

Erectogenic activity of the *Maytenus procumbens* roots extract in type 2 diabetic rats

UNIVERSITY OF ZULULAND



By

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A dissertation submitted in fulfilment of the requirement for the **Ph.D.**
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2020

DECLARATION

The experimental work described in this dissertation was conducted in the Department of Biochemistry and Microbiology, Faculty of Science and Agriculture, University of Zululand and South African Medical Research Council (SAMRC), Cape Town between June 2016 and December 2019, under the supervision of Dr. RA Mosa and Prof. AR Opoku.

I **NKOSINATHI DAVID CELE**, hereby affirm that the work entitled “Erectogenic activity of the *Maytenus procumbens* roots extract in type 2 diabetic rats” is my work. Where use was made of the work of others, it has been duly acknowledged in the text.

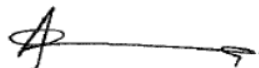
I DECLARE THE ABOVE STATEMENT TO BE TRUE.

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DEDICATION

This work is dedicated to my late parents (Mr. AM & Mrs. NM Cele), Cele family (Mlungisi, Nompilo, Zanele, Hlengiwe, Thabiso, Smiso, Luthando and Minenhle), and mostly to my sons (Melusi and Mngqobi Cele) for their special support during the period of my study.

ACKNOWLEDGEMENTS

My deepest gratitude to our Lord Jesus Christ for His Favor, Grace and Mercy upon me.

A humble thank you to the following people for their indispensable contribution to the success of this work:

Dr. RA Mosa, Prof AR Opoku and Dr. B Folusho for their invaluable guidance.

I appreciate the support given by our Biochemistry research group and Biomedical Research & Innovation Platform, MRC team. Their support has made my research easier. This research was financially supported by the University of Zululand Research Committee.

I am grateful to the National Research Foundation (NRF) for awarding me a scholarship.

Finally, KZN traditional healers for willingly sharing their valuable knowledge.

Friends and colleagues for their encouragement.

My family for their support.

General Abstract

Erectile dysfunction (ED), one of the common complications of type 2 diabetes in males, is the global health problem that markedly affects the quality of sexually active men. Since the current hypoglycemic and erectogenic drugs, lack the dual effect to ameliorate both hyperglycemia and ED respectively, the multi-target nature of medicinal plants could prove vital in the treatment of diabetes induced ED. This study investigated the erectogenic effect of the roots extracts of *Maytenus procumbens* in type 2 diabetic rats.

The plant material was separately extracted with methanol and dichloromethane. The extracts were screened for their potential antioxidant, antiprotein glycation, antidiabetic and erectogenic activities *in vitro*. The *in vivo* erectogenic effect of the methanolic extract of *M. procumbens* roots was investigated using type 2 diabetes induced erectile dysfunction in rat model. The fructose-streptozotocin model was used to induce type 2 diabetes-linked ED in sexually active male *Sprague Dawley* rats. The rats were randomly divided into normal group (normal diet and drinking tap water) and high fructose fed group (normal diet and 25% of fructose in drinking water) for 120 days. The high fructose fed group rats were then given a single intraperitoneal injection of streptozotocin solution (30 mg/kg). The diabetic ED rats were orally administered with the extract at 250 mg/kg, daily for a further 28 days. The animals were then sacrificed, and the serum, brain and penile tissues were removed for biochemical analysis and protein expression.

The *in vitro* studies revealed that methanolic extract exhibited strong antioxidant activities as they efficiently scavenged most of free radicals tested. Both methanolic and dichloromethane extracts displayed inhibitory effects on protein glycation and antidiabetic properties. Furthermore, both extracts inhibited PDE-5 (IC_{50} values ranging from 0.02 and 0.1 mg/ml), arginase ($IC_{50} = 0.1$ mg/ml), AChE, and ACE activities. The *in vivo* study further confirmed the erectogenic potential of the methanolic extract as it effectively lowered the activities of arginase, AChE and ACE along with increased serum testosterone levels in the extract treated diabetic group. While a relatively higher expression of PDE-5 and RhoA with decrease in the expression of eNOS were observed in the untreated diabetic control group, a reverse pattern in the expression of these proteins was observed in the diabetic group treated

with the extract. Both *in vitro* and *in vivo* studies further revealed the antioxidant and antiprotein glycation activities of the methanol extract.

These results support the use of *M. procumbens* roots in the treatment and management of erectile dysfunction.

LIST OF ABBREVIATIONS USED

AA	Ascorbic acid
ABTS	2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
AChE	Acetylcholinesterase
ASChi	s-acetyl- thiocholine iodide
BHA	Butylated hydroxyl-anisole
BHT	Butylated hydroxyl toluene
CAT	Catalase
DPPH	2, 2-Diphenyl-1-picryl-hydrazyl
DTNB	5, 5'-dithiobis-(2-nitrobenzoic acid)
EDTA	Ethylenediamine tetra-acetic acid
IC50	Inhibitory concentration at 50%
MDA	Malondialdehyde
NBT	Nitroblue tetrazolium
NO•	Nitric oxide radical
O ₂ ^{•-}	Superoxide anion radical
•OH	Hydroxyl radical
PDE-5	Phosphodiesterase
ROS	Reactive oxygen species
AGE	Advanced glycation end products

ACE	Angiotensin converting enzyme
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
BW	Body weight
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
cGMP	cyclic-guanine monophosphate
GTP	Guanosine triphosphate
sGC	Soluble guanylate cyclase
AMPK	Adenosine monophosphate-activated protein kinase
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
STZ	Streptozotocin
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
T2DM	Type 2 Diabetes mellitus
IDF	International Diabetes Federation
UZRC	University of Zululand Research Committee
ED	Erectile dysfunction
ANOVA	One way analysis of variance

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CHAPTER ONE

1.0 Introduction

Among different forms of male sexual dysfunction which include premature ejaculation, low sperm count and arousal difficulties, erectile dysfunction (ED) appears to be the most common form that occurs in sexual life of millions of men globally (Alin *et al.*, 2012, Singh *et al.*, 2012). Among other factors such as aging, alcohol abuse, cardiovascular disease, oxidative and psycho-social stresses, type 2 diabetes (T2D), characterized by chronic hyperglycemia, is considered one of the major contributing factor to the development and progression of ED (Thakur *et al.*, 2011).

ED has remained one of the greatest underdiagnosed yet common complication of T2D in men. This could partly be due to the fact that most diabetic men do not talk about status of their sexual activity during medical consultations (Lozano *et al.*, 2016). The underlying mechanisms of type 2 diabetes leading to the induction of ED is complex. It is related to advanced glycation end products (AGEs) formation, endothelial dysfunction, hypogonadism, neurological modifications and reduced nitric oxide synthesis (Tamola and Chitale, 2009; Zhang *et al.*, 2015). Due to the multifactorial nature of ED, the use of multi-target therapeutic agents could prove ideal towards amelioration of the condition.

There is limited information, if any, on the effectiveness of the current oral hypoglycemic drugs to exert both glycemic and erectogenic control. In an attempt to treat or manage ED, the use of pharmaceutical agents such as Sildenafil (Viagra) and Tadalafil (Cialis) has gained much more popularity and become first line therapy in men's clinics. Despite the rapid increase in number of men's clinics and use of such pharmaceutical agents, a large percentage of men, particularly in rural areas still consult traditional healers for their sexual primary health care needs.

Traditional use of medicinal plants against various ailments, including sexual dysfunctions, has been practiced since ancient times. A number of medicinal plant species have been documented as stimulants of sexual activities (Wani *et al.*, 2011; Zade *et al.*, 2013). Recently in our laboratory, the ameliorative potential of *Maytenus procumbens* roots extract against testicular dysfunction has been reported (Cele *et*

al., 2017). The ameliorative effect were associated with the regeneration of spermatogonia in the seminiferous tubules, increase serum testosterone, sperm count and mount frequency of the rats. Therefore, these findings have raised an interest to search and explore the biological activities of this plant against ED. Thus, this study aimed at investigating the erectogenic activity of the *Maytenus procumbens* roots extract in type 2 diabetic rats.

1.1 Structure of the Dissertation

The dissertation consists of seven chapters and three appendices of which the contents and information thereof are described as follows:

Chapter 1:

This chapter gives a brief background and motivation of the study. Structure of the dissertation is also provided.

Chapter 2:

The chapter presents literature review together with the aim and objectives of the study.

Chapters 3:

This chapter is prepared in a journal format. The chapter consists of three parts, A, B and C. Each of the parts include the abstract, introduction, materials and methods, results, discussion and conclusion.

Chapter 4:

The chapter gives overall discussion of the obtained results in context to literature and the research question.

Chapter 5:

This chapter gives the conclusion drawn from the obtained results. Limitations and recommendation(s) for further studies are also provided.

Appendices:

Presents all additional or supplementary information and/or documents.

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CHAPTER TWO

GENERAL LITERATURE REVIEW

2.1 Erectile dysfunction

Erectile dysfunction (ED), a common form of male sexual dysfunction, is a global health problem that markedly affects the quality of life of sexually active men (Singh *et al.*, 2012). ED is a vascular and smooth muscle disorder that is characterized by a repeated inability to initiate and maintain penile erection firm enough for normal sexual intercourse (Nayak and Buttar, 2015; Walle *et al.*, 2018). Approximately 100 million men are suffering from ED worldwide (Alin *et al.*, 2012) and this number is expected to increase to about 322 million by the year 2025 (Ismail and El-Sakka 2016). Out of this increasing global prevalence of ED, African men account for about 12% (Binmoammar *et al.*, 2016, Seid *et al.*, 2017), which is an alarming figure and a great course for concern.

Even though normal penile erection capability declines with age, increasing incidences of ED is amplified by the rising prevalence of metabolic and endocrine disorders such as diabetes mellitus, obesity and hypogonadism (Saxena *et al.*, 2012). Numerous other factors such as hypertension, cardiovascular disease, oxidative and psycho-social stresses contribute to the development of ED (Nunes and Webb, 2012; Malviya *et al.*, 2016). The underlying mechanisms of ED are very complex and they are linked to neurogenic, psychogenic, vascular and hormonal factors. Under pathophysiological conditions, ED occurs as a result of: (1) diminished functioning of the arteries and corpora cavernosa within the penile tissue, (2) an impairment of cavernosal arteries, (3) penile smooth muscle tone deficiency, and (4) defects in neuronal stimuli which lead to unsuccessful penile erection (Dean and Lue, 2005).

ED development has also been widely described in terms of insufficient nitric oxide (NO) and cyclic-guanine monophosphate (cGMP) production in the corpus cavernosal within the penile tissue (Podlasek *et al.*, 2001; Akingba and Burnett, 2001; Li *et al.*, 2014). The NO/cGMP signal transduction pathway is crucial for maintenance of smooth muscle relaxation and thus normal penile erection process (Kim and Park,

2011). Considering the multifactorial nature of ED, the use of multi-target therapeutic agents could prove ideal towards amelioration of the condition.

2.2 An overview of the mechanism of penile erection

Penile erection is a neurovascular event, dependent on the capacity of a functional vascular system, neural integrity, and healthy corpus cavernosal tissue (Ajiboye *et al.*, 2018). Penile erection requires the coordination of vasodilatation and corpus cavernosal smooth muscle relaxation (Toque *et al.*, 2013). Normally, this process involves three synergistic and simultaneous processes: (1) neurologically-mediated increase in arterial blood flow into the penile tissue, (2) cavernosal smooth muscle relaxation and (3) restriction of venous outflow of blood from the penile tissue (Bivalacqua *et al.*, 2003). The endothelium controls the contractile state of the vascular smooth muscle (VSM).

Sexual stimulation causes the release of neurotransmitters such as NO, acetylcholine (ACh) from the cavernous nerve terminals to stimulate the penis. The impulses from the brain and local nerves trigger corpora cavernosa relaxation, allowing blood to flow in and fill the expanded sinusoids. The molecules such as NO, cGMP, and ACh are considered crucial in penile erection processes (Obboh *et al.*, 2015). Thus, alteration in the levels of these cellular messengers is central to ED development. In addition, the local renin-angiotensin system that exists in the corpus cavernosum plays an important role in maintaining the corpus cavernosal tone by balancing the bradykinin (BK)-induced relaxation with the angiotensin II (Ang II)-induced contraction (Tamola *et al.*, 2009).

2.2.1 Vasodilatation and corpus cavernosal smooth muscle relaxation

A normal penile erection, which involves an increased blood flow into corporal sinusoids due to smooth muscle relaxation, is triggered by release of NO from non-adrenergic and non-cholinergic nerves (Toque *et al.*, 2011; Toque *et al.*, 2013; Ademosun *et al.*, 2019). The endothelium-derived relaxing factor, NO, is synthesized in most tissues from L-arginine in a reaction catalyzed by nitric oxide synthase (NOS) (Nunes and Webb, 2012). The constitutive form of NOS (EC: 1.14.13.39) has three isoforms, neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS).

While the nNOS and eNOS are coupled to Ca^{2+} and calmodulin, and are the principal NOS isoform involved in the induction of penile erection, the iNOS is independent of Ca^{2+} and calmodulin (Bivalacqua *et al.*, 2003).

The maximum production of NO is dependent on the availability of arginine which is also a substrate for arginase (EC: 3.5.3.1), a competitor of NOS. While NOS catalyzes the conversion of L-arginine to NO, arginase catalyzes the conversion of L-arginine to urea and ornithine, thereby reducing arginine levels that could be used for NO production by NOS. Even though arginase exist in two isoforms arginase I and II, both isoforms have similar mechanisms of action. Both isoforms are found in the endothelium of the vasculature (Oboh *et al.*, 2019). Studies have shown that arginase inhibitors improve NO production and enhance endothelium dependent vasodilation in the penile tissue (Oboh *et al.*, 2015, Adefegha *et al.*, 2017).

The endothelium-derived NO targets the heme moiety of soluble guanylate cyclase (sGC), an enzyme that stimulates generation of the second messenger cGMP from guanosine triphosphate (GTP) (Figure 2.1).

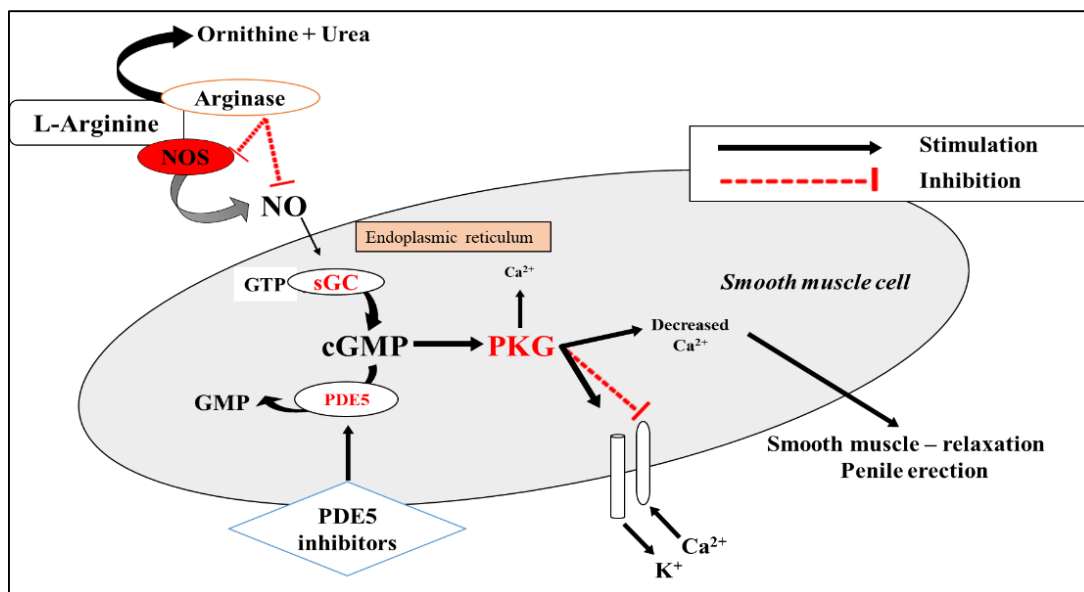


Figure 2.1: Vasorelaxant effect of NO. The relaxant effect of NO is mediated by a cellular second messenger cGMP. NO activates guanylyl cyclase which in turn converts GTP to cGMP, resulting in increased levels of cGMP. Subsequent phosphorylation of cellular membrane proteins via protein kinase G results in an efflux of calcium, which leads to smooth-muscle relaxation, vasodilatation of the penile arteries and penile sinus, and erection.

cGMP induces a cascade of reactions culminating in smooth muscle relaxation, enhanced vascular blood flow and finally penile erection (Young *et al.*, 2003; Kim and Park, 2011). The actions of cGMP are exerted through cGMP-gated cation channels and cGMP-dependent protein kinases (PKGs). The importance of cGMP signaling is not only recognized in the endothelial and vascular smooth muscles relaxation, but also in cardiac myocyte function (Tsai and Kass, 2009). The biochemical actions of cGMP are terminated by phosphodiesterase-5 (PDE-5, EC 3.1.4.17) which hydrolyzes cGMP to GMP, thus inhibiting the smooth muscle relaxation and erectile process (Campos *et al.*, 2019).

Even though there are eleven different isoforms of PDEs existing, their activities are found in every cell in the body and the distinct cellular and subcellular distribution of the eleven isoforms have provided a great potential for increasingly selective therapeutic targets (Lugnier, 2005; Boswell-Smith *et al.*, 2006). PDE-5 is mainly found in the corporal cavernosum smooth muscles, thus has become a primary target in management of ED (Corbin, 2004). PDE-5 inhibitors such as Sildenafil, Tadalafil and Udenafil are currently used as first line treatment for ED (Schellack, 2014, Okeke *et al.*, 2018). PDE-5 inhibitors have been shown to prolong the actions of cGMP, thereby amplifying the NO signal and stimulating relaxation of smooth muscle cells in the arteries, arterioles, and sinusoids of the corpus cavernosum of the penis (Francis *et al.*, 2010).

2.2.3 Role of acetylcholine in penile erection

The penile tissue is a reservoir of cholinergic nerves and ACh molecule (William *et al.*, 2012). Upon ACh release, it binds to endothelial cells receptor, thus triggering intracellular release of Ca^{2+} via Inositol triphosphate (IP3) pathway. The increase in intracellular Ca^{2+} levels has dual effect: (1) stimulation of skeletal muscle contraction and (2) activation of NOS that catalyzes synthesis of NO from L-arginine (Figure 2.2) (Wymke *et al.*, 2013). NO subsequently diffuses into adjacent smooth muscle cell, where it initiates the cascade of reactions leading to stimulation of cGMP-dependent protein kinases, and subsequent smooth muscle relaxation (Andersson, 2011).

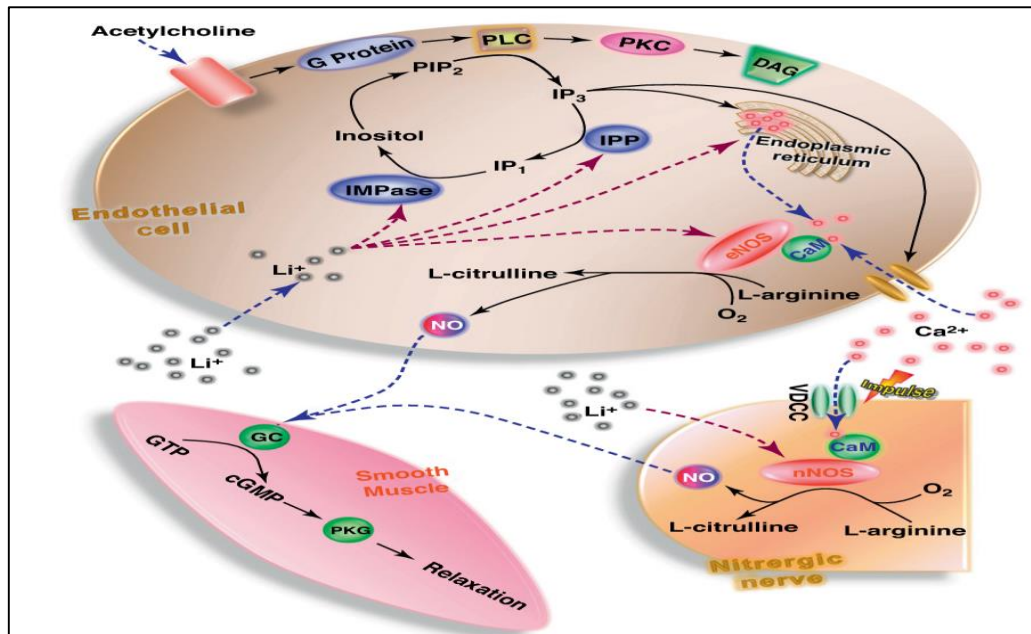


Figure 2.2: Role of ACh on smooth muscle relaxation. ACh binds to the endothelial cells receptor, triggering an intracellular release of Ca^{2+} that activates NOS and thus production of NO. The interaction of NO with sGC results in the conversion of GTP into cGMP which initiates the stimulation of cGMP-protein kinases, and subsequent smooth muscle relaxation. (Source: Mishra and Goel, 2016).

The physiological levels of ACh are regulated by acetylcholinesterase (AChE, EC: 3.1.1.7), an enzyme that hydrolyzes ACh to acetate and choline (Figure 2.3).

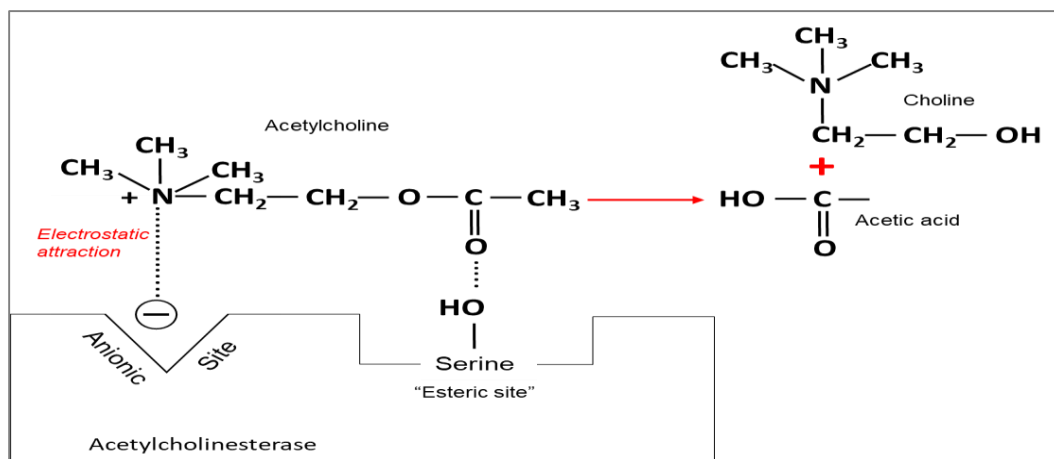


Figure 2.3: Hydrolysis of acetylcholine by acetylcholinesterase to acetic acid and choline (Source: ATSDR, 2012).

Increased activity of AChE has been implicated in the development of ED as this enzyme lowers the levels of ACh in the penile tissue, which in turn diminishes penile erection process (Li *et al.*, 2014). Thus, inhibition of AChE activity could increase

bioavailability of ACh, which could stimulate smooth muscle relaxation and restores penile erection in patients with ED (Akolomafe *et al.*, 2016, Akolomafe *et al.*, 2018). Therefore, discovery of new inhibitors of AChE could also have therapeutic effects against ED. The inhibitory effect on AChE activity has been shown to ameliorate NO production and relaxation of corpus cavernosal tissue in STZ induced diabetic rats (Ojo *et al.*, 2019).

2.3 Contribution of endocrine and metabolic disorders in ED development

ED is a sexual dysfunction of multiple pathophysiologies including hypertension, cardiovascular disease, diabetes mellitus and hypogonadism. Endocrine and metabolic disorders in the form of hypogonadism and diabetes are considered the most common and major risk factors for ED (Gonzalez *et al.*, 2015; Velez and Naranjo, 2018).

2.3.1 Hypogonadism

Androgens such as testosterone, dihydrotestosterone and androstenedione are indispensable for physical activity and normal penile erection process in men (Wilson, 1999). The mechanism through which androgens mediate the penile erection response is due to maintaining: (1) an adequate supply of NO, (2) the penile structure, and (3) the endothelial function of the corpus cavernosum (Kataoke *et al.*, 2014). Androgen deficiency, commonly known as hypogonadism is associated with a loss of libido and reduced sexual penile sensation (Kataoka *et al.*, 2017). Various factors such as aging, type 2 diabetes mellitus, pituitary and hypothalamic dysfunction contribute to the development of hypogonadism and consequent reduced testosterone levels (Julia and Khalil, 2003). Numerous studies have demonstrated the involvement of testosterone in NO mediated vasorelaxation required for normal penile erection (Anderson, 2011; Doshi *et al.*, 2012). Testosterone binds to androgen/estrogen receptors leading to the activation of eNOS that catalyzes the synthesis of NO from L-arginine (Figure 2.4) (Orshal and Khalil, 2003).

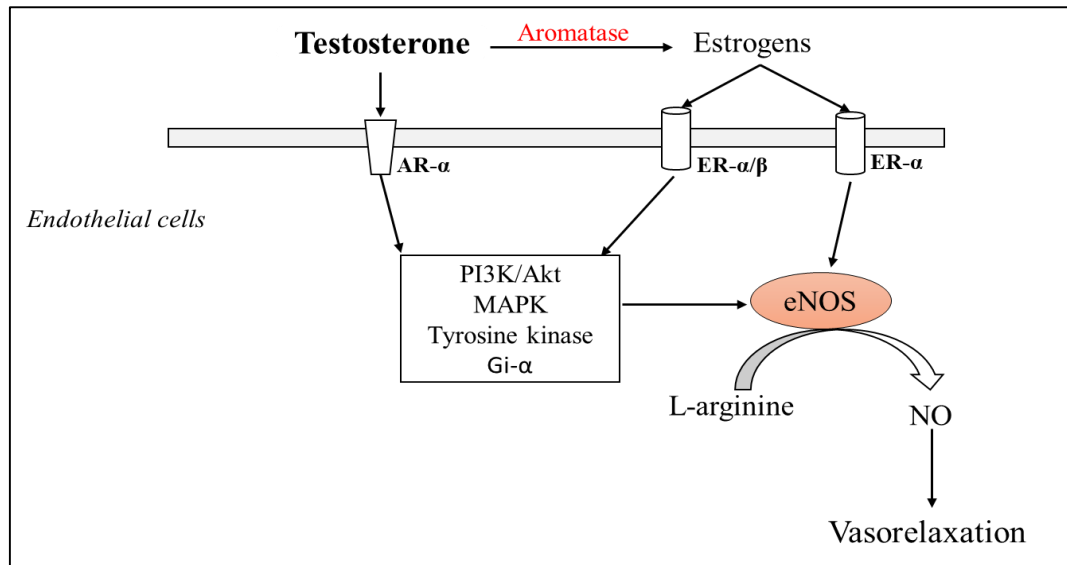


Figure 2.4. Effect of testosterone on NO production. Testosterone increases endothelial synthesis of NO through stimulation of the extracellular-signal-regulated kinase (ERK) and phos-phatidylinositol3-OH kinase (PI3K)/Akt cascades. Activation of both estrogen (ER) and androgen receptors (AR) regulates endothelial function through the mechanisms involved in the activation of the cascade of signaling proteins such as PI3K, Akt, MAPK, and tyrosine kinase, and $G_{i\alpha}$, and subsequently results in eNOS activation and NO production.

Moreover, Leydig cells and Sertoli cells, like any other steroid producing cells, synthesize testosterone from a common precursor cholesterol through steroidogenic machinery, regulated by luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Bjelic *et al.*, 2015). However, prolactin, a protein hormone produced by the pituitary gland, directly influences the steroidogenesis and spermatogenesis by acting on prolactin receptors present in Leydig and Sertoli cells in the testes and continuously decreases testosterone levels to produce primary hypogonadism and male infertility (Gonzalez *et al.*, 2015, Alagbonsi *et al.*, 2019). Physiological actions of prolactin have been shown to suppress the secretion of LH and FSH and lowers testosterone levels, resulting in decreased sexual drive in male Wister rats (Adeneye *et al.*, 2019).

Furthermore, it has been shown that androgen deficiency and upregulation of RhoA/ROCK II contribute in the pathophysiology of ED (Ismail and I-Sakka, 2016). Studies in castrated rats' model demonstrated that ED linked to hypogonadism is in part mediated through an increased expression of RhoA, preventing relaxation of penile smooth muscle tone and subsequently penile erection (Chitaley *et al.*, 2001; Karakaya *et al.*, 2019). It has also been reported that upregulation of arginase activity

due to testosterone deficiency is involved in downregulation of NOS expression in castrated rats (Wei *et al.*, 2000; Zhang *et al.*, 2001; Toque *et al.*, 2011). Therefore, inhibition on the activities of RhoA, a small GTPase which induces Rho-kinase II (ROCK II) activity, and arginase could also serve as alternative targets in the discovery of new ED therapies for patients with androgen deficiency.

2.3.2 Type 2 diabetes mellitus

Type 2 diabetes mellitus, commonly characterized by cellular insulin resistance and persistent hyperglycaemia, is one of the most dominant causes of ED. Literature reports that the risk of developing ED in diabetic men is three-fold higher when compared to that in non-diabetic men (Karakaya *et al.*, 2019). The underlying mechanisms linking type 2 diabetes with ED induction are also complex. The persistent hyperglycaemia commonly observed in diabetic patients is considered the main contributor to the development of micro-and macro-vascular complications of diabetes (Ortiz-Andrade *et al.*, 2007; Gutierrez, 2013). Hyperglycaemia-induced oxidative stress, common in the diabetic state, stimulates several glucose metabolic pathways that are associated with diabetic complications. These include the polyol pathway, formation of advanced glycation end products (AGEs), and decreased antioxidant defence, activation of protein kinase C pathway (Zychowska *et al.*, 2013; Sandireddy *et al.*, 2014). Various other factors such as endothelial dysfunction, hypogonadism, neurological modifications and reduced NO synthesis are commonly implicated (Tamola and Chitale, 2009; Zhang *et al.*, 2016).

AGEs trigger cellular damage through modification of structural proteins. By binding to the receptor of advanced glycation end products (RAGE) which are extremely expressed in endothelial cells of the peripheral nerves of diabetic rats, AGEs have been reported to reduce bioavailability of NO (Sopko *et al.*, 2014). Furthermore, interaction between AGE and endothelial cells up-regulates adhesion molecules such as ROCK II, arginase, angiotensin converting enzyme (ACE), AChE and PDE-5 that mediate vascular damage and consequently ED (Tamola and Chitale, 2009). In type 2 diabetes, elevated levels of plasma endothelin-1 induces oxidative stress and vasoconstrictor mediators such as angiotensin II (ANG II), triggering vascular endothelium damage and reduction in bioavailable NO (Velez and Naranjo, 2018; Ogrogen *et al.*, 2018).

Despite the well-known metabolic actions of insulin, studies have also shown the involvement of insulin in stimulation of NO production (Muniyappa *et al.*, 2008; Mollace *et al.*, 2015). Insulin-signalling pathway regulating vascular endothelial production of NO is dependent on phosphatidylinositol 3-kinase (PI3K). Insulin phosphorylates activates the cascade of signalling proteins such as PI3K, Akt and downstream serine phosphorylation of eNOS, which in turn results to NO production (Figure 2.5) (Montagnani *et al.*, 2001; Montagnani *et al.*, 2002).

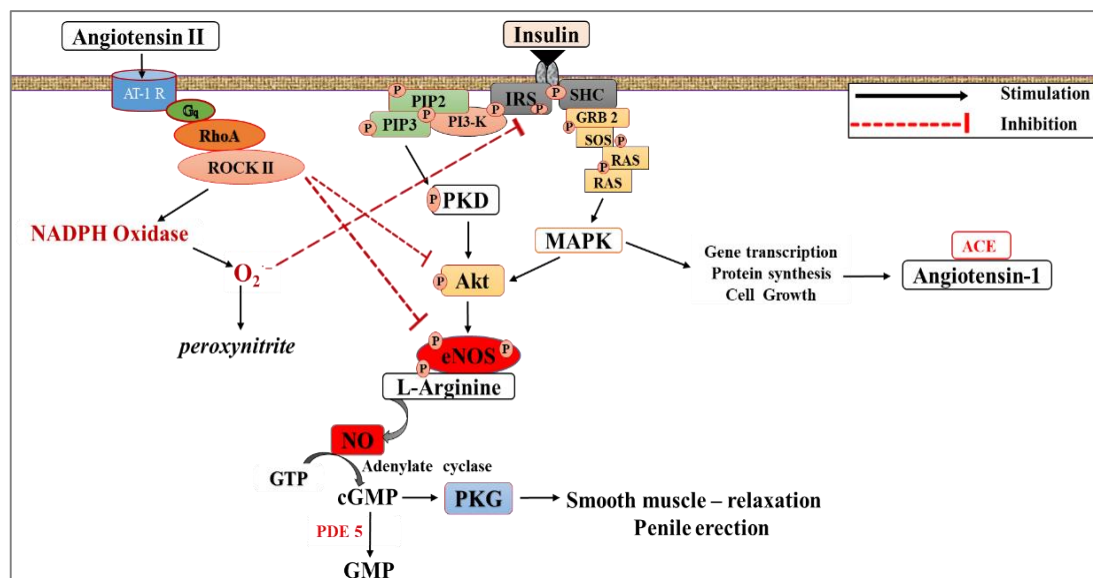


Figure 2.5: Effect of insulin on eNOS. Insulin binds to receptor substrate (IRS)-1, which then activates PI3K. Lipid products of PI3K (PI-3, 4, 5-triphosphate or PIP3) stimulate phosphorylation and activation of Akt. Akt directly phosphorylates eNOS, resulting in increased eNOS activity and subsequent NO production. The interaction of NO with sGC results in the conversion of GTP into cGMP and initiates the stimulation of cGMP-protein kinases, and subsequent smooth muscle relaxation. ACE triggers the activation of ANG II and stimulates RhoA. Activated RhoA induces NADPH oxidase and directly inhibits the expression of eNOS, leading to a decreased activity of eNOS and subsequent reduced NO production.

Insulin actions are inhibited by activation of ROCK II (Figure 2.5) (Goswami *et al.*, 2012). Activated ROCK II directly inhibits PI3K/Akt signalling and expression of eNOS, which leads to diminished eNOS activity, increased myosin light chain activation with vasoconstriction. (Figure 2.5) (Chitale *et al.*, 2001; Sowers, 2004.). Moreover, literature reports that upregulation of ROCK II in diabetic men increases the expression and activity of arginase. This in turn decreases the L-arginine concentration as the substrate for eNOS, causing eNOS uncoupling and reduced NO

production (Berkowitz *et al.*, 2003; Toque *et al.*, 2013). Thus, inhibition of ROCK II, ACE and arginase activities could also be crucial in the management of ED. The erectogenic effects as a results of a decreased expression of protein RhoA/ROCK II as well as reduced ACE activity in diabetic animals has been reported (Guagnin *et al.*, 2012; Phe and Roupret, 2012).

Furthermore, it has been shown that impaired PI3K signaling and enhanced activity of MAPK in response to insulin results in a decrease in NO production and increased secretion of endothelin-1 (ET-1), ANG II, and ROCK II, characteristics of endothelial dysfunction which are associated with ED (Muniyappa *et al.*, 2008; Goswami *et al.*, 2012). Endothelial dysfunction due to hyperglycemia causes the alterations in signal transduction responsible for the stimulation of smooth muscle relaxation, increases oxidative stress, decreases the release of endothelium-derived hyperpolarizing factors and subsequently destructs NO production (Minaz *et al.*, 2019). Experimental studies provide evidence that restoration in erectile function in diabetic animals may be associated with attenuated oxidative stress and repaired Akt activity accompanied by ameliorated endothelial function in corpus cavernosum smooth muscle cells (Li *et al.*, 2014; Fu *et al.*, 2019).

2.3.3 Oxidative stress

Oxidative stress is implicated as one of the major underlying pathophysiological causes of ED (Jeremy *et al.*, 2000). Interaction of NO with superoxide anion is considered one of the key mechanisms linked to the pathophysiological events resulting to ED (Jones *et al.*, 2002). The reaction of NO with superoxide anion produces a cytotoxic species, peroxynitrite. The peroxynitrite is known to cause a direct DNA base and protein oxidation as well as deactivate the activity of superoxide dismutase (SOD) which results to decreased removal of superoxide anions (Zou *et al.*, 1997). Increase in superoxide anions and peroxynitrite levels is known to perpetuate incidence of endothelium apoptosis and consequent endothelial dysfunction (Agarwal *et al.*, 2013). Loss of functional unity of the endothelial cells due to oxidative stress results to various vascular complications including ED (Traish *et al.*, 2008). Furthermore, under physiological conditions, the excess generated ROS is eliminated by endogenous antioxidant enzymes such as SOD, catalase (CAT) or glutathione peroxidase (GPx) (Akolomafe *et al.*, 2016). Fatani *et al.* (2015) showed the

ability of lutein to ameliorate activities of SOD, CAT and GPx as well as enhances erectile function in diabetic rats.

In the pathophysiological condition of ED, the antioxidant defense system is compromised, thus increasing chances of oxidative damage to nerve endings in the penile tissue, which results to reduced bioavailability of NO, impaired corpus cavernosum relaxation and consequently results to ED (Fu *et al.*, 2019). Studies have shown that the use of exogenous plant-derived antioxidants reverses the pathophysiological events leading to the onset and progression of ED (Yakubu and Akanji, 2010; Pomjunya *et al.*, 2017). In accordance with literature, medication with high antioxidant content could then be considered as the primary target in the management for ED, thus it may serve as a protective therapy against oxidative stress to the vasculature of the penis.

2.4 Current management of ED and future outlook

Even though male sexual dysfunctions cannot all be prevented, but some crucial measures can be considered in order to reduce the risks of ED. These include adherence to healthy lifestyles which include consumption of proper diet (rich in proteins and micronutrients), and regular exercise. Pharmaceutical agents such as Sildenafil (Viagra) and Tadalafil (Cialis) are also currently used in the management of ED. However, these pharmaceutical agents are costly, not readily available especially to men living in rural areas. They are also associated with adverse effects such as headache, muscle pain and blurred vision, which all limit their clinical use (Melynk *et al.*, 2011).

Furthermore, the fast-onset nature of these pharmaceutical agents does not always align with an individual's interest, hence some men desire a durable-response therapy that will dissociate the use of these drugs during sexual intercourse. Moreover, there is an increasing number of men who are initially refractory or become refractory to PDE-5 inhibitors, whereas combination of oral and non-oral therapy seems to salvage PDE-5 inhibitors (Nehra, 2007). Thus, due to these limitations associated with the currently available remedies, there is a continuous search for alternative drugs for the management of erectile dysfunction. Due to the multifactorial nature of ED, the use of

multi-target therapeutic agents could prove ideal towards amelioration of this condition.

A large number of men in Africa, particularly in rural areas still consult traditional healers for their sexual primary health care needs. They rely much on their cultural treatment, which commonly make use of plants as a primary source of treatment. Furthermore, an increase in number of herb marketplaces strongly indicates the high demand and probable effectiveness of medicinal plants in the treatment of various diseases. Studies have shown that drugs that are derived plants represent a significant proportion of the pharmaceutical market, particularly in ED therapy, as well as other diseases (Tarking *et al.*, 2016). Thus, researchers have turned their focus on medicinal plants as alternatives for the development of new erectogenic drugs that could target primary cause of erectile dysfunction.

2.4.1 Medicinal plants use as a promising alternative treatment for ED

The use of medicinal plant extracts, due to their multi-target nature, continue to attract attention of researchers in a bid to discover new drug regime against various diseases including ED. Medicinal plants display their therapeutic properties due to their polypharmacological interactions with phytochemical constituents. Literature reports the significant impact of secondary metabolites such as saponins, flavonoids, alkaloids, anthraquinones, sterols, and tannins in the prevention and treatment of diabetes-induced erectile dysfunction in rats (Ferreira *et al.*, 2006; Pomjunya *et al.*, 2017). Medicinal plants simultaneously target multiple pathophysiological processes through interactions between multiple compounds and cellular target proteins (Table 2.1), and thus, reversing the biological networks from illness state to wellbeing state (Gu *et al.*, 2013). Therefore, in a continuous discovery of the new pharmacologically active erectogenic agents, the erectogenic effects of various medicinal plant's extracts have been evaluated and reported. Examples of African medicinal plants with erectogenic activity are presented in Table 2.1.

Table 2.1: Some of the African medicinal plants used in the treatment of erectile dysfunction

Name of a plant	Family	Part used	Apparent mechanism of action	References
<i>Eriosema kraussianum</i>	Papilionaceae	Roots	Smooth muscle relaxation effects and inhibitory effects on PDE-5	Malvina <i>et al.</i> , 2011
<i>Citropsis articulata</i>	Rutaceae	Leaves	Increased testosterone levels and mount frequency.	Vudriko <i>et al.</i> , 2013
<i>Vernonia cinerea</i>	Asteraceae	Leaves and stem	Increased testosterone levels, improved regeneration of spermatogonia and antidiabetic effect. Increased antioxidant activity.	Pomjunya <i>et al.</i> , 2017
<i>Mondia whiteii</i>	Asclepiadiaceae	Roots	Increased NOS activity.	Martey and He, 2010
<i>Ozoroa paniculosa</i>	Anacardiaceae	Roots	Increased testosterone, Improved regeneration of spermatogonia and mount frequency. Increased antioxidant activity.	Cele <i>et al.</i> , 2017
<i>Tarenna graveolens</i>	Polygonaceae	Roots	Increased testosterone levels	Oloro <i>et al.</i> , 2016
<i>Ocimum gratissimum</i>	Lamiaceae	Leaves	Inhibitory effects on PDE-5, arginase, AChE and ACE activities. Increased antioxidant activity.	Ojo <i>et al.</i> , 2019B
<i>Ficus capensis</i>	Moraceae	Leaves	Inhibitory effects on arginase, AChE & ACE activities. Increased antioxidant activity	Akomolafe <i>et al.</i> , 2016
<i>Physalis angulata</i>	Solanaceae	Leaves	Inhibitory effects on PDE-5, arginase, AChE & ACE activities. Increased antioxidant activity.	Akomolafe <i>et al.</i> , 2018
<i>Newbouldia laevis</i>	Bignoniaceae	Leaves	Inhibitory effects on PDE-5, arginase, AChE & ACE activities. Increased antioxidant activity.	Akomolafe <i>et al.</i> , 2018

<i>Maytenus procumbens</i>	Celastraceae	Roots	Increased testosterone levels and mount frequency, Improved regeneration of spermatogonia. Increased antioxidant activity.	Cele <i>et al.</i> , 2017
<i>Garcinia Kola</i>	Clusiaceae	Fruits	Inhibitory effects on PDE-5, arginase, AChE & ACE activities. Increased antioxidant activity	Ojo <i>et al.</i> , 2019A
<i>Aframomum melegueta</i>	Zingiberaceae	Seeds	Inhibitory effects on PDE-5, arginase, AChE & ACE activities. Increased antioxidant activity	Okeke <i>et al.</i> , 2018
<i>Aframomum danielli</i>	Zingiberaceae	Seeds	Inhibitory effects on PDE-5, arginase, AChE & ACE activities. Increased antioxidant activity	Okeke <i>et al.</i> , 2018
<i>Anogeissus leiocarpus</i>	Combretaceae	Stem bark	Improve sexual behavior. Inhibitory effects on PDE-5, arginase & AChE activities. Increased antioxidant activity	Ademosun <i>et al.</i> , 2019

Literature shows that the co-inhibition of some key enzymes such as arginase, AChE, ACE, PDE-5 etc. involved in regulating biomolecules crucial to penile erection could be of pharmacological importance in the treatment of ED (Ademosun *et al.*, 2019). It is worth noting that the erectogenic effects of majority of the plants listed in Table 2.1 were associated with their antioxidant properties, ability to increase testosterone levels and inhibitory effects on arginase, ACE and PDE-5 activities. Furthermore, traditional healers from KwaZulu Natal, South Africa also use various medicinal plants including *Ozoroa paniculosa*, *Carissa bispinosa*, *Maytenus procumbens*, etc. in the treatment or management of various sexual dysfunctions. Among these plants *Maytenus procumbens* is highly recommended as the most effective plant to ameliorate sexual dysfunction, particularly erectile dysfunction.

2.4.2 *Maytenus procumbens* (L.F.) Loes

Maytenus procumbens (L.F.) Loes, also known as Dune Koko (English) and Umakhweni (Zulu), belongs to the family Celastraceae. *M. procumbens* is a densely

bushy plant with drooping branches that sometimes reach more than six meters. The plant is native to South Africa, predominant in the KwaZulu Natal province. It is also found in South America, North Africa and East Asia (Momtaz *et al.*, 2012).



Figure 2.6. *Maytenus procumbens* (L.F.) Loes.

An ethno-pharmacological survey of traditional healers in northern Kwa-Zulu Natal (South Africa) indicated that the powdered roots of *Maytenus procumbens* (Figure 2.6) are often commonly used by Zulu traditional healers to manage sexual dysfunctions including ED. In our laboratory, Cele *et al.* (2017) demonstrated the testicular dysfunction ameliorative effect of methanol roots extract of *M. procumbens* on butanol induced testicular dysfunction in *Sprague Dawley* rats. The ameliorative effect was associated with the regeneration of spermatogonia in the seminiferous tubules, increase serum testosterone, and sperm count in rats. In addition, *in vitro* studies also indicated strong antioxidant properties of the roots extract of the plant, and also exhibited phosphodiesterase and acetylcholinesterase inhibitory activities. The cytotoxic effect of the plant extract against cell lines varied from moderate to weak toxicity indicating that the plant should be used with caution (Cele, 2017). The ability of the plant extract to increase serum testosterone levels and mounting frequency as well as improving other biomarkers commonly implicated in ED, raised a great interest to further ascertain its erectogenic effect *in vivo*.

2.4.3 Common models used for investigation of erectogenic natural products

Due to the multifactorial causes of ED, various animal models are used to gain a mechanistic insight of pathophysiology of ED and to discover new effective therapy for the condition. These models include non-mammalian models (Sezen and Burnett, 2000), large animal models (Simonsen *et al.*, 1997), rodent models (William *et al.*, 2012), etc. However, some of these models are expensive and require long life cycle and a specialized facility which limit their use in the field of research. In most of these animal models, drugs/chemicals are routinely used to induce ED (Lubec *et al.*, 1998). ED induction by chemicals usually involves the initial induction of a diabetic state (a combination of either high fat diet (HDF) or fructose with streptozotocin (STZ) (Fang *et al.*, 2019). Despite its limitations (that is, monogenic models are not representative of most human ailments), the fructose/STZ is a reasonable and acceptable model, since it significantly links T2D and its late stage complication to ED (see section 2.3.2). Thus, in the current study, the combination of fructose and STZ was used to evaluate the erectogenic effects of the *M procumbens* in type 2 diabetic rats.

2.4. Aim of the study

The study was aimed to investigate the erectogenic activity of the root extract of *M. procumbens* in type 2 diabetic rats.

2.4.1 Objectives

- i. To extract the plant material separately with dichloromethane and methanol
- ii. To determine antioxidant and antiprotein glycation activity of the extracts *in vitro*
- iii. To evaluate the inhibitory activity of the plant extracts on carbohydrate digestive enzymes (α -amylase, α -glucosidase), arginase, PDE-5, and acetylcholinesterase *in vitro*.
- iv. To investigate the antihyperglycemic and erectogenic effects of the plant extract in type 2 diabetic rats.

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CHAPTER THREE

The chapter, as presented here, is prepared in a journal format. The chapter consists of three parts, A, B and C.

Part A: *In vitro* antidiabetic and antioxidant properties of the extracts of *Maytenus procumbens* roots

Part B: Roots extracts of *Maytenus procumbens* exhibit antiprotein glycation and erectogenic potential *in vitro*

Part C: Methanolic extract of *Maytenus procumbens* roots ameliorates erectile dysfunction in type 2 diabetes induced erectile dysfunction in rats

Additional supporting information or documents and detailed information on the preparation of some reagents is presented in the Appendices at the end of the dissertation

Chapter Three: Part A

***In vitro* antidiabetic and antioxidant properties of the extracts of *Maytenus procumbens* roots**

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Abstract

Hyperlipidemia and hyperglycemia continue to be the underlying factors to the onset and progression of type 2 diabetes and its related complications. Thus, this study evaluated *in vitro* antidiabetic and antioxidant properties of the extracts of *Maytenus procumbens* roots. The plant material was extracted with methanol and dichloromethane. The *in vitro* α -amylase, α -glucosidase and pancreatic lipase inhibition assays were used to investigate the antidiabetic potential of the extracts. The antioxidant activity of the extracts was determined against various oxidants such as DPPH, ABTS and hydroxyl radical. The antiprotein glycation activity of the extracts was also determined *in vitro*. The crude extracts displayed antidiabetic potential as they inhibited α -amylase and α -glucosidase as well as pancreatic lipase in a dose dependent manner. The extract further exhibited strong antioxidant activities as they effectively scavenged most of the radicals tested, with IC₅₀ values ranging from 0.04 – 8.02 mg/ml. In addition, both methanol and DCM extracts inhibited haemoglobin glycation, thus displaying the anti-protein glycation potential. The obtained results demonstrated the antioxidant and antidiabetic properties of the root extract of *Maytenus procumbens*.

Key words: *Hyperglycemia, oxidative stress, glycation.*

3A.1 Introduction

Type 2 diabetes mellitus (T2D), a metabolic disorder characterized by chronic hyperglycemia due to insulin deficiency in secretion, action or both, is exponentially increasing worldwide, and projected to affect approximately 600 million people by the year 2035 (Nowotny *et al.*, 2015; Spinola *et al.*, 2019). It is estimated that nearly 90 – 95% of all T2D cases commonly result from lack of physical activity and excessive intake of carbohydrate and/or fat-rich foods (Ho *et al.*, 2017). Unlike type 1 diabetes, an autoimmune disorder characterized by the destruction of insulin-producing pancreatic β -cells (Atkinson *et al.*, 2014), T2D is characterized by several defects in the regulation of glucose homeostasis. These defects include elevated blood glucoside levels, increased hepatic glucose production, deficient insulin secretion, insulin resistance and pancreas β -cells dysfunction (Bloomgarden, 2016).

The incapability of the cells to assimilate glucose formed via stepwise catabolism of carbohydrates with α -amylase and α -glucosidase, leads to hyperglycemia (Sancho and Pastore, 2012). Whereas, an increased activity of pancreatic lipase is implicated to a rapid lipid metabolism which triggers hyperlipidemia (Ogbunugafor *et al.*, 2014). Hence, literature has shown that hyperglycemia and hyperlipidemia continue to be the vital factors contributing to the on-set and the progression of diabetes mellitus (Oka *et al.*, 2010). The decrease in post-prandial hyperglycemia through inhibition α -amylase, α -glucosidase and pancreatic lipase has been become one of the preferred approaches in the management of T2D (Picot and Mahomoodally, 2017). The persistent hyperglycemia commonly observed in diabetic patients is considered the main contributor to the development of micro-and macro-vascular complications of diabetes (Ortiz-Andrade *et al.*, 2007; Gutierrez, 2013). Hyperglycemia-induced oxidative stress, common in the diabetic state, stimulates several glucose metabolic pathways that are associated with diabetic complications. These include the polyol pathway, formation of advanced glycation end products (AGEs), and decreased antioxidant defence (Zychowska *et al.*, 2013; Sandireddy *et al.*, 2014).

Therapeutic agents such as α -glucosidase inhibitors (acarbose) and lipase inhibitors (orlistat) are currently used as antidiabetic remedies in the management of post-prandial hyperglycemia (Sancho and Pastore, 2012). However, these drugs are associated with a variety of adverse effects which limit their use (Salehi *et al.*, 2013).

Despite the development of pharmacological agents for the treatment of T2D, the use of medicinal plants has been considered a complementary therapy for diabetes mellitus (Nickavar and Amin, 2011; Podsędek *et al.*, 2014). A number of plants with antioxidants, anti-protein glycation properties, as well as inhibitory effect on activities of carbohydrates digestive enzymes and lipid hydrolysis have been reported (Semenya *et al.*, 2012; Bolson *et al.*, 2015; Spinola *et al.*, 2019). *Maytenus procumbens* (Celastraceae) commonly known as Dune Koko (English) and Umakhweni (Zulu), is a densely bushy plant with drooping branches that sometimes reach more than six meters. The plant is native to South Africa, predominant in the KwaZulu Natal province (Momtaz *et al.*, 2012). The powdered roots of *M. procumbens* are commonly used by Zulu traditional healers to treat several diseases linked to sexual dysfunction in men (Personal communication). In this study, we evaluated an *in vitro* antidiabetic and antioxidant properties of the extracts of *Maytenus procumbens* roots.

3A.2 Materials and Methods

3A.3 Chemicals and Reagents

Unless otherwise stated, all chemicals and reagents (of analytical grade) used were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA).

3A.4 Methodology

3A.4.1 Plant collection and preparation

Fresh roots of *M. procumbens* were collected from Ndumo, KwaZulu Natal, South Africa. The plant was then confirmed by a botanist at the Botany Department, University of Zululand. (Specimen voucher number NDUZ/17 05). The plant materials were thoroughly washed, chopped, air dried and ground into powder.

3A.4.2 Preparation of methanol and dichloromethane extracts

The powdered plant material was separately extracted (1:5 w/v) with dichloromethane (DCM) and methanol for 48 hours by incubating the mixture on an orbital shaker (150 rpm, room temperature). The extracts were filtered through Whatman No. 1 filter paper. Rotary evaporator (Heidolph) was used to concentrate the extracts at 40 °C under reduced pressure. The crude extracts were stored in refrigerator (4°C) and were subsequently used for the biological assays.

3A.4.3 Phytochemical screening

The powdered plant material was screened for phytochemical composition following the standard procedure by Odebiyi and Sofowara (1978). The Common phytochemicals such as alkaloids, tannins, saponins, steroids, glycosides, flavonoids and terpenoids were screened for.

3A.4.3.1 Total phenolic content

Phenolic content of the extract was determined following the Folin-Ciocalteu reagent method (Kähkönen *et al.*, 1999). A 0.5 ml of plant extract was mixed with 1.5 ml Folin-Ciocalteu reagent diluted with distilled water (1:10) and 1.2 ml of 7.5% sodium

carbonate solution was incubated in the dark for 30 minutes. Absorbance of blue colored mixture was recorded at 765 nm against a blank which contained the Folin-Ciocalteu reagent and sodium carbonate solution. The total phenolic content of the extracts was calculated as gallic acid equivalent (GAE) from the calibration curve of gallic acid and expressed as mg/g dry plant material.

3A.4.4 *In vitro* antioxidant assays

The methanolic extract of the plant was evaluated for its (scavenging) antioxidant activity against a range of inorganic free radicals such as 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2, 2'-azinobis 3-ethylbenzothiazoline-6-sulfonate (ABTS), nitric oxide, superoxide anion and hydroxyl radical. Unless otherwise stated, ascorbic acid and butylated hydroxyl anisole (BHA) were used as standards. The experiment was replicated thrice. Free radical scavenging activity of the extract was calculated using the formula

$$\text{Scavenging activity (\%)} = ((A_c - A_t) / A_c \times 100)$$

Where, A_c is absorbance of the control sample and A_t is the absorbance of the sample in the presence of the tested extract.

Absorbance was measured with Microplate reader (Synergy HT, Biotek Instruments supplies)

3A.4.4.1 1, 1-diphenyl-2-picryl hydrazyl (DPPH) scavenging activity

DPPH radical scavenging activity of the plant extract was determined following the method of Brad-Williams (1995). A mixture (1:1) of the plant extract (0 – 0.05 mg/ml) and DPPH (0.2 mg/ml) was incubated for 30 – 60 minutes in the dark and the absorbance was read at 517 nm.

3A.4.4.2 2, 2'-azinobis 3-ethylbenzothiazoline-6-sulfonate (ABTS) scavenging activity

ABTS radical scavenging activity of the plant extract was evaluated as described by Re *et al.* (1999). ABTS radical was generated by incubating 7 mM of ABTS with 2.45

mM potassium persulfate in the dark for 16 hours. Thereafter, ABTS radical produced was diluted with methanol (1ml ABTS: 60 ml methanol) to prepare a working solution. One ml of diluted ABTS radical was added to 1 ml of various concentrations (0.0 – 0.05 mg/ml) of plant extract and incubated for 6 minutes at room temperature. The absorbance of the mixture was read at 734 nm.

3A.4.4.3 Superoxide anion scavenging activity

The method described by Nagai *et al.* (2001) was followed to investigate the superoxide anion scavenging activity of the plant extract. Briefly, 0.02 ml of each of the following solutions: 3 mM Xanthine sodium salt, sodium carbonate buffer (50 mM, pH 10.5), 3 mM EDTA, Nitroblue tetrazolium (0.75 mM, NBT), 0.15% bovine serum albumin (BSA) and plant extract (0.0 – 0.05 mg/ml) were mixed well. Xanthine oxidase (0.02 ml, 6 mU) was then added to the mixture and the reaction mixture was incubated at 25°C for 20 minutes. This was followed by addition of 0.02 ml CuCl₂ (6 mM). The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after 20 minutes. Ascorbic acid was used as the standard.

3A.4.4.4 Hydroxyl radical scavenging activity

The method of Osawa *et al.* (1997) was followed to evaluate the extract's hydroxyl radical scavenging activity. The mixture comprised of 0.2 ml of each of the following: FeSO₄.H₂O (10 mM), extract (0.0 – 0.05 mg/ml), H₂O₂ (10 mM), EDTA (10 mM), and 100 mM phosphate buffer (pH 7.4). The mixture was incubated (37°C) for 2 hours. Thereafter, TCA (2.8%) and 1% 2-thiobarbituric acid (TBA) were added. The mixture was boiled for 10 minutes and allowed to cool on ice. Absorbance was read at 520 nm.

3A.4.4.5 Nitric oxide scavenging activity

The Griess Illosvoy reaction method was adapted to evaluate the nitric oxide scavenging activity of the crude extract (Badami *et al.*, 2005). Briefly, 0.5 ml of phosphate buffered saline (0.1 M), 2 ml of sodium nitroprusside (10 mM), and 0.5 ml of extract (0.0 – 0.05 mg/ml) were thoroughly mixed. After 150 minutes of incubation at 25°C, sulphanilic acid reagent (1 ml) was added and the reaction mixture was

incubated for a further 5 minutes at room temperature to complete diazotization. Thereafter, 1 ml of Naphthyl ethylenediamine dihydrochloride (0.1%) was added, mixed well and incubated in the dark for 30 minutes. The absorbance was measured at 540 nm.

3A.4.5 *In vitro* antiprotein glycation activity

3A.4.5.1 Haemoglobin-fructose assay

The method described by Kiho *et al.* (2004) was adapted with some modification to investigate the antiprotein (haemoglobin) glycation activity of the extracts. The reaction mixture consisted of 500 µl of different concentrations (0 – 5mg/ml) of the extract, haemoglobin (0.06 %), ciprofloxacin (0.02 %) and fructose (2 %). All the constituents of the reaction mixture were prepared in 0.01 M phosphate buffer (pH 7.4). The reaction mixture was then incubated in the dark for 72 hours and absorbance was read at 443 nm. Gallic acid was used as a standard. The experiment was replicated three times. The percentage inhibition of haemoglobin glycation was then calculated using the formula

$$(\%) \text{ Hb glycation inhibition} = ((A_c - A_t) / A_c \times 100)$$

Where, A_c is absorbance of the control sample and A_t is the absorbance of the sample in the presence of the tested extract.

3A.4.6 Enzyme inhibition assay

The inhibitory activity of the extracts on carbohydrate digestive enzymes as tested against α -amylase and α -glucosidase. The inhibitory effect of the extracts was also tested on pancreatic lipase, a dietary triglycerides digesting enzyme.

3A.4.6.1 α -Amylase activity

Effect of the plant extracts on α -amylase activity was evaluated using the α -amylase activity inhibition assay described by Kandra *et al.* (2005) with some slight modifications. A reaction mixture containing 0.2 ml of each of the following, 2 units/ml of α -amylase solution and plant extract (10 – 1000 µg/ml) was incubated for 10 minutes

at 30°C. Then, 0.2 ml of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added and further incubated for 5 minutes. The reaction mixture was stopped by adding 1.0 ml of 3,5 Dinitrosalicylic acid (DNSA) color reagent (1 g of DNSA in 50 ml of reagent grade water and 30g sodium potassium tartrate tetrahydrate in 20 ml of 2 M NaOH) and the mixture was boiled for 10 minutes in a water bath at 85 – 90°C. Thereafter, the mixture was cooled down and diluted with 5 ml of distilled water. The absorbance of the mixture was measured at 540 nm. The control sample, where the plant extract was replaced with saline buffer, was considered to have 100% enzyme activity. The experiment was triplicated, and percentage inhibition was calculated using the formula

$$\text{Inhibitory activity (\%)} = ((A_c - A_t) / A_c \times 100)$$

Where, A_c is absorbance of the control and A_t is the absorbance in the presence of the tested extract.

3A.4.6.2 α -Glucosidase activity

The inhibitory effect of the extracts on α -glucosidase was estimated following the method described by Adisakwattana *et al.* (2010). The Eppendorf tubes containing a volume of 50 μ l of 10 mM phosphate buffer (pH 6.8), 10 μ l of 0.5 units/ml of α -glucosidase and 20 μ l of different concentrations (0 – 2 mg/ml) of the plant extract was incubated for 15 minutes at 37°C. Then, 20 μ l of 2.5 mM 4-nitrophenyl- β -D glucopyranoside was added. The reaction mixture was further incubated for another 20 minutes at 37°C. Afterwards, 50 μ l of sodium carbonate (0.1 M) was added into the reaction mixture. For control, the plant extract was replaced with an equal volume of the buffer. Absorbance was read at 405 nm. The experiment was replicated three times and the percentage inhibitory activity of the extracts on α -glucosidase activity was calculated using the formula in 3.4.6.1.

3A.4.6.3 Pancreatic lipase activity

The effect of the crude extracts on pancreatic lipase activity was determined following the method previously described by Slanc *et al.* (2009). A total volume of 150 μ l of Tris-HCl buffer (75 mM, pH 8, 4), 75 μ l of different concentration of plant extract (0 –

2 mg/ml), and 50 µl of lipase (10 mg/ml) were mixed and incubated at 37°C for 15 minutes. Thereafter, 25 µl of 3, 3 mM of p-nitrophenyl palmitate was added and the mixture incubated at a room temperature for further 30 minutes. The blank contained a mixture (1:1 v/v) of methanol and water while orlistat was used as the standard. The enzyme inhibitory activity of the extract was monitored by estimating the release of 4-nitrophenol at 405 nm. The experiment was replicated three times and the percentage inhibition of pancreatic lipase activity was calculate using the formula in 3.4.6.1

3A.4.7 Statistical analysis

Unless otherwise stated, the results obtained were expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett *post hoc* test and two-way ANOVA followed by Tukey's multiple comparisons test using Graph Pad Prism version 6 (V₆). The statistical difference was considered significant where $p < 0.05$.

3A.5 Results

Table 3A.1 shows the results of the preliminary phytochemical screening of *M. procumbens*. The presence of terpenoids, saponins, tannins and cardiac glycosides in plant material was noted. Even though flavonoids and steroids were not present in the plant material, it is worth noting the presence of total phenolic content (TPC).

Table 3A.1: Phytochemical analysis of roots of *M. procumbens*

Polyphenols	<i>M. procumbens</i>
Terpenoids	++
Saponins	++
Flavonoids	-
Alkaloids	+
Steroids	-
Tannins	++
Anthraquinones	+
Cardiac glycosides	++
TPC (mg/g)	0.083 ± 0.004

++ Present in high concentration.

+ Present in low concentration.

- Not detected.

3A.5.1 Antioxidant activity

The methanolic extract showed, to varying degree of efficacy, a concentration-dependent free radical scavenging activities with the IC₅₀ values ranging from 0.04 to 8.02 mg/ml (Table 3A.2). The lowest IC₅₀ values of the extract were observed on DPPH (0.04 mg/ml) and ABTS (0.13 mg/ml).

Table 3A.2: Free radical (DPPH, ABTS, superoxide radical, hydroxyl radical, nitric oxide) scavenging activities of the methanolic root extract of *M. procumbens*

Conc (mg/ml)	DPPH	ABTS•	O ₂ • ⁻	•OH	NO•
0.01	7.35 ± 0.04	20.3 ± 0.14	27.3 ± 0.02	43.9 ± 0.01	38.3 ± 0.24
0.02	23.3 ± 0.05	38.2 ± 0.01	26.5 ± 0.02	40.4 ± 0.07	43.4 ± 0.06
0.06	37.4 ± 0.02	41.4 ± 0.02	26.9 ± 0.04	29.7 ± 0.01	38.7 ± 0.20
0.08	50.6 ± 0.06	48.2 ± 0.02	27.1 ± 0.02	35.4 ± 0.02	46.4 ± 0.36
0.1	60.0 ± 0.01	50.4 ± 0.01	35.5 ± 0.01	43.6 ± 0.03	40.0 ± 0.17
IC ₅₀	0.04	0.13	< 5	< 5	< 5
AA (IC ₅₀)	0.08	4.95	1.81	2.03	0.02
BHA (IC ₅₀)	6.63	3.83	-	5.08	0.05

Values are expressed as mean ± standard deviation, (n=3).

3A.5.2 *In vitro* antiglycation activity of the *M. procumbens* root extracts

3A.5.2.1 Haemoglobin glycation

The results of the haemoglobin-fructose assay revealed that both the DCM and methanolic extracts exhibited a concentration dependent inhibitory effect against haemoglobin glycation with the IC₅₀ values of 80.3 and 75 µg/ml, respectively (Figure 3A.1).

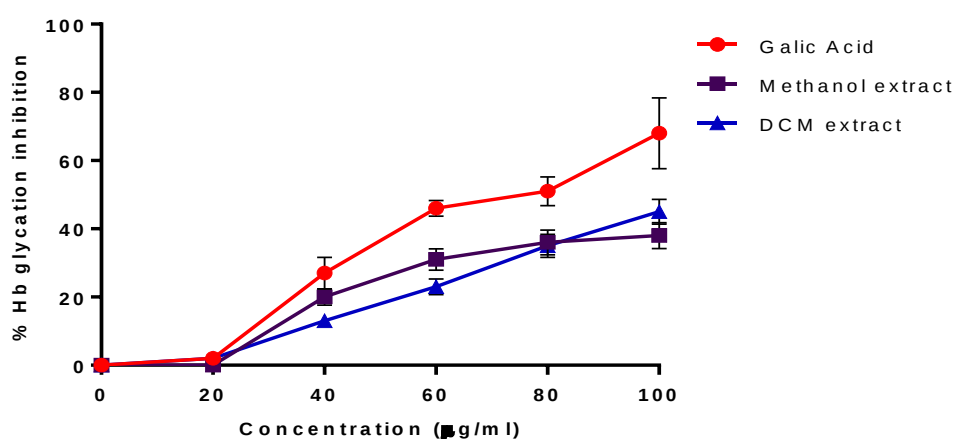


Figure 3A.1: Inhibitory effect of the methanolic and DCM extracts on haemoglobin glycation. Values were expressed as mean ± SD, (n=3).

3A.5.3 Enzyme inhibition assay

3A.5.3.1 α -Amylase and α -glucosidase activity

The results of the effect of the plant extracts on α -amylase and α -glucosidase activity are presented in Figure 3A.2. The methanolic extract exhibited a more convincing concentration dependent inhibitory effect on both enzymes with the IC₅₀ values of 90.7 μ g/ml and 84.9 μ g/ml, respectively.

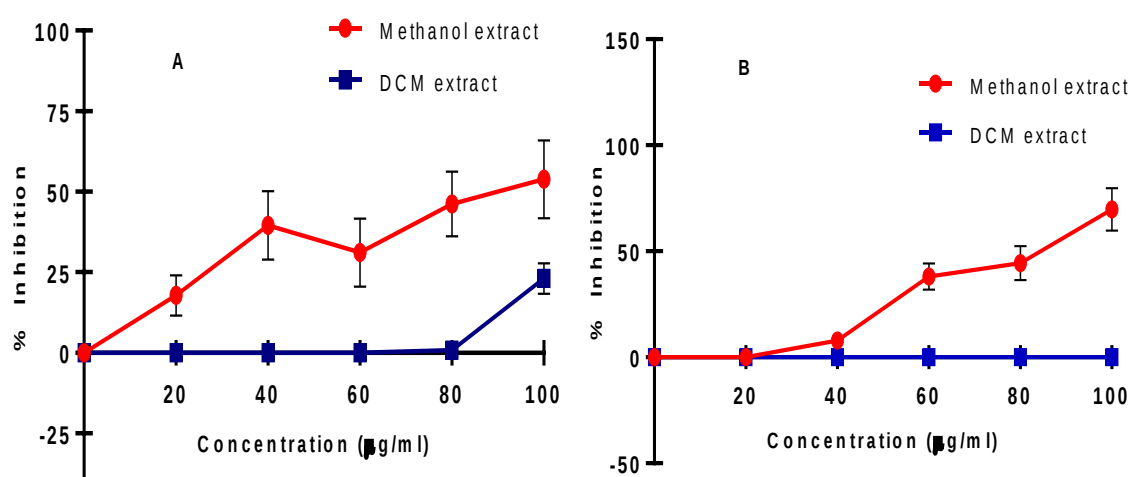


Figure 3A.2: Inhibitory effect of the roots extract of *M. procumbens* on the activity of α -amylase (A) and α -glucosidase (B). Values were expressed as mean $\pm$ SD, (n=3).

3A.5.3.2 Effects of *M. procumbens* roots extracts on pancreatic lipase activity

Figure 3A.2 presents the results of the effect of the roots of *M. procumbens* on pancreatic lipase activity. Both methanolic and DMC extracts demonstrated the inhibitory effects on lipase activity. However, the methanolic extract exhibited a twice higher IC₅₀ value (11.7 μ g/ml) against pancreatic lipase when compared to that of the standard orlistat (6.5 μ g/ml).

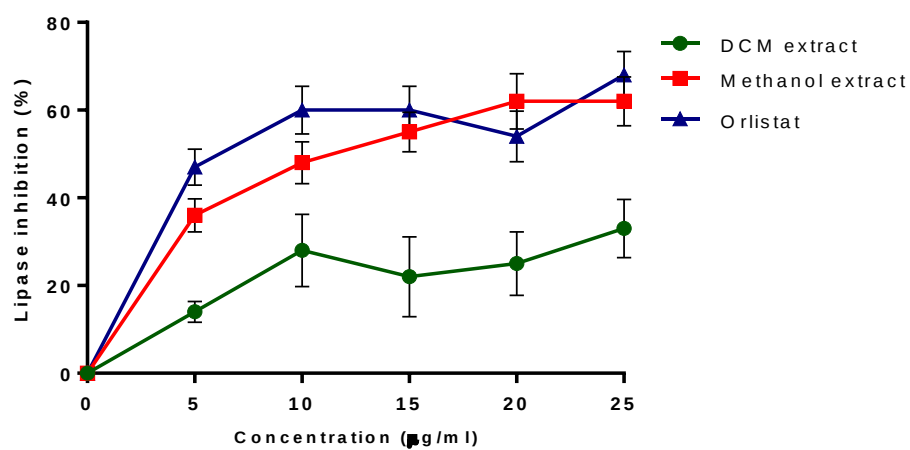


Figure 3A.2: Inhibitory effect of roots of *Maytenus procumbens* on pancreatic lipase activity. Values were expressed as mean $\pm$ standard deviation, (n=3).

3A.6 Discussion

The persistent postprandial hyperglycemia that characterizes diabetes is considered the main contributor to the development and progression of micro-and macro-vascular complications of diabetes (Ortiz-Andrade *et al.*, 2007; Gutierrez, 2013). Various approaches which include the use of acarbose, miglitol, voglibose have been considered crucial in the control of postprandial chronic hyperglycemia and thus T2D management (Van Der Laar *et al.*, 2005). Interest in the search for new antidiabetic drugs from our local flora is continuously increasing. This study investigated the antidiabetic potential of the root's extracts of *Maytenus procumbens* *in vitro*.

The observed inhibitory activity of the extracts on α -amylase and α -glucosidase (Figure 3A.2) indicated that the roots extracts of *M. procumbens* possess some antidiabetic properties. Inhibition of carbohydrates digestive enzymes which then limits carbohydrates intestinal digestion and absorption is considered one of the promising therapeutic approaches in lowering postprandial hyperglycemia in diabetic patients (Hanhineva *et al.*, 2010; Zengin *et al.*, 2015). A substantial number of other medicinal plants extracts with antidiabetic properties have shown inhibitory effects on α -amylase and α -glucosidase (Thrikawala *et al.*, 2018; Ononamadu *et al.*, 2019).

Similarly, hyperactivity of lipase increases the metabolism of lipid, which also triggers hyperlipidemia (Hanhineva *et al.*, 2010; Sompong *et al.*, 2016). Thus, hyperglycemia and hyperlipidemia remain the underlying factors to the onset and progression of T2D and its related complications (Ogden *et al.*, 2006). Pancreatic lipase catalyzes the breakdown of triglycerides into free fatty acids and monoacylglycerol in the small intestinal lumen (Yang *et al.*, 2014). Reduction of pancreatic lipase activity decreases free fatty acids availability for onward absorption from the small intestine into the blood stream, and thus prevents hyperlipidemia. The inhibitory effects shown by both extracts on lipase activity (Figure 3A.3) also supports their potential in preventing hyperlipidemia. Adisakwattana *et al.* (2012) and Spinola *et al.* (2019) have also revealed that the anti-hyperlipidemic activities of edible plant extracts are based on their interference with lipid digestion and absorption through inhibition of pancreatic lipase, consequently reducing the risk of developing diabetes. The antidiabetic properties of some other plant extracts have also been associated with their inhibitory

effect on α -amylase and α -glucosidase as well as pancreatic lipase activities (Suantawee *et al.*, 2014).

Oxidative stress is also implicated as one of the contributing factors to the development and progression of T2D and related complications (Chayaratanasin *et al.*, 2015). Hyperglycaemia stimulates the production of reactive oxygen species (ROS) through mitochondrial system. Antidiabetic effect of a number of medicinal plants has also been associated with their antioxidant properties (Ononamadu *et al.*, 2019). The free radical scavenging activity exhibited by the methanol extract of the plant (Table 3A.2) indicated its antioxidant properties. The presence of phenolic compounds such as cardiac glycosides, tannins, saponins and anthraquinones (Table 3A.1), which are well known of their antioxidant activity, could prove vital in the antioxidant properties of the roots' extract (Justino *et al.*, 2018).

Hyperglycemia-induced oxidative stress, common in the diabetic state, stimulates several glucose metabolic pathways that are associated with diabetic complications. These include the polyol pathway, formation of advanced glycation end products (AGEs), and decreased antioxidant defence (Zychowska *et al.*, 2013; Sandireddy *et al.*, 2014). AGEs trigger cellular damage through modification of structural proteins, a common event in the pathogenesis of T2D. In the current study, both methanol and DCM extracts revealed the anti-protein glycation (Figure 3A.1). The anti-protein glycation properties exhibited by both extracts of *Maytenus procumbens* roots could be attributed to the presence of phytoconstituents such as saponins, tannins, glycosides and alkaloids (Table 3A.1) which are commonly implicated in the treatment of T2D (Bartosz and Bartosz, 2016; Kato *et al.*, 2017). The antiglycation property due to saponins, glycosides, tannins, terpenoids and alkaloids present in *Coccinea grandis* methanol extract have been reported (Meenatchi *et al.*, 2016). Therefore, from the findings obtained in this study, it can be concluded that the synergistic action of the bioactive constituents present in the *Maytenus procumbens* roots extract might contributed to the observed bioactivity, thus providing evidence in support for use of this plant as significant source of antidiabetic therapy.

3A.7 Conclusion

It is concluded that the roots extract of *Maytenus procumbens* possess some vital antidiabetic and antioxidant properties. Thus, root extract of *Maytenus procumbens* could be a promising therapeutic in the prevention and management of diabetes and its related complications. However, as this is only preliminary study, *in vivo* antihyperglycemic potential of the plant extract as well as possible identification of its bioactive compound(s) still need to be explored.

3A.8 Acknowledgements

The acknowledgement goes to the Research office of University of Zululand and National Research Funding (NRF) for their financial supports.

3A.9 Contribution of authors

R.A.O. & A.R.O. designed and supervised the .project; N.D.C. carried out the experiments and wrote the manuscript, F.O.O. & M.C.O. assisted in the lab work and contributed in the writing of the manuscript.

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Chapter three: Part B

Roots extracts of *Maytenus procumbens* exhibit antiprotein glycation and erectogenic potential *in vitro*

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Abstract

Erectile dysfunction (ED) is a common form of sexual dysfunction that is rampant among type 2 diabetic men. The most common mechanism through which diabetes induces ED, is through the formation of advanced glycation end products (AGEs). This study evaluated the antiprotein glycation and erectogenic potential of *Maytenus procumbens* roots extracts *in vitro*. The methanolic and dichloromethane (DCM) crude extracts of the plant were screened for their inhibitory effect on phosphodiesterase-5 (PDE-5), arginase, acetylcholinesterase (AChE), and angiotensin converting enzyme (ACE) activities. The antiprotein glycation activity of the extracts was also determined over a period of 28 days at 7 days intervals. DCM extract exhibited a higher inhibitory activity on PDE-5 (with IC₅₀ value of 0.02 mg/ml) when compared to methanolic extract (IC₅₀ value of 0.1 mg/ml). Relatively higher inhibitory effect of the methanolic extract on arginase, AChE, and ACE activities was observed when compared to the DCM extract. Both extracts also displayed inhibitory effects on protein glycation during the 14th and 21st day of the glycation period. The results obtained suggest that the root extracts of *Maytenus procumbens* exhibit antiprotein glycation and erectogenic potential.

Key words: Erectile dysfunction, type 2 diabetes, enzyme activity.

3B.1 Introduction

Erectile dysfunction (ED) is global health problem that distinctly affects the quality of life of sexually active men (Singh *et al.*, 2012). ED is defined as the persistent failure to achieve and/or maintain penile erection required for satisfactory sexual performance (Akomolafe *et al.*, 2016). It's approximated that about 100 million of men are suffering from ED, and this figure is expected to increase roughly to 322 million men globally by the year 2025 (Binmoammar *et al.*, 2016; Seid *et al.*, 2017). Even though ED declines with age, various other factors such as alcohol abuse, endocrine and metabolic disorders, oxidative and psycho-social stresses contribute to the progression and development of ED (Nunes and Webb, 2012). Furthermore, an excessive formation of advanced glycation end products (AGEs), common in diabetic condition, has been shown as a key pathophysiological event linked to a range of human diseases (Coughlan *et al.*, 2007). Evidence from clinical studies and animal experiments with exogenous AGE infusion support the concept that AGEs are involved in the onset and development of diabetic complications such as ED (Coughlan *et al.*, 2007; Neves, 2013). The intermolecular collagen cross-linking caused by AGEs leads to thickening of blood vessels, decreased elasticity and endothelial dysfunction (Candido *et al.*, 2003).

In the corporal smooth muscle, phosphodiesterase-5 (PDE-5) hydrolyzes cyclic guanosine monophosphate (cGMP) (Agarwal *et al.*, 2006). Increased activity of PDE-5, and of other enzymes such as arginase, acetylcholinesterase (AChE), and angiotensin 1 converting enzyme (ACE) in penile tissues has been strongly linked with impaired penile erection (Adefegha *et al.*, 2018). Pharmaceutical agents known as PDE-5 inhibitors (Sildenafil, Tadalafil, etc.), are currently used as the primary oral medication for ED therapy. Despite of their adverse effects, the management of ED could not only be restricted to the inhibition of PDE-5 activity, but to some other enzymes (arginase, AChE and ACE) which involve the signal pathways importantly for vasorelaxation (Akomolafe *et al.*, 2016; Shodehinde *et al.*, 2016). Thus, consideration of bioactive compounds from plants has been become one of the preferred approaches in discovery of new drugs with erectogenic properties.

A large number of plants with AChE, PDE-5, ACE and arginase inhibitory activities have been reported (Obboh *et al.*, 2017; Odubanjo *et al.*, 2017). *Maytenus procumbens*

commonly known as Dune Koko (English) and Umakhweni (Zulu), belongs to Celastraceae family. The plant is native to South Africa, predominant in the KwaZulu Natal province. The powdered roots of *M. procumbens* are commonly used by Zulu traditional healers to treat several diseases linked to sexual dysfunction in men. Thus, this study evaluated the antiprotein glycation and erectogenic potential of *Maytenus procumbens* roots extracts *in vitro*.

3B.2 Materials and Methodology

3B.3 Chemicals and reagents

Fructose, Bovine serum albumin (BSA), aminoguanidine (AG), 5, 5'-dithiobisnitro benzoic acid (DTNB), Nitroblue tetrazolium (NBT), 1-deoxy-1-morpholino-fructose (DMF), p-nitrophenyl phenylphosphonate, arginine and 5, 5-dithiol-bis (2-nitrobenzoic) acid (DNTB), were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemical reagents used in this study were of analytical grade.

3B.4 Methodology

3B.4.1 Plant collection and preparation of extracts

The plant roots of *M. procumbens* were collected from Ndumo, KwaZulu Natal, South Africa and were confirmed at the Botany Department, University of Zululand (Specimen voucher number NDUZ/17 05). The fresh roots were thoroughly washed, chopped, air dried and ground into powder. The powdered plant material was separately extracted (1:5 w/v) with dichloromethane and methanol to obtain separate crude extracts.

3B.4.2 *In vitro* antiprotein glycation activity- BSA-fructose assay

3B.4.2.1 Bovine serum albumin (BSA)-fructose assay

The BSA-fructose assay was used to determine the antiprotein glycation potential of the extracts (Adisakwattana *et al.*, 2012). Briefly, to the test tubes, a total volume of 2 ml containing 10 mg/ml BSA, plant extract (0.25 – 1.00 mg/ml) was incubated with 0.5 M fructose in 0.1 M phosphate buffer saline (PBS, pH 7.4). Sodium azide (0.02%) was also added to the reaction mixture. The reaction mixture was incubated in the dark at 37 °C for 7, 14, 21, and 28 days. Aminoguanidine was used as a standard. The AGEs formation was determined by measuring fluorescence at excitation and emission wavelength of 355 nm and 460 nm, respectively. The percentage inhibition of BSA glycation was then calculated using the formula:

$$\text{Percentage inhibition of fluorescent AGE (\%)} = [(F_{\text{control}} - F_{\text{Tsample}}) / F_{\text{control}}] \times 100.$$

Where F_{control} represents fluorescence of the control sample and F_{Tsample} represents fluorescence of the test samples.

3B.4.2.2 Estimation of fructosamine content

After incubation for 7, 14, 21, and 28 days, the concentration of fructosamine formed in the samples in 3.4.2.1 was determined using a Nitroblue tetrazolium (NBT) assay as described by Adisakwattana *et al.* (2012). Briefly, 1 ml of glycated BSA was incubated with 1 ml of 0.5 mM NBT in 2 M sodium carbonate buffer (pH 10.4) at 37 °C for 10 – 15 minutes. The absorbance was measured at the wavelength of 590 nm. The concentration of fructosamine ($\mu\text{g/ml}$) in the respective samples was determined from the standard curve of 1-deoxy-1-morpholino-fructose (1-DMF).

3B.4.3 Enzyme inhibition assay

3B.4.3.1 Crude enzyme preparation

The rats' brain and the penile tissues were carefully removed and separately washed twice in 0.01 M phosphate buffer (pH 7.4). These tissues were homogenized with ice-cold buffer [0.1 M Tris-HCl buffer containing 1 mM CaCl_2 and 50 mM NaCl (pH 8.0)] (1/10 w/v) and centrifuged at 3000 rpm for 20 minutes. While the supernatant from the brain homogenate was collected and used as a source of AChE, the supernatant from the penile tissue homogenate was used as a source of PDE-5, arginase and ACE. The respective supernatants were used in the subsequent enzymatic assays.

3B.4.3.2 Phosphodiesterase-5 inhibition assay

The effect of the crude extracts on PDE-5 activity was investigated following the method described by Oboh *et al.* (2017). Briefly, the reaction mixture containing 200 μl of 5 mM of the substrate (p-nitrophenyl phenylphosphonate), 150 μl of supernatant as source of enzyme, 200 μl of 20 mM Tris buffer (pH 8.0) and 125 μl of different concentrations of the extract (0 – 0.5 mg/ml) were incubated at 37 °C for 10 minutes. The amount of p-nitrophenol produced was read as a change in absorbance after 5 minutes at 400 nm. This experiment was replicated three times. Caffeine was used as standard and the PDE-5 inhibitory activity of the extracts was expressed as percentage inhibition using the formula below:

$$\text{PDE-5 Inhibitory activity (\%)} = ((\text{Ac}-\text{At}) / \text{Ac} \times 100)$$

Where, Ac is absorbance of the control and At is the absorbance in the presence of the tested extract. Biotek plate reader (Synergy HT, Biotek Instrument supplies) was used to read absorbance.

3B.4.3.3 Inhibition of arginase activity

The method described by Adefegha *et al.* (2016) was followed to determine the arginase inhibitory activity of the extracts. In brief, a mixture containing 150 µl of supernatant as source of arginase enzyme and 125 µl of various concentration of extracts was pre-incubated for 5 minutes at 37 °C in 200 µl of 0.01 mM Tris-HCl buffer (pH 7.5) containing 0.05 mM MnCl₂. The reaction was initiated by addition of 50 mM L-arginine (200 µl) followed by incubation at 37°C for 10 minutes. The reaction was terminated by adding 500 µl of Ehrlich's reagent (p-dimethylaminobenzaldehyde in 3.6 N HCl). The amount of urea produced was measured spectrophotometrically at 450 nm. The control experiment was performed without the test sample or standard. The experiment was replicated three times, and arginase inhibitory activity of the extracts was calculated using the formula below:

$$\text{Arginase Inhibitory activity (\%)} = ((\text{Ac}-\text{At}) / \text{Ac} \times 100)$$

Where, Ac is absorbance of the control and At is the absorbance in the presence of the tested extract.

3B.4.3.4 Angiotensin-1 converting enzyme (ACE) inhibition assay

Effect of the extracts on ACE activity was investigated using the method of Akomolafe *et al.* (2016). The extract (0 – 5 mg/ml) and supernatant (ACE source), each at 50 µl, were mixed and pre-incubated at 37 °C for 15 minutes. The enzymatic reaction was initiated by adding 150 µl of 8.33 mM hippuryl-L-histidyl-L-leucine (HHL) prepared in 125 mM of Tris-HCl buffer (pH 8.3) to the extract-enzyme mixture and incubated at 37 °C for 30 minutes. The reaction was terminated by adding 250 µl of 1 M HCl. The hippuric acid (Bz-Gly) generated by the reaction was removed with 1.5 ml ethyl acetate and further centrifuged at 1500 rpm for 10 minutes to separate the ethyl acetate layer. Then, 1 ml of ethyl acetate was later transferred to a volumetric flask and evaporated

to dryness. The obtained residue was reconstituted in distilled water and its absorbance was read at 228 nm. For experimental control, the test sample was replaced with an equal volume of the buffer. The experiment was replicated three times and ACE inhibitory activity of the extracts was calculated from the equation below:

$$\text{ACE Inhibition Inhibitory activity (\%)} = ((\text{Ac}-\text{At}) / \text{Ac} \times 100)$$

Where, Ac is absorbance of the control and At is the absorbance in the presence of the tested extract.

3B.4.3.5 Acetylcholinesterase inhibition assay

The colorimetric method of Ellman *et al.* (1961) as described by Khan *et al.* (2012) was followed to determine effect of the extracts on acetylcholinesterase activity. The brain supernatant (source of the enzyme) was diluted (x10) with 0.1 M phosphate buffer (pH 8.0). The reaction mixture consisted of 100 µl of the enzyme, 140 µl of 0.1 M phosphate buffer (pH8.0), and 25 µl of 5, 5-dithiol-bis (2-nitrobenzoic) acid (DNTB) reagent. The reaction was initiated by adding 5 µl of acetylthiocholine iodide and absorbance of the yellow color produced was measured at 412 nm for 5 minutes, at 60 second intervals using microplate reader (Synergy HT, BioTek Instruments Supplies). The enzyme activity was calculated, and the final reading of enzyme activity was expressed as µmoles/minute/mg tissue. All experiments were done in triplicates. The AChE activity was expressed as % inhibition from the equation:

$$\text{AChE Inhibitory activity (\%)} = ((\text{Ac}-\text{At}) / \text{Ac} \times 100)$$

Where, Ac is absorbance of the control and At is the absorbance in the presence of the tested extract.

3B.4.3 Statistical analysis

Data were expressed as the mean $\pm$ standard deviation (n=3), and statistically analysed using Graph Pad Prism software (Graph Pad Prism 6, version 6.03). The results were analysed by one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. The values were considered statistically significant where $p \leq 0.05$

3B.5 Results

3B.5.1 Bovine serum albumin glycation

The antiglycation of the roots extracts of the plant was evaluated on BSA over a period of 28 days at 7 days intervals. The obtained results are presented in Figure 3B.1. Both extracts exhibited a concentration dependent inhibitory effect on BSA glycation during the 14 and 21 days of incubation. Their anti-protein glycation activity within this period was comparable to that of aminoguanidine that was used as standard.

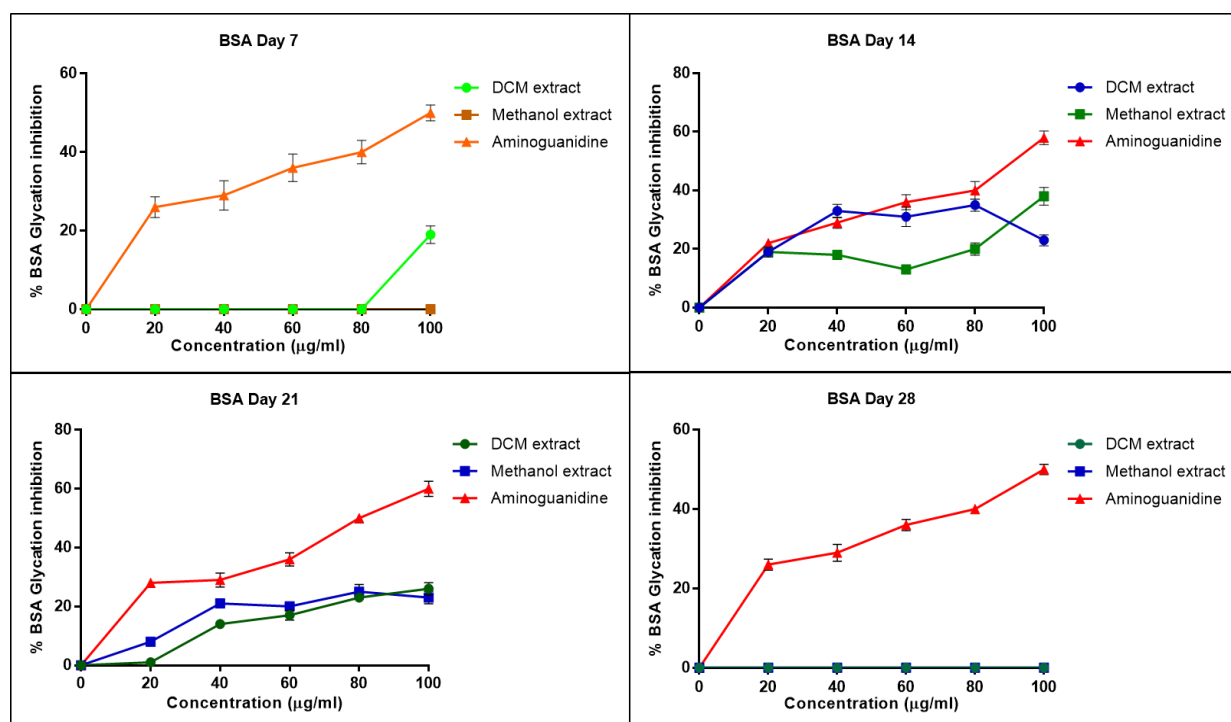


Figure 3B.1: Antiglycation activity of *M. procumbens* roots' extracts at day 7 to 28. Values were presented as mean $\pm$ standard deviation, (n=3).

3B.5.2 Fructosamine content

Results in Figure 3B.2 presents the effect of extracts on fructosamine content. The level of fructosamine in glycated BSA was gradually increased during 28 days of incubation. However, both extracts showed a reduction in fructosamine level in glycated BSA only during 14 and 21 days incubation period.

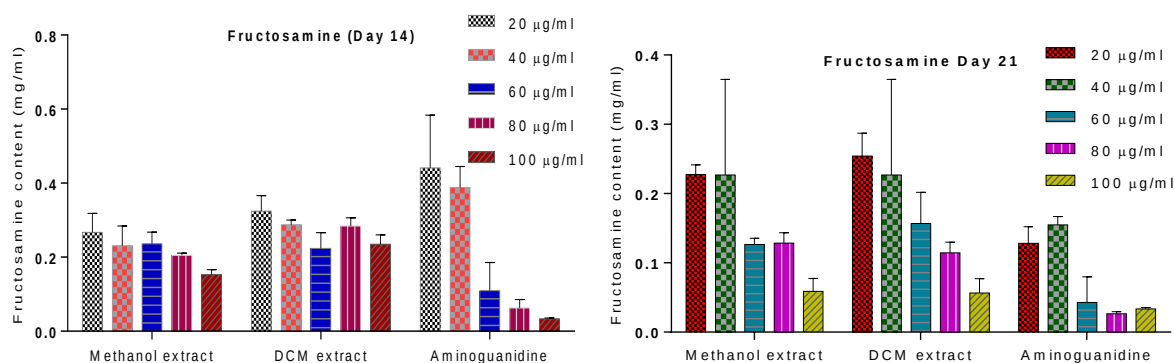


Figure 3B.2: The fructosamine content of the glycated BSA at day 14 and 21. Values were presented as mean $\pm$ standard deviation, (n=3).

3B.5.3 PDE-5 activity

Figure 3B.3 shows the results of the effect of the methanolic and DCM extracts on PDE-5 activity. Both extracts exhibited a concentration dependent inhibitory effects on PDE-5 activity. The results revealed that the DCM extract has a strong inhibitory activity ($IC_{50} = 0.02$ mg/ml) against PDE-5 when compared to the methanolic extract ($IC_{50} = 0.1$ mg/ml) and caffeine ($IC_{50} = 0.099$ mg/ml), a known phosphodiesterase inhibitor.

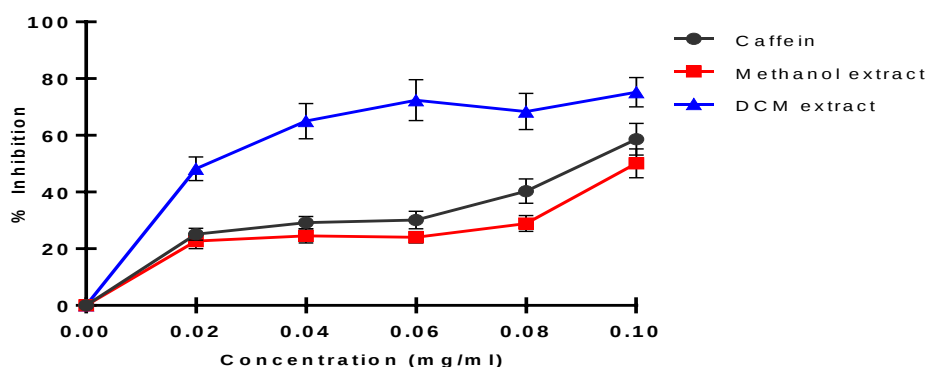


Figure 3B.3: Effect of the roots of *M. procumbens* on the PDE-5 activity. Data were expressed as mean $\pm$ SD. (n=3).

3B.5.4 Arginase activity

Figure 3B.4 shows that the methanolic extract inhibited arginase activity in a concentration-dependent manner ($IC_{50} = 0.08$ mg/ml). It is also worth noting the poor inhibitory activity shown by the DCM extract on the enzyme.

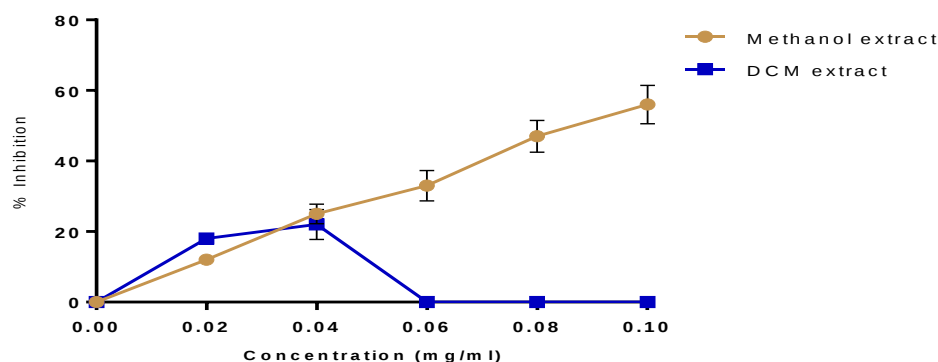


Figure 3B.4: Effect of *M. procumbens* roots extract on the activity of arginase. Data were expressed as mean $\pm$ SD. (n=3).

3B.5.5 Effect of the extracts on AChE activity

The inhibitory effect of the crude extracts on AChE activity is presented in Figure 3B.5. Both extracts exhibited inhibitory effects on AChE activity. It is worth noting the higher inhibitory (60%) effect on AChE demonstrated by the methanolic extract when compared to the DCM extract (38%).

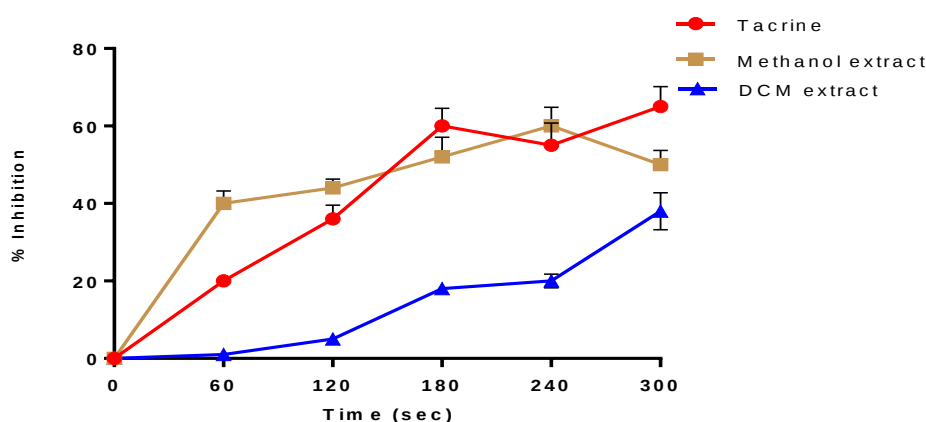


Figure 3B.5: The inhibitory property of the roots extract of *M. procumbens* on AChE activity. Data were expressed as mean $\pm$ SD. (n=3).

3B.5.6 The effect of crude extracts on ACE activity

Results in Figure 3B.6 presents the effect of crude extracts on ACE activity. Methanolic extract exhibited inhibitory effect on ACE in a concentration dependent fashion with the IC_{50} value (450 μ g/ml) higher than that of captopril (IC_{50} = 183 μ g/ml), a standard.

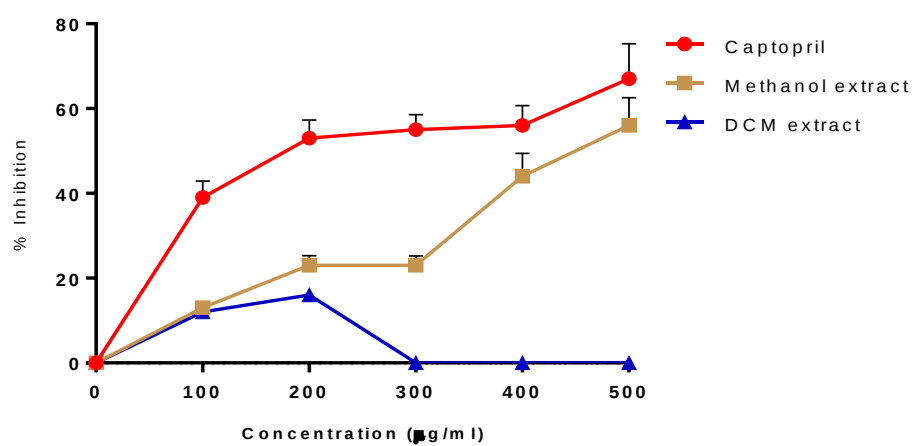


Figure 3B.6: The effect of the crude extract of the *M. procumbens* on ACE activity. Data were expressed as mean $\pm$ SD. (n=3).

3B.6 Discussion

The upregulation of PDE-5, arginase, AChE, and ACE activities in penile tissue has been strongly linked with impaired penile erection (Adefegha *et al.*, 2018). Evidence has shown an increased activities of arginase, AChE, ACE and PDE-5 in men with ED (Pavan *et al.*, 2015; Eleazu *et al.*, 2017). Thus, the control of some of these enzymes involved in the penile erection process have been considered as a potential therapeutic strategy in the management of ED (Francis *et al.*, 2010). To investigate the erectogenic potential of the extracts of *M. procumbens* roots, the inhibitory effect of the extracts was determined against the activities of PDE-5, ACE, AChE and arginase.

PDE-5 hydrolyses cGMP, a second messenger molecule crucial for activating cascade of reactions leading to vasodilation in the penile tissue (Ghofrani *et al.*, 2006). Literature shows that PDE-5 inhibitors have become the first line treatment for ED (Schellack and Agoro, 2014; Okeke *et al.*, 2018). The inhibitory effect of the extracts on PDE-5 is indicative of the potential role of the extracts in amelioration of ED. Elevated levels of arginase in patients with ED, decreases biosynthesis of NO and reduce generation of cGMP (Stefan and Schmieder, 2003). It has been shown that increased arginase activity impairs bioavailability of arginine by competing with endothelial NO synthase (eNOS) for the same substrate (arginine) which lowers the production of NO (Segal *et al.* 2012). The ability of the methanolic crude extract to inhibit arginase activity (Figure 3B.2) further supports the erectogenic potential of the extract. Thus, the crude extract could potentially be used as one effective therapeutic agent in the treatment of ED.

Furthermore, increased activity of AChE has also been implicated in the development of ED as this enzyme lowers the levels of ACh in the penile tissue. This in turn diminishes penile erection process (Kataoka *et al.*, 2017). ACh plays a vital role in the penile erection process through activation of NO-cGMP pathway (Andersson, 2011). Thus, inhibition of AChE activity could increase bioavailability of ACh, which could stimulate smooth muscle relaxation and restores penile erection in patients with ED (Akomolafe *et al.*, 2016, Akomolafe *et al.*, 2018). These findings are in accordance with those of Odubanjo *et al.* (2017) who also demonstrated the erectogenic properties of the extracts from *Myristica fragrans* and *Moringa oleifera* on AChE, ACE and

arginase activities. In addition, Ojo *et al.* (2019) recently reported the inhibitory properties of the *O. gratissimum* leaf extract on AChE activity to enhance sexual performance.

Moreover, the rennin–angiotensin system (RAS) is not only a risk factor of hypertension but also implicated in the pathophysiology of ED (Fraga-Silva *et al.*, 2013). An increase in angiotensin-II (Ang II) production due to elevated ACE activity is associated with ED development. ACE catalyzes the conversion of angiotensin I (Ang I) to Ang II, a potent vasoconstrictor which controls various pathways including muscle cell contraction in the corpus cavernosum of the penile tissue (Obboh *et al.*, 2017). Inhibitory activity of the methanolic extract on ACE indicated its potential ameliorative effect not only against ED, but also on other vascular diseases such hypertension. Previous experimental investigations have revealed that ACE inhibitors and angiotensin receptor blockers are effective therapeutic agents in the treatment of cardiovascular diseases including ED (Doumas and Douma, 2006; Jin, 2009). Therefore, inhibition on the ACE activity could ameliorate erectile dysfunction by reducing angiotensin II production and enhance smooth muscle relaxation of the corpus cavernosum (Ferrario and Levy, 2002; Shodehinde *et al.*, 2016).

Furthermore, it is well known that diabetes mellitus is a major contributory factor to the onset and progression of ED. Among the mechanisms through which diabetes induces ED, formation of advanced end products (AGEs) is considered the most common mechanism. In diabetic state, highly activated polyol pathway stimulates the non-enzymatic glycation of proteins and consequent AGEs formation. AGEs mediate their effect both directly and indirectly via receptor-dependent mechanisms (Aronson, 2003). AGEs affect the structural integrity of extracellular matrix components and form covalent bonds with vascular collagen, leading to thickening of blood vessels, decreased elasticity, endothelial dysfunction and subsequently result to ED (Candido *et al.*, 2003).

In the current study, the glycation of BSA was attenuated in the presence of both extracts as shown in Figure 3B.1. Thus, indicating that both extracts exhibited inhibitory effect on the formation of AGEs in the late stage of the glycation process. The observed antiprotein glycation activity of the extracts was further supported by the ability of both extracts to attenuate the formation of late-stage Amadori products

“fructosamine” during glycation period (Figure 3B.2). Our results are in agreement with those of Meng *et al.* (2011) who showed the *Melissa officinalis* to exhibit high inhibitory effect on the formation of advanced glycation end products in the late stage of the glycation process. Even though the underlying mechanism(s) through which the extracts exert its erectogenic effects still need to be elucidated and documented. However, in accordance with literature, the findings in the present study suggest that the erectogenic effects exhibited by both the extracts is partly linked to antiprotein glycation.

3B.7 Conclusion

Root extracts of *Maytenus procumbens* exhibited antiprotein glycation and erectogenic potential *in vitro*. Even though both the methanolic and DCM extracts showed an inhibitory effect on arginase, ACE and AChE, DCM extract exhibited more inhibition on PDE-5 activity. In addition, the results from this study partly revealed the possible mechanism of action of the *M procumbens* roots in the management of ED. However, *in vivo* studies are strongly recommended to confirm the erectogenic properties of the plant and the possible underlying mechanism(s).

3B.8 Acknowledgements

This study was financially supported by University of Zululand Research Funding and National Research Funding (NRF).

3B.9 Contribution of authors

R.A.M. & A.R.O designed and supervised the .project; N.D.C. carried out the experiments and wrote manuscript; F.O.O. & M.C.O. assisted in the lab work and contributed in the writing of the manuscript.

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Chapter three: Part C

Methanolic extract of *Maytenus procumbens* roots ameliorates erectile dysfunction in type 2 diabetes induced erectile dysfunction in rats

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Abstract

Erectile dysfunction (ED) due to diabetes mellitus remains difficult to treat despite advances in pharmacotherapeutic approaches in the field. Therefore, this study investigated the erectogenic effect of the methanolic extract of *Maytenus procumbens* roots on type 2 diabetes in rats. The fructose-streptozotocin model was used to induce type 2 diabetes-linked ED in male rats. The sexually active male *Sprague Dawley* rats were randomly divided into two major groups; normal group and high fructose fed group for 120 days. After 120 days, the high fructose fed group rats were given a single intraperitoneal injection of a freshly prepared streptozotocin solution (30 mg/kg). The diabetic ED rats were orally administered with the extract at 250 mg/kg, daily for 28 days. The serum, brain and penile tissues were removed for biochemical analysis and protein expression. Increased testosterone level, mounting frequency, reduced blood glucose level and serum fructosamine content were observed after 28 days of treatment in diabetic rats. Methanolic extract also exhibited an inhibitory effects on arginase, AChE and ACE activities. The crude extract further downregulated proteins PDE-5, RhoA and increased expression of eNOS in the diabetic ED treated rats. The results obtained indicate that the methanolic extract of *Maytenus procumbens* roots ameliorates erectile dysfunction in type 2 diabetes induced erectile dysfunction in rats.

Key words: *Medicinal plants, erectile dysfunction, anti-glycation.*

3C.1 Introduction

Erectile dysfunction (ED), the most common form of sexual dysfunction in sexually active men, is defined as the repeated inability to attain and maintain an adequate erection for satisfactory sexual intercourse. ED results from the loss of penile vascular and smooth muscle relaxation due multiple pathophysiological conditions such hypogonadism, hypertension, type 2 diabetes mellitus, etc. (Minaz *et al.*, 2019). Approximately, 90% of diabetic men worldwide have been reported to have ED (Kaya *et al.*, 2015). However, studies have shown that most clinicians do not enquire about sexual dysfunction during medical consultations and the prevalence of self-reported ED is also very low (Kandeel *et al.*, 2001). Thus, ED remains one of the greatest underdiagnosed yet common complication of diabetes in men.

Diabetes induces ED through multiple underlying factors such as increased formation of advanced glycation end products (AGEs), deficiency in the nitric oxide-cyclic guanosine monophosphate (NO-cGMP) signaling pathway, dysregulation of endothelial function, nervous lesions and hypogonadism (Thakur *et al.*, 2008; Fu *et al.*, 2019). Among these factors, AGEs formation is considered the most common and well understood underlying mechanism. AGEs form covalent linkages with vascular collagen, causing a vascular thickening, reduced elasticity, increased vascular stiffness and subsequently endothelial dysfunction (Singh *et al.*, 2001). Biomolecules such as testosterone, cGMP, NO, and acetylcholine (ACh) also play a crucial role in penile erection processes (Ademiluyi *et al.*, 2018). For instance, the continuous decreased activity of endothelial nitric oxide synthase (eNOS) which lead to a decreased NO production, depleted cGMP in the penile tissue and vasoconstriction and consequently stimulate ED. The upregulation of RhoA, a small GTPase, directly inhibits expression of eNOS (Goswami *et al.*, 2012). Furthermore, literature also suggests that increased activities of acetylcholinesterase (AChE), arginase and angiotensin converting enzyme (ACE) are some other contributing factors to the development of ED (Tsai and Kass, 2009; Shah *et al.*, 2009).

ED is currently treated with Sildenafil (Viagra) and Tadalafil (Cialis), which are all PDE-5 inhibitors. However, these drugs are associated with adverse effects such as headache, muscle pain and blurred vision (Melynk and Marcone, 2011), also a large

number of diabetic men suffering from ED are refractory to these ED therapies (Kizilay *et al.*, 2016). Thus, the other approaches with less adverse effects could be preferred as alternative new ED therapies. Despite the broad research on ED therapy for many decades, an effective cure modality is still to be achieved. The use of medicinal plant extracts, due to their multi-target nature, continue to attract attention of researchers in a bid to discover new drug regime against various ailments, including ED.

Maytenus procumbens (Celastraceae), which is predominantly found in KwaZulu Natal province of South Africa, is commonly used by Zulu traditional healers to treat several diseases linked to sexual dysfunction in men (Momtaz *et al.*, 2012; Cele, 2017). The ameliorative effect of methanol roots extract of *M. procumbens* on butanol induced testicular dysfunction in *Sprague Dawley* rats has been reported (Cele *et al.*, 2017). The ameliorative effect was associated with the regeneration of spermatogonia in the seminiferous tubules, increased serum testosterone, sperm count and mount frequency of the rats. However, the molecular mechanism associated erectile performance remains to be elucidated. Thus, in this study we investigated the erectogenic effect of the methanolic extract on type 2 diabetes-linked erectile dysfunction in rats.

3C.2 Materials and Methodology

3C.3 Chemicals and reagents

All chemicals and reagents used in this study were of analytical quality and all were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO, USA.

3C.4 Methods

3C.4.1 Extract preparation

Maytenus procumbens roots were collected from Ndumo and confirmed by a qualified botanist at the Botany Department, University of Zululand, Kwa-Zulu Natal, South Africa (Specimen voucher number NDUZ/17 05). The roots were thoroughly washed, chopped, air dried and ground into powder. The powdered plant material was extracted (1:5 w/v) with methanol to obtain methanolic crude extract. Thereafter, the methanolic extract was filtered through Whatman No. 1 filter paper. The methanol extract was then concentrated under reduced pressure at 40 °C using a rotary evaporator (Heidolph). The crude extract was used for the biological assays.

3C.4.2 Animals

Ethical clearance (UZREC 171110-030 PGD 2018/230) for approval of procedures and use of laboratory animals was obtained from the University of Zululand Research Ethics Committee (UZREC). *Sprague-Dawley* rats (06 – 12 weeks of age) were obtained from the laboratory animal unit of Biochemistry and Microbiology Department, University of Zululand. Rats were kept under standard laboratory conditions (23 ± 2 °C, 60% humidity and day-night cycle under light) as outlined in the institutional and national guidelines for handling and caring of science laboratory animals. The rats were allowed to acclimatize for at least five days with free access to enough normal rat feed and drinking water before the experiment commenced.

3C.4.3 Induction of type 2 diabetes and erectile dysfunction

Type 2 diabetes was induced using the method previously described by Pereira *et al.* (2014) with some modifications. Briefly, *Sprague Dawley* rats were randomly divided

into two major groups; normal group (normal diet and drinking tap water) and high fructose fed group (normal diet and 25% of fructose in drinking water). The animals were put of high fructose for a period of 120 days. After 120 days, the high fructose fed group rats were fasted overnight and they were then given an intraperitoneal injection of a low single dose (30 mg/kg) of a freshly prepared streptozotocin (STZ) solution. After five days of the STZ injection, blood glucose levels were measured with a glucometer (Accutrend glucometer; Roche Diagnostics, Mannheim, Germany) from the blood collected from the tail tip. Animals with blood glucose levels equal to or above 09 mmol/L were considered diabetic and used in the study.

3C.4.4 Investigation of the erectogenic effect of the plant extract

At the end of the induction of type 2 diabetes, estrous was induced in a group of female rats (150 – 200 g). These female rats received three consecutive subcutaneous injections of progesterone (7.5 mg/kg, at 24-hour intervals) 96 hours before being introduced to male rats for determination of baseline sexual behavior of the male rats. After that, the normal group was subdivided into two groups while the diabetic group was also subdivided into four groups of five rats per group as shown in Figure 3C.1.

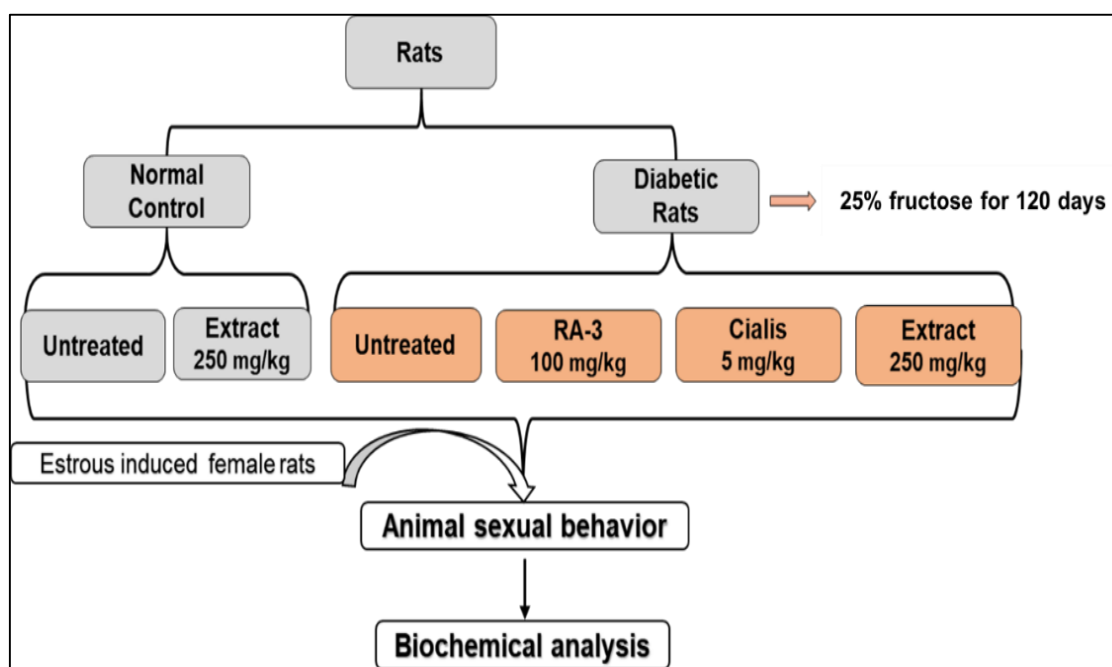


Figure 3C.1. Schematic representation of the erectogenic effect of the methanolic roots extract on type 2 diabetic rats.

Thereafter, the experimental groups were orally administered daily with the extract at 250 mg/kg b.w for 28 days. Normal control and negative control (Diabetic ED control) received a carrier solvent (water) while positive controls received RA-3 (100 mg/kg b.w) and Cialis (5 mg/kg b.w), respectively. Thereafter, estrous induced group of female rats (induced as described above), were then introduced to male rats for sexual animal behavior, each male rat from various treatment groups was individually subjected to the estrous female rats (n=3) in a separate cage for 30 minutes and the number of mounts was recorded.

3C.4.4.1 Biochemical analysis

At the end of the experimental period, all the male rats were fasted for 8 hours and euthanized under anaesthesia. The loss of sensation was confirmed by the pedal withdrawal reflex and assessed by pinching of the tails, prior to blood collection. Blood was immediately collected by cardiac puncture and the penile tissues as well as brains were removed for analysis of significant biochemical assay. The collected blood was allowed to clot and centrifuged for 10 minutes at 3,500 rpm at 4 °C, and serum was collected. The brains were weighed and homogenized in 0.1 M phosphate buffer saline (pH 8.0). The serum and brain homogenates were kept at -80°C until required. The serum levels of some antioxidants (SOD, CAT), MDA content, arginase and ACE activities as well as AChE activity from brain supernatant were estimated using respective commercial activity assay kits (Sigma-Aldrich), suitable for rat samples, following manufacturers' instructions. The serum testosterone, HDL and uric acid levels were determined using standard laboratory procedures (Global Laboratory & Viral Laboratory, Richards Bay).

3C.4.4.2 Estimation of serum fructosamine concentration

Fructosamine concentration was determined in the serum of the rats using nitro-blue tetrazolium (NBT) assay as previously described by Adisakwattana *et al.* (2012). Briefly, 125 µl of serum samples from the different groups was incubated with 125 µl of 0.5 mM NBT in 0.1 M sodium carbonate buffer (pH 10.4) at 37 °C for 15 minutes. The absorbance was measured at the wavelength of 590 nm. The concentration of fructosamine (mg/ml) in the respective samples was determined from the standard curve of 1-deoxy-1-morpholino-fructose (1-DMF).

3C.4.4.3 Determination of protein thiol group

The Ellman's assay was used to also determine the level of protein thiol groups (Adisakwattana *et al.*, 2012). The serum samples (125 µl) from different groups were incubated with 125 µl of 5 mM 5, 5'-Dithiobis (2-nitrobenzoic acid, DTNB) solution for 15 minutes at 37 °C, followed by measuring the absorbance at the wavelength of 410 nm. The free thiol concentration of samples was calculated based on the standard curve prepared by using various concentration of L-cysteine.

3C.4.4.4 Western blot analysis

Western blot analysis of some proteins involved in the erection process were performed. Snap frozen penile muscle tissue (100 mg) was defrosted on ice and lysed in lysis buffer (Pierce Biotechnologies, Rockford, CA, USA) using a Tissue lyser. Thereafter, the samples were centrifuged for 15 minutes at 13,000 rpm at 4 °C. Supernatant was collected and stored at -20 °C until required for use. Protein sample was mixed with an equal volume of 2x Laemmli sample buffer before it was denatured at 95 °C for 5 minutes. The denatured protein samples (30 µg) were loaded on the 12 % SDS-polyacrylamide gel (Bio-Rad, Hercules, CA, USA) and run for 1 hour at 150 V in Tris/Glycine/SDS-PAGE Buffer 1X. The proteins from the gel were then transferred to the polyvinylidene fluoride membrane (PVDF) (Bio-Rad, Hercules, CA, USA) (Johnson *et al.*, 2017). The membranes (containing the proteins) were incubated at 4 °C for 16 hours with the following primary antibodies: anti- PDE-5 (1:500), RhoA (1:500) and eNOS (1:10000) (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). The membranes were washed and incubated with the appropriate horseradish peroxidase conjugated secondary antibody at room temperature for 90 minutes. All proteins were normalized to a loading control (β-Actin) (1:500) (Santa Cruz Biotechnology, Dallas, TX, USA). Chemidoc-XRS imager and image lab version 06 software (Bio-Rad Laboratories, Hercules, CA, USA) was used to detect and quantify the proteins.

3C.4.5 Statistical analysis

Data were expressed as the mean ± standard error of mean (SEM) for n = 5. Data were subjected to one way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Statistical analysis of the differences between mean values

obtained from experimental groups were calculated using GraphPad prism (v6.01). $p \leq 0.05$ was regarded as statistically significant.

3C.5 Results

3C.5.1 Fasting blood glucose (FBG), Mounting frequency and serum testosterone levels

The results of the fasting blood glucose levels, mounting frequency and serum testosterone levels in diabetes induced ED rats, after 28 days of treatment, are shown in Table 3C.1. The untreated diabetic rats presented with persistently higher fasting blood glucose levels. However, a slight decrease in the fasting blood glucose level, when compared to the untreated diabetic group, was observed in the diabetic rats treated with the extract for 28 days. In addition, a remarkable decrease in fasting blood glucose levels was also observed in the rats treated with an in housed control RA-3. Relatively lower testosterone levels, and mounting frequency, when compared to the normal group, were also observed in the untreated diabetic ED group. However, an increase ($p < 0.001$) in the tested parameters was evident in the animals treated with the extract as well as positive control Cialis.

Table 3C.1. Effect of extract on FBG, mounting frequency and serum testosterone levels of the experimental rats

Groups	FBG Baseline (mmol/L)	FBG Week 21 (mmol/L)	Mount frequency (In 30 mins)	Testosterone (nmol/L)
Normal control	5.27 ± 0.32	5.28 ± 0.05	29 ± 1.02	5.66 ± 0.17
Model Diabetic ED control	12.77 ± 0.56	18.77 ± 0.16	09 ± 1.00	2.23 ± 1.01
Diabetic ED + Cialis (5mg/kg)	13.13 ± 0.43	15.46 ± 0.08	33 ± 1.01	8.69 ± 2.94
Diabetic ED + RA-3 (100 mg/kg)	16.37 ± 0.54	7.63 ± 1.52***	31 ± 3.11	8.31 ± 1.57
Diabetic ED + extract (250 mg/kg)	11.71 ± 0.03	9.11 ± 0.34	42 ± 1.20	10.01 ± 2.58***
N. Control + extract (250 mg/kg)	5.32 ± 0.01	5.12 ± 0.02	46 ± 1.11	11.93 ± 2.61***

Results are expressed as the mean ± SEM, n = 5. Significantly at *** $p < 0.001$ vs. Diabetic control.

3C.5.2 Serum fructosamine, protein thiol group content and uric acid

Figure 3C.2 shows the results of the effect of the extract on serum fructosamine, protein thiol group content and uric acid of the diabetes induced ED rats after 28 days of treatment. Elevated serum levels of fructosamine and lower protein thiol group were

observed in the untreated diabetic animals or diabetic animals treated with Cialis, respectively, when compared to the normal control group. However, lower serum levels of the fructosamine content accompanied by a significant increase in protein thiol group concentrations were observed in the diabetic rats treated with the extract or RA-3 when compared to the untreated diabetic animals (Figure 3C.2). Furthermore, a decreased uric acid level was also observed in the diabetic ED group treated with the extract or RA-3 when compared with an increase uric acid level in the untreated diabetic ED group.

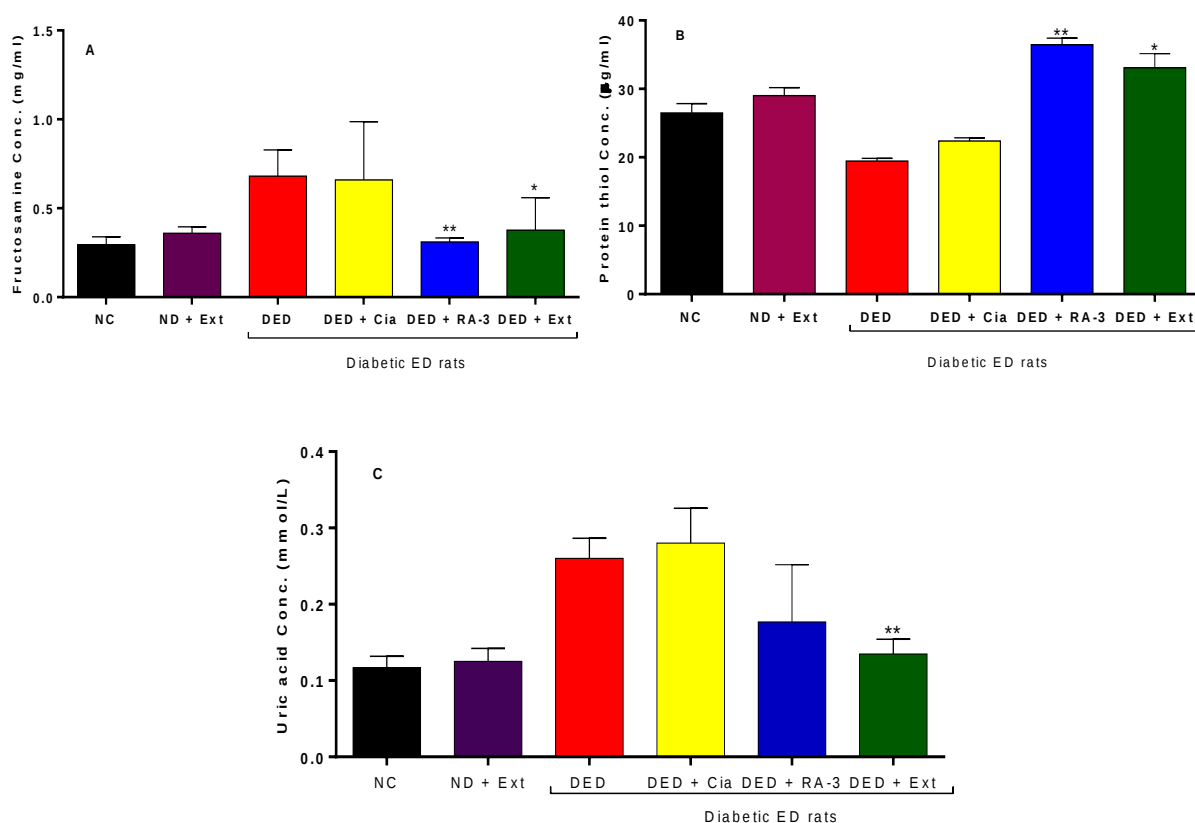


Figure 3C.2. Effect of the methanol extract on fructosamine (A); protein thiol (B) contents and uric acid levels (C). Results are expressed as the mean $\pm$ SEM, (n = 5). *p<0.01, ** p<0.001 vs. Untreated diabetic induced ED control. NC – Normal control, ND + Ext – Nondiabetic + extract, DED – diabetic ED control, DED + Cia – Diabetic + Cialis, DED + RA-3 – Diabetic ED + RA-3, DED + Ext – Diabetic ED + extract.

3C.5.3 Effect of the extract on serum antioxidant status

Table 3C.2 shows the results of the effect of the extract on some serum antioxidant levels in the diabetes induced ED rats. Significantly lower SOD and CAT levels along

with a relatively higher MDA level were observed in the untreated diabetic group animals when compared to the normal control group. However, treatment of the diabetic rats with either extract or RA-3 displayed a significantly ($p \leq 0.001$) increased SOD and CAT levels in comparison to the untreated diabetic animals. This was accompanied by a significant ($p \leq 0.01$) decrease in the MDA levels in the extract or RA-3 treated groups.

Table 3C.2. Effects of methanol extract on serum antioxidants levels of the diabetic animals.

Groups	SOD (Inhibition rate %)	CAT (mmoles/min/ml)	MDA levels (nmol/ μ L)
Normal Control	66.02 $\pm$ 1.18	1690.7 $\pm$ 295.3	0.03 $\pm$ 0.02
Diabetic ED Control	30.03 $\pm$ 2.01	344.0 $\pm$ 81.2	0.44 $\pm$ 0.04
Diabetic ED + Cialis (5 mg/kg)	38.88 $\pm$ 1.29	650.7 $\pm$ 83.3	0.32 $\pm$ 0.01
Diabetic ED + RA-3 (100 mg/kg)	62.08 $\pm$ 2.62**	1210.7 $\pm$ 88.1***	0.04 $\pm$ 0.00***
Diabetic ED + Extract (250 mg/kg)	65.36 $\pm$ 1.18***	1105.3 $\pm$ 109.6***	0.09 $\pm$ 0.01**
N. Control + Extract (250 mg/kg)	60.82 $\pm$ 0.31	1386.67 $\pm$ 268.4	0.04 $\pm$ 0.01

Results are expressed as the mean $\pm$ SEM, n = 5. Significantly at ** $p < 0.01$, *** $p < 0.001$ vs. Diabetic control.

3C.5.4 Effect of the extract on the serum levels of arginase, ACE and AChE

The results of the effect of methanolic roots extract on ACE, AChE and arginase activities are presented in Figure 4C.3. A significantly ($p \leq 0.001$) higher enzyme activities were observed in the untreated diabetic ED rats when compared with the normal control group. However, administration of the extract to the diabetic ED rats for 28 days, significantly ($p \leq 0.001$ and $p \leq 0.0001$) decreased the enzyme activities when compared to the untreated diabetic ED group. Furthermore, a slight decrease in the tested parameters was observed in animals treated with Cialis when compared to the untreated diabetic ED group.

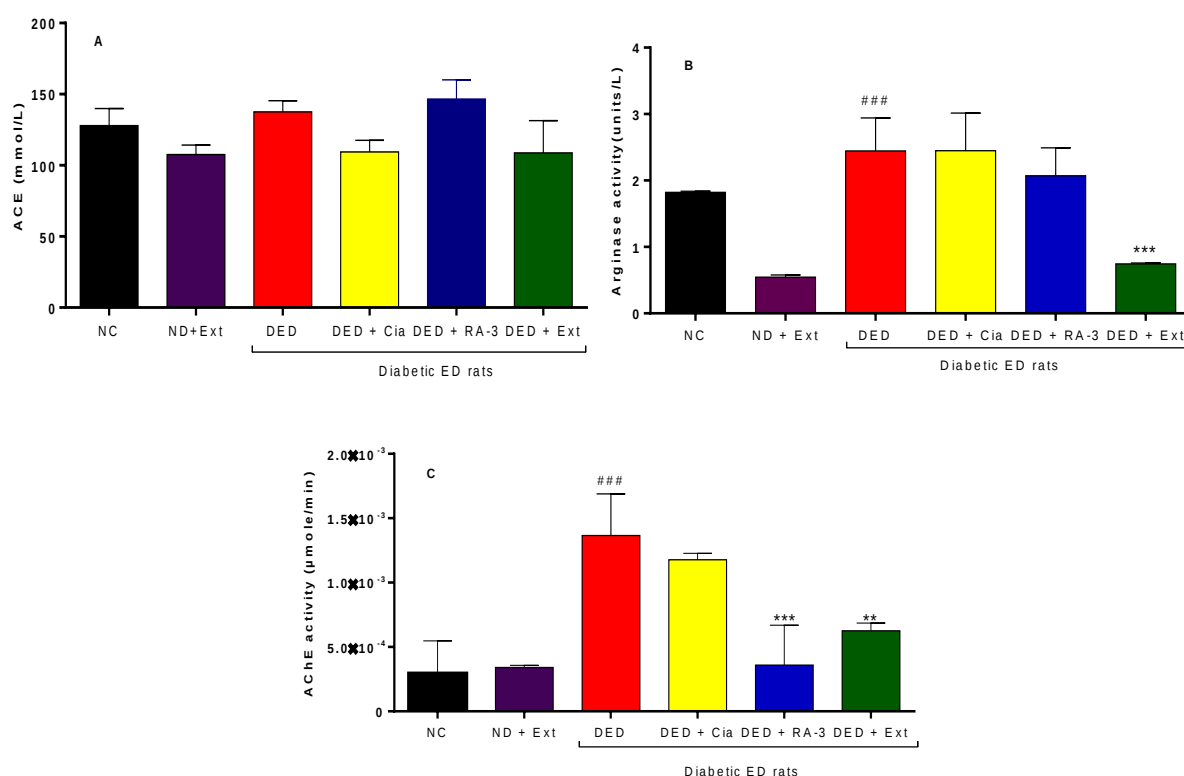


Figure 3C.3. Effect of the methanol extract on ACE (A), arginase (B) and AChE (C) activities. Results are expressed as the mean $\pm$ SEM, (n = 5). Significant at ### $p < 0.001$ vs. normal control, *** $p < 0.0001$, and ** $p < 0.001$ vs. Untreated diabetic control. NC – Normal control, ND + Ext – Nondiabetic + extract, DED – untreated diabetic ED control, DED + Cia – Diabetic + Cialis, DED + RA-3 – Diabetic ED + RA-3, DED + Ext – Diabetic ED + extract

3C.5.5 Effect of the extract on the expression of PDE-5, RhoA and eNOS

Effect of the plant extract on the expression of some proteins involved in penile erection process was also investigated. The upregulation of PDE-5 (Figure 3C.4A) and RhoA (Figure 3C.4C) accompanied by down regulated eNOS (Figure 3C.4B) were observed in the untreated diabetic ED group when compared to the normal rats. A significantly lower expression of PDE-5 and RhoA along with an increased expression of eNOS were observed in the penile tissues of the diabetes induced ED group treated with the extract. Furthermore, a significantly decreased expression of PDE-5 and RhoA in the diabetic ED groups treated with positive control RA-3 or Cialis observed was comparable to that nondiabetic animals treated with extract.

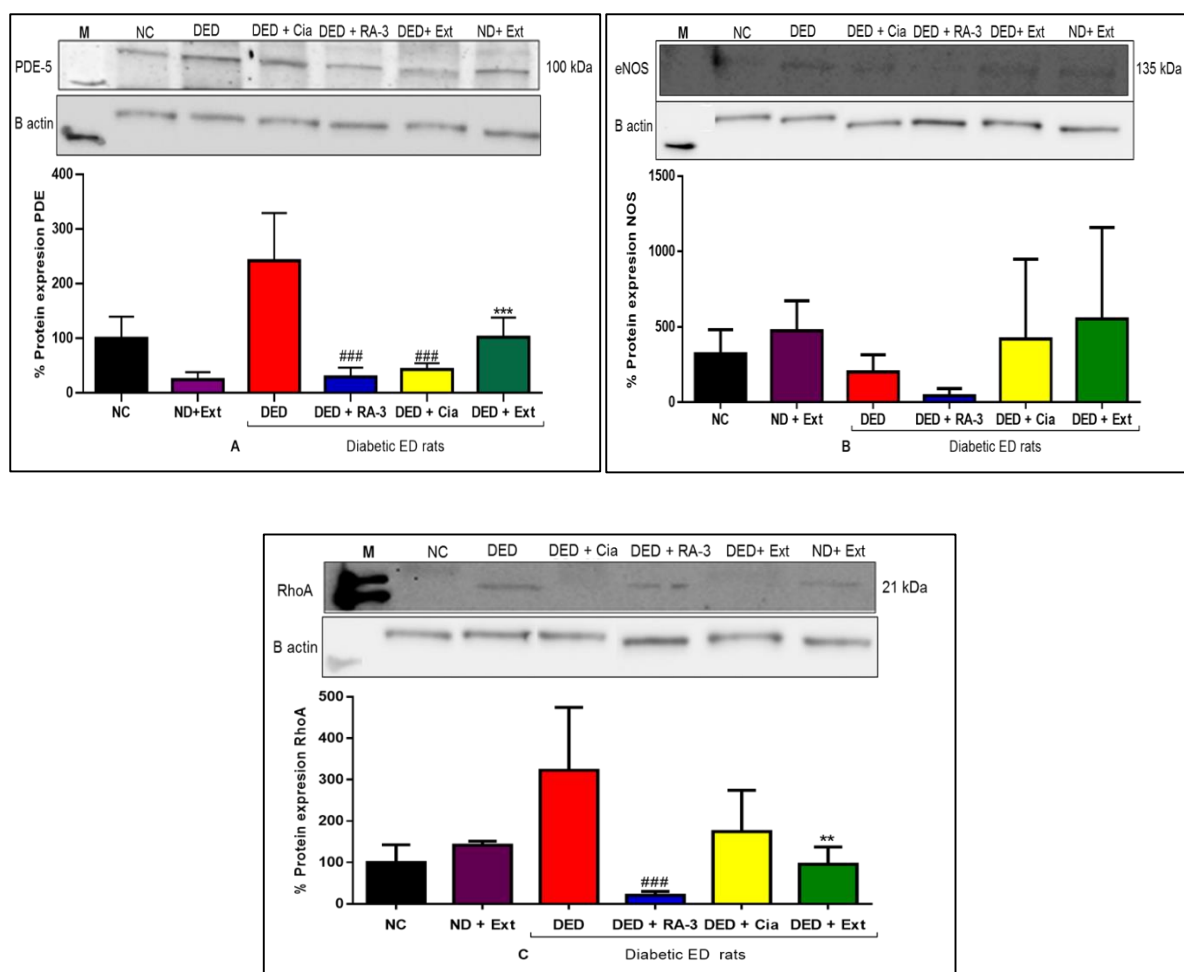


Figure 3C.4. Effect of the methanol extract on PDE-5 (A), eNOS (B) and RhoA (C) expression in type 2 diabetic rats. Values are expressed as the mean $\pm$ SD (n=4). ** $p \leq 0.01$ vs. diabetic control, ### $p \leq 0.001$ vs. diabetic control and normal control. NC – Normal control, ND + Ext – Nondiabetic + extract, DED – diabetic control, DED + Cia – Diabetic ED + Cialis, DED + RA-3 – Diabetic + RA-3, DED + Ext – Diabetic + extract.

3C.6 Discussion

The increasing incidence of type 2 diabetes in men contributes to the increasing number of men with ED (Minaz *et al.*, 2019). An increasing body of evidence supports the use of medicinal plants in the management of diabetes related complications including ED (Adefegha *et al.*, 2017; Ademosun *et al.*, 2019). In this study, the erectogenic effect of the methanolic extract from the roots of the *M. procumbens* in type 2 diabetic rats is reported.

The recorded elevated fasting blood glucose in the diabetes-induced groups confirmed induction of diabetes state in the animals. While mounting behaviour characterises sexual provocation and penile orientation (Minaz *et al.*, 2019), testosterone regulates nearly every component of erectile function. In addition to increasing sexual desire, testosterone also stimulates synthesis of NO, which activates cascade of reactions leading to vasodilation in the penile tissue (Vudriko *et al.*, 2013). In the present study, the reduced mounting frequency and serum testosterone levels in the model diabetic ED rats indicated a decreased sexual desire/libido, a characteristic of ED. The observed increase in mounting frequency and testosterone levels following treatment of the diabetic rats with the extract indicated the erectogenic potential of the extract. In addition, augmented levels of testosterone and increased mounting frequency observed in the normal animals treated with extract further supported its erectogenic boosting ability.

The decreased serum levels of AChE, arginase and ACE in the extract treated groups further supported the erectogenic effect of the plant extract. Increased activities of AChE and arginase, commonly observed in diabetic state, diminishes the synthesis and bioavailability of NO, required for efficient penile erection process (Corbin, 2004; Vargas *et al.*, 2004). Furthermore, the inhibitory effect of the extract on ACE activity, an enzyme that catalyzes the conversion of the angiotensin I to the active vasoconstrictor angiotensin II (John and Schmieder, 2003), indicates a potential to ameliorate hypertension-induced ED. Hence, the control of arginase, AChE and ACE have been reported as potential therapeutic strategy in the management or treatment of ED (Akomolafe *et al.*, 2018). The enzymatic inhibitory activity exhibited by the extract could be attributed to the phytochemical composition of the plant, which

includes saponins, tannins and alkaloids. These phytochemicals are known to inhibit the activities of arginase, AChE, ACE and PDE-5 (Oboh *et al.*, 2015; Odubanjo *et al.*, 2017, Ojo *et al.*, 2019).

The ability of the plant extract to lower the serum uric acid levels in the extract treated animals further supported its erectogenic potential. Uric acid, an end product of dietary and endogenous purine metabolism, modulate the physiological functions of various physical systems (Gao *et al.*, 2017). However, the link between serum uric acid level and ED is still controversial. Various reports have provided evidence that elevated uric acid level is a powerful scavenger of reactive oxygen species (ROS), thus acts as an antioxidant (Waring *et al.*, 2006; Fabbrini *et al.*, 2014). Whereas other studies holding the opposite view have revealed that elevated level of uric acid is a potential risk factor of ED (Seidman and Roose, 2000; Chaudhari *et al.*, 2010). In the current study we found that increased level of uric acid is associated with ED (Figure 3C.2), hence, the lowered uric acid level observed on the extract treated rats could partly explain its erectogenic property.

Moreover, understanding the molecular mechanisms that underlie the development and progression of ED is also a vital objective in the management and treatment of the ailment. On one hand, PDE-5 hydrolyses cGMP, a second messenger molecule crucial for activating cascade of reactions leading to vasodilation in the penile tissue (Ghofrani *et al.*, 2006). On the other hand, RhoA kinase pathway directly suppress the expression of eNOS, which leads to diminished eNOS activity and reduced bioavailability of NO, thus inhibiting the smooth muscle relaxation and erectile process (Chitaley *et al.*, 2001). The significant downregulation of PDE-5 and RhoA along with an increased expression of eNOS observed in the extract treated group provided support and an insight into the molecular basis of the erectogenic effect of the extract. Our results are similar to those reported by Corbin *et al.* (2004) and Bivalacqua *et al.* (2004), who showed the ability of the crude extracts to downregulate RhoA and increase eNOS expression and thus ameliorate erectile dysfunction in diabetes induced ED. Oboh and co-workers (2015) have also shown that downregulation of PDE-5 ameliorates erectile dysfunction in diabetic rats .

The ability of the extract to also lower the fasting blood glucose levels (Table 3C.1) and serum fructosamine (Figure 3C.2A), a substance clinically used as a marker of short-term glycemic control in diabetic patients, demonstrated its antihyperglycemic property. The elevated blood glucose levels are known to promote AGEs formation, which accumulate in the corpus cavernosal tissue, particularly in the endothelial and smooth muscle cells, and contribute to the pathogenesis of diabetic ED (Seftel *et al.*, 1997; Sandireddy *et al.*, 2014). AGEs form covalent bonds with vascular collagen, which leads to vascular thickening, reduced elasticity, endothelial dysfunction and increased vascular stiffness (Singh *et al.*, 2001). The obtained results from the current work thus show that the crude extract and RA-3 (our in house hyperglycemia control compound (Mosa *et al.*, 2015; Mabhida *et al.*, 2017)) partly possess a comparable hypoglycemic effect when compared to untreated diabetic ED rats and/or Cialis treated animals, respectively.

Chronic elevated blood glucose also induces oxidative stress which underlies various complications of diabetes including endothelial dysfunction and consequent ED development. Increased activity of SOD and CAT accompanied by decreased MDA levels (a measure of lipid peroxidation) in the extract treated diabetic ED group indicated the extract's potential to enhance antioxidant defence system. The increased protein thiol group content in the extract treated diabetic rats (Figure 3.2B) further indicated the ability of the extract to enhance endogenous antioxidant status. Protein thiol groups are very vulnerable to oxidation and considered as one of the most valuable body sacrificial antioxidants (Zabihi *et al.*, 2018; Smith and Thornalley, 1992). The antioxidant property of the extract could play a crucial role in ameliorating diabetes-induced erectile dysfunction incidence. Various plants with antioxidant properties have been reported to improve erectile function in diabetic-ED rats (Akomolafe *et al.*, 2018; Fu *et al.*, 2019; Ojo *et al.*, 2019).

3C.7 Conclusion

In conclusion, the present study provides evidence that methanolic roots extract of the *Maytenus procumbens* ameliorates diabetes-induced erectile dysfunction, suggesting that the plant could potentially be used in the management of diabetic ED. The ameliorative effects of the extract were due to an increased testosterone levels and mount frequency accompanied by inhibitory effect on arginase, ACE and AChE activities in the diabetic ED treated group. In addition, the mechanism through which the extract exerts its erectogenic potential could be attributed to its ability to downregulate PDE-5 and RhoA accompanied by upregulated eNOS. Nevertheless, the effect of the extract on smooth muscle contraction and the histopathological analysis of the penile tissue from the diabetic induced ED in rats is recommended to confirm the its erectogenic potential.

3C.8 Acknowledgements

The study received funding from the University of Zululand Research Committee. The authors are grateful to NRF-South Africa for studentship to N. D. Cele and the team of South African Medical Research Council (SAMRC) for their technical assistance.

3C.9 Contribution of authors

R.A.M. & A.R.O. designed and supervised the .project; N.D.C., performed the experiments and wrote the manuscript; S.E.M.M., T.N., S.E.M. & K.Z. experimental designed and interpretation of western blot analysis. Also they all contributed in the writing of the manuscript.

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CHAPTER FOUR

4.1 General Discussion

Erectile dysfunction (ED) is a vascular disorder characterized by an inability to retain penile erection firm enough for normal sexual interaction (Walle *et al.*, 2018). Even though normal penile erection declines with age, other factors such as diabetes mellitus, hypogonadism, oxidative stresses, etc. contribute to the effectual increase in PDE 5 which activity leads to the onset and progression of ED. See Figure 4.1. (Saxena *et al.*, 2012; Malviya *et al.*, 2016). Considering the multifactorial nature of ED, the multi-target nature of extracts from medicinal plants (as used by traditional healers) could prove to be an effective alternative therapeutic approach in the amelioration of ED.

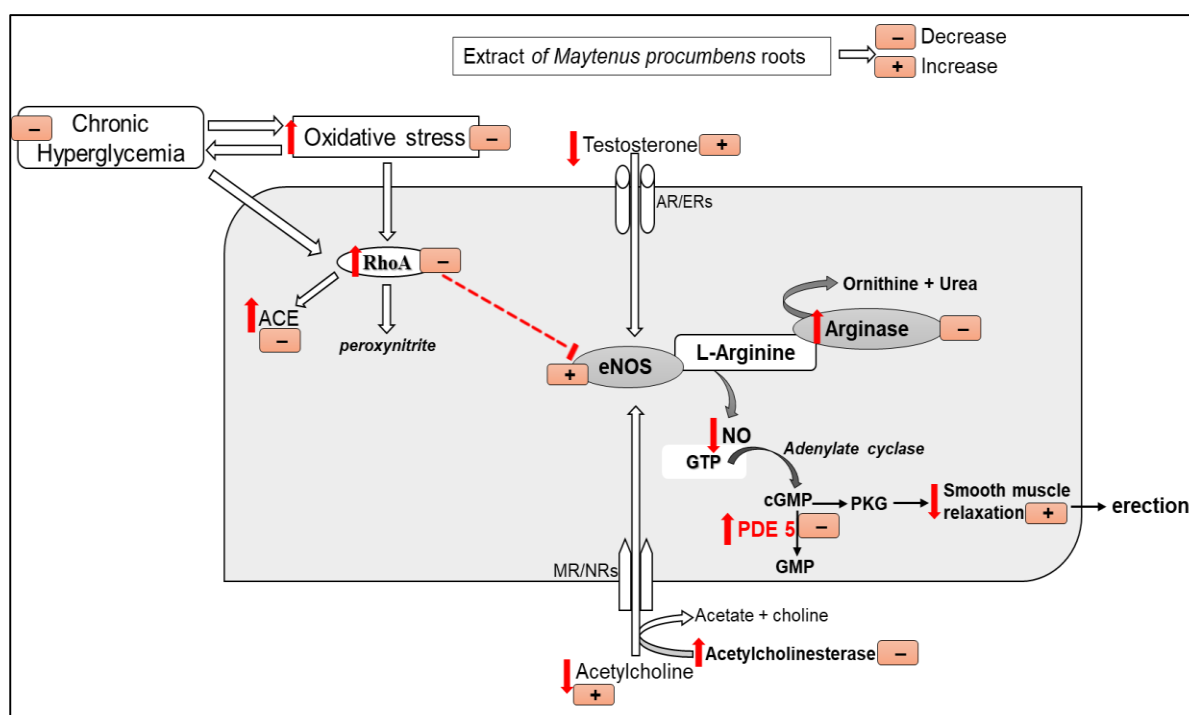


Figure 4.1: An overview mechanism of penile erection. Under physiological condition, several biomolecules including testosterone and acetylcholine aid in the expression and activity of eNOS which lead to production of nitric oxide. Thereafter, NO diffuses into soluble adenylate cyclase where it triggers a cascade of reactions which results to vasodilation and smooth muscle relaxation and subsequently into penile erection. However, under pathophysiological conditions, hyperglycemia and oxidative triggers the activation and increased protein RhoA expression, which aids in vasocontraction and lead to impaired eNOS expression accompanied by upregulation of PDE-5 in the penile tissue.

It is apparent from the findings obtained in the present study that the extract of *M. procumbens* roots does contain constituents that exhibit erectogenic potential. The antioxidant properties exerted by the roots extract (Table 3A.2) demonstrated an efficiency against a wide variety of free radicals. ROS have been implicated as causative agent of various diseases (Chayaratanasin *et al.*, 2015), and are capable of causing endothelial cell damage (Moreland, 2013). In the case of ED, ROS accumulation stimulates the production of the RhoA protein which subsequently reduces eNOS. Hence, prevention of radical generation through redox property (scavenging) as demonstrated by the extract could be important in the management of ED. It is noted that the ability of the extract to scavenge nitric oxide (NO), which is essential for erection process was poor ($IC_{50} > 5\text{mg/ml}$) when compared to other free radicals. It is apparent therefore that the plant extract will have little effect on the available concentration of NO. The antioxidant activity of the extract of *M. procumbens* roots as observed might be attributed to its phenolic content (Table 3A.1). Phenolic compounds possess anti-oxidant properties which make them a potential candidates capable of protecting cells against the deleterious effects of free radicals (Ghasemi *et al.*, 2009). A similar effect of the *Eucommia ulmoides* leaf extract on serum antioxidant status in diabetic rats has recently been reported to be strongly linked with improved erectile function (Fu *et al.*, 2019).

Chronic hyperglycemia and consequent AGEs formation progressively diminish normal erectile function in males (Murray *et al.*, 1992; Yakubu *et al.*, 2010). AGEs deposited in the collagen of the corpus cavernosum of diabetic men, reportedly (Yakubu *et al.*, 2010) caused an increased vascular stiffness. The antidiabetic properties of the plant's extract could be correlated with the observed inhibition of α -amylase, α -glucosidase and pancreatic lipase *in vitro* (Figure 3A.2). The digestive activities of these enzymes do contribute to the overall blood glucose levels. Even though the *M. procumbens*' extract could not significantly reduce fasting blood glucose levels in the rats, the reduced fructosamine content accompanied by increased thiol concentration observed in the diabetic ED treated rats (Table 3C.1 and Figure 3C.2) indicated a potential anti-hyperglycaemic properties of the extract.

It is speculated that overall regulation of hyperglycemia through inhibition of glycoside and lipid hydrolases enzymes as well as protein glycation could be one mechanism through which the extract improves diabetes associated ED. Literature has shown that

the antihyperglycemic effects of plant extracts is usually concomitant with the increased antioxidant levels and decreased lipid peroxidation (MDA) (Elmarakby and Sullivan, 2012). The antihyperglycemic effect of *M. procumbens* roots could also be attributed to the observed increase in antioxidant levels with concomitant decrease in lipid peroxidation (MDA) in the extract treated diabetic ED animals (Table 3C.2).

The up regulation of arginase, AChE and ACE activities in penile tissue of the diabetic men are strongly linked to diabetic ED (Oboh *et al.*, 2015). The inhibitory effect exerted by the extract on the activities of these enzymes (Figures 3B.3, 3B.4, and 3B.5) possibly contributes to explain the erectogenic properties of the extract. Such inhibitory properties of the extract on arginase, ACE and AChE could be attributed to presence of saponins and tannins components of the extract (Table 3A.1). Saponins and tannins have been reported to possess inhibitory effect on arginase, ACE and AChE activities (Da-Silva *et al.*, 2012; Oboh *et al.*, 2018).

The presence and action of cyclic nucleotide signaling in cellular processes have been well documented (Nunes and Webb, 2012). Such cGMP activities include the induction of a cascade of reactions culminating in smooth muscle relaxation, enhanced vascular blood flow and finally penile erection (Young *et al.*, 2003; Kim *et al.*, 2011). The biochemical actions of cGMP are terminated by phosphodiesterase-5 (PDE-5, EC 3.1.4.17) which hydrolyzes cGMP to GMP, thus inhibiting the smooth muscle relaxation and erectile process (Campos *et al.*, 2019). The down regulated protein expression of PDE-5 accompanied by up regulation of eNOS expression have been reported to ameliorate erectile function in diabetic rats (Garcia-Cardoso *et al.*, 2009; Ghasemi *et al.*, 2009). Similarly, Abbasnezhad *et al.* (2018) demonstrated the ability of extract of *Nigella sativa* to ameliorate smooth muscle relaxation through increased up regulation of eNOS protein content accompanied by decreased protein PDE-5 expression in diabetic animals. Similar activity has been observed with the extract of *M. procumbens*. It is apparent that the erectogenic properties exhibited by the plant extract could be linked to its ability to up regulate eNOS and down regulate protein PDE-5 expression in diabetic ED treated animals.

In addition, the ability of the extract to down regulate RhoA expression while up regulating the eNOS expression (Figure 3C.4) and ameliorates NO-cGMP dependent protein kinases observed also confirms its erectogenic effect. Prolonged

hyperglycaemia triggers the activation and increased protein RhoA expression, which aids in vasocontraction and lead to impaired eNOS expression in the penile tissue (Goswami *et al.*, 2012). These observations are also in agreement with the study reported by Bivalacqua *et al.* (2004) and Peng *et al.* (2008) who demonstrated the inhibition of RhoA expression to ameliorate eNOS protein content and activity thus restoring normal penile erection in diabetic rats. Thus, in accordance with literature, the obtained results from the current study illustrated the erectogenic properties and the antidiabetic effect of *M. procumbens* roots in type 2 diabetic rats.

The observed increase in testosterone (Table 3C.1), the hormonal stimulant and a vital marker for normal penile erection (Doshi *et al.*, 2012; Adeneye *et al.*, 2019), and the associated increase in mount frequency (Table3C.1) adequately add to the overall erectogenic property of *M. procumbens*. While most available drugs focus on the inhibition of PDE 5, the crude extract of *M. procumbens* exhibits properties that seem to control ED (diabetic-induced) in multiple fronts. The overall effect of which contribute to the use of the plants by traditional healers to manage ED.

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CHAPTER FIVE

5.0 General conclusion

In conclusion, the results obtained in the present study demonstrate that crude extract of *Maytenus procumbens* possesses erectogenic potential. The plant extract exhibited antioxidant properties; the extracts effectively scavenged free radicals. In addition, the ability of the extract to lower elevated blood glucose levels while decreasing fructosamine content and inhibiting intestinal glycosides and lipid hydrolases activities demonstrated its antihyperglycemic property. Since hyperglycemia induced oxidative stress activates various metabolic pathways including AGEs formation and decreased cellular antioxidant defence, the antioxidant property of the extract could be vital in preventing oxidative cellular damage.

In addition, fructose-streptozotocin induced diabetes altered normal penile function possibly by initiating and accelerating the factors such as hypogonadism, AGEs formation, etc. leading to development and progression of diabetes induced ED. The ability of the extract to reverse these contributing factors and subsequently increase mount frequency and testosterone levels in the diabetic rats further supported its erectogenic potential. The erectogenic effect of the plant was supported by the observed increase in AChE, ACE and arginase activities in the treated diabetic ED group. The erectogenic potential of the extract could be linked to its upregulation of eNOS accompanied by decreased protein PDE-5 and RhoA content in the diabetic treated rats. The results obtained from this study support the traditional use of *M. procumbens* roots in the treatment and management of erectile dysfunction.

5.1. Limitations

Due to limited funding to outsource equipment and expertise, analysis of histomorphological changes of the corpus cavernosum to confirm the prevention of cell death or degeneration of smooth muscle cells could not be conducted. Evaluation of the extract's effect on smooth muscle relaxation/contraction to further support its underlying mechanism of penile erection could not also be done. However, the

biochemical analysis conducted provided significant evidence for the erectogenic potential of the root extract of *Maytenus procumbens*.

5.2 Recommendation for future studies

- Histopathological analysis of the penile tissue from diabetic induced rats is recommended to confirm the protective effect of the extract.
- It also necessary to evaluate the effect of the roots extract on corpus smooth muscle relaxation.
- Isolation of new active compound(s) from the plant extract is also recommended.

APPENDIX A

Reagent Details

A.1 Sulphanilic acid reagent

Sulphanilic acid (0.33%) was dissolved in glacial acetic acid (20 %).

A.2 ABTS

The mixture containing potassium persulfate (2.45 mM), ABTS (7 mM) and ionized water was incubated at 25 °C in a dark for 16 hours which resulted in radical cation ABTS production which was then diluted with methanol (1:60 v/v).

A.3 Phosphate buffer (pH 7.4)

Disodium hydrogen orthophosphate (0.2 M, 40 ml) was prepared and mixed with 50 ml of 0.2 M sodium dihydrogen orthophosphate (0.2 M, 50 ml). The mixture was topped up to 100 ml with distilled water.

A.4 Tris-buffer (pH 7.0)

Tris-HCL (7.88 g), EDTA (2.79 g) and 10.227 g of NaCl (10.23 g) was dissolved in deionised water and the solution was made up to 1 L.

A.5 TBA

Glacial acetic acid (50 ml) and 1 g of TBA was mixed deionized water (50 ml) and the solution was topped up to 100 ml with deionized water.

APPENDIX B

Details of Methodology

B.1 Phytochemical Analysis

Alkaloids test: 1% aq. HCL (5.0 ml) was mixed with plant extract (0.5 ml) and the resulting mixture was stirred on the steam bath and filtered, the filtrate (1 ml) was mixed with (1 ml) Mayer's reagent and Dragendorff's reagent, respectively. Turbidity indication of the presence on alkaloids.

Tannins test: plant extract (5 g) was mixed with the water (10 ml) and the resulting mixture was stirred, filtered and the filtrate (2 ml) was mixed with few drops of FeCl_3 (0.1%) solution. The presence tannins (Blue-black, green or blue-green precipitate)

Test for saponins: plant powder (0.5 g) was boiled with water (10 ml) and filtered. The filtrate was let to cool and shaken vigorously. The resulting mixture was let to stand 15-20 minutes. The presence of saponins (The froth).

Anthraquinone test

a) Anthraquinone: plant powder (0.5 g) was dissolved in deionised water, shaken with benzene (5 ml), filtered and ammonia solution (10 %, 5 ml) was added to the filtrate and shaken. The presence of free anthraquinones (A pink color in the ammonia layer).

b) Combined anthraquinone: plant powder (0.5 g) was dissolved in deionised water, boiled with ferric chloride solution and hydrochloric acid (5 ml) for 10 minutes. The resulting mixture was cooled and filtered. Thereafter ammonia solution (10 %, 5 ml) was added to the filtrate and shaken. The presence of anthraquinone (A pink color in the ammonia layer).

Flavonoids test

(a) Lead acetate method: The plant extract (1 ml) was mixed with lead acetate (10%) (Reddish brown color)

(b) Ferric Chloride method; Plant extract (1 ml) was mixed with ferric chloride (10%) (Dark brown or dirty brown precipitate).

(c) Sodium Hydroxide method; Plant extracts (1 ml) was mixed with diluted sodium hydroxide (1 ml). (Golden yellow precipitate)

Steroids test: Acetic anhydride (2 ml) was added to plant extract (0.5 g) of each sample H_2SO_4 (2 ml). (The color changed from violet to blue or green)

Test for Cardiac glycoside

(a) Lieberman's test; mixture containing of extract (0.5 ml), acetic anhydride (2 ml), H_2SO_4 (1 ml) was added. (Color change from violet to blue or green).

APPENDIX C

C1. Ethic clearance

**UNIVERSITY OF ZULULAND
RESEARCH ETHICS COMMITTEE**
(Reg No: UZREC 171110-030)



RESEARCH & INNOVATION

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ETHICAL CLEARANCE CERTIFICATE

Certificate Number	UZREC 171110-030 PGD 2018/230					
Project Title	ERECTOGENIC ACTIVITY OF THE MAYTENUS PROCUMBENS ROOTS EXTRACT IN TYPE 2 DIABETIC MALE RATS					
Principal Researcher/ Investigator	ND CELE (200903795)					
Supervisor and Co-supervisor	Dr RA Mosa			Prof A Opoku		
Department	Biochemistry					
Faculty	Science and Agriculture					
Type of Risk	Med Risk – No data collection from people					
Nature of Project	Honours/4 th Year		Master's		Doctoral	x Departmental

The University of Zululand's Research Ethics Committee (UZREC) hereby gives ethical approval in respect of the undertakings contained in the above-mentioned project. The Researcher may therefore commence with data collection as from the date of this Certificate, using the certificate number indicated above.

Special conditions:

- (1) This certificate is valid for 1 year from the date of issue.
- (2) Principal researcher must provide an annual report to the UZREC in the prescribed format [due date-17 March 2020]
- (3) Principal researcher must submit a report at the end of project in respect of ethical compliance.
- (4) The UZREC must be informed immediately of any material change in the conditions or undertakings mentioned in the documents that were presented to the meeting.

The UZREC wishes the researcher well in conducting research.

Professor Gideon De Wet
Chairperson: University Research Ethics Committee
Deputy Vice-Chancellor: Research & Innovation
18 March 2019

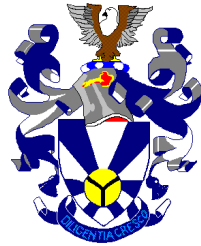
CHAIRPERSON
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ETHICS COMMITTEE (UZREC)
REG NO: UZREC 171110-30

18-03-2019 P

RESEARCH & INNOVATION OFFICE

APPENDIX D

D1. Research Questionnaire



Research Questionnaires for Traditional Healers

Date:

Questionnaire No.

Name of the Interviewee:

Particulars of the area

GPS reading:

Name of the Area:

Name of the Village (Precise place):

Sociodemographic data

Gender:

Male	
Female	

Age:

15-24	
25-34	
35-44	
45-54	
55-64	
64 ≤	

Plant Species particulars

Zulu name:

Plant

1: _____

Plant

2: _____

Plant

3: _____

Plant

4: _____

Scientific name:

Plant

1: _____

Plant

2: _____

Plant

3: _____

Plant

4: _____

English name:

Plant

1: _____

Plant

2: _____

Plant

3: _____

Plant

4: _____

Source of plant material:

Collected from the wild	
Cultivated (home-garden)	

What are the other uses of the plant?

Plant usage and collection

Question	Usage
Which part(s) used?	
Are the plants sold?	
In which state are the plants sold? (fresh or dry)	
If collected from the wild, when? (season)	
Any specific time of collection during the day?	

What places does the plant prefer to grow in? (wetland, dry land, forests, old fields, as weeds among the plants)	
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Preparation Method:

- a) How is the medicine take (e.g. by mouth or as enema)?
- b) How is the medicine prepared?

Storage Method:

Dosage:

a) What is the dosage (e.g. one cup three times a day)? _____

b) For how many days is the medicine taken? _____

c) Are there any age restrictions?

yes	No

If yes:

Age group	Age	Dosage
Infants		
Children		
Adults		

d) Are there any known side effects? _____

e) Where did the knowledge come from (e.g. grandmother, relative)?

APPENDIX E

Additional results

E1. Tables

The results on the effect of the extract on the food intake and ΔBW in the diabetic rats after the 28 days of treatment are presented in Table E.1. The results revealed a remarkable difference in food consumption in the diabetic animals compared to the normal control group. The diabetic animals consumed more food than the normal control rats. However, a reduction in food intake was observed in extract treated diabetic group when compare to the diabetic rats. The normal control group, subjected to a normal diet, displayed 58.6% increase in body weight. A similar effect on body weight was observed in the extract treated groups with an increase of 58.9% as compared to the diabetic control which displayed a low weight gain of 41.1%

Table E1: Effect of extract on body weight (BWT) and body weight change (ΔBW) of the experimental rats.

Groups	Food intake (g/day)	BWT Initial (g)	BWT Final (g)	ΔBW (%)
Normal Control	77.07 $\pm$ 1.09	126.9 $\pm$ 4.31	306.4 $\pm$ 17.85	58.6
(Diabetic Control	103.23 $\pm$ 0.24	128.3 $\pm$ 2.16	217.7 $\pm$ 22.7	41.1
Diabetic + Cialis (5 mg/kg)	100.03 $\pm$ 0.24	129.7 $\pm$ 7.71	225.8 $\pm$ 24.69	42.5
Diabetic + RA-3 (100 mg/kg)	76.66 $\pm$ 1.32	136.3 $\pm$ 4.03	301.1 $\pm$ 20.35	54.7
Diabetic + Extract (250 mg/kg)	83.03 $\pm$ 0.07	128.6 $\pm$ 2.16	312.7 $\pm$ 10.32	58.9
N. Control + Extract (250 mg/kg)	78.33 $\pm$ 0.55	129.5 $\pm$ 2.64	315.4 $\pm$ 28.68	58.9

Results are expressed as the mean $\pm$ SEM, n = 5.

The effect of the extract on prolactin and LDH of the diabetic rats after 28 days of treatment is shown in Table E2. A noticeable decreased prolactin levels in the animals treated crude extract when compared to diabetic rats was also observed. A significantly increased ($p \leq 0.01$) serum levels of HDL were also observed in the diabetic group treated with the extract when compared with the decreased levels of the lipoprotein (HDL) in the untreated diabetic group (Table E2).

Table E2: The effect of the extract on prolactin and LDH of the diabetic rats

Groups	Prolactin (ng/mL)	LDH
Normal control	8.76 ± 0.89	0.12 ± 0.02
Untreated diabetic ED	13.41 ± 0.14	0.26 ± 0.03
Diabetic ED + Cialis (5mg/kg)	14.04 ± 0.63	0.18 ± 0.07
Diabetic ED + RA-3 (100mg/kg)	14.6 ± 0.63	0.17 ± 0.05
Diabetic ED + extract (250mg/kg)	5.20 ± 0.27***	0.19 ± 0.07
N. Control +extract (250mg/kg)	6.30 ± 0.46***	0.21 ± 0.05

Results are expressed as the mean ± SEM, n = 5.

E2. Graphs

