

**Effect of methanolic extract of *Maytenus procumbens* and *Ozoroa paniculosa* on testicular dysfunction**

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**Effect of methanolic extract of *Maytenus procumbens* and *Ozoroa paniculosa* on testicular dysfunction**

**By**

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**A dissertation submitted in fulfilment of the requirement for the Degree of Masters of Science in the Department of Biochemistry and Microbiology, Faculty of Science and Agriculture, University of Zululand, KwaDlangezwa, South Africa**

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## **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 at the School of Chemistry, University of KwaZulu-Natal, Westville Campus between November 2015 and March 2016, under the supervision of Dr. R.A. Mosa and Prof. A.R. Opoku.**

**This study represents the original work by the author. 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 family (Cele family), who have been my pillar of support through all the challenging times.

## **ACKNOWLEDGEMENTS**

My deepest gratitude to our Lord Jesus Christ for His favour, grace and mercy upon me.

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

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My family for their support.

## ABSTRACT

Testicular dysfunction and related disorders continue to pose serious social and pathological health threats. Concoctions of *Maytenus procumbens* and *Ozoroa paniculosa* are commonly used by Zulu traditional healers to treat sexual dysfunction. This study aimed at investigating the effect of the methanolic roots extracts of *M. procumbens* and *O. paniculosa* against testicular dysfunction in male rats. Crude methanolic extracts of the plants were screened for their antioxidant activity, acetylcholinesterase and phosphodiesterase inhibitory activities *in vitro*. The cytotoxicity of the extracts was evaluated against normal embryonic kidney (HEK293) and cancer (HT29 and MCF7) cell lines. *n*-Butanol-induced testicular dysfunction model was used to investigate the effect of the extracts against testicular dysfunction in male rats. Rats were divided into different groups and the testicular dysfunction was induced by a single subcutaneous injection of *n-butanol* (25 mg/kg). A single dose of the extract (50 and 250 mg/kg body weight) daily for 28 days. The extracts showed to a varying degree of efficacy, concentration dependent antioxidant, acetylcholinesterase and phosphodiesterase inhibitory properties. A moderate (30.3-330.2 µg/ml) to weak (200.8-438.4 µg/ml) cytotoxicity level (LC<sub>50</sub>) was observed on cancer and normal cells, respectively. The animals treated with the extracts (50 and 250 mg/kg) showed improved testicular function which was manifested by an increased testicular weight, improved sexual performance, increased serum testosterone, a higher sperm count and better sperm quality when compared to the untreated group. The groups treated with the extracts (250 mg/kg) showed regular arrangement and increased number of spermatogonia in the seminiferous tubules comparable to the normal control group. An increase in the tissue antioxidant status (increased catalase activity and glutathione content) along with a decrease in malondialdehyde content in the extract treated groups was observed. A relatively higher serum creatinine level was also observed in the animals treated with the extracts. These results support the use of *M. procumbens* and *O. paniculosa* in folk medicine for the management of testicular dysfunction and its related disorders. However, the observed potential cytotoxicity and renal toxicity of the extracts indicate that medicinally, plants should be used with caution.

## LIST OF ABBREVIATIONS USED

AA	Ascorbic acid
ABTS	2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
AChE	Acetylcholinesterase
ALT	Alanine aminotransferase
ASChi	s-acetyl- thiocholine iodide
AST	Aspartate aminotransferase
BHA	Butylated hydroxyl-anisole
BHT	Butylated hydroxyl toluene
CA	Citric acid
CAT	Catalase
DPPH	2, 2-Diphenyl-1-picryl-hydrazyl
DTNB	5, 5'-dithiobis-(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetra-acetic acid
GSH	Glutathione
IC <sub>50</sub>	Inhibitory concentration at 50%
KZN	KwaZulu Natal
LC <sub>50</sub>	Lethal concentration at 50%
MDA	Malondialdehyde
NBT	Nitroblue tetrazolium
NO <sup>·</sup>	Nitric oxide radical
O <sub>2</sub> <sup>-·</sup>	Superoxide anion radical
<sup>·</sup> OH	Hydroxyl radical

PDE	Phosphodiesterase
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
UZ	University of Zululand
WHO	World Health Organization



## CONTRIBUTION TO KNOWLEDGE

### **Manuscript submitted for publication consideration**

**Cele N.D.**, Sangweni N.F., Penduka D., Lazarus G.G., Singh M., Mosa R.A., Opoku A.R. (2016). Antioxidant and cytotoxic effects of some Zulu medicinal plants used in treating erectile dysfunction. EXCLI Journal.

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# CHAPTER ONE

## 1.0 Introduction

The existence of the human race and offspring continuation is mostly dependent on reproduction. Since the testes are responsible for the production of male gametes and sex hormones, they play a crucial role in the male reproductive system. Any disorders that may develop in the testes can lead to various complications such as infertility, hormonal imbalances and sexual dysfunction (Nunes *et al.*, 2012). A decline in levels of testosterone the main hormonal mediator of men's sexual desire is responsible for most cases of male sexual dysfunction (Guay *et al.*, 2003). Sexual dysfunction (SD) is a serious social and medical problem that occurs in about 10-52% of men worldwide (Alin *et al.*, 2012). Erectile dysfunction appears to be the most common form of male sexual dysfunction that occurs in the sexual lives of millions of men globally (Singh *et al.*, 2012). Various risk factors such as ageing, diabetes mellitus, oxidative stress, poor diet; smoking and cardiovascular diseases contribute to increasing incidences of testicular dysfunction (Nunes and Webb 2012). Thus, restoration and/or improvement of testicular functions is important in ameliorating male sexual disorders.

Preventative measures of testicular dysfunction include regular exercise and healthy diet (food rich in proteins and micronutrients). The number of men's clinics is rapidly increasing and various pharmaceutical agents are currently used to manage testicular dysfunction and its related disorders. Despite their efficacy, these drugs are costly, not readily available and are associated with some side effects (Melynk and Marcone. 2011). Therefore, there is a need to search for alternative new forms of treatment that are preferably of natural origin. The medicinal plants-based treatment of various ailments including sexual dysfunction has long and extensive history (Patil *et al.*, 2014). There is currently a resurgent interest in the use of plant-derived therapeutic agents against various ailments including sexual disorders (Raja *et al.*, 2012). A large number of medicinal plants have been reported to be effective against testicular dysfunction and related sexual disorders (Palaniyappan *et al.*, 2013; Bashir *et al.*, 2015; Bhattacharya *et al.*, 2015). Zulu traditional healers also use various medicinal plants such as *Maytenus procumbens* and *Ozoroa paniculosa* to manage testicular dysfunctions. However, the



traditional use of these medicinal plants lacks scientific rationale and their toxicity levels are unknown. Screening of these plants, as used by the traditional healers against testicular dysfunction and its related disorders, is vital. This study is aimed at evaluating effect of methanolic extracts of *M. procumbens* and *O. paniculosa* on testicular dysfunction in male rats.

## **1.1 Outline of the thesis**

This dissertation consists of six chapters and appendices:

### **Chapter One**

Gives a brief background and motivation for the study.

### **Chapter Two**

Gives the literature review of the study. It also describes the aim and objectives of the study conducted.

### **Chapter Three**

Describes the materials and methods that were used to conduct all the experiments involved in the study.

### **Chapter Four**

This chapter presents results obtained in the study

### **Chapter Five**

Discussion of the results obtained in the study.

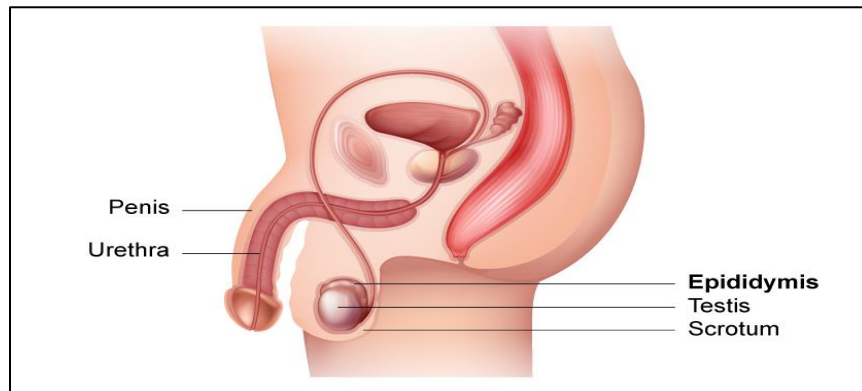
### **Chapter Six**

Comprises the conclusions drawn from the results obtained in the study as well as suggestions for further studies.

## CHAPTER TWO

### 2.0 Literature review

Reproduction is the integral part of human race existence and offspring continuation. The male reproductive system comprises of the penis, testes and epididymis (Figure 2.1), and their location is important for maintaining the testicular temperature (approximately 1.5-2.5 °C below body temperature), required for sperm production ((DUHS).



**Figure 2.1:** Male reproductive system.

The testes which are oval-shaped organs are responsible for the production of male gametes and sex hormones (DUHS). While the produced sperms are temporarily stored and mature in the epididymis, the penis serves as an external intromittent organ as well as urinary duct (DUHS). Inside each testis there is a dense network of fine-diameter tubes known as seminiferous tubules (DUHS). Sertoli cells form the walls of a seminiferous tubule (DUHS). Sertoli cells which the walls of the seminiferous tubules, nourish, support, and protect developing germ cells to form spermatozoa (immature sperm). Testosterone is key to all male reproductive organs which include fluid secretion, protein synthesis, cell growth and division (DUHS). Androgens are responsible for male sexual response and they also stimulate male secondary sex characteristics such as facial hair growth, skeletal muscle development, deepening of the voice, enlargement of the penis, testes and scrotum (Berk, 2012). When testicular functions are impaired, for any reason, serious social and medical conditions occur. Incidences of testicular dysfunction lead to complications such as low sperm count, altered sperm morphology, hypogonadism and erectile dysfunction (Alin *et al.*, 2012; Singh *et al.*, 2012).

## 2.1 Testicular function

In adult male, the testes are mainly responsible for testosterone synthesis and sperm production (Amory and Bremner, 2001). Testicular functions are under strong hormonal control. Gonadotropin releasing hormone (GnRH) from the anterior pituitary gland stimulates the synthesis and secretion of follicle stimulating hormone (FSH) and luteinising hormone (LH). FSH and LH in turn activate receptors on Sertoli cells, and Leydig cells respectively, which stimulate spermatogenesis and testosterone synthesis (Amory and Bremner, 2001). About 95% of testosterone in males is synthesized in the testes while only about 5% is produced by the adrenal cortex as the derivative of androstenedione and dehydroepiandrosterone (DHEA) (Shalet, 2009).

When either one or both primary functions of the testes is impaired, it results in a condition known as testicular dysfunction (Guay *et al.*, 2003). Various environmental factors either external or internal are known to compromise testicular functions. These factors include exposure to toxic substances, alcohol abuse, electronic radiation, smoking and lifestyle etc. (Cemile and Cigdem, 2016). Other risk factors such as diabetes mellitus, oxidative stress, poor diet, cardiovascular diseases and also aging contribute to incidences of testicular dysfunction (Nunes and Webb, 2012). Testicular dysfunction may lead to a decline in fertility and androgen (testosterone) deficiency (Singh *et al.*, 2012). Testosterone deficiency has debilitating effects in the quality of life of men. These include hypogonadism, infertility, low libido, osteoporosis, poor muscle strength and erectile dysfunction (Karagiannis and Harsoulis, 2005; Singh *et al.*, 2012; Ali *et al.*, 2012). Erectile dysfunction, defined as the repeated inability to obtain and maintain penile erection for naturally satisfying sexual intercourse (Singh *et al.*, 2012), is one of the most common complications of testicular dysfunction in men. The harmful effects of alcohol on the testis have been ascribed to a change in mitochondrial structure leading to irregularity in the diameter of the seminiferous tubules which results in low sperm count, altered morphology and motility and reduced testosterone level (Dosumu *et al.*, 2014; Oremosu and Akang, 2015). Ethanol induced testicular dysfunction rats have been reported to have an adverse effect on the sperm maturation due to a decrease in testosterone levels and epididymal secretory products (Bhargavan *et al.*, 2015).

### **2.1.1 Spermatogenesis**

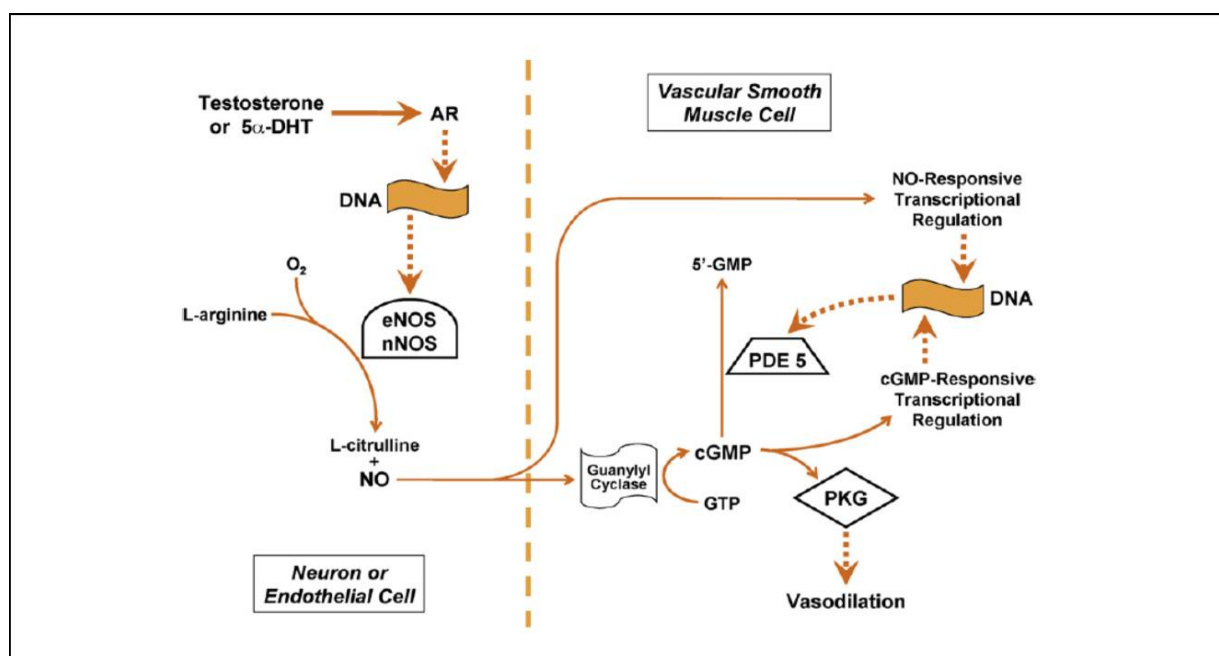
Normal sperm development is essential in enabling reproductive capacity (Govin *et al.*, 2004). Spermatozoa develop fertility and motility in the lumen of epididymis (Sati and Huszar, 2015). The epididymal lumen is a complex microenvironment in which various post-translational modifications of the sperm proteins undergo to produce a mature spermatozoa (Sati and Huszar, 2015). Studies have shown that chemicals such as alcohol and heavy metals interact with chromatin protamines preventing normal sperm chromatin condensation, which induces changes in the sperm genome and affect male fertility (Lee *et al.*, 2015).

Sperm cell evaluation is mostly employed to evaluate the biological potential of a sperm cell to access and fertilise an ovary (Parra-Forero *et al.*, 2015). Normal sperm cell is characterised by a single long tail and an oval head. Sperm morphology, number and motility are all crucial factors for male fertility and any alterations such as poor sperm quality, low sperm count or both, result to infertility (Kourouma *et al.*, 2015). Pharmaceutical Diazepam intake showed interference with spermatogenic processes in the seminiferous tubule, epididymal function and testosterone level that resulted in an increased abnormal sperm morphology and low sperm count in male rats (Mohammed and Zainab, 2015). Recent studies have shown that damage to spermatozoa by reactive oxygen species (ROS) affect sperm function thus perpetuating male infertility (Kourouma *et al.*, 2015).

### **2.1.2 Testosterone**

Testosterone, a steroid hormone derived from cholesterol, is very important for the development of male secondary sexual characteristics and production of sperms (Chauhan *et al.*, 2014). In males, large amounts of testosterone are secreted by testes while, to a lesser extent by the adrenal cortex in females (Mehta and Josephs, 1998). Approximately 50% of the total testosterone in the blood is bound to albumin (carrier protein), 45% to sex hormone binding-globulin (SHBG) and 5% is unbound, and this “free” testosterone is biologically active (Lee *et al.*, 2014). Albumin bound testosterone is biologically inactive whereas SHBG bound testosterone is biologically active. Thus, high levels of SHBG reduce the availability of free testosterone (Lee *et al.*, 2014).

In addition to sperm production, testosterone is essential in the maintenance of sexual desire and has an important role in regulating penile erection capacity in males (Julia *et al.*, 2013). In healthy men, testosterone has been reported to increase libido and inflexibility of nocturnal penile tumescence that results in more fixed spontaneous penile erection for longer periods (Orshal and Khalil, 2003). Like all hormones, testosterone is secreted by a gland (testis), travels through the blood stream and it is metabolised, in many tissues, by 5 – alpha- reductase to form 5-alpha-dihydrotestosterone (5 $\alpha$ -DHT). The 5 $\alpha$ -DHT then binds to the androgen receptor (AR) of the cell where it stimulates synthesis of nitric oxide (NO), an endothelial relaxing factor, from L-arginine (Figure 2.2). The NO is an important molecule that acts via cGMP-activated protein kinase cascade to relax the musculature wall of blood vessels.



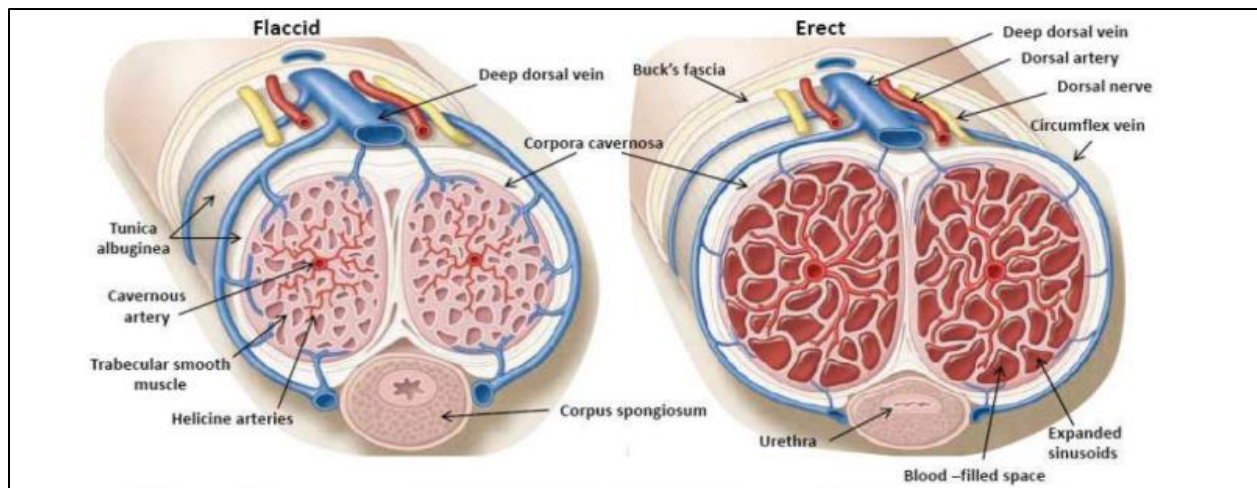
**Figure 2.2:** Mechanism of testosterone in NO synthesis. Testosterone binds to androgen receptor (AR) leading to the activation of nitric oxide synthase (NOS) that catalyses the synthesis of NO from L-arginine. NO diffuses into the vascular smooth muscle cell where it induces vasodilation via cGMP-activated protein kinase cascade (Source: Orshal and Khalil, 2003).

Deficiency of steroid hormones such as testosterone and dihydroxytestosterone may results in male infertility, and a variety of diseases and disorders such as hypogonadism, osteoporosis, cardiovascular disease and metabolic syndrome (Greco *et al.*, 2006; Doshi

*et al.*, 2012). High levels of testosterone have also been documented to have the neuroprotective effect by enhancing antioxidant (SOD and CAT) activities in Caucasians aged men (Cunningham *et al.*, 2014). Testosterone deficiency showed a trend towards reduced eNOS phosphorylation, while the administration of testosterone at different doses increased eNOS phosphorylation above castrated levels (Harris and Burnett, 2013).

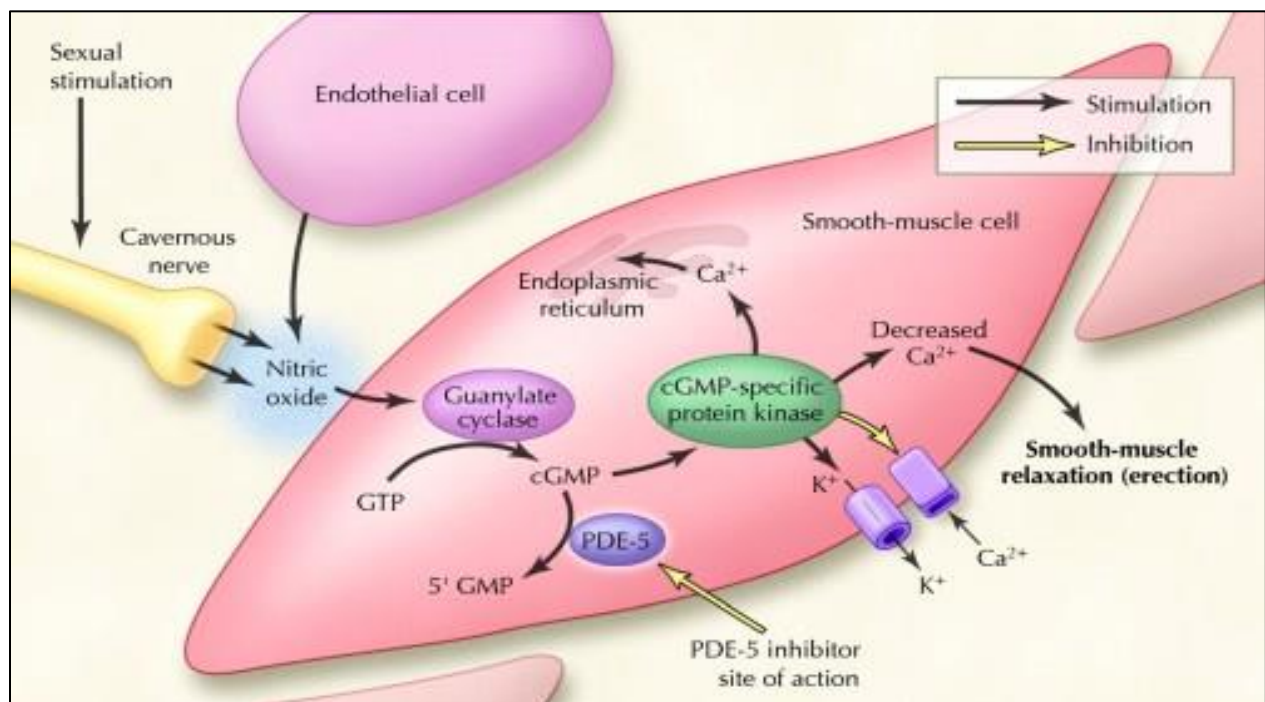
### 2.1.3 Role of nitric oxide (NO) in vasorelaxation and penile erection

NO is considered one of the main endothelial-relaxing factors that play a crucial role in the penile erection process (Nunes *et al.*, 2012). The synthesis of NO from L-arginine, a reaction catalysed by nitric oxide synthase (NOS), occurs in most tissues. The constitutive form of NOS has three isoforms, inducible NOS (iNOS), endothelial (eNOS) and neuronal NOS (nNOS) (Nunes and Webb, 2012). The eNOS and nNOS function is coupled to the  $\text{Ca}^{2+}$  calmodulin complex and the two are principal isoforms key to the induction of penile erection, whereas the iNOS functions independent on the  $\text{Ca}^{2+}$  calmodulin complex (Bivalacqua *et al.*, 2000). NO produced from non-adrenergic, non-cholinergic (NANC) nerves have been reported to be essential for the initiation of cavernosal smooth muscle relaxation (Figure 2.3) which allows more blood to flow into the penile tissue and subsequent erection (Nunes and Webb, 2012).



**Figure 2.3:** The flow of blood in the penile tissue. Helicine arteries supply blood flow to the penile tissue during vasodilation and cavernosal smooth muscle relaxation, causing the engorgement of blood in the corpora and results in erection (Source: Van Driel, 2015).

Penile erection is a complex neurovascular response that involves the integration of central nervous system activity and the release of vasodilator factors from nerves supplying the penis and the endothelium of the penile arteries (Lasker *et al.*, 2013). This process is initiated by auditory, visual, olfactory signals and local stimuli of the penis (Kataria *et al.*, 2012). Upon sexual stimulation, the release of NO targets the heme moiety of soluble guanylate cyclase (sGC), an enzyme that stimulates generation of second messenger cGMP from GTP (Figure 2.4). The actions of cGMP are exerted through cGMP-gated cation channels and cGMP-dependent protein kinases (PKGs). Activation of cGMP induces a cascade of reactions resulting to smooth muscle relaxation, increased vascular blood flow and eventually penile erection (Young *et al.*, 2003; Kim and Park, 2011).



**Figure 2.4:** Role of nitric oxide on erectile function. The interaction of NO with sGC results in the conversion of GTP into cGMP which through series of reaction relaxation of smooth muscle around the penile tissue (Source: Nunes and Webb, 2012).

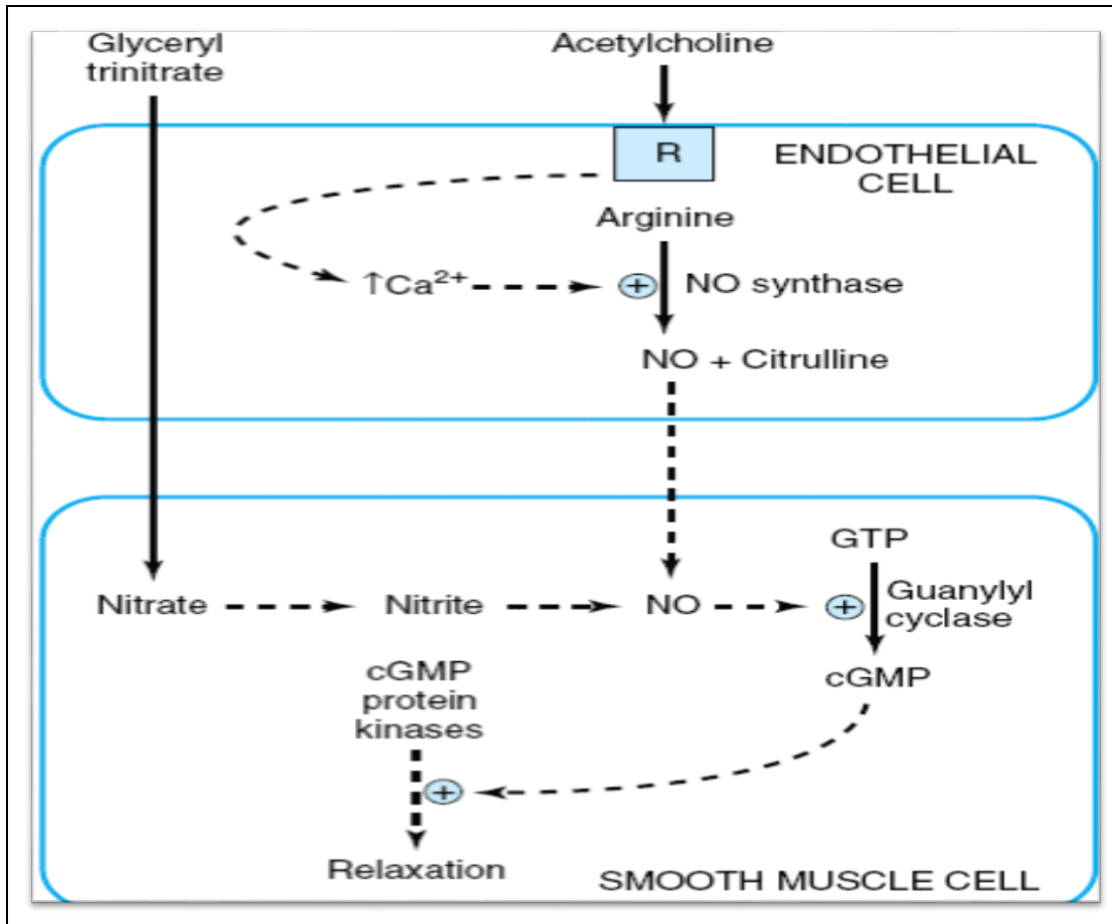
The cGMP signalling is also vital to other physiological events such as cardiac myocyte function (Tsai and Kass, 2009). Phosphodiesterase (PDE) isoenzymes (PDE1 through

PDE11) catalyse conversion of cyclic nucleotides (active cAMP or cGMP) to their non-cyclic forms (inactive). Inactivation of the intracellular mediators of signal transduction is important in cellular functions (Ghosh *et al.*, 2009). In the penile erection process, PDE-5 isoform hydrolyses cGMP to the inactive 5' GMP (Figure 2.4) and thus, inhibits smooth muscle relaxation and subsequent penile erection. As a result, PDE-5 is a prominent target in the management of erectile dysfunction. Therefore, reduced NO activity leads to endothelial dysfunction, a systemic event that precedes the development of various cardiovascular diseases and erectile dysfunction.

#### **2.1.3.1 Role of acetylcholine on penile erection**

Skeletal muscles around penile tissue (for firm erection), the cavernous smooth musculature, the arteriolar and arterial smooth muscles walls, all play a crucial role in the penile erection process. The smooth muscles are tonically contracted in the flaccid state, while in the erect state they are relaxed allowing in flow of blood to the penile tissue (Figure 2.3). Acetylcholine (ACh) is a neurotransmitter that is used at the neuromuscular junction to stimulate muscle contraction. The effects of ACh are regulated by two classes of receptors; the nicotinic receptor (ion channels) and muscarinic (M) receptors (Wymke *et al.*, 2013). When ACh is released by cholinergic nerves, it binds to endothelial cells receptor, thus triggering intracellular  $\text{Ca}^{2+}$  release via the inositol triphosphate (IP3) pathway. The increase in intracellular  $\text{Ca}^{2+}$  levels has dual effect: (1) the stimulation of skeletal muscle contraction and (2) the activation of NOS that catalyses the synthesis of NO from arginine. The NO subsequently diffuses into adjacent smooth muscle cell, where it initiates the cascade of reactions (Figure 2.5) leading to stimulation of cGMP-dependent protein kinases, and subsequent smooth muscle relaxation (Andersson, 2011).





**Figure 2.5:** Mechanism of acetylcholine. When ACh is released by cholinergic nerves, it binds to the endothelial cells receptor, thus triggering an intracellular  $\text{Ca}^{2+}$  release which result in the activation of NOS and production of NO. The interaction of NO with sGC results in the convection of GTP into cGMP and initiates the stimulation of cGMP-protein kinases, and subsequent smooth muscle relaxation (Source: Andersson, 2011).

The physiological levels of ACh are controlled by the activity of acetylcholinesterase (AChE), an enzyme that catalyses the breakdown of ACh into choline and acetate, subsequently preventing over contraction of the skeletal muscles and vasorelaxation of smooth muscles (Shah *et al.*, 2009). It is important to maintain the activity of this enzyme within physiological levels since its hyperactivity is associated with numerous pathophysiological events (Mukherjee *et al.*, 2007).

Acetylcholinesterase inhibitors (AChEI) prevent the AChE from breaking down ACh, thereby increasing both the level and stimulus duration of the neurotransmitter (ACh).

This results in increased stimulating effects with respect to smooth muscle relaxation, sexual arousal and sensation (Baumhakel *et al.*, 2008). Increased activities of AChE have been demonstrated to inhibit production of NO (Maheswari *et al.*, 2014). Donepezil, galantamine and rivastigmine are currently formulated as AChE inhibitors. However, adverse effects associated with these drugs have limited their use. Several studies have developed the use of AChE inhibitors from plants to reverse abnormally elevated AChE activity in patients with Alzheimer's disease (AD), thereby repairing ACh levels and improving AD and related complications including penile erection (Kim *et al.*, 2016; Olsen *et al.*, 2016).

### **2.1.3.2 Erectile dysfunction**

Penile erection is dependent on an increased flow of blood in the pudendal arteries to the penile tissue. Based on the physiological events leading to penile erection (Figure 2.3), erectile dysfunction (ED) is considered predominantly a disease of vascular origin (Bivalacqua *et al.*, 2003). Penile erectile dysfunction is defined as the repeated inability to obtain and maintain penile erection for naturally satisfying sexual intercourse (Singh *et al.*, 2012). Approximately 11.5% of men in Africa suffer from ED (Singh *et al.*, 2012). Complications of ED in men include misery, silent suffering, disturbed interpersonal relationship and even divorce (Singh *et al.*, 2012). Reported incidences of ED have dramatically increased in men with diabetes mellitus, hypercholesterolemia and cardiovascular disease (Melynk and Marcone, 2011). Several other factors such as abusive alcohol intake, neurological diseases, oxidative stress, poor diet (diet low in proteins, minerals and antioxidants), and old age contribute to the development of ED (Nunes and Webb, 2012; Sati and Huszar, 2015).

Studies have shown that PDE-5 plays a key role in the NO/cGMP signal transduction pathway and it functions to restrain the smooth muscle cells relaxation and penile erection process (Kim and Park, 2011). Thus, PDE-5 has been a target in the treatment of ED. PDE-5 (Figure 2.4) hydrolyses cGMP (active form) to GMP (inactive form) which consequently terminates the physiological actions of cGMP. PDE-5 inhibitors such as Sildenafil, Tadalafil and Udenafil are currently used as the first-line treatment for ED (Schellack and Agoro, 2014).

#### 2.1.4. Oxidative stress

Free radicals are endogenously produced as by-products of normal biochemical reactions and it is thus vital to maintain their concentrations within physiological levels (Gobbo *et al.*, 2015). Over production of these reactive oxygen species (ROS) and/or nitrogen reactive species (NRS) results in oxidative stress (Birben *et al.*, 2012). Oxidative stress (OS) is implicated as one of the major underlying pathophysiological causes of testicular dysfunction and its related complications (Gobbo *et al.*, 2015; Oboh *et al.*, 2015). Studies have reported that testes are vulnerable to oxidative stress due to higher level of polyunsaturated fatty acids (PUFA) and presence of potential ROS generating system (Saleh and Agarwal, 2002; Aitken and Roman, 2008).

It has been reported that an increased ROS level in seminal plasma may cause morphological deformity in sperm cells (Singh *et al.*, 2015). The interaction between nitric oxide (NO) and ROS is considered one of the key mechanisms implicated in the pathophysiological events resulting in ED (Jones *et al.*, 2002; Stavniichuk *et al.*, 2014). The reaction of NO with superoxide ions produces a cytotoxic species, peroxynitrite. The cytotoxic effect of peroxynitrite is due when peroxynitrite react with tyrosyl protein-residues, which inhibits the activity of superoxide dismutase (SOD) and leads to a reduced removal of superoxide ions (Zou *et al.*, 1997). An increase in superoxide anions and peroxynitrite levels have been reported to increase incidence of endothelium apoptosis and consequent endothelial dysfunction (Agarwal *et al.*, 2013).

The increased production of free radicals has been proven to attack germ cells within the seminiferous tubules leading to extensive necrosis and the disruption of spermatogenesis (Dosumu *et al.*, 2012). Changes in seminiferous tubules have been shown to result in low sperm count due to suppressed testosterone synthesis on diazepam induced oxidative stress (Taher and Anber, 2015). The destructive effect of free radicals is mediated through the oxidation of lipids, proteins and DNA (Dosumu *et al.*, 2012). A major product of lipid peroxidation, malondialdehyde (MDA), is usually measured as an indicator of oxidative stress *in vitro*. Several studies support the benefits of antioxidants in protecting the testis against oxidative damage (Bhargavan *et al.*, 2015; Hoseinpouran *et al.*, 2015). Fatani *et al.* (2015) showed the beneficial effects of using lutein to stimulate enzymatic

activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in diabetes-induced testicular damage. However, the testes have developed a sophisticated array of anti-oxidant systems comprising of both enzymatic and non-enzymatic constituents. These antioxidants (SOD, CAT and GPx) play a vital role in protecting the testes against oxidative stress (Khaki, 2015). The significant roles of SOD, CAT and GPx in the testes have demonstrated to maintain the functional integration of the cell membrane spermatogenesis, sperm morphology and motility (Olasantan *et al.*, 2015).

Exogenous antioxidants such as vitamin E, vitamin C, flavonoids, carotenoids etc. play a crucial role in the maintenance of healthy male reproductive organs and the survival of spermatids (Huy *et al.*, 2008). Studies have shown that vitamin E enhances the development of testes by increasing epididymal weight, epididymis ductus, seminiferous diameters and interstitial cell density which improve spermatogenesis (Cheah and Yang, 2011). This suggests that medication with high antioxidant content should be considered as the first line of treatment for testicular dysfunction, thus it may protect testes against oxidative stress during spermatogenesis and steroidogenesis.

### **2.1.5 Management of testicular dysfunction**

There is a high rate of testicular dysfunctions worldwide, however, most men remain undiagnosed. In early treatment, nutritional therapy (a diet rich in protein and micronutrients) and regular exercise have proven beneficial in ameliorating testicular dysfunction and its complications. Testicular dysfunction cannot be attained by only nutritional therapy, thus pharmacology intervention is required. Oral pharmaceutical agents such as levothroid, clomiphene citrate and Sildenafil or Tadalafil are used to treat testicular dysfunctions. Each of these pharmaceutical agents has its specific function, for instance Sildenafil inhibits the action of PDE-5 and are often called PDE-5 inhibitors (Tsai and Kass, 2009). In the inhibition of the cellular enzyme, PDE-5 lowers the breakdown of cGMP (Abdelwahab *et al.*, 2012). However, these pharmaceutical agents are associated with side effects such as headache, pounding heartbeat, insomnia, fever, appetite change etc. (Melynk and Marcone, 2011).

Despite an increasing number of men's health clinics worldwide and the use of pharmaceutical agents, a substantial number of men particularly living in rural areas continue to consult traditional healers to improve their sexual functioning (Drewes *et al.*, 2002; Ojewole *et al.*, 2006). Treatment options offered by traditional healers are based on medicinal plants use. The use of these plants by the traditional healers often lacks scientific validation, thus their proper scientific documentation can also expose the risks associated with their medicinal uses.

## **2.2 Medicinal plants**

Medicinal plants have been used throughout human history for the treatment of various diseases including sexual dysfunctions. Medicinal plant-based traditional healing is still widely used for the treatment of diseases in developing countries where the cost of conventional medicines is a burden to the population (Rates, 2001). Medicinal properties of the plants are attributed to their phytochemical constituents. The basic common phytochemicals include tannins, alkaloids, saponins, steroids, flavonoids and terpenoids. These plant components have no nutritive value to the plant, but they are responsible for protecting the plant against biotic and abiotic stressors (Doughari, 2012).

There is growing scientific evidence on the significant potential use of medicinal plants as crude extracts or their pure isolated compounds against testicular dysfunction and its related complications (Guillaume *et al.*, 2010; Raja *et al.*, 2012; Patil *et al.*, 2014). Studies have demonstrated the significant impact of phytochemical constituents such as saponins, flavonoids, alkaloids, anthraquinone, sterols, and tannins in the prevention and treatment of diabetes-induced endocrine and testicular disorders (Rahman, 2007; Palaniyappan *et al.*, 2013; Zade *et al.*, 2013; Ogunmodede *et al.*, 2015). Nurudeen and Ajiboye (2012) documented the effect of aqueous roots extract from *Lecaniodiscus cupanioides* to enhance steroidogenesis and increase activity of gamma-transferase on paroxetine induced-testicular dysfunction in the male rats experimented on. Similar findings were obtained by Toyin and Olaide. (2012) who reported on the potential effect of aqueous root extract of *Cnestis ferruginea* to improve the synthesis of nitric oxide synthase and testosterone on paroxetine induced sexual dysfunction in male rats. The ethanolic extract from Date Palm Pollen (DPP) has been recorded to have a stimulatory

effect on steroidogenic enzymes as well as to improve the production of sperms on thyroid disorder-induced testicular dysfunction in rats. The apparent steroidogenic and spermatogenic effects of the DPP ethanolic extract has been associated with its phenolic content and trace elements (El-Kashlan *et al.*, 2015). Ogunmodede *et al.* (2015) demonstrate the antioxidant potential from the ethylic leaf extract of *Dracaena arborea* to possess an anti-diabetic property which improved diabetes-induced hyperglycemia and oxidative stress-mediated testicular disorders in alloxan-induced diabetic rats.

Literature supports the stimulatory effect of *Tribulus terrestris* on Sertoli and Leydig cells to enhance testosterone secretion and sperm production which improves testicular function (Bashir *et al.*, 2015; Bhattacharya *et al.*, 2015). Adeiza and Minka. (2011) described a toxic effect of methanol extract of *Ximenia americana* to cause defects in sperm morphology, decreased sperm count and testicular weight loss in male rats. Patil *et al.* (2014) reported that the methanolic root and leaf extracts of *Cocculus hirsutus* enhances sexual performance and also increases vigour and vitality. Various triterpenes such as protodioscin from *Tribulus terrestris*, icariin from Horney goat weed and Crocin from *Crocus sativus* have been reported to enhance sexual performance (Melnik and Marcone, 2011). Even though plants (and plants' products) that are used to improve testicular dysfunction could be exerting their therapeutic effects through various mechanisms. The beneficial properties of various medicinal plants are either due to its stimulatory and/or inhibitory effects or due to its richness in antioxidants (Khlif *et al.*, 2016). Most plants are rich in antioxidants that could contribute to their effectiveness (Cemile and Cigdem, 2016). For example, the protective effects of *Artocarpus altilis*' extract against cadmium-induced testicular damage, altered sperm parameters and hormonal imbalance have been linked to its antioxidant activity (Adaramoye and Akanni, 2015; El-Shahat *et al.*, 2009; Moniem *et al.*, 2010; Castillo *et al.*, 2015).

Traditional healers continue to use various medicinal plants to manage sexual dysfunctions. An ethno-pharmacological survey of traditional healers in northern Kwa-Zulu Natal (South Africa) indicates that powdered roots of *Maytenus procumbens* (Figure 2.6) and *Ozoroa paniculosa* (Figure 2.7) are often used by Zulu traditional healers to manage sexual dysfunctions including ED. Different concoctions prepared from these

plants at the dose of 250 ml are orally administered to men in order to improve their sexual performance (Personal Communication with Tembe. 2015).

### **2.2.1 *Maytenus procumbens* (L.F.) Loes**

*Maytenus procumbens* (L.F.) Loes, commonly known as Dune Koko (English) and Umakhweni (Zulu), belongs to the family Celastraceae (Momtaz *et al.*, 2012). *M. procumbens* is a densely bushy plant with drooping branches that sometimes reach more than six meters in height. The plant is native to South Africa, predominantly in the KwaZulu Natal Province. It is also found in South America, North Africa and East Asia (Momtaz *et al.*, 2012). The Zulu traditional healers often use the powdered roots of *M. procumbens* to treat erectile dysfunction and infertility in males (Personal Communication). Leaves from *M. procumbens* possess anti-cancer activity (Momtaz *et al.*, 2012).



**Figure 2.6:** Picture of *Maytenus procumbens* (L.F.) Loes.

### **2.2.2 *Ozoroa paniculosa***

*Ozoroa paniculosa* (Sond.) R & A Fernandes, commonly known as resin tree (English) and Umfico (Zulu), is from the Anacardiaceae family. *O. paniculosa* is an evergreen, semi deciduous, small to medium sized single stemmed tree (up to nine meters in height) with a round crown (Hutchings *et al.*, 1996). The powdered roots of *O. paniculosa* are

traditionally used to treat erectile dysfunction and increase sperm concentration (Personal Communication). Hutchings *et al.* (1996) reported that the powdered stem bark of *O. paniculosa* is used by Zulu traditional healers to treat acute inflammatory condition of the chest. The stem bark of *O. paniculosa* has also been reported to have inhibitory effects against Gram-negative bacteria (Motlhanka, 2008). Leaves and the roots of *O. paniculosa* possess antioxidant activity and lipoxygenase inhibitory effects (Motlhanka and Mathapa, 2012; Ahmed *et al.*, 2013).



**Figure 2.7:** Picture of *Ozoroa paniculosa*

### **2.2.3 Cytotoxicity of medicinal plants extracts and/or their derivatives**

Despite the immense popularity (80% of people in developing countries) of traditional healing (Reddy *et al.*, 2015), the perception of most people is that it is heathen and that there are no documentations of dosage and possible side effects. It is also that traditional medicinal practices have no reproducible methods of preparation and preservation (Rates, 2001). The bioactivities of plants are attributed to the phyto-chemical content of plants (Rates, 2001). Nonetheless, some secondary metabolites of plants are reportedly toxic: Toxicities such as allergic reactions, irritation of the gastrointestinal tract, destruction of red blood cells and carcinogenicity have been reported to be associated with the use of medicinal plants (Nondo *et al.*, 2015; Chew *et al.*, 2014; Sajjadi *et al.*,



2015). For example, studies indicate that *Aristolochia* Spp. contain aristolochic acid that cause renal failure. *Drimys sanguinea* and *Bowiea volubilis* which are traditionally used for headache, infertility and bladder problems contain cardio-toxic bufadienolides (Debelle *et al.*, 2008; Van der Bijl and Van der Bijl, 2012). The evaluation of efficacy, proximate mechanism of action and toxicity of medicinal plants is of paramount importance as it could reveal some likely risks to be associated with use of these plants.

Various assays are used for the research of potential toxicity of herbal extracts. These are usually based on different biological models such as the brine shrimp lethality assay (Meyer *et al.*, 1982) and the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Mosmann, 1983). Cytotoxicity testing using cell cultures is a rapid, sensitive, standardized, and inexpensive means to determine whether a plant material contains significant quantities of biologically harmful extractable components (Miret *et al.*, 2006).

The Brine Shrimp bioassay is a convenient system for monitoring the biological activities of various plant species. More commonly known as sea monkeys, they are crustaceans that live in saline environments. One indicator of the toxicity of a substance is lethal dose or concentration ( $LD_{50}$ ) of a substance that kills 50% of the test organisms (Meyer *et al.*, 1982). The toxicity potential of a Brine Shrimp bioassay is calculated by counting the survived nauplii after 24 hours of exposure to the tested sample. The toxicity assessment of extracts or isolated compounds is classified in the following order: extracts or active compound with  $LC_{50}$  above 1000  $\mu\text{g/ml}$  are non-toxic,  $LD_{50}$  of 500 - 1000  $\mu\text{g/ml}$  are low toxic,  $LD_{50}$  of 100 - 500  $\mu\text{g/ml}$  are medium toxic and extracts with  $LD_{50}$  of 0 - 100  $\mu\text{g/ml}$  are highly toxic (Clarkson *et al.*, 2004). The Brine Shrimp assay has number of advantages including experimental simplicity, ease of handling, short exposure time, lack of continuous culturing and lower costs. The main disadvantage is that one cannot generalise the results to toxicity in mammals (McGaw and Eloff, 2005). Although, this method does not provide any adequate information regarding the mechanism of toxic action, it is a very useful method for the assessment of the toxic potential of various plant extracts and isolated compounds.

The MTT assay is based on the ability of a mitochondrial dehydrogenase from viable cells to cleave the tetrazolium rings of the MTT (pale yellow). The reaction produces dark blue formazan crystals which are largely impermeable to cell membranes. As a result of their impermeability, the formazan crystals accumulate within healthy cells. Exposure of the cells to a detergent leads to liberation of the solubilized crystals. The MTT assay is rapid, sensitive, versatile, quantitative and highly reproducible. It is also adaptable to a large-scale screening relevant for most cells. Reduction of MTT correlates to indices of cellular protein and viable cell number (Mosmann, 1983; Miret *et al.*, 2006)

Several models such as cell based cytotoxicity, toxicokinetics methods, toxicogenomic screening methods, etc. are used in screening toxicity of plants, where the cell-based cytotoxicity method is the most commonly employed method (Mosmann, 1983). In this model, the toxicity of the plant is predicted using cultured cells which may be normal or transformed cells.

Several number of scientists have worked with cell lines using the MTT method to determine toxicity of plants (Senthilraja and Kathiresan, 2015; Chan *et al.*, 2015; Sharma *et al.*, 2016; Mansoor *et al.*, 2016). The ability of plant extracts to inhibit cellular growth and viability, whereas the ability of the cells to survive a toxic insult is observed as an indication of its toxicity (Ifeoma and Oluwakanyinsola, 2013; Asha *et al.*, 2015). Toxicity of crude extracts or pure compound is categorised into strongly toxic ( $LC_{50} = 10 - 20 \mu\text{g/ml}$ ), moderate toxicity ( $LC_{50} = 20 - 100 \mu\text{g/ml}$ ) or weakly toxic ( $LC_{50} > 100 \mu\text{g/ml}$ ) (Magadula, 2014).

## **2.3 Scope of the work**

### **2.3.1 Aim**

The aim of this study is to investigate the effect of methanolic root extracts of *Maytenus procumbens* and *Ozoroa paniculosa* on testicular dysfunction in rats.

### **2.3.2 Objectives**

- i. To collect and extract the plants material with methanol
- ii. To evaluate the phytochemical composition of the plants material.
- iii. To determine the cytotoxicity and antioxidant activity of the extracts *in vitro*.
- iv. To evaluate the phosphodiesterase and acetylcholinesterase inhibitory activity of the extracts.
- v. To investigate the effect of the extracts on testicular dysfunction in rats.

## CHAPTER THREE

### 3.0 Materials and Methodology

The chapter lists the major reagents and equipment that were used in the study. Description of the methods used to prepare plant extracts and carry-out biological assays is also given. Details of the preparation of some reagents and some methods are supplied in Appendices A and B, respectively.

### 3.1 Materials

#### 3.1.1 Chemical reagents

The following chemicals were purchased from Sigma-Aldrich Co. Ltd (Steinheim, Germany) - 1.1-Diphenyl-2-picryl hydrazyl (DPPH), Dragendroff's reagent, 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS), DNA, Butylated hydroxyl toluene (BHT), 4,4'-[3-(2-pyridinyl)-1,2,4-triazine-5,6-diy] bisbenzene sulphonate (ferrozine), Butylated hydroxyl-anisole (BHA), caffeine, Ascorbic acid (AA), Gallic acid, Tris-HCl, Sodium nitroprusside, Ethylenediaminetetra-acetic acid (EDTA), Thiobarbituric acid (TBA), Folin-Ciocalteu Phenol reagent, Viagra, Ferrous ammonium sulfate, Xanthine oxidase, 5,5'-dithiobis-(2-nitrobenzoic acid)(DTNB), Xanthine, Bovine Serum Albumin (BSA), Ammonium molybdate, Hydrogen peroxide, s-acetyl-thiocholineiodide (ASChI), methanol, *n*-butanol, tacrine, and Nitro blue tetrazolium salt.

### 3.2 Methodology

#### 3.2.1 Collection and extraction of plant material

Fresh roots of *Maytenus procumbens* and *Ozoroa paniculosa* were collected from Ndumo and Mhlabayalingana, respectively, in the KwaZulu-Natal province of SA. The plants were identified and confirmed by botanists, Dr. N.R. Ntuli and Dr. T.H.C. Mostert, at the Botany Department, University of Zululand. Voucher specimens were prepared and deposited at the University herbarium. The plant materials were cleaned of dirt, washed with water, chopped, air dried and ground into powder (Grinding machine IKA-Werke, Polychem suppliers). The powdered plant materials were separately extracted (1:5 w/v) with methanol for 48 hours at room temperature on an orbital shaker (150 rpm, Labcon Polychem suppliers). The extracts were filtered through Whatman No. 1 filter paper and concentrated at 40°C under reduced pressure using a rotary evaporator (Heidolph). The

obtained crude extracts were stored in sterile brown bottles to avert light interference until use.

### **3.2.2 Phytochemical screening**

Qualitative phytochemical analysis of the plants material was performed following the standard procedures described by Odebiyi *et al.* (1978). The basic common phytochemicals such as tannins, alkaloids, saponins, steroids, flavonoids and terpenoids were screened for.

#### **3.2.2.1 Total phenolic content**

The phenolic content of the crude extracts was colourimetrically quantified following the Folin-Ciocalteu reagent method (Kujala *et al.*, 2000). Crude extract (0.2 mg/ml) was well mixed with 1.5 ml of Folin-Ciocalteu reagent and 7.5% v/w sodium carbonate (1.2 ml). Following 30 min incubation in the dark, absorbance of blue coloured mixture was read at 765 nm using plate reader (Synergy HT, Biotek Instrument supplies). Absorbance was measured at 765 nm. Gallic acid was used as standard and the total phenolic content of the plant extract was calculated as gallic acid equivalent from a calibration curve of gallic acid and it was expressed as mg/g dry plant material.

#### **3.2.2.2 Total flavonoid content**

The method described by Ordoñez *et al.* (2006) was followed to colourimetrically determine the flavonoid content of the extracts. Crude extract (0.2 mg/ml) and 0.5 ml of alcoholic aluminium chloride (2%) w/v were mixed together thoroughly. After incubation (at 25°C, 1 h), Biotek plate reader (Synergy HT, Biotek Instrument supplies) was used to read absorbance of the yellow coloured mixture at 420 nm against the blank containing alcoholic aluminium chloride. Quercetin was used as a standard and the total flavonoid content of the extract was calculated from a calibration curve of quercetin and it was expressed as mg/g dry plant material.

### **3.3 *In vitro* antioxidant assays**

The methanolic extracts of the plants were separately evaluated for their antioxidant activity against a range of inorganic free radicals. Unless stated otherwise, butylated hydroxyl-anisole (BHA) and ascorbic acid (AA) served as standards. All experiments

were replicated at least three times and radical scavenging or inhibitory activity of the extracts were 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.  $IC_{50}$  of the extracts was determined using GraphPad Prism version 5.03. Biotek plate reader (Synergy HT, Biotek Instrument supplies) was used to read absorbance.

### **3.3.1 Free radical scavenging activity**

#### **3.3.1.1 1,1-Diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity**

The method of Brad-Williams (1995) was followed to determine the DPPH scavenging activity of the extracts. DPPH (0.02 mg/ml) was mixed (1:1) with the crude extract (0-0.05 mg/ml). Following 30-60 mins incubation period, absorbance was spectrophotometrically read at 514 nm. Methanol and ascorbic acid were used as negative and positive controls, respectively.

#### **3.3.1.2 2,2-Azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical scavenging activity**

ABTS<sup>+</sup> scavenging ability of the crude extracts was investigated by following Re *et al.* (1999)'s method. The radical (ABTS<sup>+</sup>) was generated by incubating the mixture of 2.5 mM potassium persulfate and 7 mM of ABTS for 16 h. The ABTS<sup>+</sup> solution was diluted (1:60) with methanol and then mixed with the extract (0-0.05 mg/ml) in a 1:1 ratio. Absorbance was read at 734 nm. Ascorbic acid and methanol were used as positive and negative controls, respectively.

#### **3.3.1.3 Superoxide anion scavenging activity assay**

Superoxide anion ( $O_2^{\cdot-}$ ) scavenging activity of the plant extracts was investigated by the method of Nagai *et al.* (2001). Briefly, 0.02 ml of each of the following solutions: sodium 3 mM Xanthine sodium salt, 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 (0-0.05 mg/ml) were well mixed together. Xanthine oxidase (0.02 ml, 6 mU) was then added. The reduction of NBT was monitored at 630 nm.

#### **3.3.1.4 Hydroxyl radical (OH<sup>•</sup>) scavenging activity assay**

The method of Osawa *et al.* (1997) was followed to evaluate the extracts' OH<sup>•</sup> scavenging activity. The mixture comprised FeSO<sub>4</sub>.H<sub>2</sub>O (10 mM), extract (0-0.05 mg/ml), H<sub>2</sub>O<sub>2</sub> (10 mM), EDTA (10 mM), and 100 mM phosphate buffer (pH 7.4). These were mixed well and incubated (37 °C) for 2 h. Activity of the extract was measured spectrophotometrically at 520 nm.

#### **3.3.1.5 Nitric oxide (NO<sup>•</sup>) scavenging activity**

The method of Badami *et al.* (2005) was followed to evaluate the crude extracts' NO<sup>•</sup> scavenging activity. Briefly, phosphate buffered saline (0.1 M), sodium nitroprusside (10 mM), and extract (0-0.05 mg/ml) were thoroughly mixed. After 150 mins at 25 °C, sulphanilic acid reagent and 1-naphthylamine (5%) w/v were then added. Activity of the extract was spectrophotometrically measured at 540 nm.

#### **3.3.2 Reducing power**

Reducing potential of the extracts was tested using the method of Oyaizu (1986). Briefly, 2.5 ml of each of extract (0-0.05 mg/ml), potassium ferricyanide (1 %) w/v and phosphate buffer (200 mM, pH 6.6) were mixed and incubated for 20 mins at 50 °C. This was followed by centrifugation (1000 rpm) for 10 mins. Ferric III chloride (FeCl<sub>3</sub>, 0.1 %) was added to supernatant and the extract's reducing potential was measured at 700 nm.

#### **3.3.3 Metal ion (Fe<sup>2+</sup>) chelating activity assay**

The ability of the plant extracts to chelate Fe<sup>2+</sup> was investigated by following the Decker and Welch (1990)'s method. The mixture comprised 5 mM ferrozine, 2 mM FeCl<sub>2</sub>, extract (0-0.05 mg/ml) and deionized water. The mixture was incubated at 25 °C for 10 min. EDTA was used as standard. Absorbance was read spectrophotometrically at 562 nm.

#### **3.3.4 Effect of extract on acetylcholinesterase activity**

Effect of the extracts on acetylcholinesterase activity was determined colourimetrically using the method of Ellman *et al.* (1961). Rat brain homogenate (20 mg/ml) was prepared in 0.01 M phosphate buffer (pH 8). The homogenate was centrifuged (Rotofix mini-centrifuge machine) at 1000 X g for 20 min. Supernatant (0.4 ml) was diluted with 2.6 ml of the buffer. The reaction was initiated by 5 mM s-acetyl- thiocholine iodide (ASChI). 5,5'-

dithiobis-(2-nitrobenzoic acid)(DTNB) reagent (100 µl) was then added and mixed well. Tacrine was used as the standard. The absorbance was read at 412 nm for 5 min at 60 second intervals. The percentage inhibition was calculated.

### **3.3.5 Phosphodiesterase activity inhibition assay**

The method described by Corbin *et al.* (2002) was followed to determine the phosphodiesterase inhibitory activity of the extracts. The reaction mixture consisted of 0.2 ml each of 0.5 mM p-nitrophenyl 5'-thymidine monophosphate, 0.1 M Tris - HCl (pH 8.7), and the extract (0.1-1 mg/ml). The reaction was initiated by the addition of 0.25 ml of the enzyme (2 units). The mixture was in incubation 37 °C for 15 min. The reaction was stopped by the addition of 0.25 ml of 0.2 N NaOH. Caffeine was used as standard. Absorbance was read at 400 nm.

### **3.4 Cytotoxicity assay**

The cytotoxicity of the plants extract was determined using the MTT assay as described by Mosmann, (1983). The cytotoxicity of the plant extracts was evaluated against HEK 293, MCF 7 and HT 29 human cell lines. The cells were plated in a 96-well plate with cell suspensions of  $1.8 \times 10^4$  cells/ml concentrations. The cells were allowed to attach for 48 hours before being seeded with different concentrations from the crude extracts (100 mg/ml) using serial dilutions, administered in media containing 1 % of FBS and returned to the incubator for 48 hours. After 48 hours of incubation, the cell viability was determined by removing the old medium and adding the (Merck) tetrazolim salt as cytotoxicity indicator. Medium (100 µl) was added to each well and incubated at 37 °C for 4 hours. The media with MTT were then aspirated from the wells and the formed formazan crystals were solubilised in 100 µl of dimethyl sulfoxide (DMSO). The optical density of the solutions was measured at 570 nm using a Mindray-96A micro plate reader. Percent inhibition of cell viability was calculated using the formula:

$$\% \text{ cell death} = [(Ac - At) / Ac \times 100]$$

where Ac represents absorbance of control and At represent absorbance in the presence of the tested extract.



### **3.5 Animal experiment**

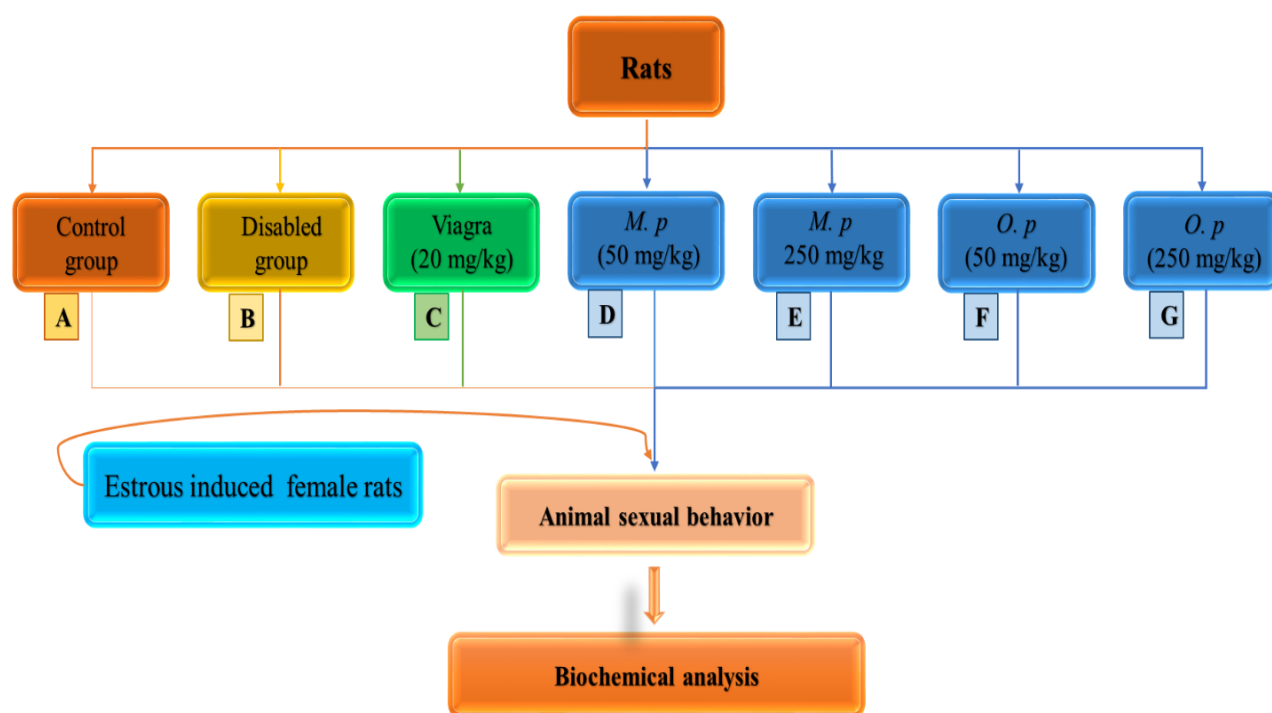
Ethical clearance for the use of laboratory animals and approval of procedures was issued by the University of Zululand Research Ethics Committee (UZREC 171110-030 PGM 2015/198; see Appendix C). Standard procedure for caring and handling of laboratory animals was strictly adhered to. The animals (Sprague-Dawley rats) of either sex were obtained from the animal unit of the Biochemistry Department, University of Zululand. Rats were kept under standard laboratory conditions (at  $23 \pm 2^{\circ}\text{C}$ , humidity and day-night cycle). The animals had access to enough fresh food and drinking water at leisure on daily basis for the duration of the experiment. The rats were separated according to gender (maximum of four rats per cage) and were allowed to acclimatise for at least five days before the experiment commenced.

#### **3.5.1 Effect of the extract on testicular function in Sprague Dawley rats**

The effect of plants extracts on testicular function was evaluated following the method previously described by Garza *et al.* (2015) with some modifications. The sexually active male Sprague Dawley rats (200 – 260 g) were randomly divided into seven groups (A-G) of four rats per group (Figure 3.1). The animals received an intraperitoneal injection of *n*-butanol at 50 mg/kg body weight for four days at two days intervals to induce testicular dysfunction. After the induction of testicular dysfunction, the animals were allowed to stand for three days before they were mated with estrous female rats to evaluate their baseline sexual performance. Animals with poor sexual behavior were considered to have testicular dysfunction.

Experimental groups (D-G) were orally administered daily with different concentrations (50 - 250 mg/kg body weight) of methanolic roots extract for 28 days (Figure 3.1). Normal control group (A) received (water) a carrier solvent while negative control (B) and positive control (C) received carrier solvent and sildenafil (Viagra 20 mg/kg) orally, respectively. Food and water intake were recorded daily for the 28 days of the experimental period. Body weight changes of the male rats were recorded for 28 days at seven days intervals. A group of female rats (150-200 g) received two consecutive subcutaneous injections of progesterone (7.5 mg/kg), at 48 hours intervals for 96 hours to induce estrous before

being introduced to male rats. To assess the rats' sexual behavior, each male rat from various treatment groups was individually subjected to one estrous female rat in a separate cage for 30 min. The number of mounts and intromission was recorded and compared between the groups. At the end of the experimental period, the male rats were euthanised under anaesthesia, blood was immediately collected by cardiac puncture and the testis were also removed and weighed. The collected tissue samples were used for biochemical parameters and histological analysis, respectively.



**Figure 3.1:** Experimental design illustrating the effect of methanol roots extract on *n*-butanol induced testicular dysfunction in Sprague Dawley male rats. Group A: Normal rats received water only; Group B: Negative control received 50 mg/kg *n*-butanol and water; Group C: Positive control received 20 mg/kg sildenafil *n*-butanol; Group D: received *M. p* 50 mg/Kg *n*-butanol; Group E: received *M. p* 250 mg/kg *n*-butanol; Group F: received *O. p* 50 mg/kg *n*-butanol and Group G: received *O. p* 250 mg/kg *n*-butanol. (*M. p* – *Mytenus procumbens*) and (*O. p* – *Ozoroa paniculosa*).

### **3.6 Biochemical assays**

Blood samples were centrifuged at 1200 rpm for 10 min in a Rotofix mini-centrifuge machine. The obtained serum was stored at  $-80^{\circ}\text{C}$  for subsequent biochemical assays. Testis and epididymis were rinsed in ice-cold 0.01 M phosphate buffer (pH 7.4) and kept at  $-80^{\circ}\text{C}$  for sperm count and morphology.

#### **3.6.1 Biochemical estimation of testosterone, creatinine and liver function enzymes**

Serum levels of testosterone, creatinine, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed using standard laboratory procedures (Global laboratory & Viral Laboratory, Richards Bay).

#### **3.6.2 Estimation of serum nitrite concentration**

Nitrite accumulation in the serum was estimated following the method previously described by Lidija *et al.* (2004). Briefly, the production of NO was determined spectrophotometrically with Griess reagent (0.1% N-1-naphthylethylenediamine dihydrochloride, 1% sulphanilic acid and 2.5% phosphoric acid). Equal volumes of Griess reagent and sample serum (125  $\mu\text{L}$ ) were mixed and plated in a 96-well microplate. The mixture was incubated for 10 min at room temperature and the absorbance was measured using Biotek plate reader (Synergy HT) at 546 nm. The serum nitrite level was determined from the standard curve of sodium nitrite and was expressed in mg/ml.

#### **3.6.3 Antioxidant status**

Serum glutathione (GSH) and malondialdehyde (MDA) contents as well as activities of antioxidant enzymes (superoxide dismutase, SOD; catalase, CAT) were estimated using respective commercial activity assay kits (Sigma-Aldrich) following manufacturers' instructions.

#### **3.6.4 Sperm count**

Sperm count was estimated following the method described by D'Souza (2003) and Adeiza and Minka (2011) with some modifications. The epididymis (0.2 – 0.3 g) were minced and placed in 1 ml of 0.1 M phosphate buffered saline (PBS, pH 7.2). The suspension was filtered and filtrate was mixed with 100  $\mu\text{L}$  of 1% aqueous nigrosine-eosin

Y (10:1 v/v) and allowed to stand for 30 min at room temperature for the staining of sperms. Thereafter, sperm suspension (10 µl) was placed on the microscope slides (1 cm<sup>2</sup>) and sperm count was performed as per standard procedure in an optical microscope with 400X magnification. The total number of sperm cells was determined using the following formula:

$$S = C \times V \times CF$$

where C = number of counted spermatozoa; S = Sum total per animal; V = dilution (10<sup>4</sup>) and FC = factor of the camera (1.25).

### **3.6.5 Sperm morphology**

Sperm morphology was analyzed microscopically following the method described by Lee *et al.* (2014). Sperm suspension mixed with 1% aqueous eosin Y (10:1) and let to stand for 30 min. Slides were then prepared with 20 µl of the mixture. The slides were air-dried, briefly rinsed in methanol excess and then cover-slipped with composition of the mounting medium. The cells' morphological abnormalities were examined under a light microscope (Carl Zeiss). Each sample was replicated twice and the abnormalities were classified as abnormal tail, abnormal neck, abnormal head, headless sperm, and double tailed sperms.

### **3.6.6 Histological analysis**

Testicular tissues (2.8–3.4 g) were removed and were stored in 40% formalin. The tissues were processed routinely and stained with haematoxylin and eosin (H&E). Histopathological analysis was carried out by a qualified pathologist (Vetdiagnostix Laboratories, Pietermaritzburg) with no prior knowledge of the animal groups and their treatment.

### **3.7 Statistical analysis**

The data were given as mean ± SD. The results were analyzed with one-way ANOVA using GraphPad Prism (v6.01). Where  $p < 0.05$ , the differences were regarded as statistically significant. The LC<sub>50</sub> or IC<sub>50</sub> values were determined using the cheburator version 1.2.0 software.

## CHAPTER FOUR

### 4.0 Results

This chapter presents results that were obtained from the evaluation of phytochemical analysis, antioxidant, anti-acetylcholinesterase and anti-phosphodiesterase activities of the *Maytenus procumbens* and *Ozoroa paniculosa* root extracts. The results of the cytotoxicity of the extracts and their effect on testicular dysfunction in male rats have also been presented.

### 4.1 Percentage yield

Powdered roots of *M. procumbens* and *O. paniculosa* were separately extracted with methanol. The yield from each plant material was 27% and 23% (w/w) for *M. procumbens* and *O. paniculosa*, respectively.

### 4.2 Phytochemical screening

Table 4.1 shows results of the preliminary phytochemical screening of *M. procumbens* and *O. paniculosa*. The presence of terpenoids, saponins, and cardiac glycosides in both plants' material was worth noting

**Table 4.1:** Phytochemical analysis of *M. procumbens* and *O. paniculosa*

Phytochemical	<i>M. procumbens</i>	<i>O. paniculosa</i>
Terpenoids	++	+
Saponins	++	+
Flavonoids	-	++
Alkaloids	+	-
Steroids	-	+
Tannins	++	-
Anthraquinones	++	+
Cardiac glycosides	++	++

++ Present in high concentration.

+ Present in low concentration.

- Not detected.

#### 4.2.1 Total phenolic and flavonoid contents

Table 4.2 shows the results of total phenols and flavonoids in the methanolic extract of *M. procumbens* and *O. paniculosa*. Though the qualitative (Table 4.1) and the quantitative tests (Table 4.2) indicated no presence of flavonoids in *M. procumbens* extract, the extract showed a relatively higher total phenolic content (0.083 mg/g) than the *O. paniculosa* extract (0.063 mg/g).

**Table 4.2:** The total phenolic and flavonoid content of *M. procumbens* and *O. paniculosa* extracts

Plant material	Phenolic content (mg/g)	Flavonoid content (mg/g)
<i>M. procumbens</i>	0.083 ± 0.012	0.00 ± 0.000
<i>O. paniculosa</i>	0.063 ± 0.003	0.102 ± 0.009

Data were expressed as mean ± SD, n=3. p<0.05

#### 4.3 Antioxidant activity

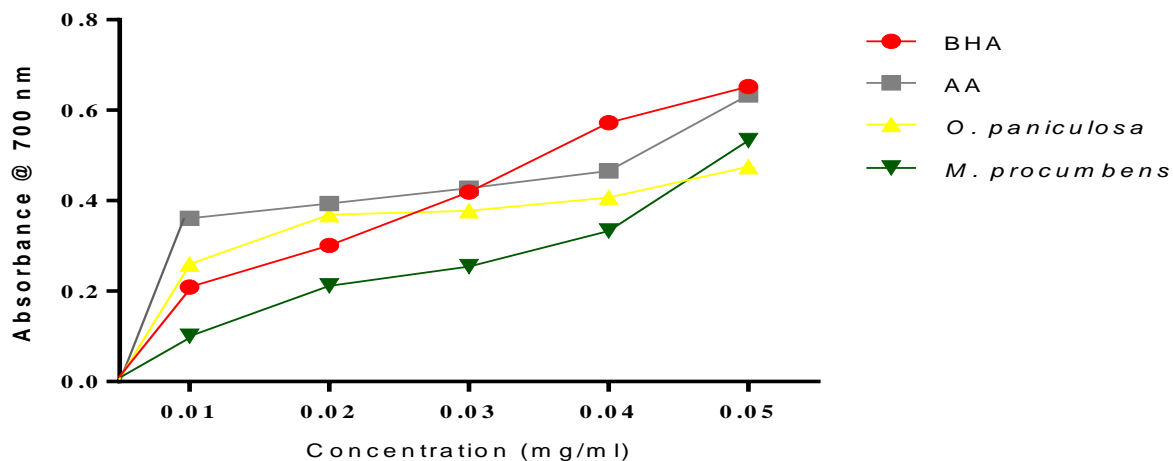
The antioxidant activity of the crude extracts was evaluated and the results are shown in Table 4.2. The extracts showed to varying degree of efficacy, a concentration-dependent free radical scavenging and Fe<sup>2+</sup> chelating activities. The IC<sub>50</sub> values of the extracts ranged from 0.04 – 8.02 mg/ml and 0.03 – 8.33 mg/ml on the free radical scavenging and metal ion (Fe<sup>2+</sup>) chelating activities, respectively. The lowest IC<sub>50</sub> values from the extract of *O. paniculosa* were observed on NO• (0.02 mg/ml) and ABTS (0.04 mg/ml). The metal ion chelating activity of *O. paniculosa* (IC<sub>50</sub> = 0.03 mg/ml) was comparable to that of EDTA (IC<sub>50</sub> = 0.03 mg/ml).

**Table 4.3:** Free radical (DPPH, ABTS<sup>•</sup>, superoxide radical, hydroxyl radical, nitric oxide) scavenging and metal ion (Fe<sup>2+</sup>) chelating activities of *M. procumbens* and *O. paniculosa* root extracts. (Figures under each radical scavenging are presented in percentage)

Extract (mg/ml)	DPPH	ABTS <sup>•</sup>	O <sub>2</sub> <sup>•-</sup>	•OH	NO <sup>•</sup>	Fe <sup>2+</sup>
<i>M. procumbens</i>						
0.01	1.95 ± 0.04	24.8 ± 0.14	27.3 ± 0.02	43.9 ± 0.01	38.3 ± 0.24	44.4 ± 0.09
0.02	13.6 ± 0.05	38.2 ± 0.01	26.5 ± 0.02	40.4 ± 0.07	43.4 ± 0.06	44.4 ± 0.05
0.03	37.4 ± 0.02	40.4 ± 0.02	26.9 ± 0.04	29.7 ± 0.01	38.7 ± 0.20	59.8 ± 0.16
0.04	48.8 ± 0.06	48.1 ± 0.02	27.1 ± 0.02	35.4 ± 0.02	46.4 ± 0.36	59.4 ± 0.26
0.05	60.3 ± 0.01	45.6 ± 0.01	35.5 ± 0.01	43.6 ± 0.03	40.0 ± 0.17	72.8 ± 0.22
IC <sub>50</sub>	0.04	4.82	8.02	6.34	6.46	1.96
<i>O. paniculosa</i>						
0.01	17.8 ± 0.06	27.5 ± 0.09	35.8 ± 0.03	44.2 ± 0.02	32.3 ± 0.47	53.3 ± 0.06
0.02	21.2 ± 0.06	19.7 ± 0.10	27.3 ± 0.05	40.4 ± 0.02	52.4 ± 0.20	63.3 ± 0.29
0.03	36.4 ± 0.05	50.3 ± 0.04	17.9 ± 0.13	42.3 ± 0.01	38.6 ± 0.17	79.4 ± 0.04
0.04	48.4 ± 0.06	66.4 ± 0.07	27.4 ± 0.05	36.8 ± 0.01	51.3 ± 0.14	49.6 ± 0.00
0.05	41.3 ± 0.06	67.9 ± 0.02	24.2 ± 0.04	32.3 ± 0.13	53.4 ± 0.35	64.1 ± 0.00
IC <sub>50</sub>	0.04	4.01	8.33	7.11	0.02	0.03
AA (IC <sub>50</sub> )	0.03	4.95	1.81	2.03	0.02	-
EDTA (IC <sub>50</sub> )	-	-	-	-	-	0.03
BHA (IC <sub>50</sub> )	6.63	3.83	-	5.08	0.05	-

Data were expressed as mean ± SD, n=3. p<0.05

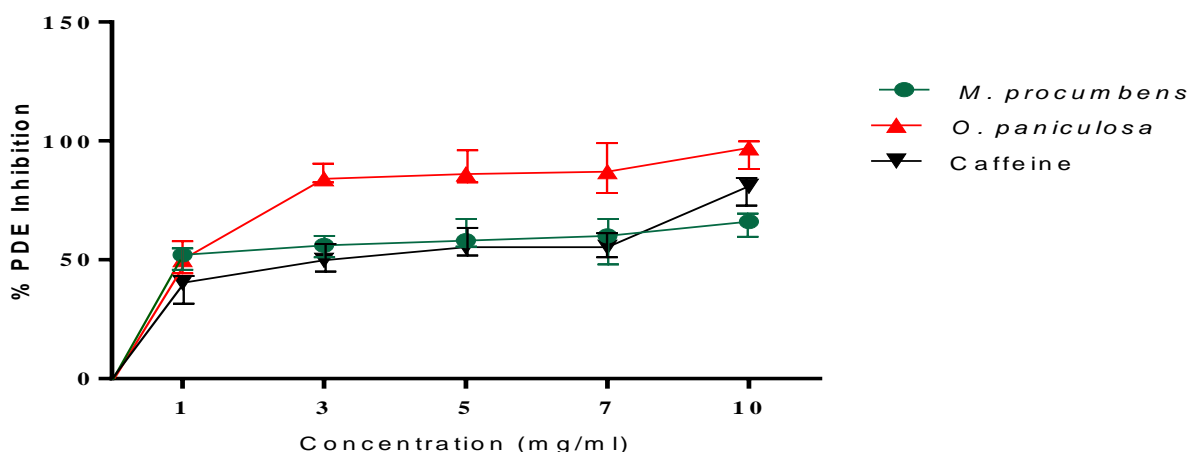
The reducing power of the extracts was also determined and the results are given in Figure 4.1. The extracts showed a concentration dependent reducing power. However their activity was relatively less than that of the standards (AA and BHA) used.



**Figure 4.1:** Reducing power of *M. procumbens* and *O. paniculosa* extracts. Ascorbic acid (AA) and Butylated hydroxyl anisole (BHA) were used as standards. Data were expressed as mean  $\pm$  SD, n=3.  $p < 0.05$

#### 4.4 Effect of the extracts on phosphodiesterase activity

Figure 4.2 presents the *in vitro* effect of the extracts on phosphodiesterase activity. The results revealed that *O. paniculosa* has a strong inhibitory activity ( $IC_{50} = 1.03$  mg/ml) against phosphodiesterase when compared to *M. procumbens* ( $IC_{50} = 0.96$  mg/ml) and caffeine ( $IC_{50} = 2.03$  mg/ml), a standard phosphodiesterase inhibitor.

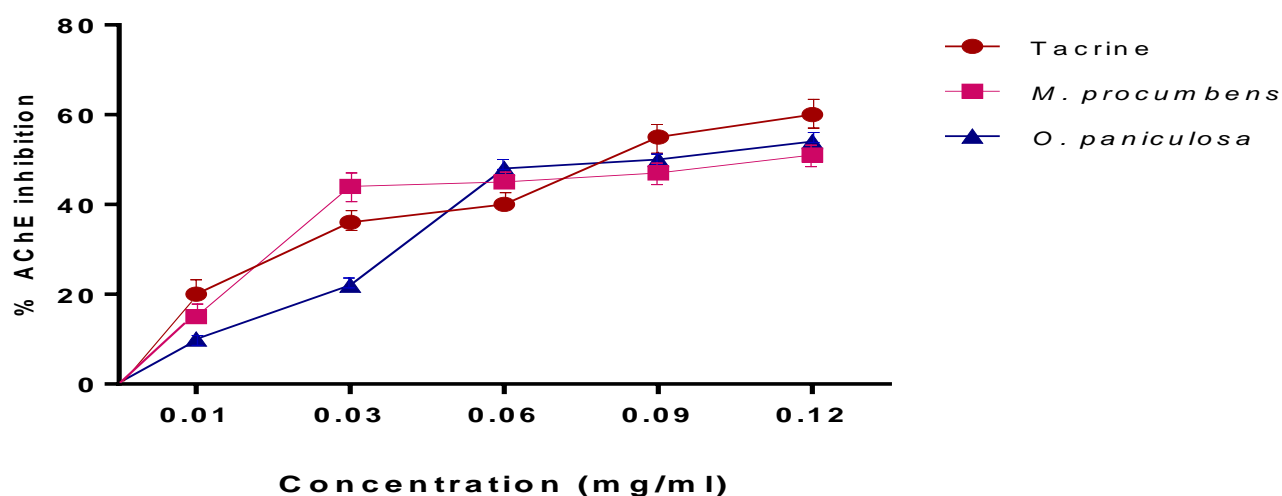


**Figure 4.2:** Effect of *M. procumbens* and *O. paniculosa* on phosphodiesterase activity. Caffeine was used as standard. Data were expressed as mean  $\pm$  SD, n = 3.  $p < 0.05$

#### 4.5 Effect of the extracts on acetylcholinesterase activity

Figure 4.3 shows the results of the effect of the extracts on acetylcholinesterase activity. Both *M. procumbens* and *O. paniculosa* extracts, at the concentration of 0.12 mg/ml, were able to inhibit more than 50% of enzyme inhibitory activity





**Figure 4.3:** Effect of *Maytenus procumbens* and *Ozoroa paniculosa* on acetylcholinesterase activity. Tacrine was used as standard. Data were expressed as mean  $\pm$  SD, n=3.  $p < 0.05$

#### 4.6 Cytotoxicity (MTT assay)

The results of the cytotoxic activity of the crude extracts against three human cell lines (MFC7, HT29 and HEK293) are presented in Table 4.4. Both crude extracts showed moderate toxicity effect on HT29 cells with lethality ( $LC_{50}$ ) values ranging from 30.3  $\mu$ g/ml and 80  $\mu$ g/ml and weak toxicity on cancer cells with  $LC_{50}$  values ranging from 230.3  $\mu$ g/ml to 438.4  $\mu$ g/ml, respectively.

**Table 4.4:**  $LC_{50}$  values ( $\mu$ g/ml) of *Maytenus procumbens* and *Ozoroa paniculosa* against MFC7, HEK293 and HT29.

Extracts	HEK 293 cells	MFC7 cells	HT29 cells
<i>M. procumbens</i>	438.4	330.2	80
<i>O. paniculosa</i>	356.1	230.3	30.3

Data were expressed as mean  $\pm$  SD, n=3.  $p < 0.05$

#### 4.7 In vivo study on the effect of the extracts on testicular dysfunction

##### 4.7.1 Effect of the extracts on serum levels of liver function enzymes and creatinine

Table 4.6 shows the results of effect of the extracts on serum levels of creatinine and liver function enzymes (AST and ALT). A decrease, relative to the normal control and disabled

groups, in the levels of ALT and AST was observed in the rats treated with the extracts for 28 days following induction of testicular dysfunction. High levels of creatinine were observed in both crude extracts treated groups.

**Table 4.5:** Effect of the extracts on serum levels of creatinine, AST and ALT.

Group	Creatinine ( $\mu$ moles/L)	AST (IU/L)	ALT (IU/L)
Normal Control	16 $\pm$ 2.58	164 $\pm$ 8.04	91 $\pm$ 6.39
Disabled	16 $\pm$ 2.95	181 $\pm$ 12.02	83 $\pm$ 2.79
Viagra (20mg/kg)	30 $\pm$ 2.34	149 $\pm$ 14.84	76 $\pm$ 7.60
<i>O.p</i> (50 mg/kg)	27 $\pm$ 0.47	185 $\pm$ 19.32	62 $\pm$ 1.65
<i>O.p</i> (250 mg/kg)	21 $\pm$ 0.70	136 $\pm$ 5.42*	54 $\pm$ 0.70*
<i>M.p</i> (50 mg/kg)	21 $\pm$ 1.25	150 $\pm$ 23.85	72 $\pm$ 1.73
<i>M.p</i> (250 mg/kg)	34 $\pm$ 2.31	155 $\pm$ 3.90	58 $\pm$ 3.59

Data were expressed as mean  $\pm$  SD, n=3. \*p<0.05 compared to disabled group.

#### 4.7.2 Effect of the extracts on tissue antioxidant status

The effect of the extracts on the tissue antioxidant status was determined and it was evident that treatment of the animals with the extracts increased their antioxidants status (Table 4.5). While *M. procumbens* at 250 mg/kg improved activity of catalase, both extracts (at 50 and 250 mg/kg) effectively increased GSH levels when compared to negative control (disabled). The administration of *O. paniculosa* (250 mg/kg) and *M. procumbens* at both concentrations (50 and 250 mg/kg) significantly decreased serum MDA levels.

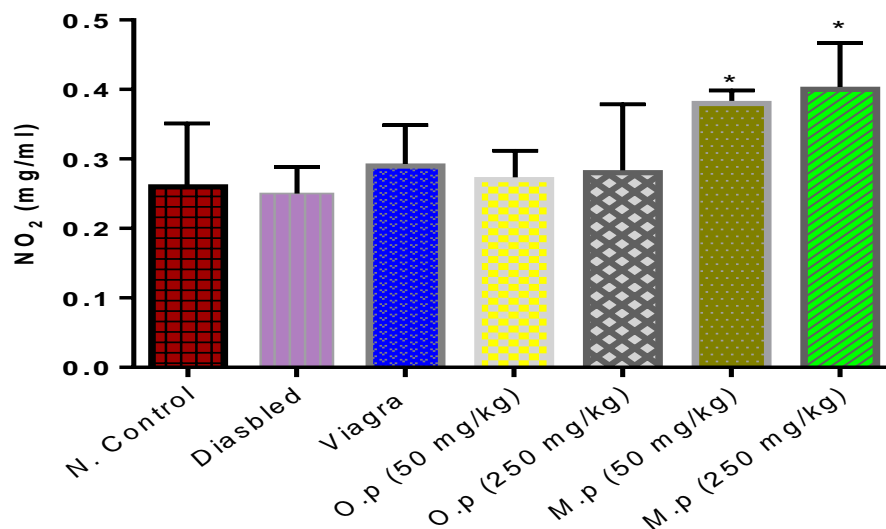
**Table 4.6:** Effect of the extracts on serum levels of SOD, CAT, GSH and MDA in the testicular dysfunction induced rats.

Group	SOD (units/ml)	CAT ( $\mu$ moles/min/ml)	GSH (nmoles/ $\mu$ l)	MDA (nmoles/ml)
Normal Control	18.9 $\pm$ 0.08	6.13 $\pm$ 0.02	7.3 $\pm$ 0.04	0.08 $\pm$ 0.01
Disabled	16.2 $\pm$ 0.02	3.37 $\pm$ 0.00	4.4 $\pm$ 0.02	0.16 $\pm$ 0.02
Viagra (20mg/kg)	13.3 $\pm$ 0.01	3.33 $\pm$ 0.00	4.9 $\pm$ 0.03	0.14 $\pm$ 0.01
<i>O.p</i> (50 mg/kg)	12.8 $\pm$ 0.00	3.36 $\pm$ 0.09	6.8 $\pm$ 0.01	0.17 $\pm$ 0.04
<i>O.p</i> (250 mg/kg)	9.6 $\pm$ 0.00	3.01 $\pm$ 0.04	9.8 $\pm$ 0.02*	0.04 $\pm$ 0.01
<i>M.p</i> (50 mg/kg)	10.6 $\pm$ 0.00	3.73 $\pm$ 0.00	3.2 $\pm$ 0.06	0.06 $\pm$ 0.02
<i>M.p</i> (250 mg/kg)	11.1 $\pm$ 0.07	4.27 $\pm$ 0.05*	5.4 $\pm$ 0.01	0.03 $\pm$ 0.00

Data were expressed as mean  $\pm$  SEM, n=3. \* p<0.05 compared to normal control.

#### 4.7.3 Effect of plant extracts on serum nitrite levels

Figure 4.5 shows the results of effect of the extracts on serum nitrite levels. The results revealed that *M. procumbens* (50 and 250 mg/kg) treated groups effectively increased serum nitrite levels (0.38 and 0.42 mg/ml) as compared to the disabled group. There was no noticeable effect of *O. paniculosa* even at its highest concentration relative to the disabled group.



**Figure 4.4:** Effect of *Maytenus procumbens* and *Ozoroa paniculosa* on serum nitrite levels. Data were expressed as mean  $\pm$  SEM, n=4. \* p<0.05 vs normal control group.

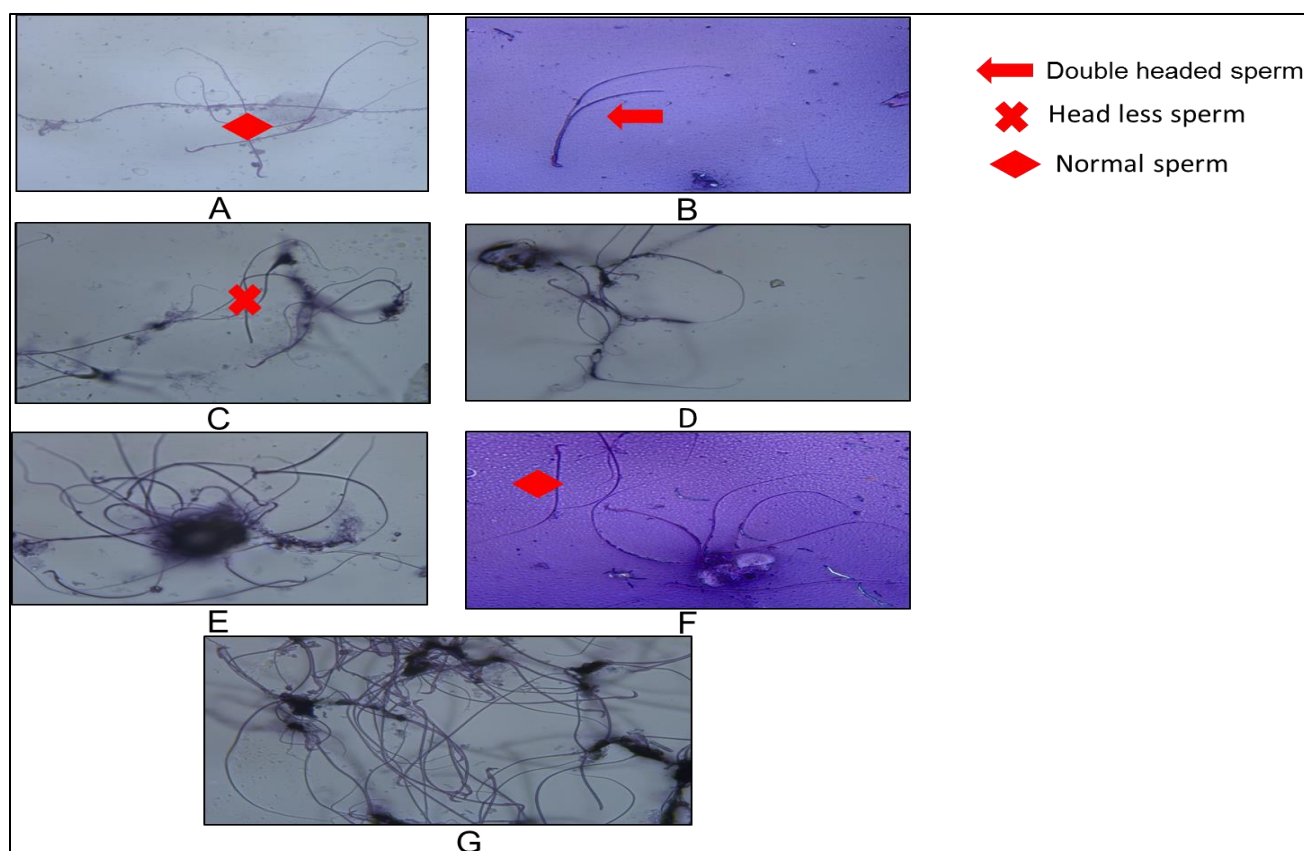
#### 4.7.4 Effect of the extracts on testicular weight, serum testosterone levels, sperm count and morphology

The effect of the extracts on testicular weight, serum levels of testosterone and sperm count was evaluated and the results are given in Table 4.7. An increase in body weight as well as testicular weight was recorded following 28 days of rats' treatment with the extracts. While there was a notable decrease in serum testosterone levels in the untreated animals, a significant increase in the serum testosterone levels was observed in the animals treated with the extracts at 250 mg/kg. A relatively higher total sperm count in the extracts treated groups was also recorded when compared to the untreated group (disabled). The sexual activeness of the animals treated with the extracts was shown by an increased mounting frequency of up to 18 and 46 times per 30 min for *O. paniculosa* and *M. procumbens*, respectively. Only a minimum mounting frequency (9 times per 30 min) was observed in the untreated (disabled) group. The effect of the extracts treatment was also determined based on the sperm morphology of the rats and the results are shown in Figure 4.6. A smaller percentage of abnormal sperm cells (headless, tailless) in the extracts treated groups was recorded when compared to the greater percentage of the abnormal cells observed in the untreated (disabled) group. Clustering of sperms was observed in the treated rats.

**Table 4.7:** Effects of plant extracts on body weight, testicular weight, serum testosterone levels, sperm count, morphology and mounting frequency.

Group	BWC (g)	TW (g)	Testosterone (nmol/L)	TSC (10 <sup>4</sup> )	AS (%)	NS (%)	MF (per 30 min)
<b>Normal control</b>	49 ± 16.26	3.40 ± 0.48	6.99 ± 3.09	47 ± 5.81	17	82.9	19 ± 1.02
<b>Disabled</b>	39 ± 16.23	2.76 ± 0.08	3.01 ± 2.82	35 ± 7.94	51	48.5	9 ± 1.00
<b>Viagra (20 mg/kg)</b>	50 ± 15.44	3.55 ± 0.41	14.93 ± 8.34	37 ± 5.92	47	54.1	64 ± 1.01
<b><i>O.p</i> (50 mg/kg)</b>	65 ± 17.47	3.33 ± 0.20	2.12 ± 1.54	53 ± 20 17	24	75.4	16 ± 3.09
<b><i>O.p</i> (250 mg/kg)</b>	97 ± 12.26	3.62 ± 0.62	6.60 ± 3.51	74 ± 10.56	18	82.4	18 ± 3.54
<b><i>M.p</i> (50 mg/kg)</b>	44 ± 11.60	4.21 ± 0.41	2.58 ± 0.71	46 ± 2.56	34	65.2	32 ± 3.11
<b><i>M.p</i> (250 mg/kg)</b>	48 ± 13.23	4.29 ± 0.11	10.95 ± 3.42*	49 ± 14.13	20	79.5	46 ± 1.20

BWC - body weight change; TW - testicular weight; TSC - Total sperm count; AS -Abnormal sperms; NS - Normal sperms; MF – Mount frequency; (*M.p*) – *Maytenus procumbens*; (*O.p*) – *Ozoroa paniculosa*. Data were expressed as mean ± SEM, n=4. \* p<0.05 compared to disabled group.



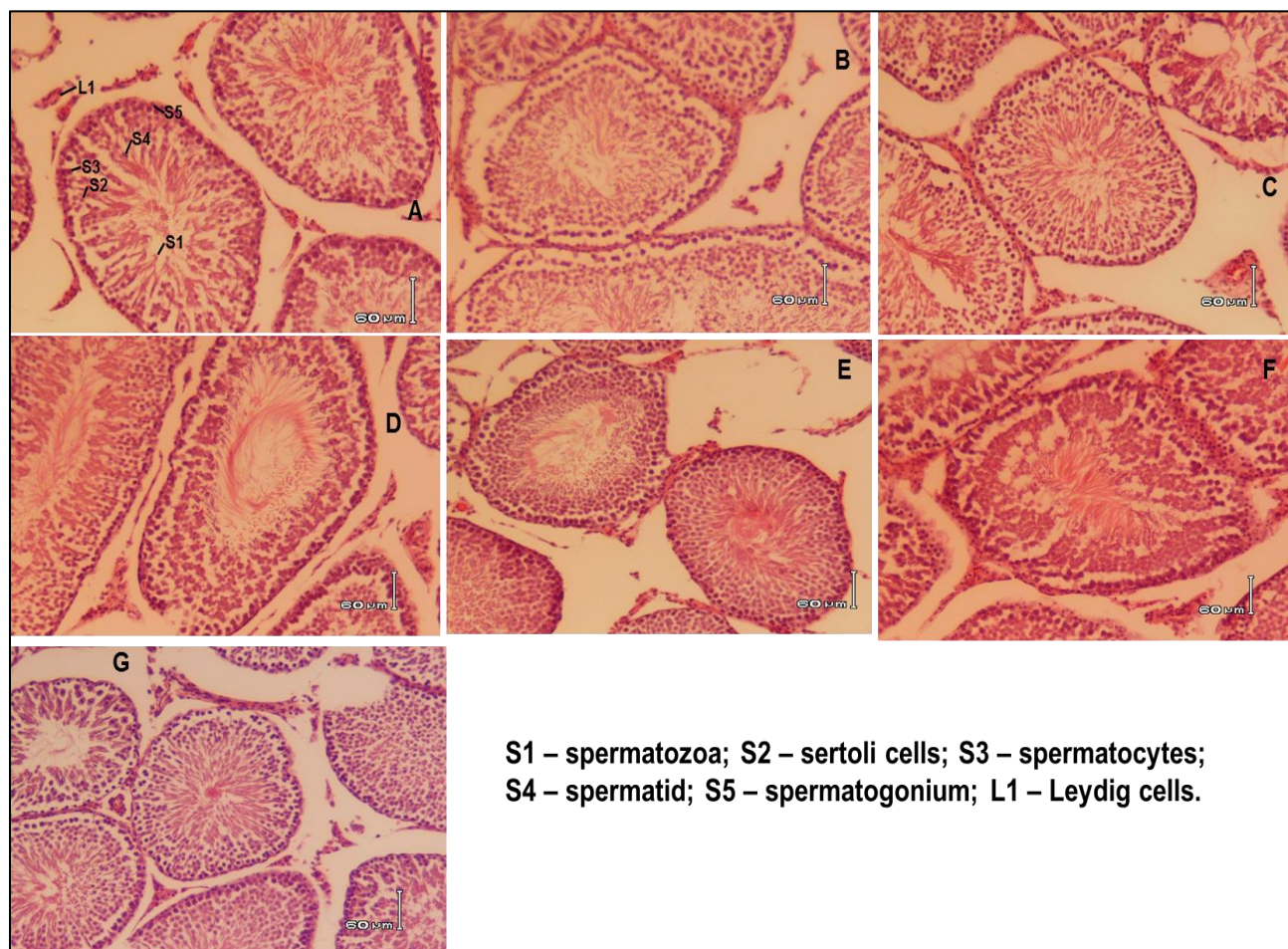
**Figure 4.5:** Micrographs showing morphological changes in sperm cells from testicular dysfunction-induced rats (Magnification 40x). **A-** sperms from normal control rats; **B-** sperms from untreated testicular dysfunctional (disabled) rats; **C-** testicular dysfunctional rats treated with Viagra (20 mg/kg); **D-** testicular dysfunctional rats treated with *M. procumbens* (50 mg/kg); **E-** testicular dysfunctional rats treated with *M. procumbens* (250 mg/kg); **F-** testicular dysfunctional rats treated with *O. paniculosa* (50 mg/kg); **G-** testicular dysfunctional rats treated with *O. paniculosa* (250 mg/kg). Magnification X400

#### 4.7.5 Histopathological analysis of the testes

Figure 4.6 shows photomicrographs of seminiferous tubules of the rats treated with the extracts. The histopathological analysis revealed various morphological changes in the untreated group (disabled) when compared to the normal control group. A complete differentiation of the seminiferous tubules with regular arrangement of cells was observed in the normal control. In addition to the degeneration of the basal layer of the germinal epithelium which resulted to decreased numbers of spermatogonia, loose and irregular arrangement of cells in the seminiferous tubules was observed in the disabled group



(Figure 4.6B). This was also accompanied by shedding of cellular material from the seminiferous epithelium. However, the histomorphological changes broad about by the treatment of the animals with the extracts (at 250 mg/kg) were comparable to those of the normal control group animals. A densely packed spermatogonia and Sertoli cells that rested on the basement membrane was observed following the animals' treatment with the extracts. These cells were surrounded by a concentric myofibroblast layer (Figure 4.6 E and 4.6 G).



**Figure 4.6.** Histological evaluation of the testes. All specimens were prepared as 60  $\mu$ m thick section stained with haematoxylin and eosin. **A** – Testis' cross section from normal control; **B** – section from untreated testicular dysfunctional (disabled) rats; **C**- testicular dysfunctional rats treated with Viagra (20 mg/kg); **D**- testicular dysfunctional rats treated with *M. procumbens* (50 mg/kg); **E**- testicular dysfunctional rats treated with *M. procumbens* (250 mg/kg); **F**- testicular dysfunctional rats treated with *O. paniculosa* (50 mg/kg); **G** – testicular dysfunctional rats treated with *O. paniculosa* (250 mg/kg). Magnification: X200.

## CHAPTER FIVE

### 5.0 Discussion

Normal testicular function is crucial in males' reproductive systems (Amory and Bremner, 2001). Sexual dysfunction, due to either endogenous or exogenous factors, may result in a decline in male sexual activeness, androgen synthesis and fertility (Karagiannis *et al.*, 2005). In this study, testicular dysfunction was induced through butanol. Studies have shown that chronic alcohol consumption affect testicular function by causing histological changes in the seminiferous tubules resulting in suppression of spermatogenesis (Bhargavan *et al.*, 2015). Atrophy of seminiferous tubules which, presented as decreased in testosterone levels and loss of spermatogenic cells, indicates the failure of spermatogenesis (Bhargavan *et al.*, 2015). In this study butanol-induced testicular dysfunction was confirmed by decreased testicular weight, serum testosterone levels and low sperm cells. It was also through histological examination by marked necrosis and degeneration of seminiferous tubules.

The restoration and/or improvement of testicular functions is important in ameliorating male sexual disorders (Julia *et al.*, 2003). With the resurgent interest in the use of plant-derived therapeutic agents against various ailments including sexual disorders, a number of medicinal plants have been reported to be effective against testicular dysfunction and related sexual disorders (Palaniyappan *et al.*, 2013; Bashir *et al.*, 2015; Bhattacharya *et al.*, 2015). Ethanolic roots extract of *Withania somnifera* have been reported to improve testicular dysfunction in ethanol-induced rats (Bhargavan *et al.*, 2015). Powdered roots of *Maytenus procumbens* and *Ozoroa paniculosa* are often used by Zulu traditional healers to manage sexual dysfunctions. It is apparent that these plants have the potential to ameliorate testicular dysfunction: The plants' extracts were able to increase the testicular weight, sperm count, and serum testosterone levels (Table 4.7) following the disabled animals' treatment with the extracts. Testes are responsible for testosterone synthesis and sperm production (Nunes *et al.*, 2012). Although the observed increase in testicular weight could be attributed to scar tissue, the observed increase in sperm count, improved sperm quality (Figure 4.5), and higher levels of testosterone on the treated groups is an indication of the extracts' potential steroidogenic and spermatogenic activity.



The ability of the extracts to also stimulate regeneration of spermatogonia in the seminiferous tubes of the rats treated with the extracts (Figure 4.6 E and G) and thus restoring spermatogenesis, further support the spermatogenic activity of the extracts.

Testosterone synthesis is key to the development of male secondary sexual characteristics, enhancement of sexual desire, penile erection and also in sperm production (Chauhan *et al.*, 2014). Table 4.7 and Figure 4.5 indicated the extracts' ability to improve sperm quality, and stimulate sexual libido. Similar amelioration of the seminiferous tubules, which enhance regeneration of germ cells and Sertoli cells have been reported (Adaramoye and Arisekola. 2013). The presence of flavonoids and alkaloid from extracts of *Artemisia Anna* has been reported to enhance regeneration of spermatogonia in the seminiferous tubule which correlate with increased in testosterone levels (Ajah and Eteng. 2010). Other documents have demonstrated the effect of the flavonoids, saponins, cardiac glycosides and tannins in improving the histological damage of the testicular tissue in rats (Adejuwon *et al.*, 2014; Bakare *et al.*, 2015). It is worth noting that similar phytochemicals were observed to be present in the plants studied in this study (Table 4.1). The presence of alkaloids, flavonoids, cardiac glycosides and tannins from *Allium sativum*'s extract have demonstrated the ameliorative potential in testicular dysfunction on lead-induced Wistar rats (Ayoka *et al.*, 2016).

Oxidative stress has been implicated in the pathogenesis of sexual dysfunction. Testes, due to their high content of polyunsaturated fatty acids, are highly vulnerable to oxidative stress (Aitken and Roman, 2008). Increased ROS level in seminal plasma may also cause morphological deformity in sperm cells (Singh *et al.*, 2015). Most medicinal plants traditionally used to manage testicular dysfunctions are known to have high antioxidant capacity (Wani *et al.*, 2011; Palaniyappan *et al.*, 2013; Zade *et al.*, 2013). The *in vitro* and *in vivo* antioxidant properties exhibited by the plants extracts under study (Tables 4.3 and 4.6) e.g. their ability to improve tissue antioxidant status with lowered MDA levels in the treated rats (Table 4.6) could partly be attributed to the potential curative effect of the plants. The antioxidant activity of the extracts in this study could be linked to their high total phenolic and/or flavonoid content (Table 4.2).

Literature also suggests that the limited bioavailability of NO, and increased activities of PDE and AChE are some causative agents of erectile dysfunction, a common form of male sexual disorder (Tsai and Kass, 2009; Shah *et al.*, 2009). While NO activates a cascade of cGMP-dependent reactions leading to the relaxation of smooth muscles around the penile tissue, PDE-5 and AChE antagonise this effect (Ghiadoni *et al.*, 2008; Shah *et al.*, 2009). The PDE and AChE inhibitory activity exhibited by the two plants' extracts (Figure 4.3 and 4.4) together with the observed higher serum NO concentration in *M. procumbens* treated rats (Figure 4.6) could support the erectogenic potential of the extracts. Erectogenic potential of other medicinal plants such as *Clerodendron capitatum* (Abdelwahab *et al.*, 2012) and *Kaempferia parviflora* (Temkitthawon *et al.*, 2011) has been linked to their ability to inhibit PDE-5, and PDE and AChE (Oboh *et al.*, 2015).

Scientific documentation of the cytotoxic effects of the plants used by traditional healers for medicinal purposes is crucial. Toxicity of crude extracts is categorised into highly toxic ( $LC_{50} \leq 20 \mu\text{g/ml}$ ), moderate toxicity ( $LC_{50} = 21 - 200 \mu\text{g/ml}$ ) or weakly toxic ( $LC_{50} = 201 - 500 \mu\text{g/ml}$ ) (Geran *et al.*, 1972). The cytotoxicity results from this study (Table 4.4) ranged from moderate to weak cytotoxic on cancer and normal cells, respectively. The relatively higher toxicity levels of the extracts on the cancer cells, particularly HT29 cells, than normal cells (HEK293) indicate their cell selectivity.

Furthermore, in *in vivo* studies, serum levels of the liver function enzymes (ALT and AST) and creatinine are commonly used as diagnostic biomarkers of liver and kidney functions. Their increased levels indicate organ damage. Creatinine is an end product of creatine metabolism. It is synthesised in the liver and passes on into the circulation and excreted in urine by the kidney.

The observed decrease in activity of the liver function enzymes in the extracts treated groups indicated their hepatoprotective potential. However, the relatively higher serum creatinine levels recorded in the extracts treated groups (Table 4.5) suggested potential renal toxicity of the plants' extracts. Thus, the observed potential cytotoxicity and renal toxicity of the plants indicate that the plants be medicinally used with caution.

## CHAPTER SIX

### 6.0 Conclusion

A Large body of evidence supports the use of medicinal plants in the management of testicular dysfunctions and related health threats. The results obtained from this study suggest that the extracts of *M. procumbens* and *O. paniculosa* have the potential to ameliorate testicular dysfunction. Their ameliorative effects could be supported by the observed increase in serum testosterone levels, libido, sperm count and sperm quality following the treatment of the animals with extracts. The antioxidant properties exhibited by the extracts as well as their ability to improve tissue antioxidant capacity could suggest the apparent partial mechanism through which the extracts exert their therapeutic effect. Even though antioxidant property was observed in both plants' extract, *O. paniculosa* was more efficient in antioxidant activity while *M. procumbens* enhanced sexual performance. Traditional healers use the combination of the two plants in the management of sexual disorders. These results support the use of *M. procumbens* and *O. paniculosa* in folk medicine in the management of testicular dysfunction and its related disorders. However, in addition to their efficacy, the observed potential cytotoxicity on both normal and cancer cells, and renal toxicity of the extracts indicate that the plants be medicinally used with caution.

### 6.1 Suggestions for further studies

- I. *In vivo* erectogenic effect of the extracts and also isolation of their active compounds are recommended.
- II. It is recommended to investigate the anti-cancer activity of *M. procumbens* and *O. paniculosa* methanolic root extracts.

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## **APPENDIX A**

### **Details of Reagents Preparation**

#### **A1. Tris-buffer (pH 7.0)**

Tris-HCL (7.88 g), 2.79 g of EDTA and 10.227 g of NaCl were mixed and dissolved in distilled water. The solution was made up to 1 L with distilled water.

#### **A2. TBA**

Glacial acetic acid (50 ml) was diluted with 50 ml and 1 g of TBA was added into the diluted acid. The solution was made up to 100 ml with distilled water.

#### **A3. Formalin**

Sodium dihydrogen orthophosphate ( $\text{NaH}_2\text{PO}_4$ , 1.75 g) and 3.25 g of di-sodium hydrogen orthophosphate ( $\text{Na}_2\text{HPO}_4$ ) were dissolved in 25 ml of boiling water. Formalin (50 ml, 40%) was added to the mixture. The solution was made up to 400 ml with distilled water.

## APPENDIX B

### Details of Methodology

#### B1. Phytochemical screening

##### B1.1 Alkaloids test

Powdered plant material (0.5 g) was mixed with 5 ml of 1% aq. HCl. The mixture was stirred on the steam bath and then filtered using Whatman No.1 filter paper. The obtained filtrate was divided into two parts. One part (1 ml) of the filtrate was mixed with Mayer's reagent and the other part (1 ml) was mixed with Dragendorff's reagent. The turbidity formed indicated presence of alkaloids.

##### B1.2 Tannins test

The powdered plant material (0.5 g) was mixed with 10 ml of water, stirred and filtered. Few drops of 0.1% FeCl<sub>3</sub> solution were added to 2 ml of the filtrate. Development of green, blue-green or blue-black precipitate was an indication of the presence of tannins.

##### B1.3 Saponins test

The powdered plant material (0.5 g) was boiled with 10 ml of water and filtered. The filtrate was allowed to cool at room temperature. This was then shaken vigorously and let to stand for 15-20 min. The formation of froth indicated presence of saponins.

##### B1.4 Anthraquinone test

**a) Free anthraquinone:** The plant powder (0.5 g) was dissolved in a small volume of distilled water. This was followed by addition of 5 ml of benzene. The mixture was thoroughly mixed and then filtered. Ammonia solution (5 ml, 10%) was then added to the filtrate and thoroughly mixed by shaking. Formation of a pink colour in the ammonia layer indicated the presence of free anthraquinones.

**b) Combined anthraquinone:** About 0.5 g of plant powder was dissolved in a small volume of distilled water. Ferric chloride solution (10%) and 5 ml of hydrochloric acid were then added and the mixture was boiled for 10 min. The mixture was filtered hot and the filtrate was allowed before being treated with 5 ml of 10% ammonia solution. The mixture

was well shaken and pink color in the ammonia layer was taken as evidence for the presence of combined anthraquinone.

### **Flavonoids Test**

**(a) Lead acetate test:** powdered plant material (0.5 g) was mixed with 10 % of lead acetate. Reddish brown coloration (or precipitate) was an indication of the presence of flavonoids.

**(b) Ferric Chloride test:** powdered plant material (0.5 g) was mixed with 10 % of ferric chloride. Dark brown (dirty brown precipitate) was an indication of the presence of flavonoids.

**(c) Sodium Hydroxide test:** powdered plant material (0.5 g) was mixed with 1 ml of diluted sodium hydroxide. Formation of a golden yellow precipitate was taken as an indication of the presence of flavonoids.

### **Test for steroids**

Powdered plant material (0.5 g) was mixed with 2 ml of acetic anhydride and 2 ml of  $\text{H}_2\text{SO}_4$  was carefully added to the mixture. Change of colour from violet to blue or green indicated the presence of steroids.

### **Test for Cardiac glycoside**

**(a) Lieberman's test:** the plant powder (0.5 g) was mixed with 2 ml of acetic anhydride on ice and 1 ml of  $\text{H}_2\text{SO}_4$  was also carefully added. Colour change from violet to blue or green was taken as an indication of steroidal nucleus i.e. the aglycone portion of the cardiac glycosides.

**(b) Salkowski test:** the plant powder (0.5 g) was extracted with 2 ml of chloroform. This was kept on ice and 2 ml of concentrated  $\text{H}_2\text{SO}_4$  was also added. Reddish-brown color at the interface indicated the presence of steroidal nucleus i.e. the aglycone portion of the cardiac glycosides.

### **Test for flavonoids**

**(a) Lead acetate test;** 1 ml of extract was mixed with 10 % of lead acetate. Reddish brown colouration (or precipitate) was an indication of the presence of flavonoids.

**(b) Ferric Chloride test;** 1 ml of extract was mixed with 10 % of ferric chloride. Dark brown (dirty brown precipitate) was an indication of the presence of flavonoids.

**(c) Sodium Hydroxide test;** 1 ml of extract was mixed with 1 ml of diluted sodium hydroxide. Golden yellow precipitate an indication of the presence of flavonoids.

**Test for steroids:** 2 ml of acetic anhydride was added to 0.5 g extract of each sample with 2 ml H<sub>2</sub>SO<sub>4</sub>. The color changed from violet to blue or green in some samples indicating the presence of steroids.

### **Test for Cardiac glycoside**

**(a) Lieberman's test:** 0.5 ml of extract was mixed with 2 ml of acetic anhydride, 1 ml of H<sub>2</sub>SO<sub>4</sub> was also added in the presence of ice. Color change from violet to blue or green indicates the presence of steroidal nucleus i.e. the aglycone portion of the cardiac glycosides.

**(b) Salkowski test;** 0.5 ml of extract was mixed with 2 ml of chloroform, 2 ml of concentrated H<sub>2</sub>SO<sub>4</sub> was also added in the presence of ice. Reddish-brown color at the interface indicated the presence of steroidal nucleus i.e. the aglycone portion of the cardiac glycosides.

**(c) Keller-killiani test;** 0.5 ml of extract was dissolved in 2 ml of glacial acetic acid containing 1 drop of 10 % ferric chloride solution underplayed with 1 ml of H<sub>2</sub>SO<sub>4</sub>. Brown ring at the interphase indicated the presence of a deoxy sugar characteristics of cardenoids, a violet ring appeared below the brown ring also indicated the presence of cardiac glycosides and the greenish ring appeared from just above the brown ring (i.e. in

the acetic acid layer) and gradually spread throughout this layer indicated the presence of cardiac glycosides.

## **B.2 Antioxidant activity**

**B.2.1 Assay of 2,2-diphenyl-1-picryl hydrazyl (DPPH)**, 2 mg/100 ml was mixed with 2 ml of CH<sub>3</sub>OH extract (0-5 mg/100 ml) after 1 hour the absorbance (517 nm) was measured against CH<sub>3</sub>OH (Brad-Williams, 1995).

**B.2.2 Assay of 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS)**, 7 mM of ABTS (10 mg) was prepared in H<sub>2</sub>O (3 ml) with potassium persulfate (2.45 mM) incubated at room temperature in the dark for 16 hours and then diluted (1 ml ABTS: 60 ml CH<sub>3</sub>OH). 1 ml of ABTS was mixed with 1 ml of CH<sub>3</sub>OH extract (0-5 mg/100 ml) after 6 min the absorbance (734 nm) was measured against CH<sub>3</sub>OH (Re *et al*, 1999).

**B.2.3 Assay of superoxide radical scavenging activity:** The assay was based on the capacity of the extracts to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) in the riboflavin–light–NBT system (Beauchamp & Fridovich, 1971). The method used by Martinez *et al.* (2001) for determination of superoxide dismutase was followed after modification. To test tube(s) 0.02 ml each of the following, Bovine serum albumin 0.15%, 3 mM Xanthine, 3 mM EDTA, NBT (0.75 mM), extract (0-5 mg/100 ml) and 0.48 ml sodium carbonate buffer (pH 10.5) was mixed together and incubated at 25 °C for 20 min and 0.02 ml Xanthine oxidase (6 µM) was added then the mixture was incubated at 25 °C for 20 min and 0.02 ml CuCl<sub>2</sub> (6 Mm) was added. The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after 20 min. Ascorbic acid was used as the standard.

**B.2.4 Assay of hydroxyl radical scavenging activity:** the assay is based on benzoic acid hydroxylation method, as described by, Osawa *et al.* (1997). In a screw capped tube, 0.2 ml of FeSO<sub>4</sub>.7H<sub>2</sub>O (10 mM) and 0.2 ml EDTA (10 mM) were added. Then 0.2 ml extract (0-5 mg/100 ml), 0.2 ml DNA (10 mM) and 1 ml phosphate buffer (pH 7.4, 0.1 mol.) were added. Finally, 200 µl of an H<sub>2</sub>O<sub>2</sub> solution (10 mM) was added. The reaction

mixture was then incubated at 37 °C for 4 hours (internal mixing). After this, 1 ml of TCA (2.8%) and 1 ml of TBA (1%) were added and the mixture was boiled for 10 min and allowed to cool on ice. Absorbance was determined at 520 nm and Inhibition of lipid peroxidation by the extract was calculated.

**B.2.4 Assay of NO- scavenging activity:** 2 ml of sodium nitropruside (10 mM), 0.5 ml of phosphate buffer saline (0.01 M; pH 7.4) was mixed with 0.5 ml extract (0-5 mg/100 ml) and incubated at 25 °C for 150 min. 0.5 ml of reaction mixture pipetted into different test tubes, 1 ml sulphanilic acid reagent (0.33% in 20 % glacial acetic acid) was added and incubated at room temperature for 5 min; 1 ml 1-naphthylamine (5%) was added and allowed to stand for 30 min in diffused light. The absorbance was determined spectrophotometrically at 540 nm. (Garraat, 1964)

**B.2.5 Reducing power:** The method reported by Oyaizu. (1986) was used to measure reducing power of plant extract (0-5 mg/100 ml). 1 ml of CH<sub>3</sub>OH extract was mixed with 2.5 ml (0.2 M) of phosphate buffer (6.6 pH) and 1% potassium ferricyanide (2.5 ml) then incubated at 50 °C for 20 min. 10% Trichloroacetic acid (TCA) was added to the mixture, the mixture was centrifuged (1000 rpm : 10 min). 2.5 ml of supernatant was mixed with 2.5 distilled H<sub>2</sub>O and 0.5 ml of ferric chloride (FeCl<sub>3</sub>) (0.1%). The absorbance (700 nm) was measured against CH<sub>3</sub>OH (Oyaizu, 1986). BHA and ascorbic acid were used as standards.

**B.2.6 Chelating Activity on Fe<sup>2+</sup>:** The method reported by Decker and Welch. (1990) used to measure the chelating activity of plant extracts on Fe<sup>2+</sup>. 1 ml of plant extract (0 5 mg/100 ml) was diluted with 3.75 ml of deionize water. This was mixed with FeCl<sub>2</sub> (2 mM, 0.1 ml) and 4, 4 1- [3-(2-pyridinyl)-1, 2, 4-triazine-5, 6-dryl] bisbenzene sulphonic acid (ferrozine) (5 mM, 0.2 ml), after 10 min the absorbance was measured. Ethylenediaminetetra-acetic acid (EDTA) and citric acid were used as standards.

## APPENDIX C

### C1. Ethic clearance

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



#### RESEARCH & INNOVATION

Website: <http://www.unizulu.ac.za>  
Private Bag X1001  
KwaDlangezwa 3886  
Tel: 035 902 6887  
Fax: 035 902 6222  
Email: [ManqeleS@unizulu.ac.za](mailto:ManqeleS@unizulu.ac.za)

### ETHICAL CLEARANCE CERTIFICATE

Certificate Number	UZREC 171110-030 PGM 2015/198						
Project Title	Effect of methanolic extract of <i>Maytenus procumbens</i> and <i>Ozoroa paniculosa</i> on testicular functions						
Principal Researcher/ Investigator	ND Cele						
Supervisor and Co-supervisor	Dr. RA Mosa				Prof AR Opoku		
Department	Biochemistry and Microbiology						
Nature of Project	Honours/4 <sup>th</sup> Year		Master's	x	Doctoral		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 proposal and the documents listed on page 2 of this Certificate.

**Special conditions:**

- (1) The Principal Researcher must report to the UZREC in the prescribed format, where applicable, annually and at the end of the project, in respect of ethical compliance.
- (2) Documents marked "To be submitted" (see page 2) must be presented for ethical clearance before any data collection can commence.

The Researcher may therefore commence with the research as from the date of this Certificate, using the reference number indicated above, but may not conduct any data collection using research instruments that are yet to be approved.

Please note that the UZREC must be informed immediately of

- Any material change in the conditions or undertakings mentioned in the documents that were presented to the UZREC
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research



**Classification:**

Data collection	Animals	Human Health	Children	Vulnerable pp.	Other
X	X				
Low Risk		Medium Risk		High Risk	
				X	

The table below indicates which documents the UZREC considered in granting this Certificate and which documents, if any, still require ethical clearance. (Please note that this is not a closed list and should new instruments be developed, these would require approval.)

Documents	Considered	To be submitted	Not required
Faculty Research Ethics Committee recommendation	X		
Animal Research Ethics Committee recommendation	X		
Health Research Ethics Committee recommendation			X
Ethical clearance application form	X		
Project registration proposal	X		
Informed consent from participants			X
Informed consent from parent/guardian			X
Permission for access to sites/information/participants			X
Permission to use documents/copyright clearance			X
Data collection/survey instrument/questionnaire			X
Data collection instrument in appropriate language		Only if necessary	
Other data collection instruments		Only if used	

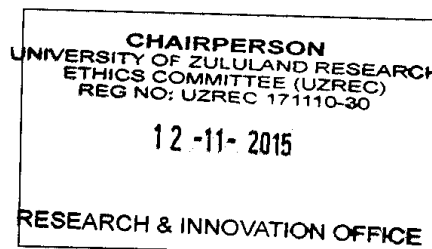
The UZREC retains the right to

- Withdraw or amend this Certificate if
  - Any unethical principles or practices are revealed or suspected
  - Relevant information has been withheld or misrepresented
  - Regulatory changes of whatsoever nature so require
  - The conditions contained in this Certificate have not been adhered to
- Request access to any information or data at any time during the course or after completion of the project

The UZREC wishes the researcher well in conducting the research.

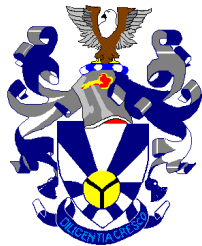


**Professor Nokuthula Kunene**  
**Chairperson: University Research Ethics Committee**  
 12 November 2015



## 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?**

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**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)	

**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? \_\_\_\_\_  
\_\_\_\_\_

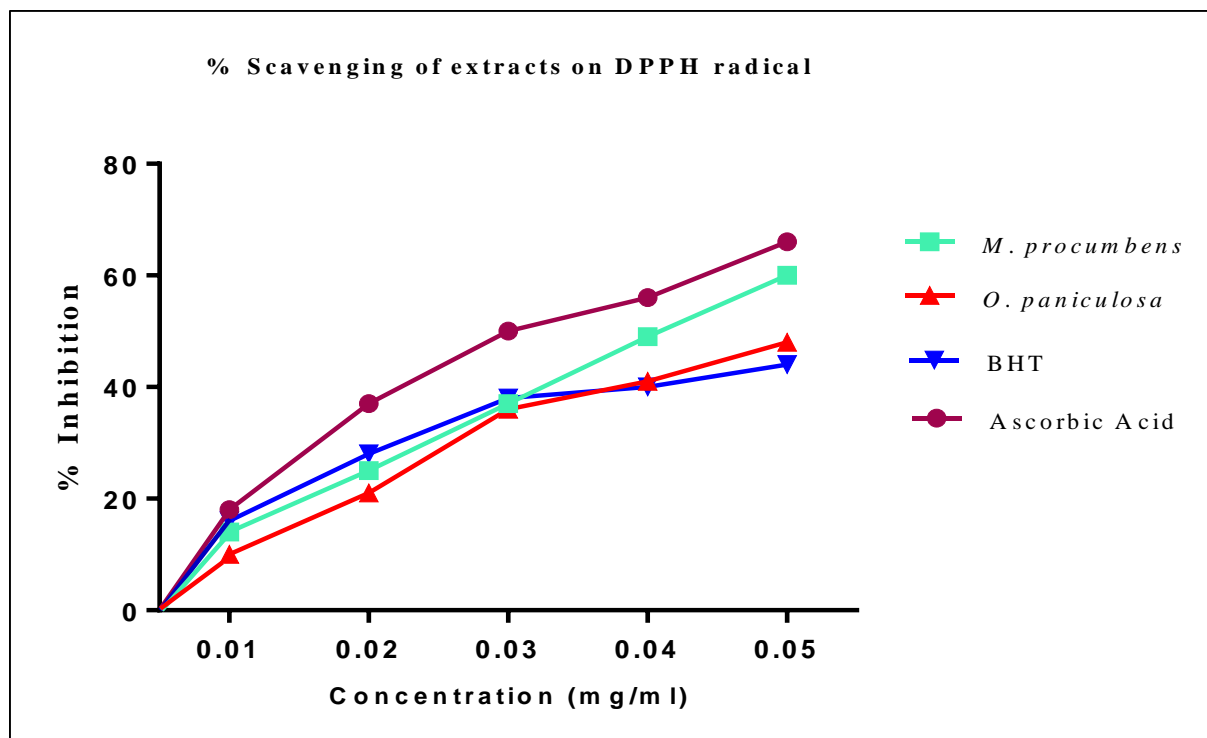
e) Where did the knowledge come from (e.g. grandmother, relative)?

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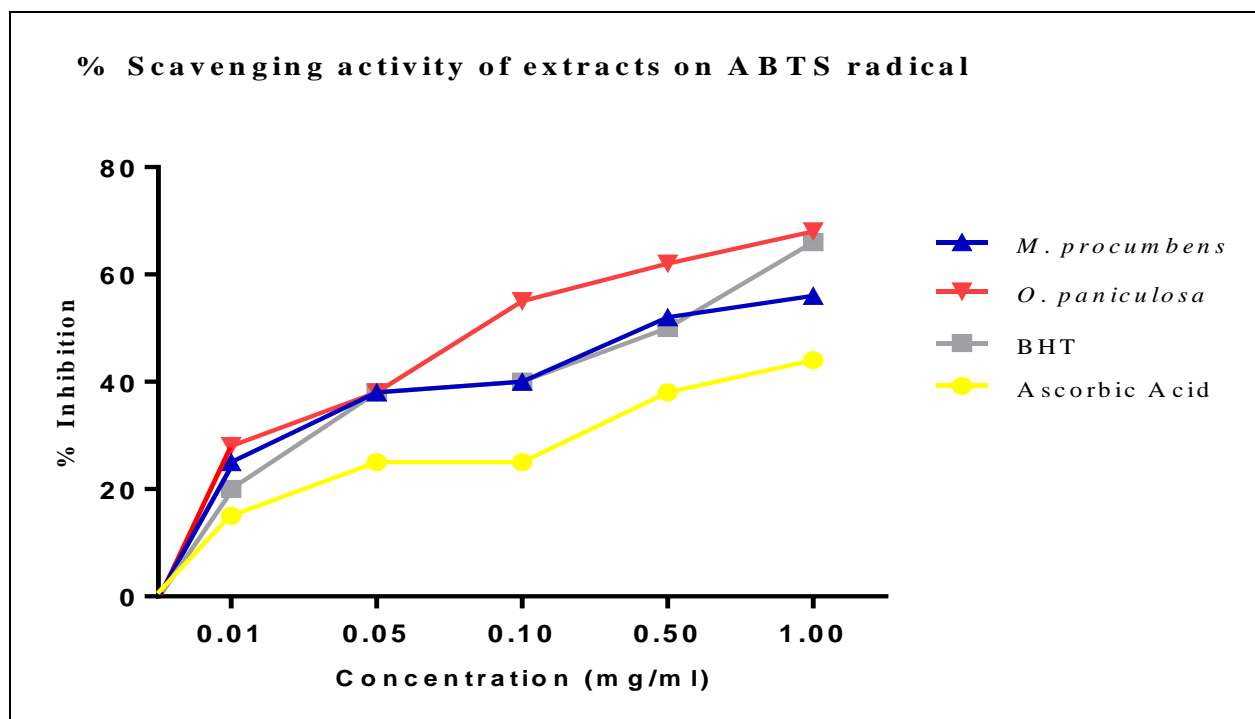
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## APPENDIX E

### E1 Graphs

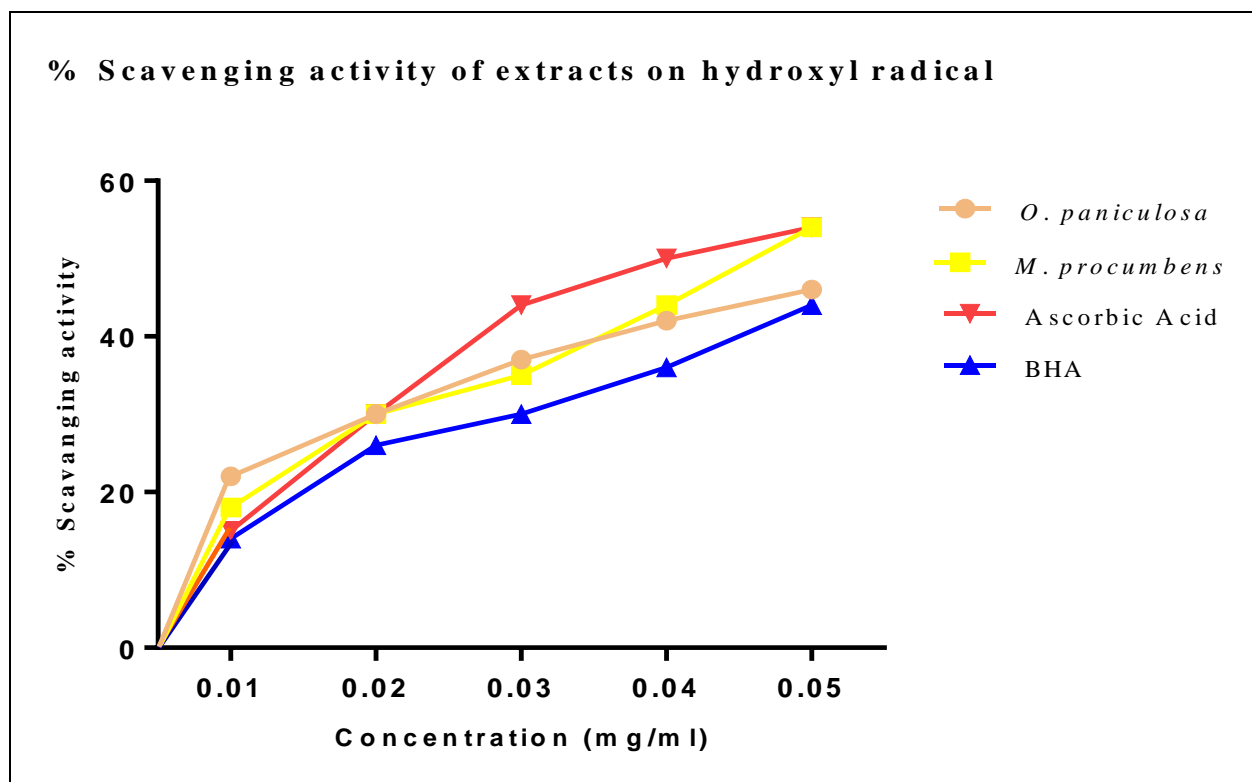


**Figure E1.1:** Percentage scavenging activity of the extracts of *M. procumbens* and *O. paniculosa* on DPPH radical.

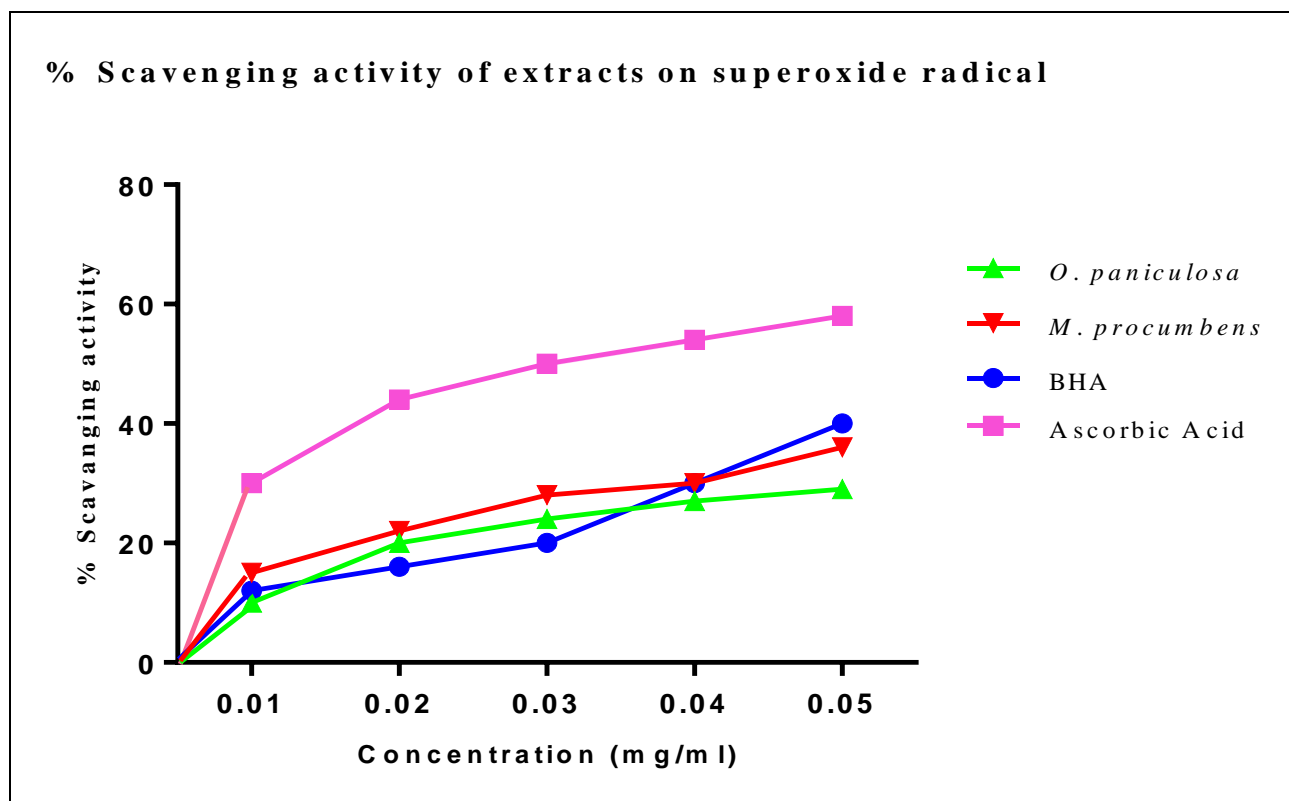


**Figure E 1.2:** Percentage scavenging activity of the extracts of *M. procumbens* and *O. paniculosa* on ABTS radical.

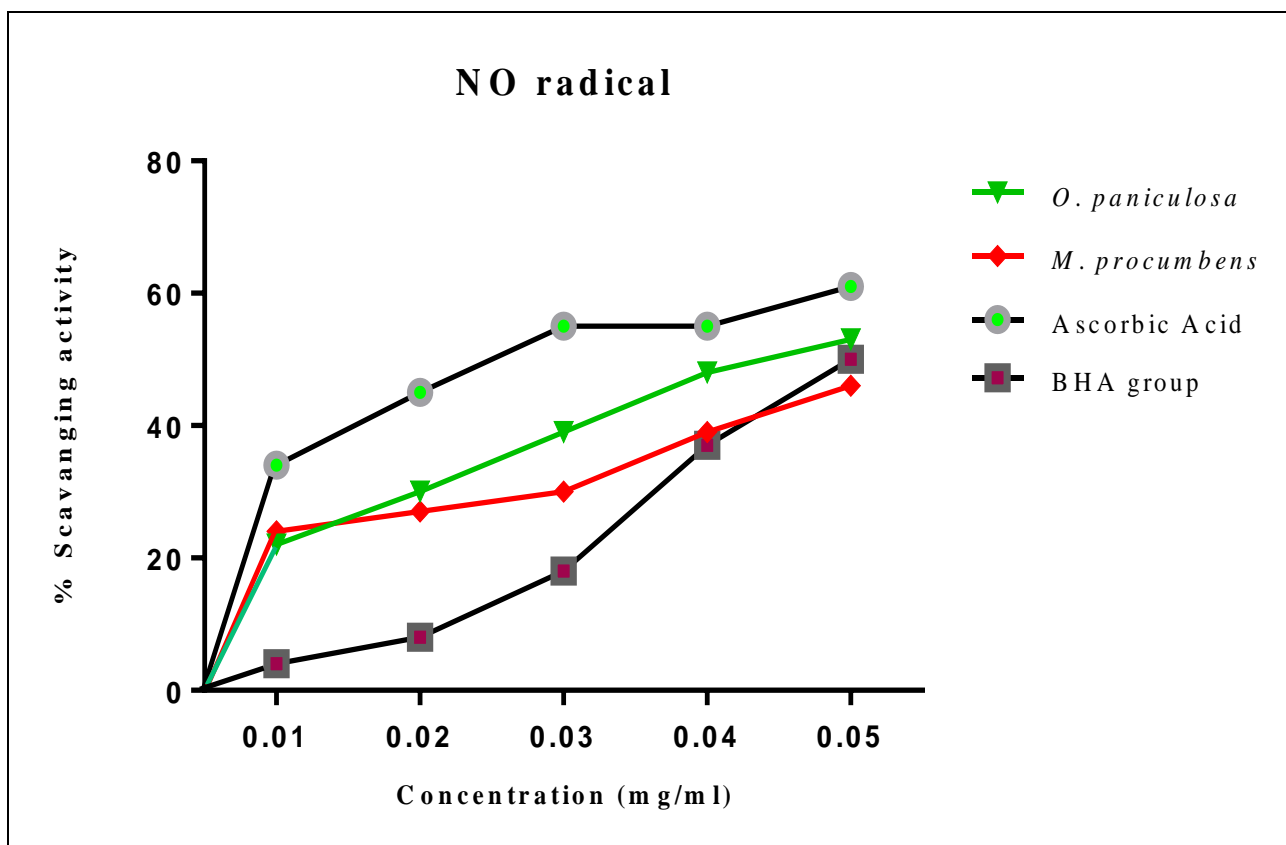




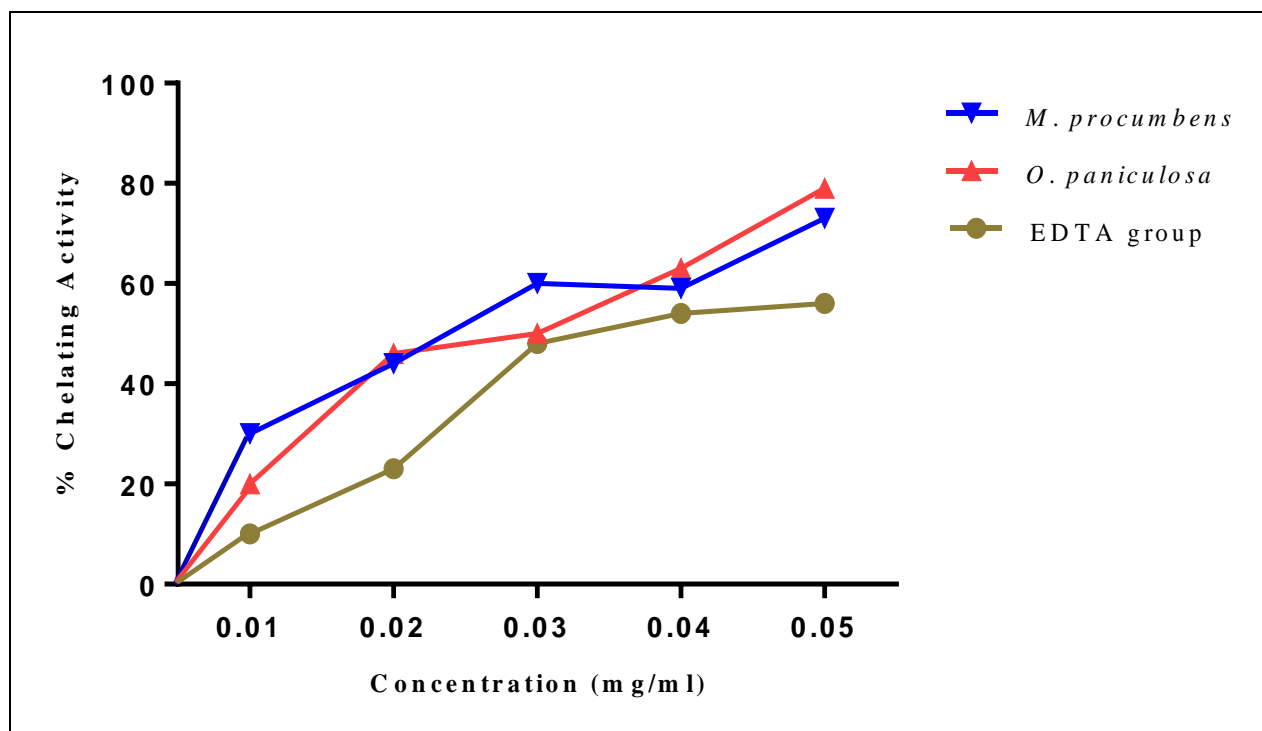
**Figure E1.3:** Percentage scavenging activity of the extracts of *M. procumbens* and *O. paniculosa* on hydroxyl radical. (BHA - Butylated hydroxyl-anisole).



**Figure E1.4:** Percentage scavenging activity of the extracts of *M. procumbens* and *O. paniculosa* on superoxide radical. (*O.p* – *O. paniculosa*; *M.p* – *M. procumbens*; BHA - Butylated hydroxyl-anisole; AA - Ascorbic acid).



**Figure E1.5:** Percentage scavenging activity of the extracts of *M. procumbens* and *O. paniculosa* on nitric oxide radical.



**Figure D1.6:** Percentage chelating activity of the roots extracts of *M. procumbens* and *O. paniculosa* on  $\text{Fe}^{2+}$ , Ethylenediaminetetra acetic acid (EDTA).

## APPENDIX F

### Contribution to knowledge



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#### Antioxidant and cytotoxic activity of some Zulu medicinal plants used to treat erectile dysfunction

**Background:** Oxidative stress is implicated as one of the major underlying pathophysiological causes of erectile dysfunction (ED). Therapeutics with high antioxidant capacity could be beneficial in the management of ED. This study investigated the cytotoxicity and antioxidant activity of the methanolic roots extract of *M. procumbens*, *O. paniculosa* and *C. bispinosa*.

**Materials and Methods:** The extracts were screened for their phytochemical composition. Their antioxidant activity was evaluated against a range of inorganic free radicals. Phosphodiesterase (PDE) inhibitory activity of the extracts was also investigated. The cytotoxicity of the extracts was evaluated against human embryonic kidney (HEK293), breast cancer (MFC7) and human colorectal (HT29) cell lines.

**Results:** The phytochemical screening showed the presence of cardiac glycosides, flavonoids, saponins and terpenoids. The extracts showed to a varying degree of efficacy, concentration dependent free radical scavenging and  $\text{Fe}^{2+}$  chelating activities. The  $\text{IC}_{50}$  values of the extracts ranged 0.006–9.44 mg/ml and 0.026–1.96 mg/ml on the free radicals scavenging and metal ion ( $\text{Fe}^{2+}$ ) chelating activities, respectively. *C. bispinosa* exhibited the lowest  $\text{IC}_{50}$  values on ABTS (0.006 mg/ml) and  $\cdot\text{OH}$  (0.008 mg/ml). The extracts also



# Indigenous Plant Use Forum

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Dear Mr Hbosinathi David Cele

The organising committee would like to inform you that your presentation

“Cytotoxicity and antioxidant activity of Zulu medicinal plants in erectile dysfunction”

has been accepted as a presentation for the 19th Indigenous Plant Use Forum 2016. The IPUF conference will take place on 4 to 6 July at Lily Lodge, in Port. St Johns, Eastern Cape. We thank you for your contribution.

We look forward to seeing you at IPUF 2016.

Kind regards

Ashton Reuters

IPUF Secretariat