# Ameliorative effect of the methanolic Crude extracts of *Inula glomerata* and *Salacia kraussii* on erectile dysfunction in Sprague Dawley rats

**By**

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## Declaration

The research project entailed in this dissertation was conducted in the department of Biochemistry and Microbiology, Faculty of Science and Agriculture, University of Zululand under the supervision of Prof. AR Opoku, Prof. GE Zharare and Dr. FO Osunsanmi.

This study represents the author's authentic work. The work of others used was duly acknowledged in the text.

I declare the above statement to be true.

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## Dedication

This work is dedicated first to God, for his divine provision, guidance and wisdom. Then to my father Mr Michael.A. Ojo, my biological mother, late Mrs Stella Manukwor Ojo and entire family (nuclear and extended) for their love and moral support.

## Acknowledgement

I want to first give God almighty all the praise and adoration. He made it happen and perfected it.

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My late biological mother for bringing me into this world and instilling in me the fear of God. I love you always mum.

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## Abstract

Erectile dysfunction (ED) is a common but multifaceted sexual disorder suffered by men with an adverse effect on their self-esteem and quality of life. Globally, the prevalence of ED has markedly increased as revealed by epidemiological studies. The use of conventional drugs has been reported to have side effects in addition to being unaffordable especially to rural dwellers. The leaves of *Inula glomerata* and roots of *Salacia kraussii* are among those herbal plants used by Zulu traditional healers to manage impotence. The study investigated the ameliorative effects of the methanolic crude extracts of the leaves of *Inula glomerata* and roots of *Salacia kraussii* on butanol-induced erectile dysfunction.

The plant materials were screened for their phytochemical's composition. The crude extracts were prepared from the plant materials by maceration using methanol. The *In vitro* antioxidant efficacy of the crude extracts was tested against DPPH and ABTS radicals. For *in vivo* studies thirty-five male Sprague Dawley rats were divided into seven groups (with five rats per group). The normal group, n-butanol, n-butanol+ *Inula glomerata* (50 and 250 mg/kg body weight), n-butanol+ *Salacia kraussii* (50 and 250 mg/kg body weight) and n-butanol+ Cialis (5 mg/kg body weight). The extracts were administered to the male rats orally by cannula every day for 28 days. Some sexual behaviour were monitored. In addition, the effect of the extracts on antioxidant status, the level of nitric oxide, testosterone and uric acid as well as acetylcholinesterase, ACE, arginase activities were assessed. The crude extracts' cytotoxicity was also determined using MTT assay.

The result showed that both plants contain tannins, flavonoids, terpenoids, and alkaloids. At a varying degree of efficiency, the crude extracts scavenged ABTS and DPPH radicals with *Salacia kraussii* ($IC_{50}$ $6.58\times 10^{-3}$ mg/ml) displaying a better scavenging activity than ascorbic acid ($IC_{50}$ $1.27\times 10^{-2}$ mg/ml). Furthermore, the results indicated that the plants boosted catalase and SOD activities and concomitantly increased the level of glutathione. The extracts also attenuated arginase, ACE and acetylcholinesterase activities at the same time elevated nitric oxide and testosterone level. In conclusion, both plants exhibited libido-boosting capacity and anti-erectile dysfunction efficacy. Nonetheless, due to their cytotoxicity, they require strict medical supervision before ingestion.

# List of abbreviations used

| | |
|---|---|
| AA | Ascorbic acid |
| ABTS | 2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) |
| ACE | Angiotensin(I)converting enzymes |
| AChE | Acetylcholinesterase |
| ALT | Alanine transaminase |
| AST | Aspartate transaminase |
| BHA | Butylated hydroxyl-anisole |
| BHT | Butylated hydroxyl toluene |
| CA | Citric acid |
| CAT | Catalase |
| DPPH | 2, 2-Diphenyl-1-picryl-hydrazyl |
| EDTA | Ethylenediaminetetra-acetic acid |
| GSH | Glutathione |
| $IC_{50}$ | Concentration to Inhibit 50% activity |
| Ig | *Inula glomerata* |
| I.glomerata | *Inula glomerata* |
| KZN | KwaZulu Natal |
| $LC_{50}$ | Lethal concentration at 50% |
| MDA | Malondialdehyde |
| $NO^{\cdot}$ | Nitric oxide radical |
| $O^{2\cdot-}$ | Superoxide anion radical |
| PDE | Phosphodiesterase |
| ROS | Reactive oxygen species |

| | |
|---|---|
| Sk | *Salacia kraussii* |
| S.kraussii | *Salacia kraussii* |
| SOD | Superoxide dismutase |
| TBA | Thiobarbituric acid |
| UZ | University of Zululand |
| WHO | World Health Organization |

# Contribution to knowledge

## Manuscripts submitted for publication

**Ojo MC,** Osunsanmi FO, Zharare GE, Mosa RA, Opoku AR. (2019). *In vitro* and *in vivo* antioxidant potentials of the methanolic crude extract from *Inula glomerata* and *Salacia kraussii*. Blacpma (under consideration).

**Ojo Mc,** Osunsanmi FO, Zharare GE, Mosa RA, Opoku AR. (2019). The effects of the methanolic crude extracts of *Inula glomerata* and *Salacia kraussii* on erectile dysfunction key enzymes. Chinese Journal of Natural Medicine (under consideration).

## Conference presentation (See Appendix E for details)

**Ojo MC,** Osunsanmi FO, Zharare GE, Mosa RA, Opoku AR. The ameliorative effect of *Inula glomerata* and *Salacia krausii* methanolic extracts on erectile dysfunction. Research in the Age of the Fourth Industrial Revolution. 25th October 2019, Faculty of Science and Agricultural Symposium, University of Zululand.

**Ojo MC**, Osunsanmi FO, Zharare GE, Mosa RA, Opoku AR. The ameliorative effect of *Inula glomerata* and *Salacia kraussii* methanolic extracts on erectile dysfunction. World Congress on Medicinal and Aromatic plants for Animal and Human Welfare (WOCMAP). November 13th-17th, Famagusta, Northern Cyprus.

# Table of Contents

## List of tables

# List of figures

# Chapter one

## 1.0 Introduction

Sexuality is regarded as the basic and physical need of every human irrespective of age and physicality/ physical state (Ramlachan and Campbell, 2014). Sexuality contributes immensely to the general well-being of an individual. It comprises of eroticism, intimacy, reproduction, sexual orientation, sexual activity, and pleasure. Sexual dysfunction, however, is self-reported conditions that affect the quality of life, psychosocial and emotional stability of males and females (Ramlachan and Campbell, 2014). Erectile dysfunction is a major and most common sexual dysfunction that is peculiar to men (Singh *et al*., 2012). Erectile dysfunction is the inability of getting and maintaining arousal for maximum sexual satisfaction (Hatzimouratidis *et al*., 2010). Its severity is known to be age-dependent. Statistical analysis by the international consultation committee for Sexual Medicine on Definitions/ Epidemiology/Risk factors for sexual dysfunction shows that men advanced in age are the most affected (Lewis *et al*., 2010). According to Ayta *et al* (1999), the number of people suffering from erectile dysfunction will skyrocket to 322 million worldwide by the year 2025 (Bacon *et al*., 2003; Aytac *et al*., 1999).

Previous studies revealed that erectile dysfunction is an early sign of coronary artery diseases and also shares common risk factors with several pathological conditions including diabetes, oxidative stress, smoking, metabolic syndrome, hypertension and dyslipidemia (Lewis *et al*., 2010; Dong *et al*., 2011; Turek *et al*., 2013). Furthermore, injury to the spinal cord and excessive use of certain medications can as well cause erectile dysfunction (Ojewole, 2007). One practical way of detecting erectile dysfunction in a man is to make a male patient complete a standardized questionnaire modeled by the

international index of erectile dysfunction- erectile dysfunction-5 (IIEF-5) (Rosen *et al*., 1999). Therapeutic approaches for the treatment of erectile dysfunction include increase biosynthesis of nitric oxide, elevated intracellular level of cyclic guanosine monophosphate (cGMP) and inhibition of phosphodiesterase-5 (PDE-5). However, contemporary treatment of erectile dysfunction employs mainly the use of oral phosphodiesterase-5-inhibitors such as Sildenafil (Viagra), Vardenafil (Levitra) and Tadalafil (Cialis). The use of these patient-friendly allopathic drugs has major setbacks due to associated side effects, inaccessibility and unaffordability by low-income earners. To this end, compounds from medicinal plants have been identified as an ideal replacement because they are cheap, ubiquitous and most importantly have little side effects if any. In Africa, precisely South Africa, male rural settlers prefer to patronize "Sangomers" for most of their health challenges including erectile dysfunction. Ethnobotanical survey of traditional healers around Kwa-Zulu Natal indicated that the roots of *Salacia kraussii* and leaves of *Inula glomerata* are used for treating erectile dysfunction.

To the best of the author's knowledge, although *S. kraussii* and *I. glomerata* are used in trado-medicine for erectile dysfunction management, they are yet to be scientifically validated. Therefore, the study evaluated the ameliorative effects of the methanolic crude extracts of these plants on butanol-induced erectile dysfunction.

### 1.1 Problem statement

Erectile dysfunction is a global health challenge that negatively impacts the psychosocial, emotional stability and quality of life of the affected individual (Ramlachan and Campbell, 2014). Men over the age of forty years are susceptible to erectile dysfunction and studies

have emphasized that erectile dysfunction becomes more severe with age advancement (Lewis *et al*., 2010). Furthermore, epidemiological studies have pointed out that erectile dysfunction is a predictor of several cardiovascular diseases including stroke, hypertension and coronary artery disease (Lewis *et al*., 2010; Dong *et al*., 2011; Turek *et al*., 2013). In South Africa, erectile dysfunction contributes to the increased divorce rate among young couples (Mohlatlole *et al*., 2017). According to the statistical report of Ayta *et al*. 1999, 322 million people worldwide have been projected to be affected with erectile dysfunction by the year 2025 (Ayta *et al*., 1999; Bacon et al., 2003).Although conventional pharmacotherapeutic drugs are efficacious, they have associated side effects including syncope, flushing, priapism, dyspepsia, colour blindness and headaches (Ojewole, 2007). Coupled with the fact that they are expensive and out of reach to peasant rural inhabitants. Furthermore, rural dwellers consult traditional healers for most of their health challenges including erectile dysfunction and as well use herbal plants to increase libido and boost their sexual performance (Ramlachan and Campbell, 2014). Sometimes, they even combine herbal medicine with orthodox medication. Ethnobotanical survey reveals that the leaves of *Inula glomerata* and roots of *Salacia kraussii* are used by “sangomers” for erectile dysfunction treatment but are without scientific backing. Therefore, the study aim at providing a scientific template on the efficacy of these plants on erectile dysfunction, the recommended doses, and cytotoxicity.

## 1.2 Hypothesis

*Inula glomerata* and *Salacia kraussii* possess the potential to reverse erectile dysfunction through the following mechanism of actions below:

- Boost nitric oxide and testosterone level
- Attenuate arginase, phosphodiesterase-5, acetylcholinesterase, and angiotensin(I)converting enzymes activities
- Modulates *in vivo* antioxidant status
- Exhibit *in vitro* scavenging free radicals

## 1.3 Scope of the work

### 1.3.1 Aim

The aim of this study was to evaluate the ameliorative effect of the methanolic crude extracts of Inula glomerata and Salacia kraussii on erectile dysfunction.

### 1.3.2 Objectives

i. To collect, botanically identify the plants, and extract with methanol.
ii. To screen the plants for their phytochemicals.
iii. To determine the cytotoxicity and *in vitro* antioxidant activity of the extracts.
iv. To investigate the *in vivo* antioxidant potentials of the plants' crude extracts using male Sprague-Dawley rats.
v. To estimate the *In vivo* inhibitory effect of the crude extracts of the plants on arginase, acetylcholinesterase, and phosphodiesterase-5 as well as the level of testosterone, nitric oxide, and uric acid.

vi. To evaluate the effect of the plants' crude extracts on liver AST and ALT.

### 1.4 Structure of the dissertation

This dissertation consists of 7 chapters and appendices

**Chapter One**

Summary and motivation for the study.

**Chapter Two**

Literature review on the study as well as the aim and objectives of the study

**Chapter Three**

Materials and methods used for the experiments of the study

**Chapters 4 and 5 are written as research papers**

**Chapter Four**

*In vitro* and *in vivo* antioxidant potentials of the methanolic crude extract from *Inula glomerata* and *Salacia kraussii*

**Chapter Five**

The effects of the methanolic crude extracts of *Inula glomerata* and *Salacia kraussii* on erectile dysfunction key enzymes.

**Chapter Six**

General discussion on the whole study

**Chapter seven**

General conclusion as well as recommendations for further studies and limitations of the study.

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# Chapter Two

## 2.0 Literature review

Erectile dysfunction is the persistent difficulty in attaining and maintaining an erection for sexual satisfaction (NIH,1993; Hatzimouratidis *et al*., 2010). Although erectile dysfunction is not a life-threatening condition, it can affect self- esteem and quality of life of the sufferer (Gao *et al*., 2017). The severity of erectile dysfunction is age-dependent, as it is known to be common clinical symptoms in aging men (Lewis *et al*., 2010; Yassin *et al*., 2016). Worldwide, erectile dysfunction is prevalent and according to the statistical report of Aytac et al (1999), about 320 Million men will be affected by the year 2025 (Bacon et al., 2003; Ayta *et al*., 1999).

Previous studies have shown there is a direct relationship between age advancement and erectile dysfunction severity. Besides erectile dysfunction also shares common risk factors with cardiovascular and coronary artery diseases such as diabetes, hypertension, metabolic syndrome, sedentary lifestyle, dyslipidemia, lower urinary tract syndrome, depression, overweight, smoking and obesity (Feldman *et al*., 2000; Fung *et al*., 2004). In addition, Injury to the spinal cord (T6-L5, the cord level range), radical prostatectomy, chronic use of certain medications such as antidepressants, antipsychotics, antihypertensives, and diuretics have also been linked with the pathophysiology of erectile dysfunction (Ojewole, 2007). More so, erectile dysfunction is also regarded as an early sign of coronary artery disease, stroke and cardiovascular diseases (Lewis *et al*., 2010; Dong *et al*., 2011;Turek *et al*., 2013). One practical way of detecting erectile dysfunction in a man is to make a male patient complete a standardized questionnaire modeled by the international index of erectile dysfunction- erectile dysfunction-5 (IIEF-5).

The IIEF-5 from a full scale of IIEF-15 consists of 2, 4, 5, 7 and 15. A sum score of 21 or less is tantamount to the patient having erectile dysfunction (Rosen *et al*., 1999). In order to fully comprehend the pathogenesis of erectile dysfunction and the possible way to reverse it, it is imperative to first understand the physiology of penile erection.

### 2.1 Physiology of Penile erection

Penile erection is a coordinated neurovascular event that involves the nervous, vascular, and endocrine system as well as the penile tissues (target organ) (Anderson 2011; Fraga-Silva *et al*., 2013; Castello´-Porcar and Martı´nez-Jabaloyas, 2016). In the detumescence or flaccid state, the smooth muscle of the corpora cavernosa located in the penile tissues is tonically contracted, allowing less flow of blood basically for nutrition. When the body, however, is aroused from a sexual stimulus, acetylcholine released at the cavernous nerve terminal causes nitric oxides to be produced from the endothelial cells by nitric oxide synthase (Figure 2.1). At the same time, the non-cholinergic, non-adrenergic nerves also releases nitric oxide. The nitric oxide mediates relaxation of the smooth muscle via the NO-cGMP pathway leading to a surge of blood into the penile tissues and collapse of the tunica albuginea thereby causing venous occlusion and eventually resulting in erection (Anderson, 2011; Fraga-silva *et al*.,2013). In order to return the penis to the flaccid state, the first action is the desensitization of the cells from sexual stimulus via the breakdown of acetylcholine to acetate and choline extracellularly by acetylcholinesterase (Ojo *et al*., 2019b; Ojo *et al*., 2019c). Thereafter, intracellularly phosphodiesterase-5 catalyzes the breakdown of cGMP to $5^{1}$GMP thus restoring the intracellular level of calcium ion, and subsequently making the penis flaccid. Penile erection homeostasis is

also maintained by the antagonistic effect between contractants and relaxants component engineered by the inhibitory or excitatory/stimulatory neurotransmitters released from the central nervous system (Anderson, 2011; Fraga-silva *et al*.,2013). Uncontrolled activities of vasoconstrictors could lead to Prolong contractility of the smooth muscle thereby causing erectile dysfunction (Cassidy *et al*., 2016).

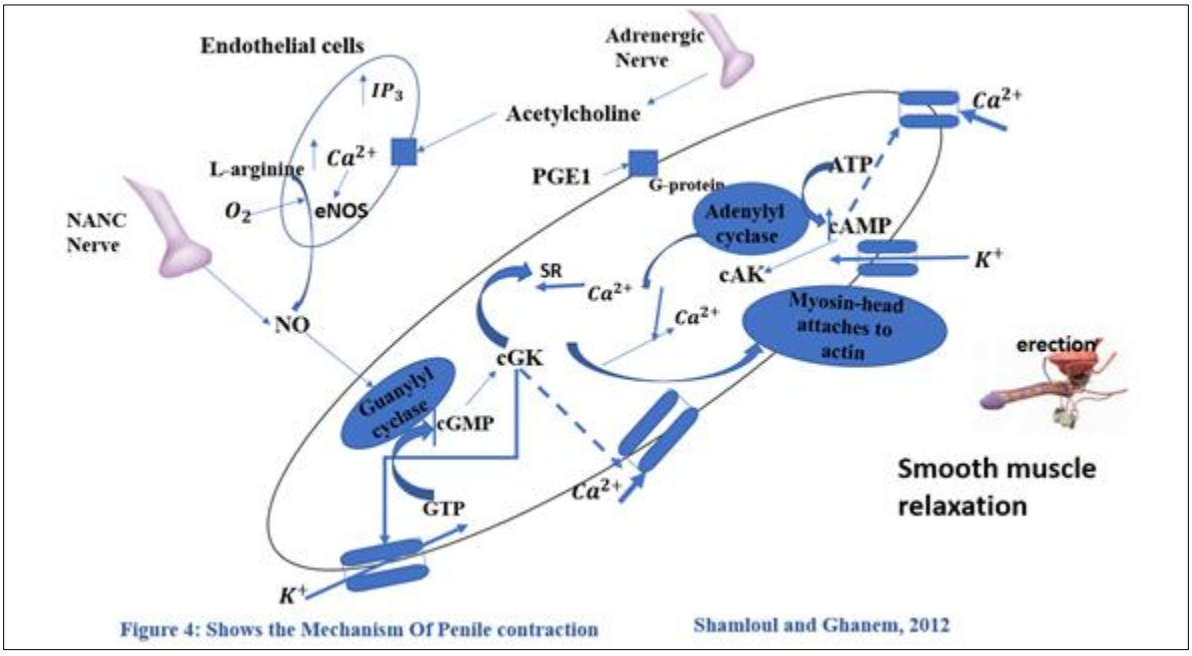


**Figure 2.1**: The mechanism of penile erection. Shamloul and Ghanem, 2012

## 2.2 Pathogenesis of erectile dysfunction

Erectile dysfunction pathogenesis is multifaceted (Figure 2.2). That is, it could result from diabetes, trauma (iatrogenic) and impediment in any of the neurovascular mechanisms involved in erection among which are: arterial (failure to fill), neurogenic (initiation abnormality) and venous (failure to store). Clinically, the etiology of Erectile dysfunction can be psychogenic (generalized or situational), organic (vasculogenic, neurogenic,

anatomic and endocrinologic) or mixed. Erectile dysfunction has been established to be more of mixed conditions however, organic causes still account for most cases of erectile dysfunction (Shamloul and Ghanem, 2013; Gareri *et al*., 2014; Bella *et al*., 2015; Olabiyi *et al*., 2017).

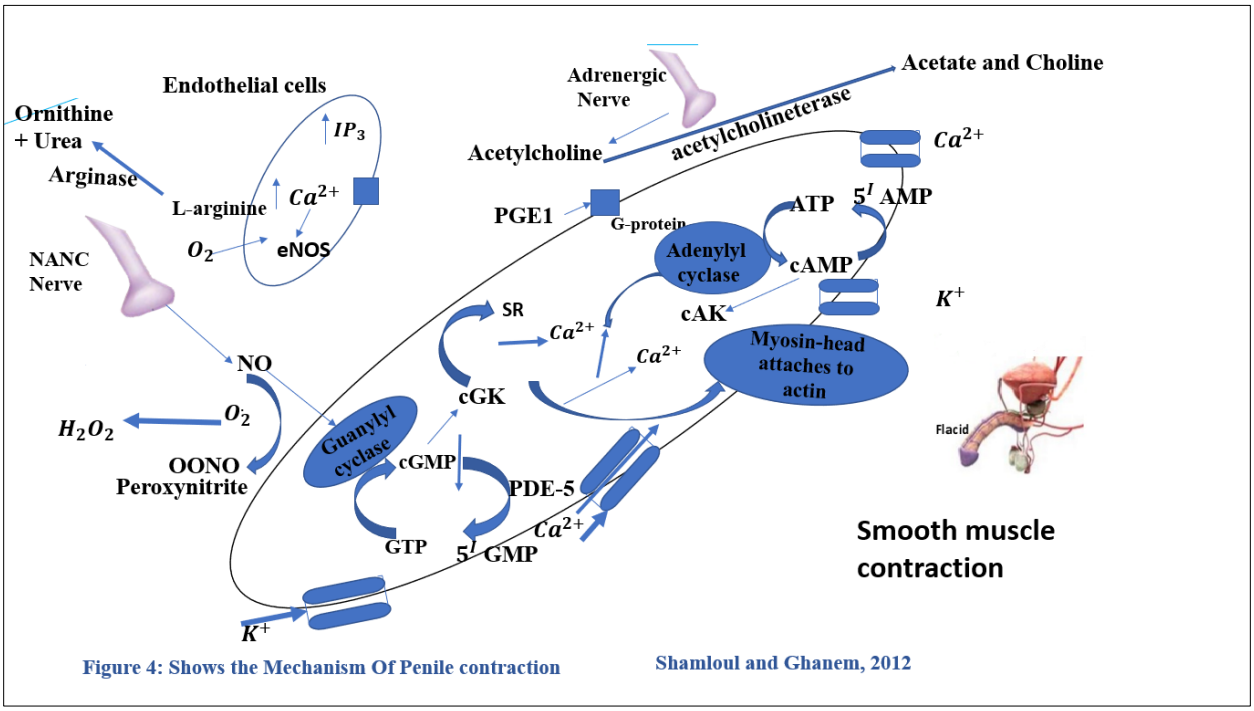


**Figure 2.2**: The mechanism of erectile dysfunction pathophysiology. Shamloul and Ghanem, 2012.

### 2.2.1 Psychogenic erectile dysfunction

These are set of predisposing, precipitating and maintaining factors that pose risks of erectile dysfunction in men such as traumatic past experiences, inadequate sex education, acute relationship problems, family or social pressures, physical or mental problems (Shamloul and Ghanem, 2013). Excitatory or Inhibitory impulses that emanate from the limbic system, hypothalamus, and cerebral cortex of the brain are responsible

for the central regulation of erection. Therefore, these impulses either facilitate or terminate erection consequently, irregularities in the type of impulses generated centrally can mediate erectile dysfunction (Bancroft, 2000). Take, for instance, a higher level of serum nor-epinephrine has been reported in men whose erectile dysfunction is psychogenic base than normal men and those with vasculogenic erectile dysfunction (Kim and Oh,1992). According to the international society of impotence research, psychogenic erectile dysfunction is further divided into generalized and situational ED (Dean and Lue, 2005).

### 2.2.2 Neurogenic erectile dysfunction

This type of erectile dysfunction result from iatrogenic or neurological disorders. Neuropathies including Parkinson's disease, stroke, Alzheimer's disease, Shy-Drager syndrome, and temporal lobe epilepsy that affects the area of the brain (medial preoptic area, paraventricular nucleus and the hippocampus) known as the integration center for penile erection leads to neurogenic ED. In addition, radical prostatectomy, perineal prostatectomy for benign disease and abdominal perineal resection, pelvic as well as spinal cord damage cause injuries to the cavernous nerves, a major underlying factor that predisposes to this kind of erectile dysfunction (Dean and Lue, 2005; Shamloul and Ghanem, 2013).

### 2.2.3 Endocrinological erectile dysfunction

Hypogonadism which refers to low level of testosterone is the hallmark of endocrine based erectile dysfunction. Testosterone controls nitric oxide synthase and phosphodiesterase-5 expression as well as maintains the ultra-structural integrity of

corpora cavernosa in penile tissues (Shabsigh et al., 2006; Castello´-Porcar and Martı´nez-Jabaloyas, 2016; Helo *et al*., 2018). Furthermore, testosterone stimulates sexual interest, increases the frequency of sexual acts and boosts nocturnal tumescence but has little or no effect on sexual arousal from vision and sexual thoughts (Dean and Lue, 2005; Shamloul and Ghanem, 2013). Therefore, factors such as hyperprolactinemia, high and low thyroid level that reduce testosterone level contributes to endocrinological erectile dysfunction (Shamloul and Ghanem, 2013).

### 2.2.4 Arteriogenic erectile dysfunction

The blockage of the arteries by atherosclerosis or traumatic arterial occlusive diseases which lead to low perfusion pressure and arterial blood flow into the corpora cavernosa and corpus spongiosum causes arteriogenic erectile dysfunction (Dean and Lue, 2005). Endothelial dysfunction a mjor contributor of arterial erectile dysfunction has been recognized as the main underlying triggers of several risk factors such as hypertension, cigarette smoking, diabetes mellitus, pelvic irradiation and blunt perineal (Shamloul and Ghanem,2013).

### 2.2.5 Vasculogenic erectile dysfunction

Impeded veno-occlusion is the main manifestation of vasculogenic erectile dysfunction. Peyronie disease and acquired venous shunts that result from surgical correction of priapism and old age are also associated with vasculogenic erectile dysfunction (Dean and Lue, 2005).

## 2.3 Treatment of erectile dysfunction

The therapeutic approaches to erectile dysfunction depend on the etiology. Counseling is mainly recommended for men suffering from psychogenic erectile dysfunction. However, oral phosphodiesterase-5-inhibitors are the first line of therapy for treating most erectile dysfunction causes. The Second line of treatment such as Alprostadil and vacuum devices are advised for patients with diabetes and nerve damage from radical prostatectomy due to unresponsiveness to oral phosphodiesterase-5- inhibitors. When all other treatment modalities fail, surgical implantation of penile prostheses becomes the last resort (Rew and Hiedelbaugh, 2016). Nevertheless, understanding the molecular mechanism of penile erection has led to the discovery of several new therapeutic targets and a plethora of medical strategies for enhancing penile erection or reversing erectile dysfunction. Among these are; upregulation of nitric oxide production, increasing the intracellular concentration of cGMP and eradicating oxidative stress (Decaluwe et al., 2014).

### 2.3.1 Upregulation of Nitric Oxide

Nitric oxide a gaseous neurotransmitter is the main mediator of penile erection. It is secreted by the neuronal and endothelial nitric oxide synthase from the nitrergic neuron and the endothelium of the arteries of the penis respectively (Thorve *et al*., 2011; Fraga-silva *et al*.,2013). Nitric oxide exerts its pharmacological effect on penile erection by relaxing the smooth muscle of corpora cavernosa via the cGMP signaling pathway (Fraga-silva *et al*.,2013; Decaluwe *et al*, 2014). The bioavailability of nitric oxide is regulated mainly by arginase activities (Berkowitz *et al*., 2003; Goswami *et al*., 2014).

Acetylcholinesterase activities, as well as the level of testosterone, also affect nitric oxide production (Oboh *et al*., 2015; Olabiyi *et al*., 2017; Ojo *et al*., 2019c). Therefore, attenuating acetylcholinesterase and arginase activities as well as administration of oral L-arginine boost nitric oxide level consequently, serve as therapeutic approaches to alleviating erectile dysfunction (Decaluwe *et al*, 2014).

### 2.3.2 Arginase Inhibition

Nitric oxide is biosynthesized endogenously from L-arginine and molecular oxygen via a reaction catalyzed by nitric oxide synthase. Arginase competes for L-arginine hence, reduces nitric oxide generation and subsequently impedes the vascular function of the penile tissues (Ilies *et al*., 2011; Segal *et al*., 2012). Hyperactivities of arginase culminate into endothelial dysfunction which is associated with the pathophysiology of erectile dysfunction (Goswami *et al*., 2014; Oboh et al., 2017). Studies have shown that inhibition of arginase help restores endothelial function, lowers collagen depositions and fibrosis hence improves erection (Bivalacqua *et al*., 2007; Toque *et al*., 2011; Segal *et al*., 2012).

### 2.3.3 Acetylcholinesterase Inhibition

Acetylcholine is a neurotransmitter that improves erection by inducing the release of nitric oxide from vascular endothelium (Adefegha *et al*., 2018). Acetylcholinesterase, on the other hand, is the enzyme that maintains acetylcholine homeostasis as a result, regulates neurotransmission and sensitization of the corpora cavernosa in penile tissues to sexual stimuli. Increase activities of acetylcholinesterase have been implicated in the pathogenesis of erectile dysfunction. This suffices that inhibition of this enzyme have

beneficial effects in maintaining an adequate erection (Adefagha *et al*., 2018; Ojo *et al*., 2019c).

### 2.3.4 Angiotensin-converting enzymes (ACE) Inhibition

Angiotensin-converting enzymes (ACE) plays a major role in renin-angiotensin system (RAS) that modulates cardiovascular homeostasis (Jin, 2009; Fraga-Silver *et al*., 2013; Adefegha *et al*., 2018). ACE converts angiotensin-1 to angiotensin-2 and degrades bradykinin, a known vasodilator. Angiotensin (II) is known to cause deleterious effects including fibrosis, proliferation, vasoconstriction and oxidative stress (Jin, 2009; Fraga-Silver *et al*., 2013). In addition, angiotensin (II) produced in erectile tissues is markedly higher than those in systemic circulation hence regulates smooth muscle contractility and tone. Therefore, hyper activities of ACE have been linked in the pathogenesis of erectile dysfunction. The use of angiotensin receptor blocker (ARB) or angiotensin-converting enzyme inhibitors are effective modalities against erectile dysfunction ((Jin, 2009; Fraga-Silver *et al*., 2013; Adefegha *et al*., 2018; Ojo *et al*., 2019c).

### 2.3.5 Oxidative stress elimination

The interplay between free radicals or reactive species and antioxidants is fundamental for the survival of living organisms (Halliwell, 2010). Free radicals are produced from endogenous (oxidative phosphorylation, auto-oxidation of unstable biomolecules and phagocytosis) and exogenous (X-ray, industrial chemicals and ozone) sources (Halliwell, 2010; Lobo *et al*., 2010). At a normal level, reactive species mediate important physiological functions including stimulating an immune response, preventing prolong inflammation and over-activation of T-lymphocytes as well as promoting adaptation to ischemia and exercise (Halliwell, 2010). However, the overproduction of free radicals

leads to oxidative stress. Oxidative stress is the imbalance between reactive species or free radicals and free radicals' scavengers tilting in the favour of free radicals' generation (Ojo *et al*., 2019a). Oxidative stress which is characterized by elevated reactive species, causes protein and DNA damage, abortive apoptosis, lipid peroxidation, and oligoasthenoteratozoospermia thus, resulting in oxidative damage (Figure 2.3) (Halliwell, 2010; Buford, 2016; Majzoub and Agarwal, 2017). The level of malondialdehyde (MDA), a known biomarker of lipid peroxidation, is increased during oxidative damage (Zhao *et al*., 2017; Oladipo *et al*., 2018). Oxidative damage contributes to the development and pathology of neurodegenerative, aging and age-related diseases including cancer, Alzheimer's, diabetes, male infertility, hypertension and erectile dysfunction (Tsao and Deng, 2004; Halliwell, 2010; Buford, 2016; Majzoub and Agarwal, 2017). Antioxidants which comprise of diet-derived and endogenously synthesized antioxidants such as superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), peroxiredoxins, flavonoids, and polyphenols, modulate reactive species' production and action, hence, prevent oxidative damage (Halliwell, 2010; Lobo *et al*., 2010; Lavanya *et al*.,2019).

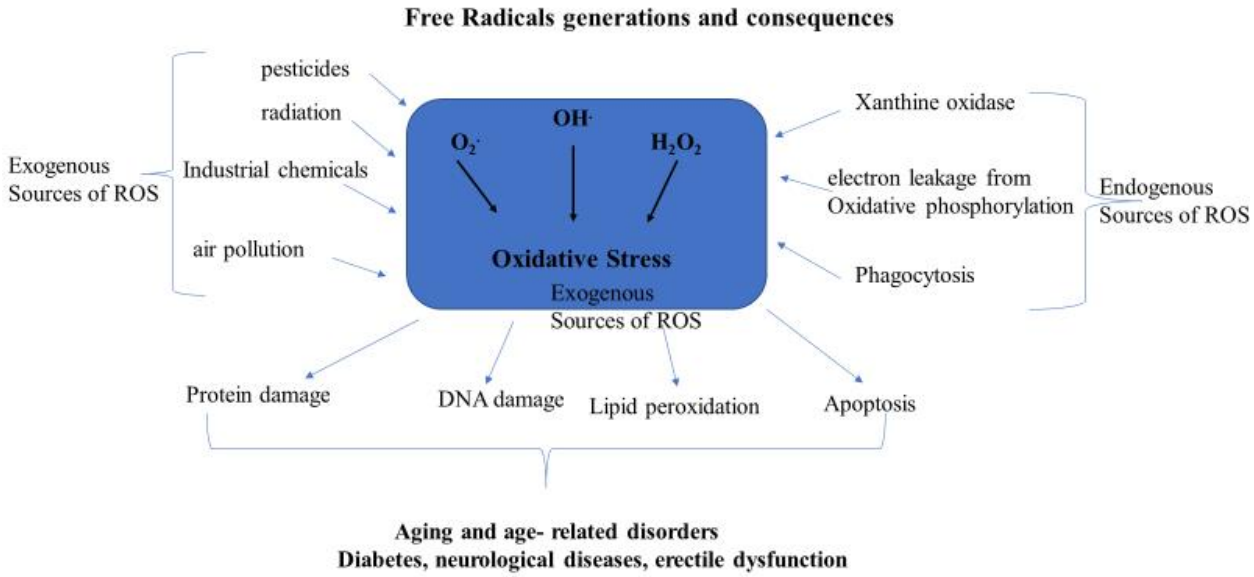


**Figure 2.3: different sources of reactive oxygen species generations leading to oxidative stress and consequently resulting in aging and age-related disorders including diabetes, neurological diseases, erectile dysfunction, cancer (Lobo *et al*., 2010; Majzoub and Agarwal, 2017).**

Despite the plethora of therapies available for the treatment of erectile dysfunction, the scourge continues to beset men of all works of life. Furthermore, the use of these therapies such as Vardenafil, Cialis, Levitra etc has encountered numerous setbacks because of the associated side effects including headaches, facial flushing, muscle pain dyspepsia, and retinal impairment. Also, these pharmaceutical drugs are unaffordable to low-income earners and inaccessible to most rural settlers. Therefore, there is a need for an alternative approach with better efficacy of which natural compounds from medicinal plants have caught the interest of researchers. Since these natural bioactive compounds are cost-effective, accessible and most importantly has little or no side effects (Noureen *et al*., 2017).

## 2.4 Medicinal Plants

Plants are predominantly used in folklore medicine for curative purposes. It is fast becoming ubiquitous in modern society as alternatives to allopathic medicine (Dey and De,2015). Phytomedicine is an ancient practice that involves the use of plants (whole or parts) which includes the rhizome, leaves, stem, flowers, fruits or roots as therapy or maintain the general well-being of an individual (Kurup *et al*.,1993; WHO,2017). Herbal medicines also known as phytomedicine is generally affordable, readily available and culturally more acceptable because of the idiosyncrasy that they cause little or no side effects than pharmacological drugs (Carlson,2002; Dey and De,2015). In Africa, a large chunk of the population makes use of medicinal plants to manage several diseases (Neuwinger,1996; NIH,2003). South Africa alone accounts for 9% of the higher plants globally with more than 30,000 plant species (Van Wyk and Gericke,2000). As a matter of fact, more than 3000 species of these plants are presently utilized in the treatment, management or cure of numerous ailments by over 200,000 traditional healers (Balogun and Ashafa,2019). In South Africa, the effort has been intensified into investigating the pharmacological potentials of medicinal plants against erectile dysfunction. A handful of plants have been scientifically elucidated and well documented to possess ameliorative efficacy against erectile dysfunction (table 2.1). The mechanism of action with which these plants exert their anti-erectile dysfunction pharmacological effect however, differs. Most medicinal plants effect their erectogenic properties by modulating the activities of key enzymes involved in erectile dysfunction such as phosphodiesterase-5, arginase, acetylcholinesterase and angiotensin-I-converting enzymes as well as boosting antioxidant status and increasing serum level of nitric oxide (Akomolafe *et al*., 2016; Oboh

*et al*., 2019; Ojo *et al*., 2019c; Ademosun *et al*., 2019). Few plants reverse testicular dysfunction, increase testosterone, follicle-stimulating and Luteinizing hormone level (Cele *et al*., 2017; Zhou *et al*., 2019). Similarly, isolated bioactive compounds of flavonoids and phenolic origins have been known to exhibit anti-erectile dysfunction properties (Oboh *et al*., 2015; Cassidy *et al*., 2016; Adefegha *et al*., 2018). Therefore, it suffices that the phytochemicals contained by plants confer on them broad bioactivities and a wide spectrum of specificities against diverse ailments or pathologies including erectile dysfunction (Adefegha *et al*., 2018).

**Table 2.1: Some medicinal plants with reported anti-erectile dysfunction efficacy**

| Medicinal plants | Parts used | Extract | Mechanism of action | Article |
|---|---|---|---|---|
| *Moringa oleifera* | Leaves | Aqueous | ACE and arginase inhibition, antioxidant property | Oboh *et al*., 2015 |
| *Ficus capensis* | Leaves | Aqueous | ACE, AChE and arginase inhibition as well as a free-radical scavenger | Akomolafe *et a*l., 2016 |
| *Maytenus procumbens* | Root | Methanol | testosterone and sperm count booster, antioxidant property | Cele *et al*., 2017 |
| *Lyscium chinensis* | Root bark | Ethanol | Antioxidant efficacy, LH, FSH and testosterone booster, sperm count, viability and motility enhancement | Zhou *et al*., 2019 |
| *Anogeisus leiocarpus* | Bark | Aqueous | PDE-5, arginase and AChE attenuation antioxidant activity | Ademosun *et al*., 2019 |
| *Spondias mombim* | Leaves | Ethyl acetate | ACE, PDE-5, and MAN inhibition | Ojo *et al*., 2019b |
| *Hunteria umbellate* | Seed | Aqueous | Arginase inhibition and NO upregulation | Oboh *et al*., 2019 |
| *Ocimum gratissium* | Leaves | Aqueous | AChE, ACE, PDE-5 and arginase attenuation with free radical scavenging activity | Ojo et al., 2019c |

**Keywords: LH-luteinizing hormone, FSH- follicle-stimulating hormone, NO- nitric oxide, PDE-5-phosphodiesterase-5, AChE-acetylcholinesterase, ACE-angiotensin(I) converting enzymes, MAN-monoamine oxidase A.**

This study, however, evaluated the ameliorative effect of two non-scientifically validated medicinal plants, *Inula glomerata* and *Salacia kraussii* used by traditional healers in Empangeni region of Kwazulu Natal province, South Africa for managing erectile dysfunction.

### 2.4.1 *Inula glomerata* Oliv. & Hiern

*Inula glomerata* Oliv. & Hiern belongs to the family Asteraceae and the genus Inula (Figure 2.4). *Inula glomerata* grows to about 2m tall with a basal rosette leaves of 45 by 20cm in size that has irregularly toothed margin and endemic in Northern South Africa, Zimbabwe, Angola and Tanzania (Burrows and Willis, 2005). The roots of *Inula glomerata* are used for treating hypertension (oral communication). However, closely related species, *Inula racemosa* Hook.f. have been known to have a lot of bioactive phytoconstituents and bioactivities including anti-microbial and activity on the cardiovascular system (Tan *et al*., 1998; Wang *et al*., 2000).

**Figure 2.4: *Inula glomerata* growing in the wild**

www.Westafricanplants.Senckenberg.de/root/index.php. (9-04-18).

### 2.4.2 *Salacia kraussii* (Harv.) Harv

*Salacia kraussii* (Harv.) Harv commonly called Ibhonsi in South Africa belongs to the genus and family of hippocrateaceae and Celastraceae respectively. It is a creeping surfactant herb that gets to 1-3m long and produces juicy fruits when matured (Figure2.5). *Salacia kraussii* is scattered in Southern Zimbabwe, South Africa (Kwazulu Natal), and Mozambique (Raimondo *et al*., 2009). *Salacia kraussii* possesses anti-diarrhoea and anti-malaria efficacy (Bandeira *et al*., 2000).

**Figure 2.5: The leaves of *Salacia kraussii* with fruit (Hyde *et al*., 2018)**

### 2.4.3 Cytotoxicity of medicinal plant crude extracts

Traditional medicine or ethnomedicine is now gaining widespread popularity globally. However, proper documentation of dosage, possible side effects, reproducibility of preparation and preservation methods are setbacks that beset traditional/ethnomedicine (Rates, 2001; Reddy *et al*., 2015). Medicinal plants exhibit broad bioactivities mainly because of their phytochemicals. Nevertheless, these phytochemicals can sometimes

lead to allergic reactions, irritation of the gastrointestinal tracts, erythrocyte and liver damage as well as hyperuricemia and carcinogenesis (Shabsigh *et al*., 2006; Nondo *et al*., 2015; Chew *et al.,* 2014; Sajjadi *et al.,* 2015). For instance, Aristolochia spp contains aristolochic acid that causes kidney failure while bufadienolides found in Drimia sanguinea and Bowea volubilis is toxic to the heart (Debelle *et al*., 2008; Van der Bijl and Van der Bijl, 2012). Therefore, it is imperative alongside with the efficacy and mechanism of action of medicinal plants to determine their cytotoxicity. In order to know if a plant extract is cytotoxic, the lethal dose or concentration must first be estimated. The lethal dose or concentration ($LD_{50}$ or $LC_{50}$) of a plant crude extract is the concentration of the crude extract of the plant that can kill 50 % of the test organism's viable cells. The Brine Shrimp and MTT bioassays are two commonly used methods to evaluate the cytotoxicity of a plant's crude extract (Meyer *et al*., 1982; Mosmann, 1983).

The Brine Shrimp also known as sea monkey, is a crustacean that thrives in an aquatic environment. The toxicity potential of a test sample in Brine Shrimp bioassay is determined by counting the number of nauplii that survived after twenty-four (24) hours of exposure. The crude extract of a plant is said to be non-toxic, less toxic and highly toxic when $LD_{50}$ corresponds to over 1000 μg/ml, 500-1000 μg/ml, 100-500 μg/ml respectively (Clarkson *et al*., 2004). The Brine Shrimp bioassay has lots of benefits including simplicity of experiment, ease of handling, short exposure time, low cost and non-continuous culturing. The main drawback, however, is that the result cannot be synchronized with mammal toxicity (McGaw and Eloff, 2005).

The MTT assay hinges on the principle that mitochondria dehydrogenase from viable cells cleaves the tetrazolium rings of MTT (pale yellow) to form a dark blue formazan crystal.

These crystals are impervious to cell membrane hence accumulate in healthy cells. MTT reduction is tantamount to the numbers of cellular proteins and viable cells present (Mosmann, 1983; Miret *et al*., 2006). Under this bioassay, the crude plant extracts are termed to be toxic when they halt the cell viability and growth (Ifeoma and Oluwakanyinsola, 2013; NK *et al*., 2015). The toxicity of a crude extract or pure isolated compound is regarded as strongly, moderately and weakly toxic when $LC_{50}$ corresponds to 10-20, 20-100 and above 100 μg/ml respectively (Magadula, 2014). Quite a number of scientists have employed MTT bioassay to estimate the plant's toxicity with cell lines (Senthilraja and Kathiresan, 2015; Chan *et al*., 2015; Sharma *et al*., 2016; Mansoor *et al.,* 2016). Other models include cell-based toxicity, toxicokinetics and toxicogenomic screening assays (Mosmann, 1983).

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# Chapter Three

## 3.0 Materials and Methods

This chapter contains a list of the reagents and equipment used for the study. It also entails the methods for preparing the plants' crude extracts and performing biological assays. Appendices A and B are detailed preparation of some reagents as well as methods.

### 3.1 Materials

#### 3.1.1 Chemicals

The following chemicals were purchased from Sigma-Aldrich Co. Ltd (Steinheim, Germany) - 1.1-Diphenyl-2-picryl hydrazil (DPPH), Dragendroff's reagent, 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS), DNA, Butylated hydroxyl toluene (BHT), 4,41- [3-(2-pyridinyl)-1,2,4-triazine-5,6-dryl] bisbenzene sulphonic acid (ferrozine), Butylated hydroxyl-anisole (BHA), caffeine, Ascorbic acid (AA), Gallic acid, Tris-HCl, Sodium nitroprusside, Ethylenediaminetetraacetic acid (EDTA), Thiobarbituric acid (TBA), Folin-Ciocalteu Phenol reagent, Ferrous ammonium sulfate, Bovine Serum Albumin (BSA), Ammonium molybdate, Hydrogen peroxide, s-acetyl-thiocholineiodide (ASChI), methanol, *n*-butanol, tacrine.

#### 3.1.2 equipment

96-well microplates- (Sigma), Eppendorf, centrifuge 5804 R – (Merck), Platform shaker (Labcon) - Polychem supplies, Rotary evaporator – (Heidolph Instruments)**,** Synergy/HT microplate reader- (BioTek).

## 3.2 Methodology

### 3.2.1 Collection and extraction of plant materials

The roots of *Salacia kraussii* (Harv.) Harv and leaves of *Inula glomerata* Oliv. & Hiern were collected from Mbazwana ($27^0$ 15'11.3" S $32^0$28'14.3" E) KwaZulu Natal, South Africa. The plants were authenticated at the Department of Botany, the University of Zululand by Dr. Ntuli. The plants with specimen number V04 and V06 have been deposited at the university's herbarium. Thereafter, the roots and leaves of *Salacia kraussii* and *Inula glomerata* respectively were cleaned, washed, air-dried and pulverized into fine powder. The pulverized samples (200 mg) were extracted with methanol (1:5 w/v) using a mechanical shaker (150 rpm; $25^0$C) for 72 h. The extract was filtered using Whatman filter paper (no.1) and concentrated using Heidolph rotary evaporator (90 rpm, $40^0$C). The crude extracts were then kept in brown sterile bottles and stored in the refrigerator until use.

### 3.2.2 Phytochemical analysis

The methods of Odebiyi and Sofowora, (1978) and Harbone, (1973) were used to Qualitatively screen for the presence of Phyto-constituents in the crude extracts. Alkaloids, Tannins, Saponins, steroids, terpenoids, and flavonoids were the basic phytochemicals assayed for.

### 3.2.3 Total phenolic content

The total phenolic contents of the crude extracts were estimated using the Folin-Ciocalteu reagent while following the method of Kujala *et al*. (2000). The Crude extract (0.2 mg/ml) was mixed with 1.5 ml dilute (1:10 v/v) Folin-Ciocalteu reagent and 1.2 ml of 7.5 % sodium

carbonate solution. The mixture was then allowed to stand for 30 min in the dark at room temperature. Gallic acid was used as standard and absorbance of the coloured mixture was read at 765 nm. The total phenolic content of the plant extract was determined as gallic acid equivalent from a calibration curve of garlic acid and expressed as mg/g dry plant material.

### 3.2.4 Total flavonoid content

The total flavonoid content of the crude plants' extracts was evaluated using the colorimetric method described by Ordonez *et al*. (2006). The extracts (0.2 mg/ml) was mixed with 0.5 ml of 2 % alcoholic aluminium chloride solution. Thereafter, the mixture was incubated for 1 hour at room temperature. Quercetin was used as standard and absorbance of the coloured mixture was read at 420 nm against the blank containing alcoholic aluminium chloride. The total flavonoid content of the extract was calculated from a calibration curve of quercetin and expressed as mg/g dry plant material.

## 3.3 *In vitro* antioxidant assays

The methanolic extracts of the plants were differently assessed for their antioxidant potential against several synthetic-free radicals. Unless when stated otherwise, all experiments were replicated at least three times and the radical scavenging activity of the methanolic crude extracts was calculated using the formula:

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

Where $A_c$ is the absorbance of the control and $A_t$ is the absorbance in the presence of the tested extract. $IC_{50}$ values of the extracts were determined using GraphPad Prism version 6.01.

### 3.3.1 Free radical scavenging activity

#### 3.3.1.1 1.1-Diphenyl-2-picryl hydrazil (DPPH) scavenging activity

The scavenging potential of the crude extracts on DPPH was investigated using Brand-Williams (1995) method. The DPPH solution (0.02 mg/ml methanol) was mixed (1:1 v/v) with the plant extract at different concentrations (0-0.05 mg/ml). The mixture was allowed to stand at room temperature for 30 mins and absorbance was read at 517 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) scavenging activity

The methanolic crude extracts of the plants' scavenging activity on ABTS was estimated using the method of Re *et al*. (1999). The mixture of 7 mM ABTS and 2.45 mM potassium persulfate was incubated in the dark for 16 hours to generate ABTS radical. Methanol (60 ml) was used to dilute 1 ml of the generated ABTS radical stock solution to give a working solution. The plant extract (0-0.05 mg/ml) was mixed (1:1) with ABTS' working solution and incubated for 10 min at room temperature. Absorbance was read at 734 nm. Methanol and ascorbic acid were used as negative and positive controls, respectively.

#### 3.3.1.3 Nitric oxide ($NO^{\cdot}$) scavenging activity

The method of Griess Illosvoy reaction (Badami *et al*., 2005), was used to calculate the number of nitrite ions produced from the reaction between Nitric oxide and molecular

oxygen. The reaction tube contained 0.5 ml each of the phosphate buffer saline (pH 7.4, 0.01 M) and different concentration (0-5 mg/ml) of the extract with 2 ml of 10 mM sodium nitroprusside. The resultant mixture was incubated for 150 min at 25 ˚C. Thereafter, 1.0 ml of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) was added to 0.5 ml pipetted from the reaction mixture (containing nitrite) and allowed to stand for 5 min for complete diazotization. Afterward, 0.1 ml of 0.1 % Naphthylethylenediamine dihydrochloride was added, then mixed and allowed to stand for 30 mins in diffused light. The absorbance was measured at 540 nm.

#### 3.3.1.4 Reducing power

The method described by Oyaizu (1986) was used to evaluate the reducing power of the crude extracts. The reaction mixture consisted of 2.5 ml each of 0.2 M phosphate buffer (pH 6.6) and 1 % potassium ferricyanide $K_3Fe(CN)_6$ with different concentration (0-5 mg/ml) of the crude extract. Afterward, the mixture was incubated for 20 min at 50˚C. 2.5 milliliter of 10 % TCA was then added after incubation and thoroughly mixed. The resultant mixture was Centrifuged at 1000 rpm for 10 mins. The supernatant (2.5 ml) was collected and mixed with distilled water (2.5 ml) and 0.5 ml 0f 0.1 % FeCl3. The absorbance was read at 700 nm. Ascorbic acid (0-5 mg/ml) and BHA (0-5 mg/ml) were used as standards.

Hint: The higher the absorbance value the stronger the reducing power.

### 3.4 Cytotoxicity assay

The cytotoxicity of the plant's methanolic extracts was investigated using the MTT assay as described by Mosman (1983). The cytotoxicity of the methanolic crude extracts was

evaluated against HEK 293 and Hela human cell lines. The cells were plated in a 96-well plate with cell suspensions of $1.8x10^4$ cells/ml concentrations. The cells were allowed to attach for 48 hours before being seeded with different concentrations of the crude extracts (100 mg/ml) using serial dilutions. After administering a media containing 1% of FBS, they were returned to the incubator for another 48 hours. Thereafter, the cell viability was estimated by removing the old medium and adding the (Merck) tetrazolium salt as a cytotoxicity indicator. The 100 µl medium was added to each well and incubated at 37$^o$C for 4 hours. The media with MTT was aspirated from the wells and the formed formazan crystals were solubilized in 100 µl of dimethyl sulfoxide (DMSO). The optical density of the solutions was measured at 570 nm using a Mindray-96A microplate reader. The Percentage inhibition of cell viability was calculated using the formula.

% cell death= $[(A_c-A_t)/A_c \times 100)]$

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

### 3.5 Animal study

Ethical clearance certificate (UZREC 171110-030 PGM 2018/576) was obtained from the University of Zululand Ethics committee for the use of laboratory animals and approval of procedures. The standard operating procedures for experimental animals were adhered to (PHS). Male and female Sprague-Dawley rats (250 g) were collected from the animal house of the Department of Biochemistry and Microbiology, University of Zululand. The male rats were kept separately from the female rats with five each in a cage. The animals were kept under conducive environmental conditions (25ºC; 12:12 light: dark cycle) with

free access to safe drinking water and pellet feeds. The animals were acclimatized for 5 days before the experiment commenced.

### 3.5.1 Effect of the crude extracts on erectile dysfunction in Sprague Dawley rats

The crude extracts' ameliorative effect on n-butanol induced erectile dysfunction was evaluated using the modified method of Garza *et al.* (2015). Thirty-five male Sprague Dawley rats (200-260 g) were acquired and after five days of acclimatization, they were divided into a normal and n-butanol group. Thereafter, the n-butanol group was administered with n-butanol (5.0mg/kg body weight) intraperitoneally for four days at two days interval to induce erectile dysfunction. Afterward, the sexual behaviours of the male rats were monitored by introducing them (one at a time) in a cage with two female rats on heat for thirty minutes in order to ascertain the baseline of their sexual activity. The heat was induced in the female rats by subcutaneously injecting each female rat with progesterone (7.5 mg/kg b.w) for 96 hours at 48 hours interval. Reduced mounting frequency in the male rats belonging to the n-butanol group as compared to the normal group was used as a confirmation that erectile dysfunction has been induced. Thereafter, the male rats in the n-butanol group were further divided into 6 groups (5 rats/group) and given daily oral medications for 28 days. Group 1, normal control, was given only water and pellet feed. Group 2, positive control, was treated with Cialis 5 mg/kg body weight (b.w). Group 3 and 4 were treated with 50 and 250 mg/kg b.w of *Inula glomerata* respectively. Group 5 was treated with 50 mg and group 6 with 250 mg/kg b.w of *Salacia kraussii*. Group 7, untreated group, received only water and feed pellets (see figure 3.1). The dosage used (50 and 250 mg) was chosen from previous studies by Cele et al. The food and water taken were recorded daily, weight change was recorded weekly

throughout the experiment. After the end of the experiment, the sexual behaviours of each rat was determined with the intromission and mounting times recorded. Thereafter, at the end of the experiment, the rats were fasted for 18 hr and then euthanized under anesthesia (pentobarbital@ 30 mg/kg body weight, intraperitoneally). Blood was immediately collected by open cardiac puncture for biochemical analysis.

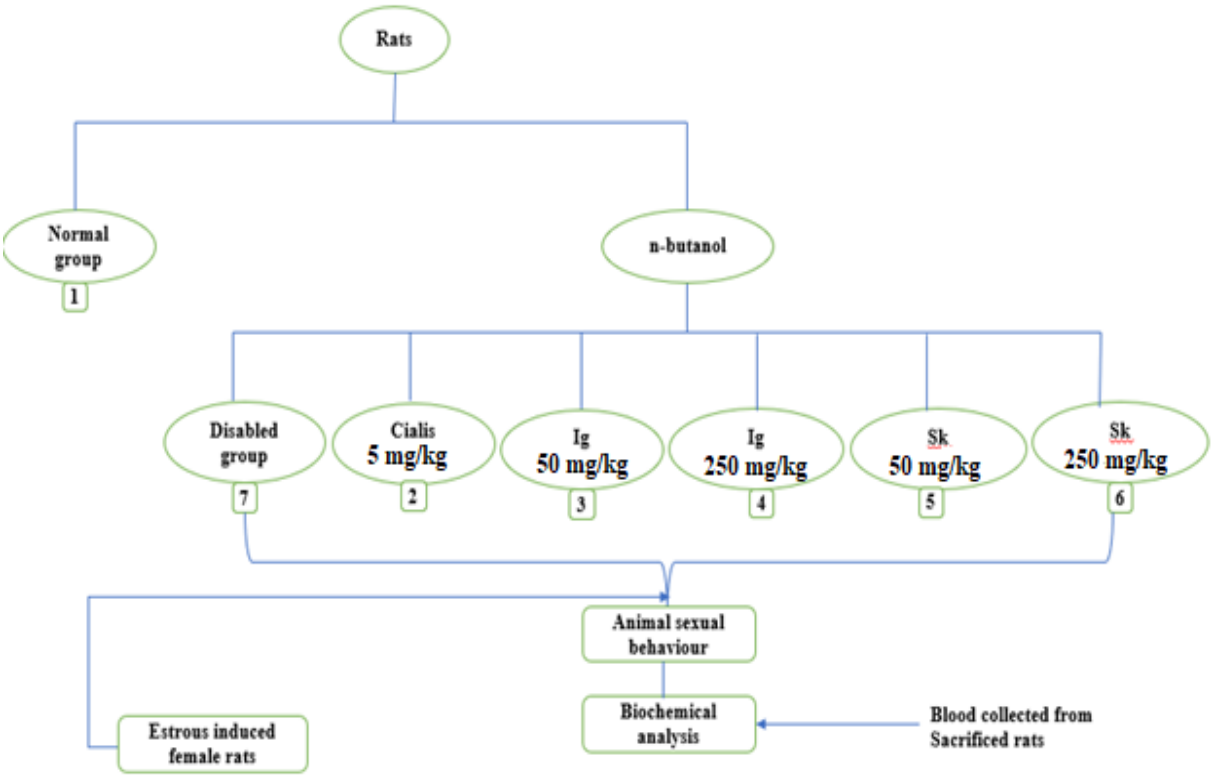


**Figure 3.1: The animal study design on the effect of the crude extracts of *Inula glomerata* and *Salacia krausii* will have on *n*-butanol induced erectile dysfunction in Sprague Dawley male rats. Keywords: Ig *Inula glomerata, Sk Salacia kraussii.***

## 3.6 Serum preparation

Blood samples were allowed to clot afterward, were centrifuged at 4000 rpm for 15 mins in Rotofix mini-centrifuge machine. The serum obtained (the supernatant) was stored at −80 °C for subsequent biochemical assays.

### 3.6.1 Biochemical analysis

The sera were analyzed at the Global laboratory and viral laboratory (Richards Bay) using standard pathological procedures for the measurements of testosterone, uric acid, alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

### 3.6.2 Determination of nitrite concentrations in blood samples

The accumulation of nitrite in the serum was determined following the method of Lidija *et al*., 2004. The serum and 125 µl of Griess reagent (0.1% N-1-napthylethyleneaminediyhdrochoride, 1% sulphanilic acid and 2.5% phosphoric acid) was mixed and plated in a 96- well microplate. Thereafter, it was incubated at room temperature for 10 mins and the absorbance was read with Biotek plate reader (Synergy HT) at 546 nm. The nitrite level in the serum was extrapolated from the standard curve of sodium nitrite and expressed as mg/ml.

### 3.6.3 Antioxidant status samples

The antioxidant enzymes: catalase, (CAT); superoxide dismutase, (SOD) activities, glutathione (GSH) and malondialdehyde (MDA) level were evaluated using commercial assay kits (Sigma-Aldrich) following manufacturer’s instructions.

### 3.7 Statistical analysis

All experiments were triplicated, and data were statistically expressed as mean and standard deviation. The results' analysis with one-way ANOVA and the calculation of the $IC_{50}$ values were done using the graph pad prism (version 6.01). Where $p< 0.05$ is taken to be statistically significant.

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# Chapter four

**Chapter four describes free radicals and oxidative stress-induced pathologies including erectile dysfunction and how *I.glomerata* and *S.kraussii* prevent it. Details of the materials and methods used in this study have been given in chapter three of this dissertation.**

**The manuscript has been formatted to Blacpma journal to which it has been submitted (article number 1768).**

**Author contributions**

**A.R Opoku and R.A Mosa designed and supervised this project; M.C Ojo, performed the experiments, analyzed the data and wrote the manuscript; G.E Zaharare and F.O Osunsanmi co-supervised the project and perfected the editing. The authors approved the final draft.**

# Chapter four

## 4.0 *In vitro* and *in vivo* antioxidant potentials of the methanolic crude extract from Inula glomerata and Salacia kraussii.

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**Abstract**

Reactive oxygen species are implicated in multiple pathological conditions such as cancer, obesity, diabetes mellitus, cardiovascular diseases, and erectile dysfunction. These diseases continue to be the leading cause of death globally. This study evaluated the *in vitro* and *in vivo* antioxidant potential of the methanolic extracts of *Inula glomerata* and *Salacia kraussii*. The plants were screened for the presence of phytochemicals using a standard protocol. Thereafter, the plant materials were pulverized and extracted with methanol. The ability of the crude extracts to scavenge free radicals (DPPH, ABTS and nitric oxide) *in vitro* was investigated. *In vivo,* antioxidant potentials of the crude extracts (50/250 mg/kg body weight) were determined using a rat model. The phytochemical

analysis revealed that both plants contain flavonoids, tannins, terpenoids, and alkaloids. The crude extracts at varying degree of efficiency, scavenged ABTS and DPPH radicals with *Salacia kraussii* ($IC_{50}$ 0.00658 mg/ml) possessing a better scavenging potency than ascorbic acid ($IC_{50}$ 0.0127 mg/ml) on ABTS. The crude extracts at low concentrations (50 mg/kg b.w) significantly ($P<0.05$) diminished the level of malondialdehyde, augmented catalase activities and elevated glutathione levels. However, SOD activities were significantly boosted in a dose-dependent manner by the crude extracts. Therefore, *Inula glomerata* and *Salacia kraussii* possess antioxidant properties, hence, they could serve as a therapeutic modality in the treatment of oxidative stress-induced erectile dysfunction.

**Keywords:** antioxidants, free radicals, reactive oxygen species, oxidative stress.

## 4.1 Introduction

Free radicals also known as reactive species, are chemically reactive molecules that could even be by-products of biological reactions [1],[2],[3],[4]. Under physiological conditions, free radicals enhance signaling molecules and destruction of antigens or foreign invaders consequently, boosting immunity and promoting good health [2],[5]. Nevertheless, when reactive oxygen species are overproduced or the antioxidant system has been compromised, oxidative stress occurs. Oxidative stress causes protein and DNA damage, abortive apoptosis, lipid peroxidation, and oligoasthenoteratozoospermia. These products of oxidative stress have been implicated in most pathological abnormalities such as cancer, hyperplastic diseases, diabetes, male infertility, hypertension, immune system retrogression, myocardial ischemia-reperfusion injury, build-up of cataract as well as aging process [6],[7],[8],[9]. Endogenous antioxidants'

enzymes such as superoxide dismutase and catalase and other compounds like reduced glutathione, peroxiredoxins, vitamin C, E, and beta-carotene help to cushion the deleterious effects of free radicals in cells and tissues [2],[9]. In addition, fruits, herbs, spices, and vegetables contain naturally occurring compounds that militate free radicals-induced diseases thus, further boosting the total antioxidant capacity of the body [2],[10]. Naturally occurring antioxidant compounds have gained much interest lately because of their affordability, accessibility and most importantly are devoid of adverse effects [10].

*Inula glomerata* Oliv& Hiern indigenous to Northern South Africa, Zimbabwe, Angola, and Tanzania belongs to the Asteraceae family [11]. It is about 2 m tall with a basal rosette leaves with an irregularly toothed margin. Although there is a dearth of information on the bioactivities of *I. glomerata*, its closely related species, *I. racemosa* Hook.f. exhibit anti-microbial activities and pharmacological effects on the cardiovascular system [12],[13]. However, *Inula glomerata* roots are used for treating hypertension (oral communication yet to be validated scientifically).

*Salacia kraussii* (Harv.) Harv commonly called Ibhonsi is an underground creeping surfactant herb that is of the Celastraceae family. *S. kraussii* is endemic to Southern Africa including South Africa (Kwazulu Natal) and possesses anti-diarrhoea and anti-malaria efficacy [14],[15].

The leaves of *Inula glomerata* Oliv & Hiern and roots of *Salacia krausii* (Harv.) Harv are used by traditional healers for managing erectile dysfunction, one of several free radicals mediated disorders but has not been scientifically validated. So, the study aimed at evaluating the *in vitro* and *in vivo* antioxidant capacity of the methanolic extracts of these plants.

## 4.2 Materials and Methods

Chemicals

All the chemicals and kits used in this study were purchased from Sigma Aldrich Co.Ltd (Steinheim, Germany) and are of analytical grades.

### 4.2.1 Plant Identification

The roots of *Salacia kraussii* (Harv.) Harv and leaves of *Inula glomerata* Oliv. & Hiern were collected from Mbazwana ($27^0$ 15'11.3" S $32^0$28'14.3" E) KwaZulu Natal, South Africa. The plants were authenticated at the Department of Botany, the University of Zululand by Dr. Ntuli. The plants with specimen number V04 and V06 have been deposited at the university's herbarium.

### 4.2.2 Plant Extraction

The roots of *Salacia kraussii* (Harv.) Harv and leaves of *Inula glomerata* Oliv. & Hern respectively was cleaned, washed, air-dried at room temperature and pulverized into a fine powder. Pulverized samples (200 mg) were extracted with methanol (1:5 w/v) and incubated on a mechanical shaker (150 rpm; $25^0$C) for 72 h. The extract was filtered using Whatman filter paper 1 and concentrated using Heidolph rotary evaporator ($40^o$C).

### 4.2.3 Phytochemical screening

The pulverized samples were screened for the presence of phytochemicals using the method of Odebiyi and Sofowora [16], and Harbone, [17]. Tannins, saponins, flavonoids, alkaloids, terpenoids, and steroids were the phytochemicals screened for.

### 4.2.4 Total Phenolic Content (TPC)

The phenolic content of the crude extracts was measured, using the Folin-Ciocalteu reagent as described by Kujala *et al*. [18] method. The crude extracts (0.2 mg/ml) was added to 1.5 ml diluted Folin-Ciocalteu reagent (1:10 v/v) and 1.2 ml of sodium carbonate solution (7.5%). The reaction mixture was incubated in the dark at room temperature for 30 min. Absorbance was then read at 765 nm and the total phenolic content of the crude extracts was determined as gallic acid equivalent in mg/g dry plant material.

### 4.2.5 Total Flavonoid Content (TFC)

The total flavonoid content of the crude extracts was estimated using the method of Ordonez *et al*. [19]. The crude extracts (0.2 mg/ml) was well mixed together with aluminium chloride (0.5 ml; 2%). The reaction mixture was then incubated for 1 hr at $25^0$C. Thereafter, absorbance was read at 420 nm. The total flavonoid content of the crude extracts was calculated from the quercetin calibration curve and expressed as quercetin equivalent in mg/g dry plant material.

### 4.2.6 1,1-Diphenyl-2-Picryl Hydrazyl (DPPH) scavenging activity

The method of Brand-Williams [20] was used to evaluating the scavenging activity of the crude extracts on DPPH. The total reaction mixture volume contains (1:1 v/v) different concentrations of the crude extracts (0-4 mg/ml) and DPPH (0.02 mg/ml). The mixture was made to stand for 30 min under diffused light at $25^0$C and the absorbance was read at 517 nm with the aid of a spectrophotometer. Butylated hydroxyl anisole (BHA) and Ascorbic acid (AA) served as positive controls.

### 4.2.7 2,2[1]-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) Scavenging activity

The scavenging activity of the crude extracts on ABTS was determined by the method of Re *et al*. [21]. The ABTS radical was generated overnight (16 h) by mixing 2.5mM potassium persulfate with 7 mM of ABTS. Thereafter, the ABTS solution was diluted with methanol (1:60). ABTS solution was then added to different concentrations of the crude extracts at a ratio of 1:1 (v/v). The reaction mixture was incubated for 10 min in the dark at 25$^{o}$C and absorbance was taken at 734 nm using a spectrophotometer. The positive controls were Ascorbic acid (AA) and Butylated hydroxy anisole (BHA).

### 4.2.8 Nitric oxide ($NO^{\cdot}$) scavenging activity

The scavenging activity of the crude extracts on nitric oxide radical was investigated using the method of Badami *et al*. [22]. The reaction mixture consists of 0.5 ml each of phosphate buffer saline (pH 7.4, 0.1M) and different concentrations of crude extracts (0-5 mg/ml) with 2 ml of sodium nitroprusside (10 mM). The mixture was well mixed and incubated at 25$^{0}$C for 150 min. 1 ml of sulphanilic acid reagent (0.33% sulphanilic acid in 20% glacial acetic acid) was added to 0.5 ml aliquot from the reaction mixture. The resultant mixture was allowed to stand for 5 min. 0.1 ml Naphthylethylenediamine dihydrochloride (1%) was added and mixed together then incubated under diffused light for 30 mins. Absorbance was read at 540 nm and Ascorbic acid (AA) with Butylated hydroxy anisole (BHA) served as a positive control.

### 4.2.9 Animal experiment

Ethical clearance certificate (UZREC 171110-030 PGM 2018/576) was obtained from the University of Zululand Ethics committee for the use of animal experiments. The standard operating procedures for experimental animals were adhered to according to Public Health Service policy [23]. Male Sprague-Dawley rats (250 g) were collected from the animal house of the Department of Biochemistry and Microbiology, University of Zululand. The animals were kept under conducive environmental conditions ($25^0$C; 12:12 light: dark cycle) with free access to safe drinking water and pellet feeds. The animals were acclimatized *for 5 days before the experiment commenced*.

**4.3 Animal model**

The method of Garza *et al*. [24]. with slight modification was followed to perform the animal experiment. Thirty-five male Sprague-Dawley rats were collected and asymmetrically divided randomly into two groups (normal control and n- butanol group). The n-butanol group (30) were injected interperitoneally with n-butanol (5 mg/kg b.w) for four days at two days interval to induce erectile dysfunction. Afterward, each animal was allowed to mate with two estrous female rats (injected with estradiol 7.5 mg/kg b.w for 2 days at 24 h interval) to establish the baseline of their sexual prowess. After establishing that erectile dysfunction has been induced (reduced mounting frequency observed in the n-butanol induced group when compared to the normal control group), they were further divided into 6 groups (5 rats/group) and given daily oral medications for 28 days. The dosage of the crude extracts administered to the rats (50/250 mg/kg body weight) was chosen from previous work of Cele et al. The treatment regime for 28 days was as follows:

Group 1 (normal control): received only water and pellet feeds

Group 2: treated with *Salacia kraussii* at 50 mg/kg b.w

Group 3: treated with *Inula glomerata* at 50 mg/kg b.w

Group 4 (untreated): received only water and pellet feeds (negative control)

Group 5: treated with 250 mg/kg b.w

Group 6: treated with 250 mg/kg b.w

Group 7: treated with Cialis at 5 mg/b.w (positive control).

After the experiment as elapsed, At the end of the experiment, the rats were fasted for 18 hr and then euthanized under anesthesia (pentobarbital@ 30 mg/kg body weight, intraperitoneally). The blood was immediately collected by cardiac puncture for biochemical analysis of the antioxidant status of the serum.

#### 4.3.1 *In-vivo* Antioxidant Studies

The activities of Superoxide dismutase (SOD), Catalase (CAT) as well as the level of glutathione and malondialdehyde in the sera were determined using standard analytical grade kits from Sigma Aldrich and following manufacturer's instructions.

### 4.4 Data analysis

All experiments were carried out in triplicates and data was expressed as mean ± standard deviation. Analysis of the data was done with one-way analysis of variance (ANOVA) and $IC_{50}$ values were determined using the graph pad prism. The percentage (%) inhibitions of the crude extracts were estimated using the formula; % inhibition= $(A_0-A_1)/A_0) \times 100$. Where $A_0$ is the absorbance value of the control and A1 is the absorbance of the crude extracts.

## 4.5 Results

### 4.5.1 Percentage yield

The crude extracts' yield of *Inula glomerata* was 8.5 % while that of *Salacia krausii* was 5.97 %.

### 4.5.2 Phytochemical analysis

The phytochemical screening of the plants revealed that both plants contained tannins, flavonoids, terpenoids and alkaloids as depicted in table 4.1. However, steroids were only present in *S.kraussii*

**Table 4.1: Phytochemicals of *I.glomerata* and *S.kraussii***

| Phytochemicals | *Inula glomerata* | *Salacia kraussii* |
|---|---|---|
| Alkaloids | + | + |
| Flavonoids | + | + |
| Saponins | - | + |
| Steroids | - | - |
| Tannins | + | + |
| Terpenoids | + | + |

**Sign notations:** + present, - Absent

### 4.5.2 Total Phenolic and Flavonoid Content

The phenolic and flavonoid contents of *I.glomerata* and *S.kraussii* are depicted in Figure 4.1 a and b respectively. *I.glomerata* contained significantly ($P<0.05$) higher phenolic than *S.kraussii*. In addition, the flavonoid content was higher in *I.glomerata* than *S.kraussii* however, non-significantly.

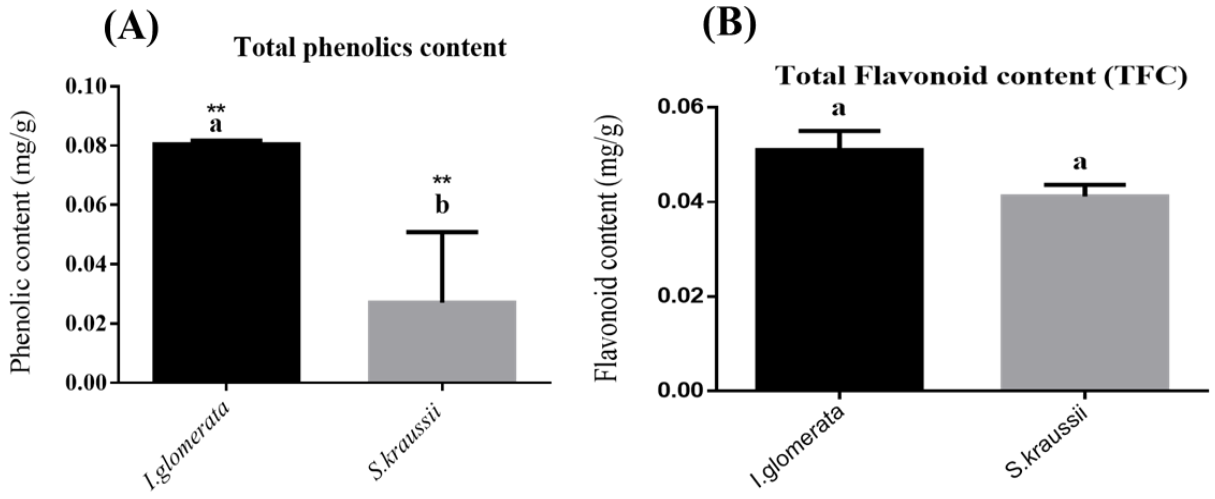



**Figure 4.1: (a) Phenolic content of *Inula glomerata* and *Salacia kraussii* (b) Flavonoid content of *Inula glomerata* and *Salacia kraussii*. Values with different alphabets (a,b) indicated significant differences (P<0.05).**

### 4.5.3 DPPH scavenging activity

The result showed that both plants' crude extracts displayed concentration-dependent scavenging activities on DPPH that was in overall poor as shown in figure 4.3a.

### 4.5.4 ABTS Scavenging activity

The scavenging activity of both plants 'crude extracts on ABTS is depicted in figure 4.3b. The result showed that both crude plants' extracts scavenged ABTS at varying degrees of efficiency that was concentration-dependent but *Salacia kraussii* exhibited better scavenging activity ($IC_{50}$ 0.00658 mg/ml) than ascorbic acid ($IC_{50}$ 0.0127 mg/ml).

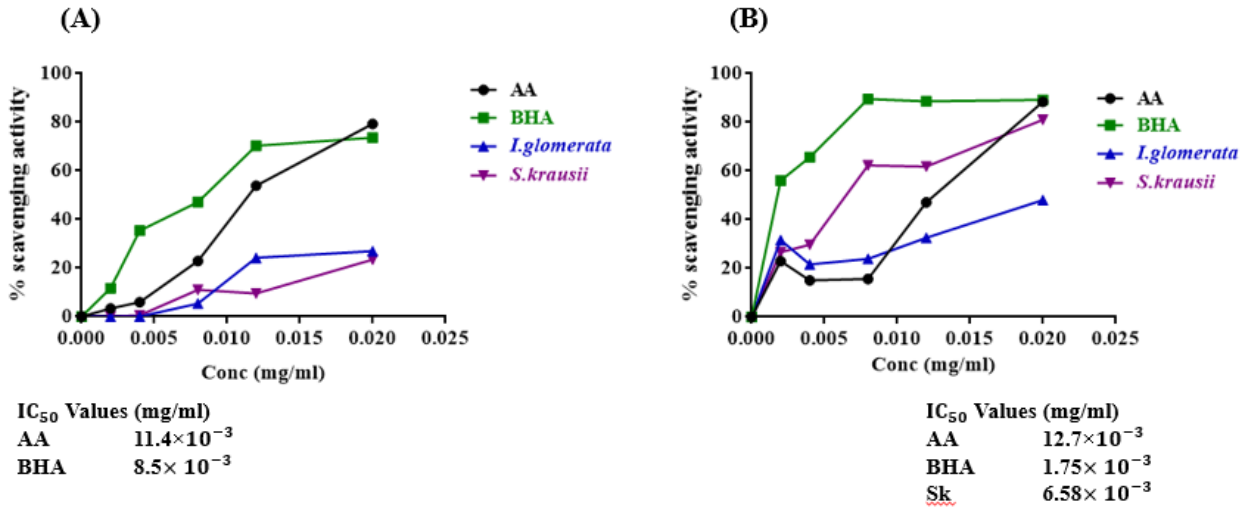



**Figure 4.2: Scavenging effects of *Inula glomerata* and *Salacia kraussii* on (a) DPPH (b) ABTS. Sign notations: AA ascorbic acid, BHA Butylated hydroxyanisole, Sk *Salacia kraussii*.**

### 4.5.5 Nitric oxide (NO˙) scavenging activity

Figure 4.3 depicted the scavenging activity of the crude extract of the plants on nitric oxide radicals. The result indicated that *Inula glomerata* and *Salacia kraussii* showed minimum nitric oxide radical scavenging activities at lower concentrations and had no effect at higher concentrations. Overall, both crude extracts are poor scavengers of nitric oxide radicals.

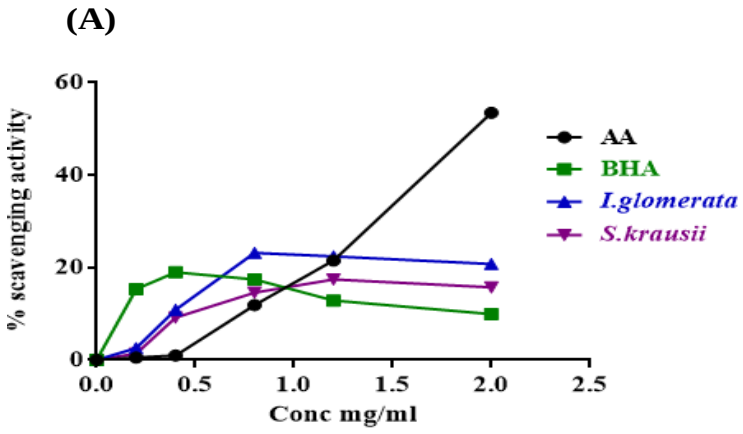



**Figure 4.3: Scavenging activity of *Inula glomerata* and *Salacia kraussii* on nitric oxide radical.**

### 4.5.6 Level of glutathione in the serum

The level of glutathione is depicted in figure 4.4a. The level of glutathione was significantly diminished in the untreated group when compared with the normal. However, at low concentration (50 mg/kg b.w) only *I. glomerata* markedly increased glutathione level more than the n-butanol induced rats (figure 4.4a).

### 4.5.7 Malondialdehyde level in the serum

The crude extracts of the plants at both concentrations markedly reduced the malondialdehyde level as seen in figure 4.4b. However, at low concentration (50 mg/kg b.w), the crude extracts, significantly and non-significantly decreased malondialdehyde level when compared with the n-butanol induced and normal rats respectively. In addition, the crude extracts also exhibited the same effect on the malondialdehyde level as Cialis, the positive control (figure 4.4b).

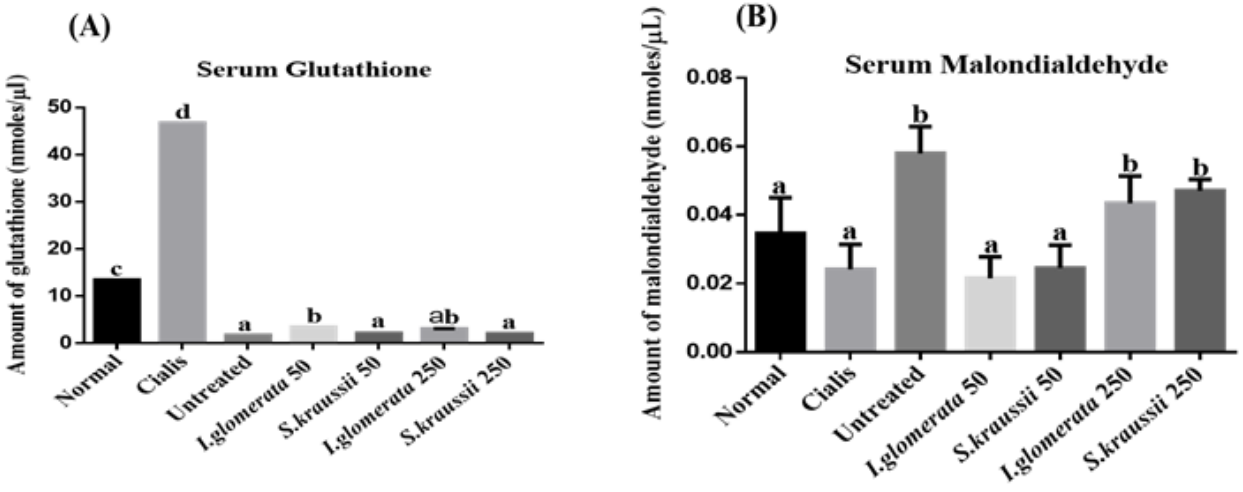


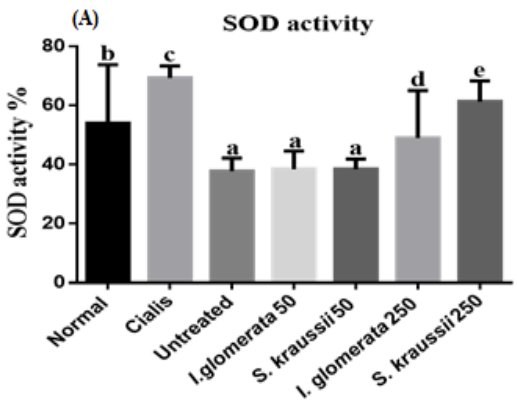

**Figure 4.4: Effect of the crude extracts on (a) glutathione (b) malondialdehyde level in the serum. Bars with different alphabets indicate a significant difference (P<0.05). The values are mean ± SD (n=5).**

### 4.5.8 Effect of crude extracts on SOD activities in the serum

Superoxide dismutase (SOD) activities were compromised in the n-butanol induced rats as represented in figure 4.5a. however, the crude extracts at low concentrations (50 mg/kg b.w) had no effect on SOD activities whereas at high concentrations (250 mg/kg b.w), significantly augmented SOD activities with *S.kraussii* having the greater impact (between both plants) and almost on par compared to the positive control (Cialis).

### 4.5.9 Effect of crude extracts on CAT activities of Catalase in the serum

Figure 4.5b depicted the effect of the crude plants' extracts on catalase (CAT) activities. The activities of CAT were significantly reduced in the n-butanol induced rats compared to the normal rats. However, *I. glomerata* at both concentrations (50 and 250 mg/kg b.w) significantly elevated CAT activities compared to the untreated. Furthermore, *I. glomerata* at low concentration (50 mg/kg b.w) exhibited the greatest positive effect on CAT activities. Contrastingly, *S. kraussii* only at low concentration (50 mg/kg b.w) markedly increased CAT activities.



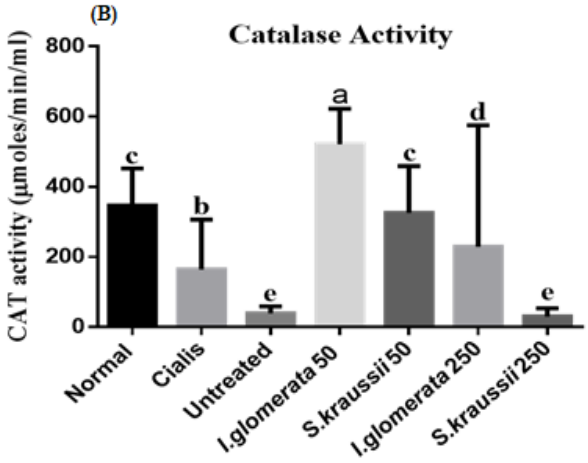



**Figure 4.5: Antioxidant status modulation of the crude extracts on (a) SOD (b) CAT activities in the serum. The bars with different alphabets denote a significant difference (P<0.05). Bars with different alphabets indicate a significant difference (P<0.05). The values are mean ± SD (n=5).**

## 4.6 Discussion

Free radicals mediated cellular damages such as lipid peroxidation, alteration of membrane fluidity, DNA disruption and impaired platelet aggregation play prominent role in the development and pathogenesis of several disease conditions including cancer, cardiovascular diseases, diabetes mellitus and erectile dysfunction [7],[8],[9]. Compounds with antioxidant potentials are becoming increasingly prominent in the combat of these diseases.

DPPH and ABTS are artificial free radicals with a similar mechanism of actions as lipophilic radicals that initiate chain reactions through lipid autoxidation [1]. Hence, are used to evaluate the scavenging efficacies of crude extracts [8],[9]. It is apparent from this study that the crude extracts of the plants under study exhibited poor free radicals scavenging potentials (Figures 4.2 and 4.3) hence, could exert their antioxidant properties as pro-oxidants. Halliwell [2], Leiser and Miller [25], as well as Kaspar *et al*. [26] have argued that poor antioxidants (pro-oxidants) could augment endogenous antioxidants thereby providing better antioxidant defense in humans.

Superoxide dismutase (SOD) and catalase (CAT) are the first lines of defense of cells and tissues against endogenous and exogenous free radicals [27]. Therefore, boosting endogenous antioxidants can help to prevent free radicals induced pathologies [28],[29],[30]. It was recorded in this study that the crude extracts augmented SOD and CAT activities (Figure 6 a and b). The elevated glutathione (figure 5a) and concomitantly

markedly diminished malondialdehyde level (Figure 5b) attest to the *in vivo* antioxidant potential of the plants. Such properties could contribute to the bioactivities of these medicinal plants as used by traditional healers.

Nitric oxide radical reacts with superoxide anions ($O2^{\cdot -}$) and hydroxyl radicals ($OH^{\cdot}$) to generate peroxynitrites. These nitrogen reactive species induce cellular structural changes that eventually lead to oxidative stress and cell deaths [1],[29]. The observed poor nitric oxide scavenging activities of the plants' extracts (Figure 4.3) implies that these crude extracts might not prevent deleterious effects associated with peroxynitrites accumulation. However, because of the biological significance of nitric oxide in penile erection physiology [29],[31],[32], it is apparent that a poor nitric oxide scavenger (as exhibited by the plants under study) could contribute to the management of erectile dysfunction since nitric oxide play a biological significance role in the physiology of penile erection.

It can be inferred that the phenolics and flavonoids are responsible for the antioxidant properties displayed by the crude extracts. These compounds have polyunsaturated heterocyclic ring structures that are stabilized through resonance effect when they terminate free radicals progressive lipophilic attack through abstract proton [9],[28,[33].

In conclusion, the plants boosted endogenous antioxidants, therefore, can serve as a natural antioxidant source with the potential to prevent oxidative stress-related pathologies including erectile dysfunction, consequently, promote health and vitality.

**Conflicts of Interest**

The authors declare that they have not been conflicts of interest.

## Author contributions

A.R Opoku, And R.A Mosa designed and supervised this project; M.C Ojo, performed the experiments, analyzed the data and wrote the manuscript; G.E Zaharare and F.O Osunsanmi co-supervised and perfected the editing. The authors approved the final draft.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# Chapter five

**Chapter five looked into the key enzymes involved in erectile dysfunction pathophysiology and the possible of action *I. glomerata* and *S. kraussii* extracts in abating erectile dysfunction.**

**Details of the materials and methods used in this study have been given in chapter three of this dissertation.**

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**Author contributions**

**A.R Opoku and R.A Mosa designed and supervised this project; M.C Ojo, performed the experiments, analyzed the data and wrote the manuscript; G.E Zaharare and F.O Osunsanmi co-supervised the project and perfected the editing. The authors approved the final draft.**

# Chapter five

## 5.0 Effects of *Inula glomerata* and *Salacia kraussii* methanolic crude extracts on erectile dysfunction key enzymes.

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**Abstract**

Erectile dysfunction (ED) is a common but multifaceted sexual disorder suffered by men with an adverse effect on their self-esteem and quality of life. Globally, the prevalence of ED has markedly increased as revealed by epidemiological studies. The use of conventional drugs has been reported to have side effects in addition to being unaffordable especially to rural dwellers. Traditional healers use extracts of medicinal plants to manage ED and the leaves of *Inula glomerata* (Ig) and roots of *Salacia kraussii* (Sk) are among those used by traditional healers to reverse impotence. The study aimed at determining the ameliorative effect of the methanolic crude extracts of Ig and Sk on butanol-induced erectile dysfunction. The crude extract was prepared by maceration using methanol. An animal study was conducted whereby thirty-five male Sprague

Dawley rats were used. After five days of acclimatizing the rats, they were divided into seven experimental groups: normal group, n-butanol treated group, n-butanol+ Ig (50 and 250 mg/kg), n-butanol+ Sk (50 and 250 mg/kg) and n-butanol+ Cialis (5 mg/kg). The experiment lasted for 28 days, after which the sexual behaviour of the rats was tested. Blood collected from the animals was analysed for various biochemical including acetylcholinesterase, ACE, and arginase activities; testosterone and uric acid levels. The cytotoxicity of the crude extracts was also determined. The results revealed that n-butanol induced erectile dysfunction in the rats by decreasing mounting frequency, testosterone, and nitric oxide level and simultaneously elevated the activities of arginase and acetylcholinesterase. The plants' extracts, however, inhibited arginase and acetylcholinesterase when compared to the untreated. The extracts at low concentrations, the plants' extracts were able to increase the level of testosterone while *S. kraussii* enhanced the activity of angiotensin (I) converting enzymes at higher concentrations. In conclusion, it can be inferred that both plants could be promising natural therapy for erectile dysfunction. Nonetheless, the plants' extracts exhibited high cytoxicity and hence should be taken with caution.

## 5.1 Introduction

Sexual stimulation leads to the release of nitric oxide from the neural and endothelial nitric oxide synthase respectively. Nitric oxide through the cyclic guanosine monophosphate (cGMP) pathway facilitates trabecular smooth muscle relaxation and veno-occlusion thereby resulting in penile engorgement (Anderson 2011; Fraga-Silva *et al*., 2013). Penile erection is a coordinated neurovascular process that involves the nervous, endocrine, vascular system and the penile histo-architecture (Anderson 2011; Fraga-Silva *et al*.,

2013; Castello´-Porcar and Martı´nez-Jabaloyas, 2016). Therefore, impediments or changes in the neural, vascular and fibroblast structure of the penile tissues have been implicated in the pathogenesis of erectile dysfunction (Ojewole, 2007; Rew and Heidelbaugh, 2016). Erectile dysfunction affects mostly men above the age of 40 years and becomes more severe with increasing age (Lewis *et al*., 2010; Yassin *et al*., 2016). Previous studies show that erectile dysfunction shares common risk factors with cardiovascular diseases including diabetes, smoking, chronic alcohol consumption and obesity (Feldman *et al*., 2000; Fung *et al*., 2004). Although there are accumulations of evidence that erectile dysfunction is a combination of organic and psychogenic causes, most cases however, are attributed to organic causes (Gareri *et al*., 2014; Bella *et al*., 2015; Olabiyi *et al*., 2017). A number of pathological conditions including hyperactivities of arginase, acetylcholinesterase through a common denominator known as endothelial dysfunction lead to erectile dysfunction (Deluwe *et al*., 2014; Morris, 2009, Oboh *et al*., 2015; Olabiyi *et al*., 2017). Hypogonadism (low testosterone) and increased actions of ACE, an enzyme that degrades bradykinins and promotes angiotensin [II] production also contribute to erectile dysfunction (Jin, 2009; Fraga-Silver *et al*., 2013; Castello´-Porcar and Martı´nez-Jabaloyas, 2016; Helo *et al*., 2018; Adefegha *et al*., 2018).

The mainstay treatment and management of erectile dysfunction has been the use of phosphodiesterase 5 inhibitors, but their associated side effects have limited their use (Whittaker 2010; Saxena *et al*., 2012; Oboh *et al*., 2015). However, the exploration of medicinal plants as an alternative therapy has continued to gain much interest in recent times mostly because of they have little to no side effect and availability to rural communities (Carlson,2002; Dey and De,2015).

*Salacia kraussii* is a wild fruit with runner underground stems that are commonly distributed in the Kwazulu Natal province of South Africa. It is popularly called Ibhonsi and it belongs to the family known as Celastraceae and the genus Hippocrateacea (Raimondo *et al*., 2009). This plant possesses anti-diarrhoea and anti-malaria potential (Bandeira *et al*., 2000). *Inula glomerata* belongs to the genus Inula and the family Asteraceae. *Inula glomerata* is 2 m tall with a basal rosette leaves of 45 by 20 cm in size that has irregularly toothed margin and endemic to Northern South Africa, Zimbabwe, Angola and Tanzania (Burrows and Willis, 2005). Much of this plant is unknown, however, a closely related species, *Inula racemosa* Hook.f. have a lot of bioactive phytoconstituents and bioactivities including anti-microbial and promoting normal functioning of the cardiovascular system (Tan *et al*., 1998; Arumugam *et al*., 2012). The root of *Salacia krausii* and leaves of *Inula glomerata* are used by traditional healers in Kwazulu Natal for treating erectile dysfunction but have not been validated scientifically. Therefore, this research focuses on evaluating the inhibitory effect of the methanolic extracts of these plants on arginase and acetylcholinesterase, their modulatory capacity on the level of testosterone, nitric oxide and uric acid as well as their cytotoxicity.

## 5.2 Materials and Method

Chemicals

Analytic grade chemicals and kits used in this study were purchased from Sigma Aldrich Co.Ltd (Steinheim, Germany).

### 5.2.1 Plant Identification

The roots of *Salacia kraussii* (Harv.) Harv and leaves of *Inula glomerata* Oliv. & Hiern were collected from Mbazwana ($27^0$ 15'11.3" S $32^0$28'14.3" E) KwaZulu Natal, South Africa. The plants were authenticated at the Department of Botany, the University of Zululand. The plants with specimen numbers V04 and V06 respectively have been deposited at the university's herbarium.

### 5.2.2 Plant Extraction

The plants were rinsed with tap water and air-dried at room temperature. Pulverized plants samples (200 mg) were macerated with methanol (1:5 w/v) with the aid of a Labcon orbital mechanical shaker (150 rpm; $25^0$C) for 72 h. The extracts were filtered (Whatman filter paper no 1) and the filtrate concentrated *in vacuo* (Heidolph rotary evaporator $40^0$C).

### 5.2.3 Phytochemical screening

The methods of Odebiyi and Sofowora (1978), as well as Harbone (1973), were used to analyze the pulverized samples for the presence of phytochemicals. The phytochemicals screened for were Tannins, saponins, flavonoids, alkaloids, terpenoids, and steroids.

### 5.2.4 Cytotoxicity Assay

Cytotoxicity study for the crude extracts was determined against the HEK293 and Hela cell lines using the MTT assay as described by Mosmann, 1983. The cells were first plated in 96-micro plate well with a cell suspension of $1.8\times10^4$ cells/ml. Thereafter, they were

seeded with different concentrations of the crude extract (25, 50, 100 and 200 µg/ml). 1 % FBS was then added into the medium and incubated for 48 hours for the cells to attach. Afterward, tetrazolium salt was added as a cytotoxicity indicator after the old medium had been removed. 100 µl of the medium was then added to each well and incubated for 4 hours at 37 $^{0}$C. The Formazan crystals formed from the aspirated media were solubilized in 100 µl of dimethyl sulfoxide. The absorbance was read at 570 nm with a mindray-96A microplate reader. The percentage inhibition of cell viability was calculated using the formula:

% cell death= [(Ac-At)/Ac x 100)]

where Ac and At represent control and sample absorbance respectively.

### 5.2.5 Animal experiment

Ethical clearance certificate (UZREC 171110-030 PGM 2018/576) was obtained from the University of Zululand Ethics committee for the use of animal experiments. The standard operating procedures for experimental animals were adhered to (PHS, 2000). Thirty-five male Sprague-Dawley rats (250 g) were collected from the animal house of the Department of Biochemistry and Microbiology, University of Zululand. The animals were kept under conducive environmental conditions (25$^{0}$C; 12:12 light: dark cycle) with free access to safe drinking water and pellet feeds. The animals were acclimatized for 5 days before the commencement of the experiment.

### 5.2.6 Animal model

The method of Garza *et al* (2015) with slight modification was followed to perform the animal experiment. Thirty-five male Sprague-Dawley rats weighing between 200-300g were collected and randomly divided asymmetrically into seven groups (5 rats/group). Erectile dysfunction was induced with n-butanol which was injected interperitoneally (5 mg/kg b.w) for four days at two days interval. Afterward, each animal was allowed to mate with two estrous female rats (injected with estradiol 7.5 mg/kg b.w for 2 days at 24 h interval) to establish the baseline of their sexual prowess. Group 1, normal control, was not disabled with n-butanol and did not receive any treatment. Group 2, positive control, was treated with Cialis 5 mg/kg b.w. Group 3 and 4 were treated with 50 and 250 mg/kg b.w of *Inula glomerata* respectively. Group 5 was treated with 50 mg and group 6 with 250mg/kg b.w of *Salacia kraussii*. Group 7, disabled but untreated group. All the animals had free access to feed (commercial ret chow) and water throughout of the experiment period. The dosage used for the crude extracts (50 and 250 mg) was chosen due to previous studies by Cele *et al*., 2017. The medication was given orally with the aid of a gavage daily for 28 days. After the end of the experiment, the rats were fasted for 18 hr and then euthanized under anesthesia. Blood was immediately collected by open cardiac puncture into a 50 ml centrifugation tube. After the blood had clotted, it was centrifuged at 1200 rpm for 10 min in a Rotofix mini-centrifuge machine. The obtained serum was stored at −80 °C for subsequent biochemical assays.

#### 5.2.6.1 Biochemical estimation of liver function biomarkers (ALT and AST), testosterone and uric acid

The level of testosterone and uric acid in the serum were analyzed using standard pathology laboratory procedures (Global laboratory & Viral Laboratory, Richards Bay).

#### 5.2.6.2 Biochemical estimation of arginase and acetylcholinesterase

The serum activities of arginase and acetylcholinesterase were determined using the respective standard commercial assay kit (Sigma- Aldrich), following the manufacturer's instructions.

#### 5.2.6.3 Determination of serum nitric oxide level

The accumulation of nitrite in the serum was determined following the method of Lidija *et al*., 2004. Griess reagent (0.1% N-1-napthylethyleneaminediyhdrochoride, 1% sulphanilic acid and 2.5% phosphoric acid, 125 µl) and the serum was mixed and plated in a 96- well microplate. Thereafter, the mixture was incubated at room temperature for 10 mins and the absorbance was read with Biotek plate reader (Synergy HT) at 546 nm. The nitrite level in the serum was extrapolated from the standard curve of sodium nitrite and expressed as mg/ml.

## 5.3 Sexual Behaviour Protocol

Estrous female rats were used to investigate the sexual behavior of the male rats in different groups. Estrous was induced in the female rats with progesterone at 7 mg/kg b.w for two days at a twenty-four hours interval. Before the commencement of the sexual behavioural studies, the behaviours of the female rats were observed for four hours after progesterone administration. In order to monitor the sexual behaviours of the male rats, a male rat was introduced into a cage with two estrous female rats in a separate room for

thirty minutes. Mounting number (the number of mounts without intromission from the time of introduction of the female rats to the male rat), mount latency (time from introduction of the female rats until the first mount with pelvic thrusting), intromission number (the number of intromissions from the time of introduction of the female rats until the end of the experiment), intromission latency (time from introduction of the female rats until the first mount with pelvic thrusting and vaginal penetration) were parameters monitored by a video recording with an IPAD, and used to determine the sexual behaviours of the rats.

## 5.4 Data analysis

All experiments were carried out in triplicates and data was expressed as mean ± standard error of the mean. The data were analyzed with a one-way analysis of variance (ANOVA) and $IC_{50}$ values were determined using the graph pad prism.

## 5.5 Results

### 5.5.1 Cytotoxicity test

The cytotoxicity of the extracts on HEK293 and Hela cell lines is indicated in table 5.1. The result showed that both plants' extracts were cytotoxic but *Salacia kraussii* exhibited stronger cytotoxicity.

**Table 5.1: The outcome ($IC_{50}$ values µg/ml) of the crude extracts on HEK293 and Hela cells viability**

| Crude extract | HEK 293 (µg/ml) | Hela (µg/ml) |
|---|---|---|
| *Inula glomerata* | $23.3 \pm 0.036^{a}$ | |
| *Salacia kraussii* | $18.7 \pm 0.042^{a}$ | $19.3 \pm 0.054^{b}$ |

Data are presented as mean ± SD, n= 3, The same alphabets denotes no significant different P>0.05

### 5.5.2 In vivo effects of crude extracts on n-butanol induced erectile dysfunction rats

#### 5.5.2.1 Sexual behaviour of n-butanol induced erectile dysfunction rats

The mounting frequency was lowered in the n-butanol induced rats (untreated) compared to normal as illustrated in table 5.2. Both plants significantly increased the mounting frequency compared to the untreated and normal rats. However, *Inula glomerata* was more effective in boosting the mounting frequency of both plants.

**Table 5.2: Effect of I.glomerata and S. kraussii on the sexual behaviour of n-butanol induced erectile dysfunction rats .**

| Groups | Mounting frequency |
|---|---|
| Normal control | $20.00 \pm 0.00^{a}$ |
| n-butanol + Cialis | $35.67 \pm 0.33^{b}$ |
| n-butanol + *Inula glomerata* 50 mg b.w | $26.33 \pm 0.33^{c}$ |
| n-butanol + *Salacia kraussii* 50 mg b.w | $23.33 \pm 0.33^{d}$ |
| n-butanol + *Inula glomerata* 250 mg b.w | 0.00 |
| n-butanol + *Salacia kraussii* 250 mg b.w | 0.00 |
| n-butanol (Untreated) | $10.33 \pm 0.33^{e}$ |

The values represent mean ± SD (n = 5). b.w body weight, different alphabets show a significant difference, $P<0.05$.

#### 5.5.2.2 Effect of crude extracts on arginase activity

Increased arginase activity was observed in the n-butanol induced rats as depicted in figure 5.1. *Inula glomerata* significantly lowered arginase action in a dose-dependent manner and *Salacia krausii* only at high concentration diminished arginase activity.

Although both plants inhibited arginase activities better than Cialis, *Salacia krausii* exhibited the best inhibitory efficacy against arginase.

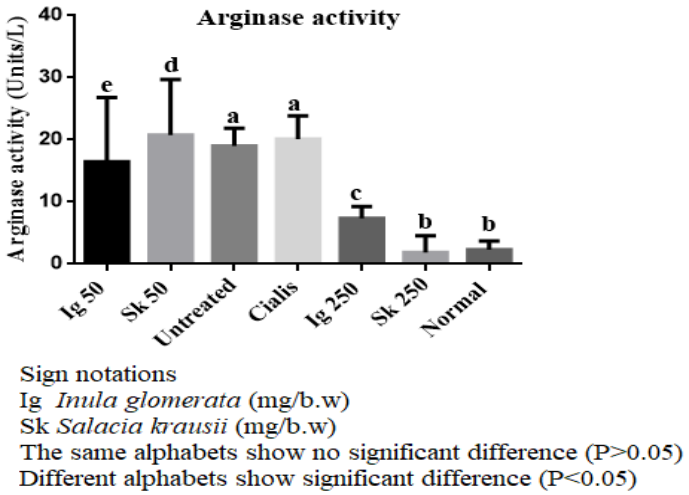


**Figure 5.1: Effect of crude extracts on arginase activity. Data expressed as mean ± SD, n=5.**

#### 5.5.2.3 Effects of crude extracts on Acetylcholinesterase activity

Acetylcholinesterase inhibitory effects of the plant's extracts are presented in figure 5.2. Crude extracts of both plants significantly reduced the action of acetylcholinesterase in a concentration-dependent manner. However, acetylcholinesterase inhibitory efficacy of *Inula glomerata* was more effective at lower concentration as shown in figure 5.2.

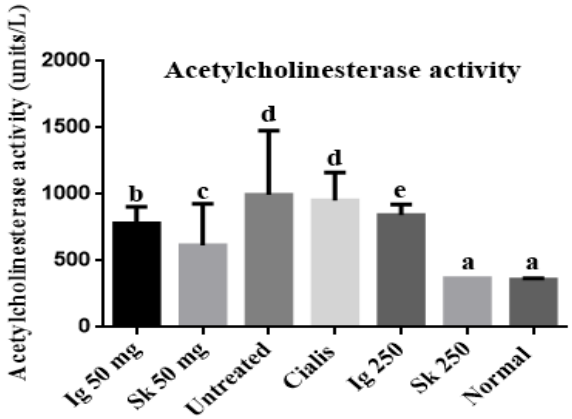

**Figure 5.2: Inhibitory effect of the crude extracts on acetylcholinesterase activity. Data expressed as mean ± SD, n=5. The same alphabets show no significant difference (P>0.05) while different alphabets show a significant difference (P<0.05).**

#### 5.5.2.4 Effects of crude extracts on Angiotensin (1) Converting Enzymes (ACE) activity

ACE activities were increased in the n-butanol induced rats but were inadvertently reduced by the dose of the crude extract independently as revealed in figure 5.3. In addition, at low concentrations (50 mg/ml) both crude extracts reversed ACE activity to normal and there was no significant difference with Cialis.

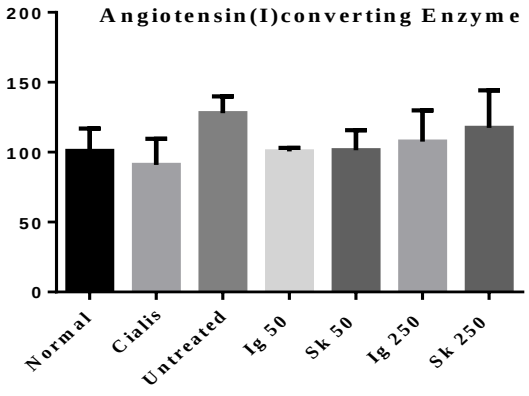


**Figure 5.3: The inhibitory effect of the crude extracts on the activity of ACE. Data expressed as mean ± SD, n=5.**

#### 5.5.2.5 Effects of Crude extracts on testosterone level

Testosterone levels dropped in the n-butanol induced (untreated) rats compared to the normal as depicted in Figure 5.4. However, *Inula glomerata* and *Salacia kraussii*'s crude extracts dose-dependent and independently increased testosterone level respectively. In addition, *Salacia kraussii* at both concentrations (50 and 250 mg/kg b.w) reversed the level of testosterone to normal hence exhibited better testosterone boosting effect.

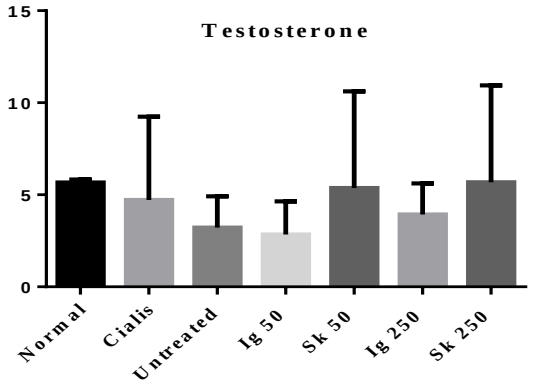


**Figure 5.4: The boosting effect of the crude extracts on testosterone level. Data expressed as mean ± SD, n=5. The same alphabets show no significant difference (P>0.05).**

#### 5.5.2.6 The effect of the crude extracts on nitric oxide level

The level of nitric oxide was drastically reduced in the n-butanol induced rats in comparison to the normal rats but the crude extracts non-significantly elevated nitric oxide level (figure 5.5). Although the crude extracts could not restore nitric oxide to normal, there was no significant difference in the nitric oxide level among the normal, Cialis and extracts treated rats. Furthermore, both extracts at low concentrations displayed equal nitric oxide boosting efficacy. However, *Salacia kraussii* at high concentration had no effect on nitric oxide as depicted in figure 5.5.

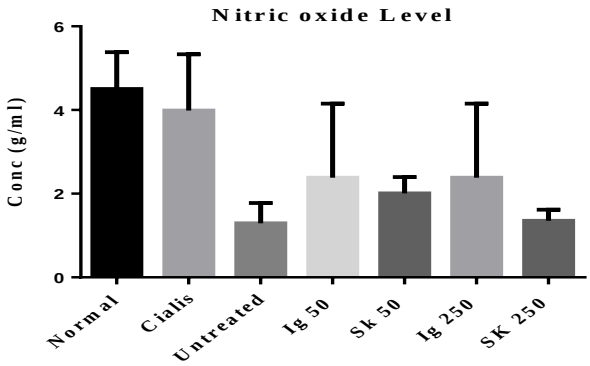

**Figure 5.5: Boosting effect of the crude extracts on nitric oxide level. Data expressed as mean ± SEM, n=5.**

#### 5.5.2.7 Effects of the crude extracts on uric acid level

The result as illustrated in figure 5.6 showed that uric acid was elevated in untreated rats compared to the normal. The extracts had no effects on uric acid at low concentrations (50 mg/kg b.w) but at high concentrations uric acid level was significantly and non-significantly raised by *Inula glomerata* and *Salacia kraussii* respectively using normal rats as a yardstick.

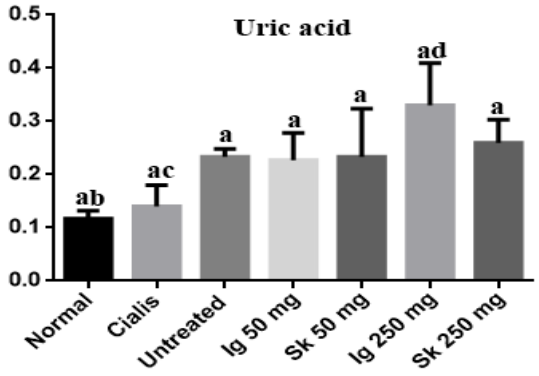


**Figure 5.6: Effect of crude extracts on the uric acid level. Data expressed as mean ± SD, n=5. The same alphabets show no significant difference (P>0.05) while different alphabets denote significance difference (P<0.05).**

#### 5.5.2.8 Effect of crude extracts on Liver function enzymes

Figure 5.7 illustrate the modulatory effect of the crude extracts on liver function enzymes which are aspartate (AST) and alanine transaminases (ALT). The crude extracts had no effect on the enzymes at high concentrations (250 mg/kg b.w) but increased the level of AST and ALT in the serum at low concentrations (50 mg/kg b.w) compared to the normal.

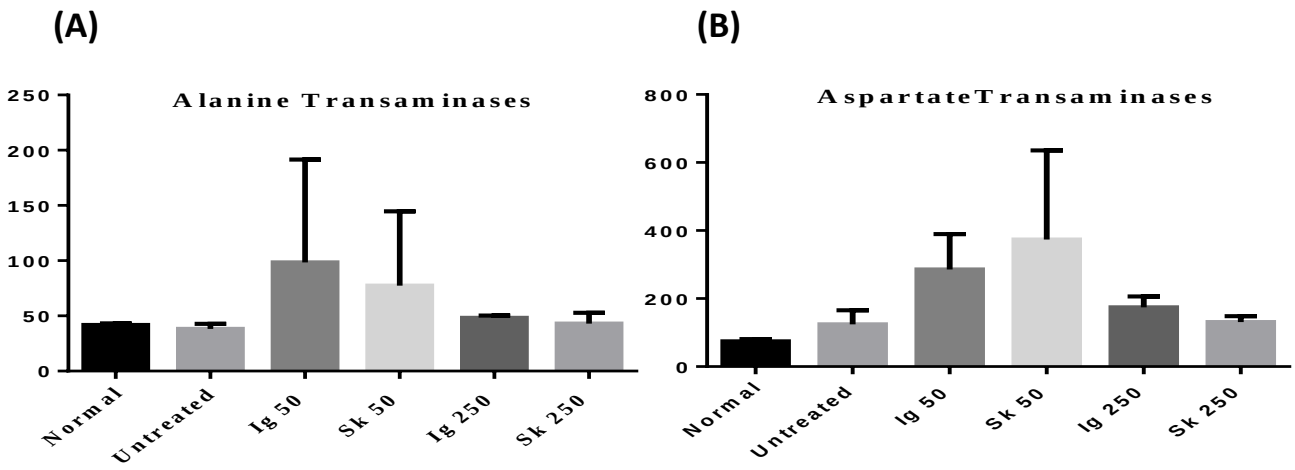


**Figure 5.7: Modulatory effect of the crude extracts on liver function enzymes: (A) Alanine transaminase (B) Aspartate transaminase. Data expressed as mean ± SD, n=5.**

## 5.6 Discussion

The pathophysiology of erectile dysfunction is multifaceted with several predisposing factors including oxidative stress, diabetes and chronic alcohol. Chronic alcohol causes erectile dysfunction through testicular atrophy, abnormal changes in the histo-architecture of the corpus cavernosum, lowering testosterone and nitric oxide level (Grover *et al*., 2014; Choi *et al*., 2017; Cele *et al*., 2017). In our laboratory, Cele and colleagues successfully used n-butanol to mediate testicular dysfunction, hypogonadism and oxidative stress (Cele *et al*., 2017). It was observed in this study that the crude extracts restored the mounting frequency that had been diminished by butanol in the butanol treated groups (Table 5.2). This indicated that butanol induced hypogonadism (testosterone deficient) dependent erectile dysfunction in rats as suggested by Cele *et al*. (2017). The increased level of testosterone (Figure 5.4) also supports the enhanced sexual activities and libido boosting efficacy of the crude extracts. Hence, justifies the inclusion of these plants in traditional medicine for improving sexual activities. This finding

is asserted by previous studies that some medicinal plants possess aphrodisiac effect (Oboh *et al*., 2019).

Elevated activities of arginase and acetylcholinesterase result in endothelial dysfunction, a condition characterized by low nitric oxide bioavailability, is a fundamental cause of erectile dysfunction. In this study, the crude extracts attenuated arginase (Figure 5.1) and acetylcholinesterase activities (Figure 5.2). The augmentation of nitric oxide by the crude extracts (Figure 5.5) also corroborates the fact that arginase activities regulate normal endothelial production of nitric oxide (Oboh *et al*., 2015; Olabiyi *et al*., 2017). This implied that the plants could correct endothelial dysfunction and therefore stimulate an adequate penile erection. This finding agreed with previous studies that medicinal plants' extracts with arginase and acetylcholinesterase activities inhibitory property would exhibit a therapeutic effect on erectile dysfunction (Oboh et al., 2019; Ojo *et al*., 2019b; Ojo *et al*., 2019c).

Furthermore, it was evident in this study that the crude extracts lowered ACE activities (Figure 5.3). Thus, alluding to the fact that the crude extracts could facilitate smooth muscle relaxation hence, boost erection as suggested by Fraga-Silver and colleagues, (2013) and Adefegha and team, (2018) that inhibition of ACE could serve as an alternative therapeutic potential to reversing erectile dysfunction.

High levels of uric acid, AST and ALT(liver function enzymes) are prognosis for renal and hepatic damage (Nakagawa et al., 2006; Yap and Aw, 2010). Increased level of uric acid, as well as AST and ALT, observed in the extract-treated rats is a pointer to the fact that indiscriminate use of these plants have the potential to damage the kidney and liver. This was further buttressed by the cytotoxicity of *Inula glomerata* and *Salacia* kraussii against

HEK 273 and Hela cell lines (Table 1). MTT assays are the most used assays to evaluate the cytotoxicity of the crude extract (Clarkson *et al*., 2004; Magadula 2014). The MTT toxicity ranges of 10-20, 20-100 and above 100 μg/ml indicating strongly, moderately and weakly toxic respectively (Magadula, 2014).

In conclusion, *Inula glomerata* and *Salacia kraussii* possess the potential to alleviate erectile dysfunction and the possible mechanism of action could be by the inhibition of arginase and acetylcholinesterase activities as well as boosting the level of testosterone and nitric oxide. However, the strong cytotoxicity and potential of renal and hepatic damage implied that the plants should be taken with caution.

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# Chapter six

## 6.0 General conclusion, Limitations and recommendations.

### 6.1 General conclusion

Sexual dysfunction affects both males and females, but erectile dysfunction, a type of sexual dysfunction is peculiar to males and its severity increases with age (Ramlachan and Campbell, 2014). The pathophysiology of erectile dysfunction is multifaceted (Hatzimouratidis and Hatzichristou, 2009; Shamloul and Ghanem, 2013). A traditional healer might view low libido, early ejaculation, infertility, weak erection, etc. as a measure of sexual dysfunctions and thus prescribe herbs for the treatment. Traditional healers' inability to distinguish erectile dysfunction from the other sexual dysfunctions, could thus make them recommend *I. glomerata* and *S. kraussii* for erectile dysfunction treatment. This study, therefore, focused on evaluating the ameliorative effect of the methanolic crude extracts of these plants on n-butanol induced erectile dysfunction in male Sprague Dawley rats.

Previous studies have revealed that chronic alcohol consumption causes erectile dysfunction (Grover *et al*., 2014; Choi *et al*., 2017). In this study, the result indicated that mounting frequency was lowered in the n-butanol treated groups (Table 5.3). This indicated that butanol effectively disabled the rats; an observation that has already been established in our laboratory by Cele and colleagues (2017). The ability of the crude extracts to increase the mounting frequency (Table 5.2) and stimulate testosterone production (Figure 5.4) supports the use of these plants in folk medicine as aphrodisiac.

This finding aligns with postulations of other studies, that medicinal plants displayed an aphrodisiac effect (Oboh *et al*., 2019).

Furthermore, the significant attenuation of the activities of arginase (Figure 5.1) and acetylcholinesterase (Figure 5.2), at the same time inhibiting ACE action (Figure 5.3) imply that the plants' extracts can restore endothelial dysfunction and improve penile erection. These findings are in accordance with several studies that inhibition of the aforementioned enzymes (arginase, AchE, and ACE) proffer therapeutic efficacy against erectile dysfunction (Phatarpekar *et al*., 2010; Zucca *et al*., 2016; Adefegha *et al*., 2017b; Adefegha *et al*., 2017a; Oboh *et al*., 2017a; Oboh *et al*., 2017 b).

Nitric oxide plays an irreplaceable role in normal penile erection initiation. Sexual stimulation leads to the release of nitric oxide from the neural and endothelial nitric oxide synthase respectively. Nitric oxide through the cyclic guanosine monophosphate (cGMP) pathway facilitates trabecular smooth muscle relaxation and veno-occlusion thereby resulting in penile engorgement (Andersson 2011; Fraga-Silva *et al*., 2013). The poor nitric oxide radicals scavenging ability of the crude extracts (Figure 4.4) could point to non-interference with nitric oxide bioavailability hence enhance penile erection physiology.

Although the crude extracts exhibited poor scavenging activities (Figure 4.2 a&b), they effectively augmented endogenous antioxidant activities (figures 4.6a, 4.6b and 4.5b). This antioxidant boosting potential could help to prevent oxidative stress-induced aging and age-related disorders including erectile dysfunction (Azadzoi and Siroky, 2009; Erukainure *et al*., 2014; Oboh *et al*., 2015).

Phtochemicals which are secondary metabolites confer on plants therapeutic properties (Ghasemzadeh and Ghasemzadeh, 2011). Flavonoids and phenolics are believed to attenuate key enzymes involved in erectile dysfunction development and progression (Oboh *et al*., 2015; Ademosun *et al*., 2019). The phytochemicals of these plants as depicted in chapter four indicated that the plants contain flavonoids (Table 4.1). Interestingly, the crude extracts inhibited arginase, AchE, and ACE activities. Their inhibitory effects could thus be attributed to their flavonoids and phenolic contents (Figure 4.2). Nevertheless, since *I. glomerata* has higher flavonoids and phenolic contents coupled with the fact that it also displayed enhanced mounting frequency better than *S. kraussii* (Table 5.2), it can be inferred that *I. glomerata* can provide more effective therapeutic potency in the treatment and management of erectile dysfunction.

Nonetheless, due to the strong cytotoxicity of the crude extracts, which is evident in the increase of liver biomarker enzymes (5 fig 7) and uric acid (5 fig 6) in the serum, their ingestion should be thoroughly scrutinized.

## 6.2 Conclusion

This study was an attempt to justify the use of the leaves of *Inula glomerata* and the root of *Salacia kraussii* in ayurvedic medicine for erectile dysfunction amelioration. It is concluded that these plants exhibit properties that support their use in folk medicine.The possible mechanism of actions could be:

- Modulation of endogenous antioxidant status
- Attenuation of arginase, acetylcholinesterase, and angiotensin (I) converting enzymes- key enzymes that control erection.
- Augmenting testosterone and nitric oxide level.

However, due to their cytotoxicity, they need to be taken under strict medical supervision.

### 6.3 Limitations of the study

This cursory study was to ascertain alternative therapeutic approaches the plants could possess to alleviating erectile dysfunction. Therefore, the scope of the work does not entail histology, vasoactivity on smooth muscle and PDE-5 inhibitory effect. In the same vein, purification and isolation of bioactive compounds and western blotting (to determine if attenuations of the enzymes were by downregulation of gene expression or inhibition of their activities) were not investigated.

### 6.4 Recommendation for further study

Recommendations for further study will be:

- To Evaluate the effect of the methanolic crude extracts on PDE-5 inhibition (*invitro* and *invivo*)
- To Investigate the vasoactivity of the methanolic crude extracts on smooth and skeletal muscle.
- To Isolate, purify and characterize bioactive compounds from the methanolic crude extracts.
- Western blotting to ascertain the degree of attenuation (either by gene expression regulation or inhibition of enzymatic rate of reaction) of the key enzmes involved in erectile dysfunction pathophysiology.

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# Appendix A

## Preparation of reagent

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

Tris-HCL (7.88 g), 2.79 g EDTA and 10.227 g NaCl were solvated in distilled water and made up to one litre with distilled water.

### A2. TBA

TBA (1g) was added into diluted acid glacial acetic acid. 50 ml glacial acetic acid was diluted with 50 ml distilled water. Thereafter, the solution was made up to 100 ml with distilled water.

### A3. Formalin

Sodium dihydrogen orthophosphate ($NaH_2PO_4$, 1.75 g) and 3.25 g of di-sodium hydrogen orthophosphate ($Na_2HPO_4$) 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

## Detailed Methodology

### B1. Phytochemical screening

#### B1.1 Alkaloids test

The methanolic crude extract (0.5 g) was dissolved in 5 ml of 1% aqueous HCl. The resultant mixture was stirred on the steam bath and then filtered using Whatman No.1 filter paper. The filtrate was then 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 Dragendroff‘s reagent. The presence of turbidity indicated the presence of alkaloids (Harbone, 1973; Odebiyi *et al.,* 1978).

#### B1.2 Tannins test

Crude extract (0.5 g) was solvated in 10 ml of water, stirred and filtered. Few drops of 0.1% $FeCl_3$ solution were added to 2 ml of the filtrate. The presence of green, blue-green or blue-black precipitate was an indication of tannins (Harbone, 1973; Odebiyi *et al.,* 1978).

#### B1.3 Saponins test

The crude extract (0.5 g) was boiled in10 ml of water. Thereafter, it was filtered. The filtrate was allowed to cool at room temperature. Afterwards, the filtrate was shaken vigorously and let to stand for 15-20 min. The presence of saponins was indicated by froth formation (Harbone, 1973; Odebiyi *et al.,* 1978).

**Flavonoids Test**

**(a) Lead acetate test**: crude extract (0.5 g) dissolved in 10 % lead acetate. The development of reddish brown colouration (or precipitate) indicated the presence of flavonoids (Odebiyi *et al.,* 1978).

**(b) Ferric Chloride test:** 10 % ferric chloride solution was added to crude extract (0.5 g). The formation of dark brown (dirty brown) precipitate indicated the presence of flavonoids (Harbone, 1973; Odebiyi *et al.,* 1978).

**(c) Sodium Hydroxide test**: 1 ml diluted sodium hydroxide solution was added to the crude extract (0.5 g). A golden yellow precipitate indicated the presence of flavonoids (Harbone, 1973; Odebiyi *et al.,* 1978).

**Test for steroids**

The mixture of 2 ml acetic anhydride and 2 ml $H_2SO_4$ was added to the crude extract (0.5 g). Colour Change of from violet to blue or green indicated the presence of steroids (Harbone, 1973; Odebiyi *et al.,* 1978).

**Test for Cardiac glycoside**

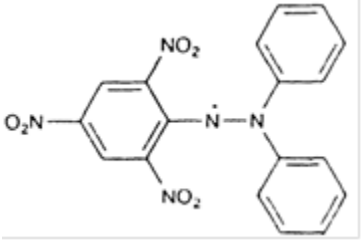
**(a) Lieberman's test**: 2 ml acetic anhydride and 1 ml of $H_2SO_4$ were mixed on ice. The resultant mixture was then added to crude extract (0.5 g). Colour change from violet to blue or green indicated the presence of steroidal nucleus which is the aglycone portion of the cardiac glycosides (Odebiyi *et al.,* 1978).

**(b) Salkowski test**: chloroform (2ml) was used to macerate crude extract (0.5 g) and then kept o ice. Afterwards, 2 ml concentrated $H_2SO_4$ was also added. The development of reddish colour indicated the presence of the aglycone portion of the cardiac glycosides. (Harbone, 1973; Odebiyi *et al.,* 1978).

**(c) Keller-killiani test:** The resultant mixture of glacial acetic acid (2 ml), 1 drop ferric chloride (10 %) solution and 1 ml H2SO4, was used to dissolve 0.5 g of the crude extract. The formation of a brown ring at the interphase indicated the presence of Carotenoids. The appearance of a violet ring just below the brown ring indicated the presence of cardiac glycosides. Lastly, the development of a greenish ring just above the brown ring (i.e. in the acetic acid layer) and gradually spreads throughout the layer also indicated the presence of cardiac glycosides (Harbone, 1973; Odebiyi *et al.,* 1978).

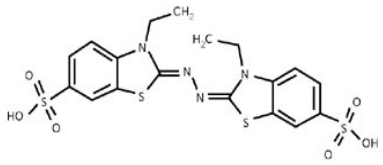
## B.2 Antioxidant activity

### B.2.1 Assay of 2,2-diphenyl-1-picryl hydrazyl (DPPH)




DPPH solution (0.02 mg/ml) was mixed (1:1 v/v) with the methanolic crude extract (0-0.05 mg/ml). After 30-60 mins absorbance was read at 517 nm while using methanol as blank (Brad-Williams, 1995).

### B.2.2 Assay of 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS)



The mixture of 7 mM ABTS (10 mg), distilled $H_2O$ (3 ml) and potassium persulfate (2.45 mM) was incubated at room temperature for 16 hours in the dark. Afterwards, it was diluted (1 ml ABTS: 60 ml methanol). ABTS solution was mixed (1:1 v/v) with the methanolic crude extract (0-0.05 mg/ml) and after 6 mins, absorbance was read at 734 nm with methanol as blank (Re *et al*, 1999).

### B.2.3 Assay of NO- scavenging activity

0.5 ml crude extract (0-0.05 mg/ml) was added to 2 ml of sodium nitropruside (10 mM) and 0.5 ml of phosphate buffer saline (0.01 M; pH 7.4) and incubated at 25 ˚C for 150 min. Afterwards, 0.5 ml was pipetted from the reaction mixture into a different test tube. 1 ml sulphanilic acid reagent (0.33% in 20 % glacial acetic acid) was then added. The resultant reaction mixture was incubated at room temperature for 5 min. Thereafter, 1 ml 1-naphthylamine (5%) was added and allowed to stand for 30 min in diffused light. The absorbance was measured at 540 nm. (Samajdar *et al*., 2000).

### B.2.4 Reducing power

The method of Oyaizu. (1986) was followed to evaluate the reducing power of the plant crude extract. 1 ml crude extract (0-0.05 mg/ml) was added to 2.5 ml phosphate buffer (0.2 M, 6.6 pH) and 2.5 ml potassium ferricyanide (1%). The reaction mixture was

incubated at 50 ˚C for 20 min. Thereafter, 10% Trichloroacetic acid (TCA, 2.5 ml) was added to the mixture and then centrifuged for 10 min at 1000 rpm. The supernatant (2.5 ml) was mixed with 2.5 ml distilled $H_2O$ and 0.5 ml of ferric chloride (FeCl, 0.1 %). The absorbance was measured at 700 nm against methanol. BHA and ascorbic acid were used as standards.

# Appendix C

## C.1 Ethical clearance

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

**RESEARCH & INNOVATION**
Website: http://www.unizulu.ac.za
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### ETHICAL CLEARANCE CERTIFICATE

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Certificate Number | UZREC 171110-030 PGM 2018/576 | | | | | | |
| Project Title | THE AMELIORATIVE EFFECT OF THE CRUDE EXTRACTS OF INULA GLOMERATA AND SALACIA KRAUSIL ON ERECTILE DYSFUCTION | | | | | | |
| Principal Researcher/ Investigator | MC Ojo | | | | | | |
| Supervisor and Co-supervisor | AR Opoku | | Prof GE Zharare | | | | |
| Department | Biochemistry | | | | | | |
| Faculty | Science and Agriculture | | | | | | |
| Type of Risk | High Risk No data collection from people | | | | | | |
| Nature of Project | Honours/4th 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. The Researcher may therefore commence with data collection as from the date of this Certificate, using the certificate number indicated above.

Special conditions:

(1) This certificate is valid for 1 year from the date of issue.
(2) Principal researcher must provide an annual report to the UZREC in the prescribed format [due date- 11 March 2020]
(3) Principal researcher must submit a report at the end of project in respect of ethical compliance.
(4) The UZREC must be informed immediately of any material change in the conditions or undertakings mentioned in the documents that were presented to the meeting.

The UZREC wishes the researcher well in conducting research.

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

CHAIRPERSON
UNIVERSITY OF ZULULAND RESEARCH ETHICS COMMITTEE (UZREC)
REG NO: UZREC 171110-30
13 -03- 2019
RESEARCH & INNOVATION OFFICE

## Appendix D

### D.1 Contribution to body of Knowledge

### D.1.1 WOCMAP Conference Certificate of Attendance.