, Ac: acarbose, C1: 0.001 $\mu\text{g/mL}$, C2: 0.01 $\mu\text{g/mL}$, C3: 0.1 $\mu\text{g/mL}$, C4: 1 $\mu\text{g/mL}$, SOD: superoxide dismutase.

3.5. Sperm capacitation and acrosome reaction

The difference in capacitation and acrosome reaction amongst the sperm cell treatment groups is depicted in Figure 3. Exposure of sperm cells to high glucose conditions resulted in a significant ($P < 0.0001$) increase in AR-pattern sperm cells, a decrease ($P < 0.001$), in F-pattern sperm cells, and a decrease ($P < 0.001$) in B-pattern sperm cells. The majority of the hyperglycaemic sperm cells were capacitated and contained reacted acrosomes. There was no significant ($P > 0.05$) difference in sperm cell capacitation and acrosome reaction after treatment with the aqueous leaf extract of *A. johnsonii*. Treatment with acarbose led to a significant ($P < 0.001$) decrease in the AR-pattern cells and a significant ($P < 0.0001$) increase in F-pattern sperm cells compared to the untreated hyperglycaemic sperm cells.



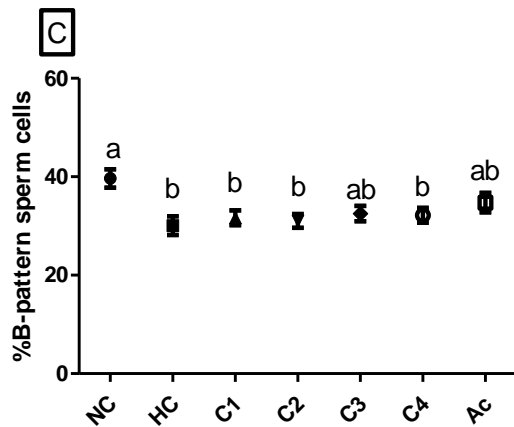


Figure 3: : The ameliorative effect of aqueous leaf extract of *A. johnsonii* on A). percentage non-capacitated with intact acrosomes (F-pattern B). percentage capacitated, acrosome-intact (B-pattern), and C). percentage capacitated and acrosome reacted (AR-pattern) in hyperglycaemic sperm cells. Data is represented as \pm SEM of 200 sperm cells. Letters of the alphabet on bars represent significance, and bars with letters different from each other show a significant ($P < 0.05$) difference while the same letters show no significance ($P > 0.05$). NC: normal control; HC: hyperglycaemic control, Ac: acarbose, C1: 0.001 μ g/mL, C2: 0.01 μ g/mL, C3: 0.1 μ g/mL, C4: 1 μ g/mL.

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 investigated the effect 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 pathway to the polyol pathway, an alternative source of free radical (Garg & 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 & 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 over-activate oxidative phosphorylation pathway, which releases excessive ROS and overloads the mitochondria (Chianese & 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 & 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. johsonii* 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. johsonii* 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 & Waqas Abdulllah, 2023). A decreased DNA fragmentation can interfere with fertilisation and can lead to male infertility (Nisa & Waqas Abdulllah, 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 ability of the sperm cells to undergo capacitation and acrosome reaction (Dahan & Breitbart, 2022). Capacitation involves physical and chemical alteration of the sperm cell and initiates the process of acrosome reaction (Chakraborty & Saha, 2022). Although fertilisation is initiated by capacitation, early/premature capacitation is detrimental (Dahan & Breitbart, 2022). The normal amount of ROS produced in the plasma contributes to normal sperm cell capacitation (Nowicka-Bauer & 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 & 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 and recommendations

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.

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O. Oguntibeju: supervision, conceptualization, resources, secured funding for the study, writing review and editing.

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CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSION

8.1. General discussion

The use of medicinal plants as an alternative treatment of different ailments is a current health research focus (Khumalo et al., 2022; Kuralkar & Kuralkar, 2021). The search for alternative ways of treatment is due to the multiple reports and study findings revealing the side effects of current synthetic drugs (Galeano et al., 2020; Witwer & Wolfram, 2021). Most synthetic drugs are expensive and not affordable by the majority of the population in developing countries (Elkordy et al., 2021). Several medicinal plants used in traditional medicine are easily accessible and exhibit minimal toxicity, hence less side effects (Pal et al., 2023). Although DM standard drugs have shown effectiveness in the treatment of DM complications, side effects and unaffordability remain a great concern and a major reason in the increase in the prevalence of the disease (Blahova et al., 2021). For instance, the use of metformin is associated with gastrointestinal side effects when used as monotherapy, and pathological hypoglycaemia when used with insulin (Ahmad et al., 2020; Grammatiki et al., 2021). The literature contains several reported ethnobotanical studies that identified a group of medicinal plants used traditionally worldwide in the treatment of DM and its complications, including male infertility (Edo et al., 2024; Mechchate et al., 2020; Singh et al., 2020). The present study shows evidence of the availability of various medicinal plants used in Limpopo, South Africa to treat DM and DM-related male infertility. This study compared the efficacy of selected medicinal plants to the standard drug ACA. Although we identified several medicinal plants reported to be effective, the majority of the plants rarely appear in the literature. In addition to this, only very few experimental studies have been reported on the medicinal plants reported in this study.

The use of medicinal plants is associated with its phytochemical and nutritional content (Nwozo et al., 2023; Radha et al., 2021; Shahrajabian et al., 2022). In our current study, two

medicinal plants used in the treatment of DM and DM-related male infertility were selected from our ethnobotanical study based on frequent use in the community. Although *A. johnsonii* and *G. livingstonei* are commonly used traditionally, very few studies on their medicinal benefit exist. The phytochemical composition of medicinal plants can assist in linking the constituents to the observed effect, and to study the mechanism of action for further therapeutic improvement. Since this study focussed on the hypoglycaemic, antioxidant, and anti-inflammatory effects, the phytochemical profiling performed on the aqueous leaf extracts of *A. johnsonii* and *G. livingstonei* was specific to phenolic compounds. Phenolic compounds are of medicinal significance due to their hypoglycaemic, antioxidant, and anti-inflammatory properties, hence their interest in the treatment of diseases associated with hyperglycaemia, oxidative stress, and chronic inflammation (Pieczykolan et al., 2021; Tatipamula & Kukavica, 2021).

Diabetes mellitus is a chronic metabolic disease characterised by hyperglycaemia and accompanied by oxidative stress and inflammation (Yaribeygi et al., 2020). The occurrence of oxidative stress and inflammation in male individuals presenting with hyperglycaemia leads to the decline in sperm parameters and threatens the ability to conceive (Ayad et al., 2022; Nna et al., 2020). The effects of hyperglycaemia in sperm parameters are well-established in this study. Prolonged hyperglycaemia on sperm cells (over 24 h) showed significantly high production of ROS compared to the normal control group. Although not many studies have focussed on the induction of hyperglycaemia on sperm cells, similar supporting studies have revealed the occurrence of oxidative stress and increased inflammation following hyperglycaemia in other cell and animal models (Iova et al., 2021; Zhao et al., 2021). Although the number of inflammatory markers could not be quantified in this study due to the sperm washing process involved in hyperglycaemia induction, the appearance of increased inflammatory markers in the semen of diabetic individuals has been reported in previous studies (Nna et al., 2020).

The treatment of hyperglycaemic sperm cells with aqueous leaf extracts of *A. johnsonii* and *G. livingstonei*, individually, led to a significant decrease in ROS. In this study, groups of phenolic compounds such as flavonoids, benzophenones, hydroxycinnamic acids were identified in the aqueous leaves of *G. livingstonei*. This supports previous phytochemical profile results of the same extract in a previous study (Abdul-Rahman et al., 2023). Like *G. livingstonei*, phenolic compounds such as hydroxycinnamic acids and flavonoids were identified in the leaf extract of *A. johnsonii*. Additionally, compounds such as gallotannins and quinic acid were identified. Similarly, a previous study reported the presence of abundant flavonoids, quinic and coumaric acids (Mfotie Njoya et al., 2024). Flavonoids, including flavanols, flavones and flavonols are a major group of phenolic compounds in medicinal plants, commonly reported as strong antioxidant compounds (Mutha et al., 2021). The presence of abundant flavonoids in both plant extracts investigated in this study is linked to the antioxidant capacity (DPPH, FRAP, TEAC) observed in both plants. Antioxidant capacity reveals the plant extract's ability to reduce or neutralise free radicals (Akbari et al., 2022). This effect is supported by the observed reduction of ROS after treatment of the hyperglycaemic sperm cells with both plant extracts in this study. During oxidative stress, the antioxidant system is compromised and the production of ROS is extremely increased, leading to the direct oxidation and damage of biomolecules (Jomova et al., 2023). The addition of antioxidants boosts the antioxidant system and neutralises the effect of ROS (Raza et al., 2020). The reduction of ROS following treatment with the plant extracts in this study is possibly due to both the neutralisation of free radicals, and the activation of antioxidant enzymes exhibited by the present identified antioxidants. However, the FRAP assay is conducted at an acidic pH (3.6), which may not accurately represent antioxidant activity in physiological conditions. This pH dependency could alter the reactivity of certain compounds and may not fully capture the antioxidant potential under neutral or basic conditions. Additionally, FRAP measures only the ferric-reducing ability, which may not reflect the full antioxidant capacity of the plant extract. It does not account for other radical scavenging mechanisms, such as peroxy radical neutralization or lipid peroxidation inhibition.

The reduction of ROS is controlled by both the neutralisation of the free radicals by non-enzymatic antioxidant compounds, and the activation of antioxidant enzymes that eradicate free radicals (Dvořák et al., 2021; Jena et al., 2021). The semen contains both catalase and SOD antioxidant enzymes in very low amounts, responsible for the reduction of ROS, preventing lipid peroxidation of spermatozoa (Ribas-Maynou & Yeste, 2020). In the occurrence of oxidative stress, the activity of antioxidant enzymes is compromised (Malik et al., 2020). In the current study, although an increase in ROS is observed, activities of catalase and SOD remain unchanged. The oxidative stress in hyperglycaemic cells is caused by a rapid increase in ROS production regardless of the functional antioxidant enzyme system. Due to this, the antioxidant effect of both *A. johnsonii* and *G. livingstonei* target the neutralisation of ROS and not the activation of the antioxidant enzymes. The amount of antioxidant enzymes in semen is extremely low which makes it difficult to see the change in activity, especially sperm washing in the laboratory (Torres-Arce et al., 2021).

Besides their antioxidant properties, previous studies have also revealed the hypoglycaemic effect associated with flavonoids (Al Duhaidahawi et al., 2021). The reduction of glucose levels in patients with hyperglycaemia is paramount in the treatment of downstream complications (Poonosamy et al., 2023). Glucose metabolism is regulated by the counteractive effect of insulin and glucagon (Tomar et al., 2022). Insulin deficiency and/or the insensitivity of insulin to its receptors leads to the overload of glucose (Khalid et al., 2021). Accumulation of glucose leads to a non-enzymatic reaction of glucose molecules with proteins forming AGEs and consequent ROS and inflammatory markers (Perrone et al., 2020; Xu et al., 2021). Excessive ROS are also produced through oxidative phosphorylation in the mitochondria (Kadenbach, 2021). Flavonoids improve the insulin signalling pathway and repairs glucose uptake and metabolism (Hussain et al., 2020). This was observed in this study, where the treatment of C3A hepatocarcinoma cells with *A. johnsonii* and *G. livingstonei* led to increased glucose uptake.

Blood glucose balance also depends on the activity of enzymes such as α -amylase and α -glucosidase which are involved in the catabolism of carbohydrates to glucose (Ghauri et al., 2021). Both α -amylase and α -glucosidase regulate the absorption of glucose from the digestive tract to the bloodstream (Jagadeesan et al., 2022). The inhibition of carbohydrate metabolism enzymes is a study target in the investigation of alternative medication for DM (Oyewusi et al., 2023; Pant et al., 2021). In the present study, the α -glucosidase inhibitory activity of the plant extracts was compared to that of a standard drug (acarbose). It is evident that the inhibitory activity of both the plant extracts against α -glucosidase was higher compared to acarbose. Flavonoids have been reported to exhibit α -glucosidase inhibitory activity and are possibly linked to the observed effect (Hussain et al., 2020). Blood glucose regulation also depends on the activity of glycogen phosphorylase, an enzyme that breaks down glycogen to glucose in the liver (Shamsudin et al., 2022). The hypoglycaemic effect of flavonoids is also associated to their inhibitory activity against glycogen phosphorylase (Shamsudin et al., 2022).

Diabetes-related male reproductive complications are also a consequent of chronic inflammation (Płaczowska et al., 2024). The increased production of ROS and oxidative damage of biomolecules prompts the inflammatory response which involves macrophage infiltration and production and release of inflammatory markers (Charlton et al., 2020). Although inflammation is an immune response against tissue damage, prolonged high levels of inflammatory markers lead to more tissue damage and more ROS production (Yousef et al., 2023). Previous studies have shown the anti-inflammatory effect of hydroxycinnamic acids present in both *A. johnsonii* and *G. livingstonei* leaves (Coman & Vodnar, 2020). Hydroxycinnamic acids reduce anti-inflammatory cytokines through the inhibition of macrophage infiltration (Sova & Saso, 2020). In the current study, the reduction of nitrite after exposure to the selected plant extracts shows their potential in the reduction of inflammation. Chronic inflammation leads to damage of insulin receptors and consequent increased insulin resistance (Li et al., 2022). The reduction of inflammatory cytokines can therefore also be

linked to the improve glucose uptake observed. However, the RAW 264.7 mouse macrophage cell line may not fully mimic human immune responses. Results obtained using murine cells might not be entirely representative of effects in human cells. Also, only nitric oxide (NO) production is measured as an indicator of anti-inflammatory activity. This single-marker approach may overlook other inflammatory mediators (e.g., TNF- α , IL-6) that could provide a more comprehensive view of anti-inflammatory effects.

The prevalence of male infertility in diabetic men is over 35% (Lotti & Maggi, 2023). The decline in male sperm parameters is a causative factor of male infertility in males with DM (Sharma et al., 2021). Hyperglycaemia-induced oxidative stress and inflammation leads to the damage of proteins, lipids and DNA of sperm cells (Krawczyk et al., 2023). Damaged sperm cells are either incapable of fertility or initiate downstream pathways leading to apoptosis (Upadhyay et al., 2022). In our present study, the subsequent oxidative stress in hyperglycaemic sperm cells led to the decline in sperm parameters such as DNA integrity, total motility, MMP, and acrosome integrity. In a previous study, the reduction of MMP in sperm cells was linked to the overload of the mitochondria with ROS, leading to mitochondrial damage (Moichela et al., 2021). Mitochondrial function in spermatozoa is necessary to produce energy in a form of ATP for sperm motility (Kumar, 2023). The importance of mitochondrial function in sperm cells is clarified in this study, as the reduction of MMP led to a significant decrease in total motility of the spermatozoa.

DNA damage caused by oxidative stress leads to DNA fragmentation (Vaughan et al., 2020). While fertilisation involves the fusion of genetic material, when spermatozoa with fragmented DNA undergo fertilisation, embryonic development is impaired leading to failed conception (Hamilton & Assumpção, 2020). In this study, the higher number of hyperglycaemic sperm cells containing fragmented DNA can be linked to oxidative damage. DNA ultimately leads to apoptosis of sperm cells leading to a decrease in total vitality and motility (Iovine et al., 2021). Oxidative stress has been reported for its apoptotic effect on different cells, and consequent

tissue damage (Arfin et al., 2021; Kong et al., 2022). In a previous study, oxidative stress in the testes was shown to cause death of testicular cells (Tian et al., 2020). A similar study concluded that oxidative stress leads to the reduction of live sperm cells (Takalani et al., 2021). These findings support our present observation. Acrosome integrity and non-spontaneous capacitation are pre-requisites of fertilisation (Dahan & Breitbart, 2022; Das et al., 2023). Oxidative stress leads to the inability of the sperm cell to undergo biochemical changes necessary for fusion with the zona pellucida of the oocyte (Ribeiro et al., 2022). Oxidative stress also leads to premature capacitation and acrosome reaction, which leads to reduced capacity to fertilise (Shi et al., 2024). In our current study, hyperglycaemia induction and oxidative stress evidently led to the decrease in acrosome integrity, and premature capacitation, regardless of absence of capacitation inducers.

The aqueous leaf extracts of *A. johnsonii* and *G. livingstonei* both had therapeutic effect against declined sperm parameters. However, the effect of the plant extracts differed in different concentrations, with *A. johnsonii* exhibiting its effect at highest concentrations while *G. livingstonei* had reduced effects as the concentration was increased. Also, *G. livingstonei* caused a significant increase in total sperm motility of hyperglycaemic spermatozoa, while *A. johnsonii* had no significant effect on total sperm motility.

8.2. Conclusion

The current study was designed to identify medicinal plants in Limpopo used to treat DM and male infertility in traditional medicine and to investigate their mechanism in the treatment of male infertility as a diabetic complication. Both selected plants (*A. johnsonii* and *G. livingstonei*) demonstrated high antioxidant capacity which can be attributed to the phenolic compound content. The selected plants were effective in reducing ROS in hyperglycaemia-induced spermatozoa. In this study, the decline in sperm parameters such as motility, vitality, DNA, MMP, capacitation, and acrosome reaction in diabetic individuals can be attributed to

excessive ROS production caused by hyperglycaemia. Although both plants demonstrated protective effects on sperm cells, *A. johnsonii* exhibited higher effects at its highest concentrations, while *G. livingstonei* achieved its maximum effect at the lowest concentrations. In addition to this, both plants exhibited maximum and hypoglycaemic effects, anti-inflammatory effects at high concentrations in a different cell test model, and less toxicity at lower concentrations. These findings reveal the importance of selection of the most appropriate concentration for treatment. This study highlights the therapeutic potential of *A. johnsonii* and *G. livingstonei* in mitigating oxidative stress, chronic inflammation, and ultimately sperm cell damage caused by hyperglycaemia. Although the current work closely focuses on aqueous extracts, comparison of the efficacy of ethanol-based extracts might offer more comprehensive insights, considering the solvent-dependent differences in the extraction of phytochemical compounds in medicinal plants. Both *A. johnsonii* and *G. livingstonei* are potentially beneficial in the treatment of DM-related male infertility, and with further studies, more effects of these plant extracts can be uncovered.

8.3. Recommendations

The effects of the selected plant extracts in this study do not offer a comprehensive context because it was solely conducted in a cell model where hyperglycaemia was induced in vitro. The utilisation of sperm cells collected from diabetic individuals could have revealed more comprehensive findings. Also, clinical in vivo studies could be conducted in the future. To help ensure the best therapeutic outcomes, further research to determine the optimal dose range for each plant is necessary. To offer a more holistic understanding of the mechanism of action of the plant extracts in improving reproductive parameters, exposure of the sperm cells with inflammatory markers such as IL-6, IL-8, and TNF- α and determining the effect of the plant extracts in reducing them could be beneficial.

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stress following hyperglycemia and hypoxia-induced vascular cell injury. *Cellular & Molecular Biology Letters*, 26(1), 40. <https://doi.org/10.1186/s11658-021-00283-8>

Addendum

Research outputs

Published articles

- Nethengwe, M., Kerebba, N., Okaiyeto, K., Opuwari, C. S., & Oguntibeju, O. O. (2024). Antioxidant, Anti-Diabetic, and Anti-Inflammation Activity of *Garcinia livingstonei* Aqueous Leaf Extract: A Preliminary Study. *International Journal of Molecular Sciences*, 25(6), 3184. <https://doi.org/10.3390/ijms25063184>
- Nethengwe, M., Okaiyeto, K., Opuwari, C. S., & Oguntibeju, O. O. (2022). A Review on Medicinal Plants Used in The Management of Male Infertility Associated with Diabetes Mellitus in Thengwe, Limpopo Province, South Africa. South Africa.

Articles submitted for publication

- Murendeni Nethengwe, Kunle Okaiyeto, Chinyerum S. Opuwari, Oluwafemi O. Oguntibeju. The ameliorative effect of *Androstachys johnsonii* aqueous leaf extract on hyperglycaemic spermatozoa. *Phytomedicine Plus*.
- Murendeni Nethengwe, Nasifu Kerebba, Kunle Okaiyeto, Chinyerum S. Opuwari, Oluwafemi O. Oguntibeju. Phenolic compounds profile and hypoglycaemic, anti-inflammatory and antioxidant properties of aqueous leaf extract of *Androstachys johnsonii*: In vitro study. *South African Journal of Botany*.
- Murendeni Nethengwe, Kunle Okaiyeto, Chinyerum S. Opuwari, Oluwafemi O. Oguntibeju The effect of *G. livingstonei* aqueous leaf extract on hyperglycaemic-

induced human sperm cell: An in-vitro study. *Clinical Traditional Medicine and Pharmacology*.

- Murendeni Nethengwe, Kunle Okaiyeto, Chinyerum S. Opuwari, Oluwafemi O. Oguntibeju The role of hyperglycaemia, oxidative stress, and inflammation in diabetes-related male infertility: Therapeutic properties of medicinal plants. *Saudi Journal of Biological Sciences*.

Conferences attended

- 35th JOHANNESBURG International Conference on “Chemical, Biological and Environmental Engineering” (ICCBEE-22) Nov. 28-29, 2022, Johannesburg (South Africa). A review on medicinal plants Used in the management of male infertility associated with diabetes mellitus in Thengwe, Limpopo Province, South Africa. Murendeni Nethengwe, Kunle Okaiyeto, Chinyerum S. Opuwari, Oluwafemi O. Oguntibeju. (Oral presentation).
- Cape Peninsula University of Technology Postgraduate Conference. Feb 2023, Bellville (South Africa) Oxidative stress biomarkers link diabetes mellitus and obesity: implications in male infertility. Murendeni Nethengwe, Kunle Okaiyeto, Chinyerum S. Opuwari, Oluwafemi O. Oguntibeju. (Oral presentation).
- Education and Research Training in Health Sciences conference. Aug. 29, 2024, Hamburg, (Germany). The treatment of diabetes-related male infertility using medicinal plants. Murendeni Nethengwe, Kunle Okaiyeto, Chinyerum S. Opuwari, Oluwafemi O. Oguntibeju. (Oral presentation).
- American Council for Medicinally Active Plants 13th Annual Conference. Oct 3-6, 2024,

Texas, (USA). "Potential impact of *Anchomanes difformis* aqueous extract on testes and epididymis of diabetic animal model". Murendeni Nethengwe, Kunle Okaiyeto, Chinyerum S. Opuwari, Oluwafemi O. Oguntibeju. (Oral presentation).