IN VITRO EVALUATION OF THE BIOACTIVITY OF *GNIDIA POLYCEPHALA* AND *SENECIO SERRATULOIDES*

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STATEMENT REGARDING INDEPENDENT WORK

I, NTAGI GERALD MARIRI, identity number [redacted] and student number [redacted], do hereby declare that this research project submitted to the Central University of Technology , Free State for the Degree MASTER OF HEALTH SCIENCES IN BIOMEDICAL TECHNOLOGY, is my own independent work ; and complies with the code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State; and has not been submitted before to any institution by myself or any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification.

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Summary

The worldwide increase in disability and death rates due to non-communicable and infectious diseases may partly be attributed to the expense, inaccessibility and severe side effects of current treatment strategies. Medicinal plants provide an avenue to discover and develop cheap, safe yet potent alternative therapies that are easily accessible and culturally acceptable. This study evaluated the bioactivity of extracts from *Gnidia polycephala* and *Senecio serratuloides* in order to assess their potential for drug development.

The acetone, methanol and aqueous plant extracts were tested for anticancer activity as well as for cytotoxicity *in vitro* using the Sulforhodamine B assay. The α-amylase and α-glucosidase inhibition assays were used to evaluate their potential as hypoglycemic agents. The antimicrobial efficacy of the plant extracts against specific bacteria was determined by means of the broth microdilution method. The phytochemical constituents of the plant extracts were detected using standard qualitative phytochemical screening techniques as well as Gas chromatography.

The extracts from *Gnidia polycephala* and *Senecio serratuloides* had weak (IC_{50}) or no anticancer activity against renal, melanoma and breast cancer cell lines. The extracts were also classified as low or weak hazard against the normal human fetal lung fibroblast cell line and were selective for the cancer cells and could therefore be safe to use. The acetone extract from *G. polycephala* showed good α-amylase inhibition at 66.34 ± 0.84%; while *G. polycephala* acetone (81.75 ± 0.86%), aqueous (54.84 ± 0.65%) and methanol (45.43 ± 0.56%) extracts and *S. serratuloides* acetone (78.86 ± 1.10%) extract showed good anti-α-glucosidase activity. Only *S. aureus* was susceptible to *G. polycephala* acetone and methanol extracts at 10 mg/ml and susceptible to *S. serratuloides* acetone and methanol

extracts at 5 mg/ml. Hydrolysable tannins were detected in extracts from both plants, while flavonoids were detected in *S. serratuloides* extracts. Few medically important compounds such as 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol and 2-Methoxy-4-vinylphenol were identified using Gas chromatography/mass spectrometry (GCMS). The *G. polycephala* extracts showed higher α-amylase and α-glucosidase enzymes inhibitory activities than the extracts from *S. serratuloides* and further fractionation of these extracts to determine which compounds are responsible for the α-amylase and α-glucosidase inhibition as well as to determine if the inhibition is as a result of the compounds acting synergistically or individually is recommended.

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List of Abbreviations

Abbreviation	Definition
NCD	Non-communicable diseases
WHO	World health organization
IDF	International diabetes federation
MIC	Minimum inhibitory concentrations
MBC	Minimum bactericidal concentration
SRB	Sulforhodamine B assay
PA	Pyrrolizidine alkaloids
ROS	Reactive oxygen species
NCI	National cancer institute
CSIR	Council for scientific and industrial research
ECACC	European collection of cell cultures
RPMI medium	Roswell park memorial institute medium
EMEM medium	Eagle's minimum essential medium
DMSO	Dimethyl sulphoxide
µg/ml	Microgram per millilitre
IC_{50}	Half maximal inhibitory concentration
SI	Selectivity index
ATCC	American Type Culture Collection
INT	*p*-iodonitrotetrazolium violet

CHAPTER 1

INTRODUCTION

Health-care systems across many disease endemic countries are noticeably weak (World Health Organization (WHO), 2012) and continue to be pressurized by the ever increasing morbidity and mortality rates associated with non-communicable (Saeed *et al.,* 2015) and infectious diseases (Mothana *et al.,* 2011). The current treatment strategies (chemotherapy, hypoglycemic agents and antibiotics) face a serious challenge of drug resistance (Saeed *et al.,* 2015) and severe side effects (Manosroi *et al.,* 2015). These strategies are also not readily available to all citizens of developing countries (Sakarkar and Deshmukh, 2011) and patients cannot afford the prescribed treatment drugs (WHO, 2012). This imposes the need to discover and develop cost effective, safe and potent alternative therapies (Sakarkar and Deshmukh, 2011). Medicinal plants have gained interest from researchers across the world as alternative sources of new drug leads (Manosroi *et al.,* 2015).

1.1 Non-communicable diseases

Over the past decade, there has been growing concern over the rapid increase in the occurrence and prevalence of non-communicable diseases (NCDs) (Reubi *et al.,* 2016) and consequently, the health and economic burdens they place on healthcare systems worldwide (Katende and Becker, 2016). NCDs are accountable for about 38 million annual deaths worldwide where cardiovascular diseases, cancers, chronic respiratory disease and diabetes cause an overwhelming 82% of the 38 million annual deaths (WHO, 2014). The current

annual death rate associated with non-communicable disease is expected to increase worldwide, and the biggest increase is predicted to occur in the low-and-middle income countries (Samoisy and Mahomoodally, 2015).

The prevalence of NCDs has been escalated to outrank infectious diseases as the world's leading cause of death (de Wet *et al.,* 2016); resulting in more annual deaths than those caused by all other diseases combined (Chintamunnee and Mahomoodally, 2012). This is a result of the globalization of unhealthy lifestyles such as the excessive indulgence in alcohol, unhealthy diets, physical inactivity, excessive body weight as well as changes due to urbanization which might cause persistent stress (Shinkafi *et al*., 2015).

Remarkable advances in the development of medicines and prevention strategies made available in the fight against NCDs have been observed. However, the management and control of NCDs has remained largely insufficient and NCDs continue to cause deaths worldwide (Samoisy and Mahomoodally, 2015). For instance, in Sub-Saharan Africa, the focus of prevention and treatment is mainly directed towards communicable diseases such as Human immunodeficiency virus (HIV), Tuberculosis (TB) and malaria, whereas the remaining non-communicable diseases are ignored. This has led to overwhelming mortality and morbidity rates (Katende and Becker, 2016).

1.1.1. Cancer

Across the world, cancer is a leading cause of death and disability and impacts more than 14 million people yearly (WHO, 2015a). It is responsible for about 8.2 million deaths reported each year (which is 13% of all mortality cases). A 70 %

increase in new cancer cases is expected over the next twenty years (WHO, 2016). Although the current global cancer rates may remain the same, the projected incidence of 8.7 million new cancer cases reported in 2008; will eventually reach 12.6 million by 2030 (WHO,2014). Further projections predict that 27 million new cancer cases and 17.5 million cancer deaths will be reported worldwide by 2050 (Senthilkumar *et al*., 2014).

More than two thirds of all cancer deaths occur in low- and middle-income countries. Lung, breast, colorectal, stomach and liver cancers together are responsible for more than half of all cancer related deaths in these countries. In high-income countries, lung cancer is the leading cause of cancer deaths among both males and females, and is followed by breast cancer amongst females and colorectal cancers amongst males. The cancer levels in low-and middle-income countries vary as compared to those in high-income countries according to the prevailing underlying risks. In low-and middle-income countries; cervical cancer, liver cancer and stomach cancer all cause a larger proportion of cancer deaths as compared to high-income countries. For instance; in sub-Saharan Africa, cervical cancer remains the leading cause of death among females (WHO, 2014).

The main methods of treatment for human cancers may involve surgery, radiation therapy and chemotherapeutic drugs, and at times a combination of all three or two of these methods are employed (Houghton *et al*., 2007). These interventions can temporarily relieve symptoms, prolong life, and occasionally cure the disease (Sakarkar and Deshmukh, 2011).

However, cancer patients have often suffered unpleasant and severe side effects associated with chemotherapy treatment (Manosroi *et al*., 2015). This is because most of the anticancer agents influence the process of cell proliferation; and not

only for the cancer cells, but for the normal cells as well. These include cells in the bone marrow, oral mucosal cells and hair follicles. The most common side effects include nausea and vomiting as a result of the damage in the epithelium covering the gastrointestinal tract caused by these anticancer agents. Some other common side effects include diarrhea, dyspepsia, and constipation, all because of the damage incurred by the intestinal epithelium. Moreover, chemotherapy is often associated with hair loss, fatigue, sexual dysfunction, anxiety, and oral ulcers which, in turn cause taste and smell dysfunctions (Krukiewicz and Zak 2016; Li *et al*., 2016). Furthermore, there is a limited survival outcome as shown by metastatic pancreatic cancer patients in the United States who were treated with the first-line chemotherapeutic drug; Gemcitabine (Sherine *et al*., 2010). Additionally, synthetic anticancer drug treatments are not easily accessible to the residents of the rural areas. Therefore, there is an urgent need for alternative treatment and management strategies of cancer (Sakarkar and Deshmukh, 2011).

1.1.2. Diabetes

About 285 million people between 20 and 79 years of age were diagnosed with diabetes in 2010 (Trinh *et al*., 2016). Furthermore, about 415 million people worldwide were diagnosed with this disease in 2015. It is predicted that diabetes will affect 552 million people by 2030. A further 642 million adults are predicted to be diagnosed with diabetes by 2040, which is one in every ten adults. In addition, 66.7% of diabetics do not know their hyperglycaemic status. About 14.2 million of the diagnoses made in 2015 were made in Africa alone; and estimates predict that in 2040; 34.2 million diagnoses would be made in Africa alone. Consequently, the management and treatment of diabetes amounts to 12% of the total global health

expenditure (International Diabetes Federation (IDF), 2015; Wannes and Marzouk, 2016).

Diabetes can be treated using the different types of synthetic oral hypoglycaemic agents and insulin. However, these synthetic agents may produce severe side effects and toxicity, and insulin cannot be administered orally (Xu *et al*., 2015). The side effects of synthetic hypoglycaemic drugs include severe hypoglycaemia, lactic acidosis, peripheral oedema and abdominal discomfort (Wannes and Marzouk, 2016). Furthermore, the use of thiazolidinediones which are widely used in the treatment of type II diabetes is associated with weight gain; which is a risk factor for many other NCDs. Again, Liraglutide, a GLP (Glucagon-like peptide) -1 analog, which is used to improve glycaemic control in type II diabetic adults, was found to cause thyroid C-cell tumours in rodents. It is still to be established whether liraglutide causes thyroid C-cell tumours in humans or not (Aguilar, 2011). As a result, there is an urgent need for the development of cheaper, safer and effective anti-diabetic agents from natural materials (Xu *et al*., 2015).

1.2. Infectious diseases

Throughout history, mankind has always been tormented by infectious diseases which have always remained a major cause of mortality and disability; making them the second leading cause of death worldwide (EL-Zawahry *et al*., 2013). Infectious diseases were responsible for the deaths of more than 8.7 million people worldwide in 2008 and have continued to kill almost 9 million people every year and have also caused enormous burdens through life-long disability. A large number of these deaths were poor residents living in low and middle-income countries, with the

majority of the deaths occurring in children under the age of five. This is commonly due to the misdiagnosis and under-detection of these diseases in healthcare systems of impoverished areas. Ultimately, infectious diseases continue to place significant health and economic burdens on poor populations across the world (WHO, 2012).

Bacterial existence in the environment and their interactions with humans is a major cause of most infections and diseases (Upadhyay *et al*., 2014). Diseases related to bacterial infections are orchestrated by a series of virulent factors which facilitate several aspects of their pathophysiology critical for disease in the host. These include amongst others, adhesins and membrane proteins that mediate bacterial attachment, colonization, and invasion of host cells. Additionally, microbial toxins result in host tissue damage, and bacterial cell wall components like capsular polysaccharides confer resistance against the host's immune system (Kao *et al*., 2016). Further virulent factors such as biofilm formation and spore forming capacity assist in the existence and persistence of pathogens in harsh environmental conditions (Upadhyay *et al*., 2014). Some common enteropathogenic microbes and the associated diseases are listed in Table 1.1.

Table 1.1: Common opportunistic and enteropathogenic microbes and associated diseases (Ahmed *et al*., 2014)

Common enteropathogenic microbes:	Gram positive/negative:	Associated human diseases or symptoms:
Escherichia coli	Gram-negative	Diarrhea, hemorrhagic colitis, hemolytic uremic syndrome and thrombocytopenic purpura.
Pseudomonas aeruginosa	Gram-negative	Infections in the urinary tract, respiratory system, soft tissue, bone and joint, gastrointestinal, dermatitis and bacteremia.
Staphylococcus aureus	Gram-positive	Mastitis, toxic shock syndrome (TSS) and staphylococcal food-poisoning (SFP). Skin infections like boils, abscesses, carbuncles and sepsis of wounds.
Enterococcus faecalis	Gram-positive	Leading cause of nosocomial infection and an infrequent cause of pneumonia, meningitis and osteomyelitis.

The development of antimicrobial resistance is a natural phenomenon. In this instance, microorganisms replicate themselves erroneously or exchange resistant traits with other microorganisms (WHO, 2015b). The inappropriate use of antimicrobial drugs further accelerates the emergence and spread of drug-resistant strains (Ahmed *et al.,* 2014; Wikaningtyas and Sukandar, 2016). This greatly threatens the human ability to treat common infectious diseases. Infections resulting from resistant microorganisms often cannot be treated with the standard treatment drugs, causing extended periods of illness, high health care expenses, and a greater risk of loss of life and disability (EL-Zawahry *et al*., 2013). The death toll in patients with serious infections associated with common bacteria treated in hospitals is almost twice that seen in patients with infections caused by the same non-resistant bacteria. For instance, patients with methicillin-resistant *Staphylococcus aureus*, (MRSA) are estimated to be 64% more likely to die than patients with a non-resistant form of the infection (Wikaningtyas and Sukandar, 2016; Aumeeruddy-Elalfi *et al*., 2015).

1.3. The link between cancer, diabetes and infectious diseases

Epidemiological research and meta-analysis centred on both comparative and cohort studies have shown an association between diabetes and the incidence and mortality as a result of cancer. Increased cancer risk in type II diabetes refers to liver, pancreatic, colorectal, kidney, endometrial and breast cancer (Tokajuk *et al*., 2015). Patients with diabetes have a higher risk of developing a number of severe health problems as compared with patients without diabetes. This is because consistently high blood glucose levels can ultimately lead to problems in the functioning of the heart and blood vessels, eyes, and the kidneys (IDF, 2015, Trinh *et al*., 2016). Additionally, people with diabetes also have a higher risk of

developing infections. Even though the risk factors for type I diabetes are still being researched, exposure to some viral infections has been directly linked to the risk of developing type I diabetes (IDF, 2015).

According to the World Health Organization (2012), infectious diseases frequently contribute to the chronic NCD burden. For instance; 28% of bladder cancer cases in Bulawayo, Zimbabwe were accounted for by urinary schistosomiasis (caused by trematodes of the genus *Schistosoma*). Similarly, there was a case reported in the east and south-eastern Asia where the outbreak of Fish-borne liver fluke infections (that trigger liver and bile duct cancers) was seen. During this period, the highest incidence of liver and bile duct cancer in the world was reported in this region. Emerging reports highlighted a link between the infections and cancers amongst Thai males (WHO, 2012). Chronic inflammation as a result of infectious disease is perceived to be responsible for above 15% of currently known cancers (Sideras and Kwekkeboom, 2013).

Evidence also suggests the direct association of the *Helicobacter pylori* infection with metabolic diseases including type II diabetes mellitus. *H. pylori* infection alters the secretion of metabolic hormones which aggravates insulin resistance (He *et al*., 2016). Furthermore, *H. pylori* infection is also reported as the most common risk factor of gastric cancer, responsible for above 70% of all gastric cancer cases (Yang *et al*., 2016).

Ultimately, NCDs add to the burden of disease for individuals, communities and countries that are already struggling to cope with the infectious disease (WHO, 2012).

1.4. The use of medicinal plants as alternative medicines

The oldest and most diverse of all known medicinal systems currently in use; is the African traditional medicine (Shinkafi *et al*., 2015). From antiquity, humans have used plants or their parts to treat most of their ailments, and this knowledge has been passed from generation to generation. Fossils provide evidence that since approximately 60 000 years ago, humans have used plants as medicine (Elgorashi *et al*., 2003).

Most residents of developing countries continue to rely on the use of traditional medicinal plants as a primary health care resource, mainly because traditional medicinal plants are widely available and affordable. Furthermore, the use of traditional medicinal plants is also generally found to be culturally and spiritually acceptable (Verschaeve *et al*., 2004).

To date, about 80% of the population in the developing world depend on and utilize plants and/or their derivatives for primary health-care (Sakarkar and Deshmukh, 2011; Sher *et al*., 2016). In South Africa, approximately 60% of the total population consults one of about 200 000 traditional healers, who mainly prescribe the use of certain plant derivatives (Fuku *et al*., 2013).

There is an estimation of about 350 000-420 000 plant species that exist on earth but, documented knowledge about their medicinal use is very limited (Pan *et al*., 2013; Sher *et al*., 2016). South Africa, in its entirety, is home to a unique and diverse botanical heritage having above 30 000 plant species (Fouche *et al*., 2008; van Vuuren, 2008) of which only about 10% are used as medicine (van Vuuren, 2008). This is a clear indication that most of the medicinal plants in South Africa are currently not sufficiently documented possibly because this knowledge is only

transmitted by oral instruction from generation to generation without the assistance of a formal writing system because most traditional healers do not keep written records (Mahwasane *et al*., 2013).

The therapeutic properties of plants are attributed to the presence of secondary metabolites (Fuku *et al*., 2013), which have been significantly applied in modern therapy (Aziz *et al*., 2016; Sher *et al*., 2016). For instance, 61% of 877 molecules used in drug development between 1981 and 2002 were naturally derived or inspired. Only the remaining 39% was truly synthetic in origin (Fuku *et al*., 2013). As a result, naturally derived plant products are the best sources for novel drug targets. Some of the modern drugs that have been developed from plants include amongst others, aspirin, metformin, morphine and quinine (Chintamunnee and Mahomoodally, 2012). Benzoin and emetine; were also isolated from plants and are known to inhibit microorganisms (Barbour *et al*., 2004). Despite this, scientific knowledge of the therapeutic potential of most plants is very limited (Pan *et al*., 2013).

1.5. The use of medicinal plants in the treatment of cancer, diabetes and infectious disease

Some of the medicinal plants involved in the treatment and management of cancer; protect the human body from cancer by enhancing the body's detoxification functions, while some are known to hinder the proliferation of cancer cells by modulating the activity of certain hormones and enzymes. For instance, phytochemicals extracted from *Aloe vera* and *Morinda citrifolia* have previously been utilized extensively in various formulations that assist the human body to more effectively fight cancer and reduce the toxic side effects associated with both

radiotherapy and chemotherapy treatments (Sakarkar and Deshmukh, 2011). Further examples of plant derived anticancer compounds currently used in patient treatment include the therapeutic vinca alkaloids (vinblastine and vincristine), camphotecins, taxanes and epipodophyllotoxins (Fuku *et al*., 2013; Solowey *et al*., 2014).

To date, more than 1200 plant species have been reported across the world as anti-diabetic. Recently, the most commonly used anti-diabetic drug which is known as Metformin; was developed based on a biguanide compound isolated from the plant *Syringa vulgaris* (French lilac). Similar bioactive compounds isolated from other medicinal plants can display multiple actions on insulin production and distinct insulin action mechanisms such as insulin sensitizing, insulin mimicking, inhibition of intestinal carbohydrate digestion and absorption. Ultimately; these actions increase glucose disposal and uptake by muscle and hepatic cells (Kadan *et al*., 2016; Wannes and Marzouk, 2016).

The combination of plant extracts and existing antibiotics aids to minimize the minimum inhibitory concentrations (MIC), enhance synergistic activity and this minimizes the multiple side effects of combining synthetic drugs. Furthermore, such combinations can be effective in the control of some bacteria that are known to exhibit consistently high resistance to antimicrobials by improving the efficacy of antibiotics against resistant bacterial pathogens (Abioye *et al*., 2017).

Medicinal plants and their natural components have different mechanisms of action to that of the conventional drugs when used to treat infections, which are related to:

- the degradation of the cell wall,

- damage to the cytoplasmic membrane and membrane proteins,
- leakage of intracellular contents,
- coagulation of cytoplasm,
- interference with active transport or metabolic enzymes,
- dissipate cellular energy in ATP form,
- depletion of proton motif force (PMF), and electron flow which can cause cell death (EL-Zawahry *et al*., 2013).

1.6. *Gnidia polycephala*

Gnidia polycephala, Gilg. (Fig 1.1) of the family Thymeleaeceae; is one of the traditional medicinal plants commonly used in the treatment and prevention of human illness in the dry regions of the Southern African countries such as Botswana, Zimbabwe and South Africa (Munkombwe *et al*., 2003). It is a small plant with numerous, erect leafless branches, which have a few flowered hairy heads, each with a few large, papery bracts below the flowers (Mothogoane, 2013). *G. polycephala* is commonly known as “makgonasotlhe” (which is Setswana for ‘having the ability to do everything’, referring to the ability of the plant to heal every human ailment), and can grow well in areas where other plants are difficult to grow and spread (Munkombwe *et al*., 2003).

Fig 1.1: *Gnidia polycephala* (Source: www.plantsystematics.org)

Preparations of *G. polycephala* are commonly taken orally to stabilize heart conditions, treat tuberculosis and tonsillitis, and its ashes are applied onto wounds (Munkombwe *et al*., 2003). However, depending on locality and season, this plant can be toxic when consumed by livestock (Munkombwe *et al*., 2003; Bhandurge *et al*., 2013). In traditional medicine, the roots of the species within the genus are used for several kinds of ailments, including constipation, boils, burns, snakebites, coughs, insanity and poor appetite, following adherence to the correct and strict procedures of preparation (Mothogoane, 2013).

1.7. *Senecio serratuloides*

Senecio serratuloides, DC. (Asteraceae) is another of the commonly used traditional medicinal plants in the treatment of human ailments. *S. serratuloides* is

a herbaceous perennial plant with an erect stem of up to a meter high, which sprouts from a woody rootstock. The leaves are about 60 mm long typically with serrated margins and bears small yellow flowers in sparse clusters towards the ends of the branches (Fig 1.2). In South Africa, it is found in KwaZulu Natal, Limpopo, and Mpumalanga Provinces, where it is commonly known as “Insukumbili” (which is isiZulu for ‘two-days’, referring to the healing effect of the plant in two days) (van Wyk *et al.,* 2009).

Fig 1.2: *Senecio serratuloides* (Source: www.ispotnature.org)

The leaves and the stem of the plant are commonly used for medicinal purposes. The leaves of *S. serratuloides* are applied over cuts, swellings, burns and sores to encourage healing which occurs in about two days (van Wyk *et al.,* 2009; Fawole *et al.,* 2010). The charred leaf extracts of *S. serratuloides* reportedly healed deep

partial thickness skin wounds in a pig model two days earlier than as was observed in the wounds treated with either activated carbon or dressing alone (Gould *et al*., 2015). The dried and powdered leaves are also snuffed to treat headaches. To purify blood for skin eruptions or swollen gums and chest pains, plant teas are taken in small doses (van Wyk *et al.,* 2009; Fawole *et al.,* 2010). Recently, anecdotal reports from the traditional healing community claim to use *S. serratuloides* in the treatment of gastric ulcers (Gould *et al*., 2015).

The hepatic, renal and pulmonary disorders related to the ingestion of *S. serratuloides* have been attributed to the presence of some of the Pyrrolizidine alkaloids (PAs) that are generally found in the species of the genus *Senecio* (van Wyk *et al.,* 2009; Gould *et al*., 2015). However, there has been no study that has to date reported specifically on the presence of these PAs in *S. serratuloides*. Following the hepatotoxicity that has been noted subsequent to accidental ingestion, *S. serratuloides* has been classified as a poisonous plant by the South African National Biodiversity Institute (SANBI, 2010) (Gould *et al*., 2015). According to Hol and Van Veen (2002), PAs cause severe liver damage in many mammalian species sometimes resulting in death. This demands thorough cytotoxicity testing of the *Senecio* species.

1.8. Aim of the study

The current study was conducted with the aim to evaluate the bioactivity of the acetone, methanol and aqueous extracts from *Gnidia polycephala* and *Senecio serratuloides*.

1.9. Objectives of the study

1. To determine the anticancer activity of the acetone, methanol and aqueous extracts form *Gnidia polycephala* and *Senecio serratuloides*.
2. To evaluate the antidiabetic activity of the plant extracts.
3. To assess the antimicrobial activity of the plant extracts against common pathogens.
4. To characterize the phytochemical composition of the plant extracts.
5. To establish the safety of the extracts from the plants in question through cytotoxicity assays.

1.10. Chapter outline

This study is presented in six chapters. Chapter two presents the anticancer activities and cytotoxic effects of the acetone, methanol and aqueous extracts from *Gnidia polycephala* and *Senecio serratuloides* using the Sulforhodamine B (SRB) assay. The ability of the plant extracts to inhibit the carbohydrate-hydrolyzing enzymes, α-amylase and α-glucosidase (antidiabetic activity) are dealt with in chapter three. The fourth chapter is an assessment of the antimicrobial activity of the plant extracts against common pathogens while the fifth chapter presents the phytochemical composition of the extracts. A general discussion of the results of the study and conclusions are presented in the final chapter.

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CHAPTER 2

ANTICANCER EFFECTS AND CYTOTOXICITY OF EXTRACTS FROM *GNIDIA POLYCEPHALA* AND *SENECIO SERRATULOIDES*

2.1. Introduction

Cancer can be described as a wide group of different diseases characterized by unregulated cell growth (Ochwang'i *et al*., 2014). Cell division and growth in a cancerous state, is uncontrollable and often results in tumors that, if malignant, can metastasize and disrupt the surrounding tissues (Tagne *et al*., 2014). Cancer cells continually divide even in conditions where normal cells would normally await a special chemical transduction signal. The cancerous cells disregard similar stop signals as they are sent out by neighboring tissues. These cancer cells are immortal even in *in vitro* conditions while normal cells cease dividing after 50-70 generations and normally undergo cell apoptosis; which is their genetically directed programmed death (Sakarkar and Deshmukh, 2011).

The World Health Organization (WHO, 2014) projects increases in the worldwide mortality rate due to cancer. Currently, the exact causes in the ever increasing cases of cancer are still unknown (Ochwang'i *et al*., 2014). Nonetheless, scientific evidence attributes a large number of cancer cases to the changes in deoxyribonucleic acid (DNA) that eradicate or reduce the normal controls over cell proliferation, maturation and apoptosis (Sakarkar and Deshmukh, 2011). In many cases, free-radical reactions that are generally important in numerous metabolic processes could also be harmful to human health. For instance, free radicals like reactive oxygen species (ROS) i.e. hydroxyl radicals, superoxide anions and hydrogen peroxide play a vital role during the development of tissue damage in humans. Scientific research relates the occurrence of cancer to oxidative damage to DNA, proteins and lipids; as caused by radicals and other carcinogens (Ahmadi

et al., 2010; Mothana *et al*., 2011; Mhaladi *et al*., 2013).

The cause of cancer in patients with certain genetic profiles or in patients infected with chronic viruses (viral hepatitis can cause liver cancer, HIV can cause lymphoma), is commonly due to exposure to carcinogenic chemicals and/or radiation, together with the inability of the immune system to eradicate the cancer cells at an early stage during their proliferation (Sakarkar and Deshmukh, 2011). Furthermore, risk factors including the use of tobacco, radiation, some infections, obesity, poor diet and environmental pollutants, are all also known to be the major factors leading to the development of cancer. These risk factors can cause damage to the genes directly or act in combination with other existing genetic disorders within body cells to cause the disease (Ochwang'i *et al*., 2014).

For decades now, chemotherapy has always been an important option and the hallmark of cancer therapy. Regardless of the outstanding role chemotherapy has played in cancer treatment, the development of drug resistance and severe side effects have limited its success in cancer therapy (Saeed *et al*., 2015). As a result, the focus has now shifted towards the discovery and development of new alternative treatments and management approaches that target specific molecules associated with the development and progression of tumors. Following this new approach, several strategies are suggested in the quest for novel antitumor drugs. These strategies consider naturally occurring compounds as a source of novel cancer therapies (Solowey *et al*., 2014; Manosroi *et al*., 2015). Naturally occurring compounds isolated from medicinal plants used in the treatment of cancer are known to possess multiple targets and therefore are favored over the mono-targeting drugs (Senthilkumar *et al*., 2014).

Over the recent decade; chemoprevention with the use of plant metabolites to

subdue, block or reverse the process of carcinogenesis is receiving great attention from researchers (Senthilkumar *et al*., 2014). Global interest for the discovery and development of new drugs especially from traditionally used medicinal plants is ever growing (Mhaladi *et al*., 2013). More and more medicinal plants have been investigated in the search for alternatives to synthetic agents. Actually, a large number of plants have been used in traditional medicines with great potency and safety (Manosroi *et al*., 2015) and such medicinal plants are perceived as a valuable source of bioactive compounds useful in the therapies of a majority of diseases including cancer; across almost all ethnic groups and for over thousands of years (Ochwang'i *et al*., 2014). Certain groups of phytochemicals found in vegetables and fruits, like terpenoids, phenolic acids, tannins, quinones, coumarins, flavonoids, carotenoids, and vitamins have been investigated and have showed great potential as chemopreventive agents in the treatment of cancer (Senthilkumar *et al*., 2014; Tagne *et al*., 2014). A few examples of the many medicinal plants that are increasingly becoming an integral component of the ethnomedical approach in the treatment of cancer include the leaves, fruits of *Moringa oleifera* Lam (Saeed *et al*., 2015), *Catharanthus roseus* L., *Aloe volkensii* Engl**.** (Ochwang'i *et al*., 2014), *Ammania baccifera* L. and *Polygala chinensis* L (Houghton *et al*., 2007).

When used in chemotherapy, compounds extracted from medicinal plants work either by targeting the rapidly dividing cells by impairing mitosis or causing apoptosis of the target cells (Ochwang'i *et al*., 2014). The success of medicinal plants as anticancer agents could also be due to their redox properties (Kilani-Jaziri *et al*., 2011). Medicinal plants have the ability of scavenging oxygen free radicals that are suspected to be involved in the development of many diseases

including cancer (Mothana *et* al., 2011) and the ability to quench singlet and triplet oxygens, or decomposing peroxides (Kilani-Jaziri *et al*., 2011). Thus, a thorough understanding of the complex synergistic interaction of various components of anticancer compounds extracted from medicinal plants can be useful in the formulations designed to attack and destroy cancerous cells without inducing any harm on other normal body cells (Sakarkar and Deshmukh, 2011).

In South Africa, a collaborative research programme between the U.S. National Cancer Institute (NCI) and the Council for Scientific and Industrial Research (CSIR) of South Africa has contributed immensely to the discovery of new naturally occurring anticancer agents (Fouche *et al*., 2008). However, scientific reports on medicinal plants used in the treatment of cancer remain limited in South Africa (Steenkamp and Gouws, 2006).

Across the world, following their continued use for long periods in traditional medicine, medicinal plants are assumed and believed to have very high healing effects and minor toxic side effects, as compared to synthetic compounds (Verschaeve and Van Staden, 2008; Zeng *et al*., 2013). The justification for the use of medicinal plants has largely relied on long-term clinical experience with very minimal or no scientific information on their efficacy and safety (Chanda *et al*., 2015).

Numerous studies on medicinal plant pharmacological properties have suggested that the use of extracts from medicinal plants can also cause harm or damage to the users (Verschaeve and Van Staden, 2008; Ene-Ojo *et al*., 2013; Mounanga *et al*., 2015). These studies further show that the aqueous extracts are less toxic than organic extracts, probably due to the different phytochemical composition of both the aqueous and alcoholic (organic) types of extracts. The relatively low toxicity of

the aqueous extracts may be associated with the fact that they include the total family of medicinal compounds (both known and unknown) as they may be found naturally and thus; pose a less risk of associated side effects (Mounanga *et al*., 2015). Medicinal plant toxicity may also be attributed to the incorrect preparation and dispensation of the plant extracts (Ene-ojo *et al*., 2013) as well as the prolonged use of some of the popular medicinal plants following the lack of regulation of the prescription and use of these medicinal plants in South Africa (Fennell *et al*., 2004).

Furthermore, the dosage prescribed with the use of medicinal plants is as important as in the use with all other medicines. In fact, the administered dose is actually proportional to the degree of toxicity. Therefore, the higher the prescribed dosage; the more the lesions caused become important particularly in cases of regular consumption. A dose that could potentially cause damage varies from one plant to another because of the differences in both the chemical compounds distributed within the plant and the quantities of these chemical compounds (Mounanga *et al*., 2015).

In the long run, many commonly used medicinal plants cause acute toxic effects (Mounanga *et al*., 2015). These toxic effects may range from diarrhea, hypersensitivity reactions, nausea or vomiting, to organ-targeted toxicity; immunotoxicity, embryo/fetal and prenatal toxicity, mutagenicity/genotoxicity, hepatoxicity, nephrotoxicity, presence of epileptogenic compounds, cardiac toxins, gastrointestinal toxins to carcinogenicity. Some of the other adverse effects may include cardiovascular, neurological and dermatologic toxic effects. The ultimate adverse effect is death (Bisi-Johnson *et al*., 2011). As a result, all products used in therapeutics; including those of natural origin, must all be subjected to efficacy and

safety testing by the same methods as used for new synthetic drugs to determine the appropriate dose of such a drug (Chanda *et al*., 2015).

This study was carried out to investigate the anticancer potential and the cytotoxic effects of extracts from *Gnidia polycephala* and *Senecio serratuloides*.

2.2. Methods

2.2.1. *Plant collection, preparation and extraction*

Gnidia polycephala and *Senecio serratuloides* were collected and identified by researchers from the National Museum, Bloemfontein. Voucher specimens (*Gnidia polycephala* NGM 001 and *Senecio serratuloides* NGM 002) were prepared and deposited in the herbarium at the National Museum, Bloemfontein, South Africa.

The plant material was thoroughly washed, air dried at room temperature and ground into fine powder. For this study, the aerial parts (stems, leaves and flowers) of *G. polycephala* and the stems of *S. serratuloides* were used. Plant sample portions of 15 g were separately shaken at 165 RPM in 400 mL acetone, 400 mL methanol and 400 mL water for 48 hours. Then the extracts were filtered through Whatman No. 1 Filter paper and each filtrate was concentrated to dryness using the Genevac Rocket evaporation system.

2.2.2. *The Sulforhodamine B (SRB) assay*

The Sulforhodamine B (SRB) (Houghton *et al*., 2007; Manosroi *et al*., 2015) assay was used to measure the growth inhibitory effects of the investigated plant extracts using a 3-cell line panel consisting of TK10 (renal), UACC62 (melanoma) and

MCF7 (breast) cancer cells. The WI38 (normal human fetal lung fibroblast) cell line was used to evaluate the cytotoxicity of the plant extracts.

The SRB assay is based on the ability of the anionic aminoxanthene dye Sulforhodamine B (Acid Red 52) to bind and form electrostatic complexes with the basic amino acid residues of trichloroacetic acid-fixed cells in a pH-dependent manner. Under mild acidic conditions, it binds to the fixed cellular protein, while under mild basic, conditions it can be extracted from cells and solubilized for measurement. The SRB Assay was performed at the Council for Scientific and Industrial Research (CSIR) in Pretoria, South Africa in accordance with the protocol as developed in the frame work of the collaborative research programme between the CSIR and the Drug Evaluation Branch of the National Cancer Institute (NCI).

The cancer cell lines TK10, UACC62 and MCF7 were obtained from NCI and were routinely maintained as a monolayer cell culture at 37ºC, 5% CO2, 95% air and 100% relative humidity in RPMI containing 5% foetal bovine serum, 2 mM L-glutamine and 50 µg/ml gentamicin. The normal Human Foetal Lung Fibroblast - WI-38 cell line; from the European Collection of Cell Cultures (ECACC) was routinely maintained as a monolayer cell culture at 37ºC, 5% CO2, 95% air and 100% relative humidity in Eagles' minimum EMEM containing 10% foetal bovine serum, 2 mM L-glutamine and 50 µg/ml gentamicin.

The dry material from each extract was then re-dissolved in dimethyl sulphoxide (DMSO) and diluted to produce five concentrations ranging from 0.01-100 µg/ml.

To carry out the screening experiments, 3 -19 passages of the cells (for the anticancer activity screening) and 21-50 passages of the cells (for the cytotoxicity screening) were inoculated in a 96-well microtiter plates at plating densities of 7-

10 000 cells per well and were incubated at 35ºC for 24 hours. Following incubation, the cells were treated with different concentrations of the re-dissolved plant extracts. Controls were prepared with cells without the plant extracts. The blank contained complete medium without cells. Parthenolide was used as a standard (because of its known anticancer properties) at concentrations of 0.01-100 µg/ml. For the cytotoxicity experiment; Emetine was used as a standard, at concentrations of 100 – 1.0 x10-2 µg/ml.

After addition of the extracts, the plates were further incubated for 48 hours and cold 50% trichloroacetic acid was added to fix viable cells to the bottom of each well. The plates were then washed, air-dried and dyed by SRB solution. Unbound dye was removed and 10 mM Tris base was used to extract protein-bound dye for optical density determination using a multiwell spectrophotometer. This determined the net cell growth percentage. The assays were done in triplicate.

2.2.3. *Statistical analysis*:

Data analysis was performed using GraphPad Prism software. The cell growth inhibition activities and cytotoxic effects of the plant extracts are reported as IC_{50} values and were divided into four categories (Table 2.1). Non-linear regression was used to determine the IC_{50}.

Table 2.1: CSIR standard criteria for anticancer activity and Cytotoxicity

Anticancer activity		Cytotoxicity	
IC_{50}, µg/ml	**Status**	**IC_{50}, µg/ml**	**Status**
> 100	Inactive	> 100	Low Hazard
< 100 > 15	Weak	< 100 > 30	Weak Hazard
< 15 > 6.25	Moderate	< 30 >5	Moderate Hazard
< 6.25	Potent	<5	High Hazard

2.2.4. *Selectivity index*

The selectivity index (SI) was calculated to estimate the potential of extracts to inhibit growth of cancerous cells without toxicity. The SI value is a measure of the extract's beneficial effects at a low dose versus its harmful effects a high dose. A high selectivity index is an indication of a large safety margin between beneficial and toxic dose (Makhafola *et al*., 2014). The SI was calculated as the ratio between the IC_{50} values of normal human foetal lung fibroblast (WI38) and cancerous (TK10, UACC62 and MCF7) cells. Extracts with SI value greater than 3 were considered to have high selectivity (Mahavorasirikul *et al*., 2010). For the purpose of calculating SI, IC_{50} values greater than 100 were taken as 100.

2.3. Results and discussion

The anticancer activity and cytotoxicity of the crude extracts from *Gnidia polycephala* and *Senecio serratuloides* against cancer cell lines and the normal human fetal lung fibroblast cell line are presented in Table 2.2. According to the CSIR criteria (Table 2.1), an extract is considered inactive if the IC_{50} for two or three

cell lines is more than 100 µg/ml. Therefore, the aqueous and the methanol extracts of *Gnidia polycephala* were inactive when tested against TK10 and MCF7 cancer cell lines. Similarly, the aqueous extracts of *Senecio serratuloides* were inactive when tested against TK10, UACC62 and MCF7 cancer cell lines.

An extract with an IC_{50} value greater than 15 µg/ml but less than 100µg/ml when tested against two or more cell lines is considered weak (Table 2.1). The acetone extracts of *Gnidia polycephala* as well as both the acetone and methanol extracts of *Senecio serratuloides* were weak against TK10, UACC62 and MCF7 cancer cell lines. The standard drug (Parthenolide) had potent anticancer activity with IC_{50} values of less than 6.25 µg/ml for all three cell lines.

Table 2.2: Anticancer activity and cytotoxicity of the extracts from *Gnidia polycephala* and *Senecio serratuloides*

Anticancer activity IC_{50} (µg/ml)							
	Gnidia polycephala			***Senecio serratuloides***			**Standard**
Cell line	**dH_2O[1]**	**Acetone**	**Methanol**	**dH_2O**	**Acetone**	**Methanol**	**Parthenolide**
TK-10[2]	>100	19.53	>100	>100	29.75	27.18	1.55
SI[3]	0.34	5.12	1	1	2.66	3.68	-
UACC-62[4]	71.81	25.33	62.35	>100	18.04	17.18	2.18
SI	0.47	3.95	1.6	1	4.39	5.82	-
MCF-7[5]	>100	56.11	>100	>100	32.99	29.67	1.9
SI	0.34	1.78	1	1	2.4	3.37	
	Inactive	**Weak**	**Inactive**	**Inactive**	**Weak**	**Weak**	**Potent**
Cytotoxicity IC_{50} (µg/ml)							
	Gnidia polycephala			***Senecio serratuloides***			**Standard**
Cell line	**dH_2O**	**Acetone**	**Methanol**	**dH_2O**	**Acetone**	**Methanol**	**Emetine**
WI-38[6]	34	>100	>100	>100	79.2	>100	- 2.66
	Weak Hazard	**Low Hazard**	**Low Hazard**	**Low Hazard**	**Weak Hazard**	**Low Hazard**	**High hazard**

[1]: distilled water, [2]: renal cancer cell line, [3]: selectivity index, [4]: melanoma cancer cell line, [5]: breast cancer cell line, [6]: normal human fetal lung fibroblast

This is the first record worldwide, of the anticancer activity screening for both *Gnidia polycephala* and *Senecio serratuloides. Gnidia* species are commonly used as medicine to treat abdominal pains, sore throats, wounds, burns and snakebites (Franke *et al*., 2002). According to the review of literature, species in this Genus are not known for their anticancer activities (Bhandurge *et al*., 2013). This is explained by the weak and inactivity of *G. polycephala* against the tested cancer lines in this study. Plants in the *Senecio* genus are known to possess compounds such as PAs, senecionine and senecionine-*N*-oxide which were previously reported to have anti-tumor, antispasmodic and inflammatory properties (Fawole *et al*., 2012). However, these were not detected in the current study.

Active compounds isolated in their pure form have proven to have higher activities than the crude compounds (Magama *et al.,* 2003; Suleiman *et al.,* 2012; Yessoufou *et al*., 2015). Parthenolide had potent anticancer activity in the experimental conditions of this study while the tested crude plant extracts had weak or inactive anticancer activity. Fractions of the plant extracts or pure compounds instead of crude preparations should be further tested for anticancer activity and perhaps greater anticancer activity may be observed especially from the acetone extracts from both plants and the methanol extracts from *S. serratuloides.* However, further investigations are only feasible for those extracts that show potent activity (IC_{50}s of less than 6.25 µl/ml) (Fouche *et al*., 2008). Additionally, it is known that different cell lines exhibit different sensitivities towards different compounds as contained by different plant extracts (Kamuhabwa *et al*., 2000). Further screening of *G. polycephala* and *S. serratuloides* extracts against more cell lines may show different inhibitory activities.

According to CSIR criteria (Table 2.1), an extract is a low hazard if the IC_{50} value

is more than 100 µg/ml. Consequently, the acetone and the methanol extracts of *Gnidia polycephala* as well as the aqueous and methanol extracts of *Senecio serratuloides* were low hazards and can be considered relatively safe. The aqueous extract of Gnidia *polycephala* and the acetone extracts of *Senecio serratuloides* can be classified as weak hazards with IC_{50} values of between 30 µl/ml and 100 µl/ml respectively. All extracts were relatively safer than the standard Emetine.

The selectivity of the anticancer activity of *Gnidia polycephala* and *Senecio serratuloides* extracts was determined by comparing the anticancer activity (IC_{50}) of each plant extract against each cancerous cell with that of the normal human fetal lung fibroblast cell line (Table 2.2). The SI of greater than 3 was considered as highly selective. As a result, the acetone extract of *Gnidia polycephala* demonstrated higher selectivity for the TK10 and UACC62 cell lines. Similarly, the acetone extract of *Senecio serratuloides* demonstrated higher selectivity for UACC62 cells, while the methanol extract of *Senecio serratuloides* demonstrated higher selectivity for all cell lines used in this study. Extracts that demonstrate higher selectivity offer the potential of safer therapy. A low SI indicates that the anticancer activity is probably due to cytotoxicity rather than activity of the tested extracts against the cancer cell lines themselves (Valdés *et al*., 2010).

2.4. Conclusion

The extracts from *Gnidia polycephala* and *Senecio serratuloides* were generally inactive or weak. Despite the potential of improving anticancer activity from the isolation of pure compounds, we do not recommend these extracts as possible leads in the development of new anticancer agents. Since some of the tested

extracts demonstrated higher selectivity for cancer cells and have proved to be relatively safe, we warrant the use of *Gnidia polycephala* and *Senecio serratuloides* in ethno-medicine.

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CHAPTER 3

ANTIDIABETIC EFFECTS OF EXTRACTS FROM *GNIDIA POLYCEPHALA* AND *SENECIO SERRATULOIDES*

3.1. Introduction

The beta (β)-pancreatic cells of the *islet of Langerhans* synthesize insulin; which is the hormone responsible for controlling the body's blood glucose levels by enhancing membrane transport of glucose; inhibiting the breakdown of glycogen to glucose; and inhibiting the conversion of amino acids or fats to glucose (Marieb and Hoehn, 2014; Olabanji *et al*., 2014). Diabetes mellitus is defined as a metabolic disease associated with high levels of blood glucose- hyperglycemia, due to defects in insulin secretion, insulin action, or both (Olabanji *et al*., 2014) or insensitivity of target organs to insulin (Mahendran *et al*., 2014). When insulin production or secretion is insufficient, the resulting type is type I diabetes mellitus; whereas; when insulin secretion is sufficient, but its effects are deficient, the resulting type is type II diabetes mellitus (Marieb and Hoehn, 2014; Yang *et al*.,2015).

In 2013, the International Diabetes Federation (IDF, 2013) reported that the number of people living with diabetes had reached 382 million. Another 316 million people were found to have impaired glucose tolerance and were at a higher risk of developing the disease. Furthermore, by December 2013, diabetes had killed 5.1 million people which is more deaths than those caused by HIV/AIDS (1.5 million), TB (1.5 million) and Malaria (0.6 million) combined (IDF, 2015). According to a recent report of the IDF; there are now more than 500 000 children aged 14 years and younger who are living with type I diabetes. The number of adults aged 20 - 79 living with diabetes has also increased from 258 million in 2010 (Trinh *et al*.,

2016) to 415 million worldwide; with 318 million adults now found to have impaired glucose tolerance. The number of people living with this disease is now expected to rise to 642 million by the year 2040 (IDF, 2015). The increase in the prevalence of diabetes mellitus may be attributed to factors including excessive body weight, dietary changes due to urbanization, and physical inactivity (Shinkafi *et al*., 2015; Yang *et al*., 2015). In particular, type II diabetes together with its associated complications has placed serious economic and health burdens on societies across the world (Yang *et al*., 2015).

The deficiency in insulin action leads to abnormalities of carbohydrate, lipid, and protein metabolism (Mahendran *et al*., 2014). Diabetes mellitus, and in particular type II diabetes; can cause complications like renal failure, cardiovascular disease, blindness or other liver diseases (Barapatre *et al*., 2015; Yang *et al*., 2015). Similarly, epidemiological research into the causes and effects of diabetes mellitus has shown an association between diabetes and the incidence and mortality as a result of cancer. Increased cancer risk in type II diabetes refers to liver, pancreatic, colorectal, kidney, endometrial and breast cancer (Tokajuk *et al*., 2015).

Correct glycemic control measures are the best possible prevention strategy that delays the progression of the disease and the development of the often associated complications (Tokajuk *et al*., 2015). The most recently adopted therapeutic measure for treating diabetes mellitus is to decrease the post-prandial hyperglycemia. This is achieved by retarding the absorption of glucose through the inhibition of the carbohydrate-hydrolyzing enzymes, α-amylase and α-glucosidase, in the gastrointestinal system. Inhibitors of these enzymes delay carbohydrate digestion time, resulting in a reduction in the rate of glucose absorption and consequently blunting the post-prandial plasma glucose increase (Ali *et al*., 2006;

Narkhede *et al*., 2011). As a consequence, more research efforts are now focused on the search for more effective and safe inhibitors of α-amylase and α-glucosidase from naturally occurring products to develop physiological functional food to treat diabetes mellitus (Wang *et al*., 2010).

Hypoglycemic drugs are mostly used as monotherapy or in combinations to yield better glycemic regulation. These include insulin and insulin analogs, insulin secretagogues (such as sulfonylureas, meglitinides, GLP-1 agonists, DPP-4 inhibitors), insulin sensitizers (such as biguanides, thiazolidinediones), and other drugs with different mechanisms of action (such as α-glucosidase inhibitors, SGLT2 inhibitors) (Tokajuk *et al*., 2015; Yang *et al*., 2015). However, most of the anti-hyperglycemia agents currently in use, like acarbose and metformin are synthetic (McCue *et al*., 2004). These synthetic drugs are associated with high costs of production and severe and adverse side effects at high doses which limits their use (Mahendran *et al*., 2014; Tokajuk *et al*., 2015). Furthermore, a number of these synthetic drugs are losing efficacy in treating this condition, showing drug resistance (Yang *et al*., 2015).

Medicinal plants exert an effect on blood glucose through different mechanisms. For instance, some of them may possess insulin kinase, while some may inhibit insulinase activity, and others may increase the reconstruction of the pancreatic β cells (Bahmani *et al*., 2014). The medicinal effects and efficacy of these plants may be attributed to the presence of various active phytochemicals (Shinkafi *et al*., 2015) such as flavonoids which have the ability to prevent the progressive impairment of pancreatic beta-cell function as a result of oxidative stress. As a result; flavonoids may reduce the occurrence of type II diabetes (Bhandari *et al*., 2008). Furthermore, the flavonoid; quercetin, is a glycation inhibitor and can

stimulate insulin secretion. Some alkaloids (casuarine 6-0-alpha-glucoside) and phenolics (7'-(3', 4'-Dihydroxyphenyl)-N-[(4 methoxyphenyl) ethyl] propenamide, 7'-(4'-hydroxy-3'methoxyphenyl)-N-[(4butylphenyl) ethyl] propenamide) are known to inhibit α-glucosidase activity (Bahmani *et al*., 2014). These phytochemicals are associated with low side-effects and low costs of production, and therefore open new avenues to explore for the treatment various diseases including diabetes mellitus (Mahendran *et al*., 2014). This chapter explores the antidiabetic effects of extracts from *G. polycephala* and *S. serratuloides* in support of the search for novel antidiabetic drug leads.

3.2. Methods

The identification, collection, and preparation of the extracts from *Gnidia polycephala* and *Senecio serratuloides* was performed as previously described in Chapter 2 (section 2.2.1). In the next step, the aqueous extracts were re-dissolved in distilled water and both the acetone and methanol extracts were re-dissolved in 10% ethanol.

3.2.1. *α-Amylase inhibition assay*

The antidiabetic effects of extracts from *G. polycephala* and *S. serratuloides* were evaluated using the α-amylase inhibition assay adopted from Narkhede *et al*., (2011) and Sabitha *et al*., (2012) with slight modifications.

In different test tubes; 30 µl of each of the different plant extracts and 10 µl of 20 mM phosphate buffer (pH 6.9) were added to 20 µl human salivary α-amylase (1 mg/ml); and incubated in a water bath at 37^0C for 30 minutes. Afterwards, 90 µl of a 1% soluble starch was added to each test tube, to act as a substrate for the

reaction, and incubated further at 37ºC for 20 minutes. Then the reaction was stopped with the addition of 50 µl DNS colour reagent (1% 3, 5-dinitrosalicylic acid and 12% potassium sodium tartrate (S6170) in 0.4 M NaOH), and heated in a boiling water bath for 5 minutes, and allowed to cool down to room temperature. The reaction mixture was then diluted with 500 µl distilled water in an ice bath. All the tests were performed in triplicate at a final concentration of 200 µg/ml (which would have been reduced in the subsequent experiments if there was potent antidiabetic activity at 200 µg/ml).

The control tubes were prepared with 40 µl phosphate buffer and 20 µl α-amylase. The blank was prepared with 60 µl of phosphate buffer without the enzyme and the inhibitor tubes with 10 µl phosphate buffer, 20 µl α-amylase and 30 µl acarbose (1 mg/ml) or α-amylase inhibitor from wheat seed (1 mg/ml). The solvent control tubes were prepared with 10 µl phosphate buffer, 20 µl α-amylase and 30 µl solvent (10% ethanol).

The extracts that showed inhibition on α-amylase were recognized by a pale brown colour as compared to the control or no colour change, which in turn determines the extent of inhibition. The contents in the tubes were then transferred to the respective wells in a 48 well plate. The absorbance was measured at 540 nm on a Tecan-Infinite 500 multiwell spectrophotometer (Tecan Group Ltd., Switzerland). The results were expressed as percentage inhibition which was calculated using the formula:

$$\%\text{Inhibition} = \frac{A540\ Control - A540\ test\ sample}{A540\ Control} \times 100$$

3.2.2. *α-Glucosidase inhibition assay*

The extracts from *G. polycephala* and *S. serratuloides* were also evaluated using

the α-glucosidase inhibition assay (Narkhede *et al*., 2011). In a 96 well plate; 20 µl of each of the different plant extracts were added to 10 µl phosphate buffer and 10 µl α-glucosidase (from *Saccharomyces cerevisiae*). Since the extracts had pigments which might interfere with the assay, extract controls were prepared with 80 µl phosphate buffer and 20 µl of the extract. The other control wells were prepared with 30 µl 20 mM phosphate buffer (pH 6.9) and 10 µl α-glucosidase (100 µg/ml). The blank was prepared with 40 µl of phosphate buffer without the enzyme and the inhibitor wells with 20 µl dimethoxymethylamphetamine (DMMA) (1 mg/ml), 10 µl phosphate buffer and 10 µl α-glucosidase.

The plate was incubated at 37°C for 30 minutes. Sixty microliters of (1%) soluble starch was added to each well and the plate incubated further for 15 minutes. All samples were done in triplicate at a final concentration of 200 µg/ml (which would have been reduced in the subsequent experiments if there was potent antidiabetic activity at 200 µg/ml). The absorbance was measured at 405 nm on a Tecan micro-plate reader (Tecan Group Ltd., Switzerland). The results were expressed as percentage inhibition which was calculated as follows:

$$\%\text{Inhibition} = \frac{A405\ Control - A405\ test\ sample}{A405\ Control} \times 100$$

3.3. Results and discussion

A total of six extracts from *Gnidia polycephala* and *Senecio serratuloides* were assessed for inhibitory activity against α-amylase and α-glucosidase, and the results are presented as Table 3.1. *G. polycephala* showed more antidiabetic potential than *S. serratuloides.* The acetone extract from *G. polycephala* showed the highest anti-α-amylase activity at 66.34 ± 0.84% inhibition in comparison to all other *G. polycephala* and *S. serratuloides* extracts which had anti-α-amylase

inhibition of less than 10%. Acetone extract from *G. polycephala* had the highest anti-α-glucosidase activity at 81.75 ± 0.86%; followed by the acetone extracts from *S. serratuloides* with 78.86 ± 1.10% inhibition. The aqueous (54.84 ± 0.65%) and methanol (45.43 ± 0.56%) extracts of *G. polycephala* had higher inhibitory effects than those of *S. serratuloides*. Although, the inhibitory potency of these extracts were still lower than that of acarbose (92.10 ± 0.91%); α-amylase inhibitor (91.12 ± 1.19%) and dimethoxymethylamphetamine (DMMA) (100.16 ± 0.011%), the observed results (Table 3.1) clearly demonstrate the potential of these crude extracts as inhibitors of α-amylase and α-glucosidase enzymes. The solvent control, 10% EtOH showed low effect on both the α-amylase (6.05 ± 0.086%) and on the α-glucosidase (7.70 ± 0.65%). This effect was subtracted from that of all extracts except for the aqueous extracts, to correct for the % inhibition due to the solvent.

Table 3.1: α-amylase and α-glucosidase inhibition by *G. polycephala* and *S. serratuloides* extracts

Extract		% Inhibition ± SD	
		α-Amylase Assay	α-Glucosidase Assay
G.polycephala	dH_2O[1]	-11.79	54.84 ± 0.65
	Acetone	66.34 ± 0.84	81.75 ± 0.86
	MeOH[2]	10.81 ± 1.32	45.43 ± 0.56
S.serratuloides	dH_2O	-0.60	28.44 ± 1.98
	Acetone	9.71 ± 0.019	78.86 ± 1.10
	MeOH	6.59 ± 0.10	22.95 ± 1.50
Controls	10%EtOH[3]	6.05 ± 0.086	7.70 ± 0.65
	Acarbose	92.10 ± 0.91	-
	α-amylase inhibitor	91.12 ± 1.19	-
	DMMA[4]	-	100.16 ± 0.011

[1]: distilled water, [2]: methanol, [3]: ethanol, [4]: dimethoxymethylamphetamine

The inhibitory effects of the tested extracts could be enhanced by adopting preparation techniques similar to those used in ethnomedicine. For instance, most of the medicinal plants used to treat diabetes are prepared in ethnomedicine as infusion or decoctions. Boiling the plant material in water to prepare these infusions

and decoctions extracts could yield more active compounds from the plants (Ezuruike and Prieto, 2014; Cruz and Andrade-Cetto, 2015). As a result, infusions and decoctions possess high concentrations of active compounds. The nature of compounds within medicinal plants is complex. This suggests that they exert their therapeutic effects either as single compounds or act synergistically and increase each other's efficiency. They can also antagonise the therapeutic effect of an otherwise bioactive compound (Komape *et al*., 2017).

3.4. Conclusion

The *Gnidia polycephala* extracts showed higher α-amylase and α-glucosidase enzymes inhibitory activities than the extracts from *S. serratuloides.* We recommend further *in vitro* experiments to ascertain the mechanism of action from the extracts with high α-amylase and α-glucosidase enzymes inhibitory activities as well as to determine if the inhibition is as a result of the compounds acting synergistically or individually. These extracts could be potentially developed as anti-diabetic drugs.

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CHAPTER 4

ANTIMICROBIAL ACTIVITY OF EXTRACTS FROM *GNIDIA POLYCEPHALA* AND *SENECIO SERRATULOIDES*

4.1. Introduction

The well-being of humankind is seriously under threat following the emergence of resistant pathogens (Komape *et al*., 2017). The emergence of bacterial resistance to the currently available antibiotics is often attributed to the indiscriminate use of antibiotics and the development of new bacterial strains which cause diseases (Khan *et al*., 2014). Antibiotics such as quinolones, carbapenems and cephalosponins have become ineffective following the emergence of anti-microbial resistance (Ahmed *et al.,* 2012; Roca *et al*., 2015). Consequently, antibiotic resistance has become an enormous therapeutic challenge and thus infections have greatly increased globally (Elisha *al.,* 2017). Over 70% of pathogens seen in hospitals in the United States of America have now developed resistance to at least one antibiotic which has led to the rate at which people die from infections acquired in hospital exceeding 14 000 patients per year (Khanam *et al.,* 2015). This problem is not only persistent in the United States of America, but has become a worldwide phenomenon (Ahmad and Beg, 2001).

The most important emerging cases of antimicrobial resistance include; the resistance of oxacillin by staphylococci, penicillin resistance in streptococci, vancomycin resistance in enterococci (and eventually staphylococci), resistance to extended-spectrum cephalosporins and fluoroquinolones in members of enterobacteriaceae, and carbapenem resistance in *Pseudomonas aeruginosa* (Ahmed *et al.,* 2012). Furthermore, opportunistic and enteropathogenic bacteria have been identified as causes of serious diseases. For example, *Pseudomonas*

aeruginosa is implicated in infections of the respiratory system as well as other systemic infections. *Staphylococcus aureus* has been associated with skin infections and sepsis of wounds (Ahmed *et al*., 2014). According to the World Health organization (WHO, 2012), infectious (including parasitic) diseases were together responsible for the deaths of more than 8.7 million people worldwide in 2008.

From ancient times, traditionally used medicinal plants have always been an abundant source of antimicrobial agents (Khanam *et al.,* 2015). Plant derived antimicrobials have immense therapeutic potential and can effectively be used in the treatment of infectious ailments (Kokoska *et al.,* 2002). This is because plants have developed various defense responses against most microbial pathogens. These include the production of small molecular mass peptides called antimicrobial peptides (Khanam *et al.,* 2015) which are grouped into the following families: thionins, defensins, cyclotides, lipid transfer proteins and hevein-like proteins (Nawrot *et al*., 2013). Phytochemicals from medicinal plants may also have a different mechanism of action from that of conventional antimicrobial agents (Mothana and Lindequist, 2005). This explains the extensive use of plant extracts to treat most infectious diseases throughout the history of mankind and why this continues as part of the primary healthcare especially in developing countries (Magama *et al.,* 2003).

As an alternative, medicinal plants are important in the discovery and consequent development of novel antimicrobial metabolites to solve the issue of antimicrobial resistance to the currently available drugs (Mickymaray *et al.,* 2016). Subsequently, the attention of researchers across the globe is now focused on traditional medicine, searching for leads to the discovery of new drugs for the

treatment of microbial infections (Srinivasan *et al.,* 2001).

South Africa has one of the richest flora in the world; with about 19 581 indigenous species, but only approximately 3 000 species are used as medicines (Nielsen *et al*., 2012), and the rest are still to be evaluated medicinally. The evaluation and subsequent documentation of medicinal plants should be seen and treated as an important matter (Srinivasan *et al.,* 2001).

The majority of the current synthetic drugs are manufactured from herbal medicines. It is estimated that no less than 119 compounds of plant origin are currently used as important clinical drugs (Khan *et al.,* 2014). There is an increased use of plants in the treatment of bacterial infections as compared to synthetic antibiotics because plants are believed to be safe and are easily accessible and generally affordable. Thus, the popularity of plant remedies has increased in comparison to the synthetic drugs (Bahmani *et al.,* 2015).

The purpose of this study was to evaluate the antimicrobial potential of *Gnidia polycephala* and *Senecio serratuloides*. *Gnidia polycephala* is used to treat tuberculosis, tonsillitis and wounds (Munkombwe *et al*., 2003). *Senecio serratuloides* is applied in the treatment of chest pains and skin eruptions, sores and cuts (van Wyk *et al.,* 2009; Fawole *et al.,* 2010). These traditional uses of the plants suggest that they may have antimicrobial properties.

4.2. Methods

4.2.1. *Preparation of plant material*

The plant extracts from *Gnidia polycephala* and *Senecio serratuloides* were prepared as outlined in Chapter 2 (section 2.2.1). The dried extracts were then re-

dissolved separately on a vortex shaker in 2% acetone, 2% methanol and distilled water to give a final concentration of 10 mg/ml as a stock solution. Thereafter, each plant extract was serially diluted two-fold into concentrations of 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml and 0.3125 mg/ml. Thus, for the experiment, the concentrations used ranged from 0.3125 to 10 mg/ml. Acetone and methanol were both used as solvents of extraction due to their ability to completely dissolve the extracts and because of their non-inhibitory effect on the organisms at 2% (Meyer and Afolayan, 1995; Madamombe and Afolayan, 2003).

4.2.2. *Microorganisms*

The bacterial species used in this investigation were supplied by the Department of Health Sciences of the Central University of Technology, Free State in Bloemfontein. The Gram positive bacteria used were *Staphylococcus aureus* (ATCC 11632), *Staphylococcus epidermis* (ATCC 12228) and *Bacillus subtilis* (ATCC 11774). Gram negative bacteria included *Proteus vulgaris* (ATCC 6380), *Pseudomonas aeruginosa (ATCC 10145)*, *Klebsiella pneumonia* (ATCC 10031), *Enterobacter cloacae* (ATCC 13047) and *Escherichia coli* (ATCC 13762). Pure cultures of each organism were streaked and maintained on Mueller-Hinton (MH) agar plates (Oxoid) and incubated at 37°C for 24 hours. Isolated colonies were aseptically inoculated in fresh sterile MH broth (Sigma) and incubated for a further 24 hours (Khanam *et al.,* 2015).

4.2.3. *Antimicrobial activity screening*

The microdilution assay was performed as prescribed by Eloff (1998); Aremu *et al.,* (2010) and Suleiman *et al.,* (2012), with slight modifications. Hundred microliters

of the diluted extracts were then added to a 96-well microtiter plate. The positive controls were prepared with 100 µl of chloramphenicol at 1mg/ml. The negative control only contained MH broth; prepared to ensure that there was no bacterial growth other than that of the inoculated bacteria. The other controls were the wells with each tested bacterial culture with MH broth, and the wells prepared with the solvent of extraction (2% acetone, 2% methanol and dH_2O) to ensure that the solvents of extraction did not inhibit bacterial growth.

One millilitre of overnight bacterial cultures previously grown in MH broth at 37°C (Section 4.2.2) was diluted with 100 ml fresh sterile MH broth (1:100 dilution) and 100 µl was added to the treatment wells and incubated for 24 hours at 37°C. Thereafter, 40 µl of *p*-iodonitrotetrazolium violet [INT] (Sigma) at a concentration of 0.2mg/ml was added to each of the microtitre wells to indicate bacterial growth by formazan formation in the presence of bacteria. The microtiter plate was then incubated for 30 minutes at 37°C and the Minimum inhibitory concentration (MIC) values were recorded as the lowest concentrations at which no bacterial growth occurred. The assay was performed in triplicate.

4.3. Results and discussion

The extracts from *Gnidia polycephala* and *Senecio serratuloides* were not active against most of the tested bacterial species at the concentrations used in this study. This was shown by the violet colour change in the wells of the microtiter plate where bacterial growth occurred (Fig 4.1). The inactivity of most antimicrobial drugs against most bacteria may be a result of the presence of the lipopolysaccharide in the outer membrane of gram negative bacteria. This

membrane presents a permeability barrier and hampers diffusion of antibiotic molecules. Consequently; gram negative bacteria are found to be relatively resistant to plant extracts (Suleiman *et al.,* 2012; Khanam *et al.,* 2015).

The methanol and acetone extracts of both *Gnidia polycephala* and *Senecio serratuloides* showed some antibacterial activity on only one bacterial species. *S. aureus* was susceptible to *G. polycephala* at 10 mg/ml and to *S. serratuloides* at 5 mg/ml. Generally, crude extracts acquired by extracting plant material with methanol have previously proved to have high success rates with regards to the variety of phytochemicals as compared with water (Magama *et al.,* 2003). Water extracts from several plant species barely have antimicrobial activity (Dzoyem *et al.,* 2016) because water is not effective in extracting antimicrobial compounds from plants (Makhafola and Eloff, 2012). This may explain the inactivity of the water extracts of both *Gnidia polycephala* and *Senecio serratuloides* against the tested bacteria. Furthermore, acetone has also been found to be the best solvent of extraction for most plant species when investigating their antimicrobial activity (Makhafola and Eloff, 2012; Dzoyem *et al.,* 2016) primarily because of its ability to extract a wide variety of compounds with different polarities (Elisha *et al.,* 2017).

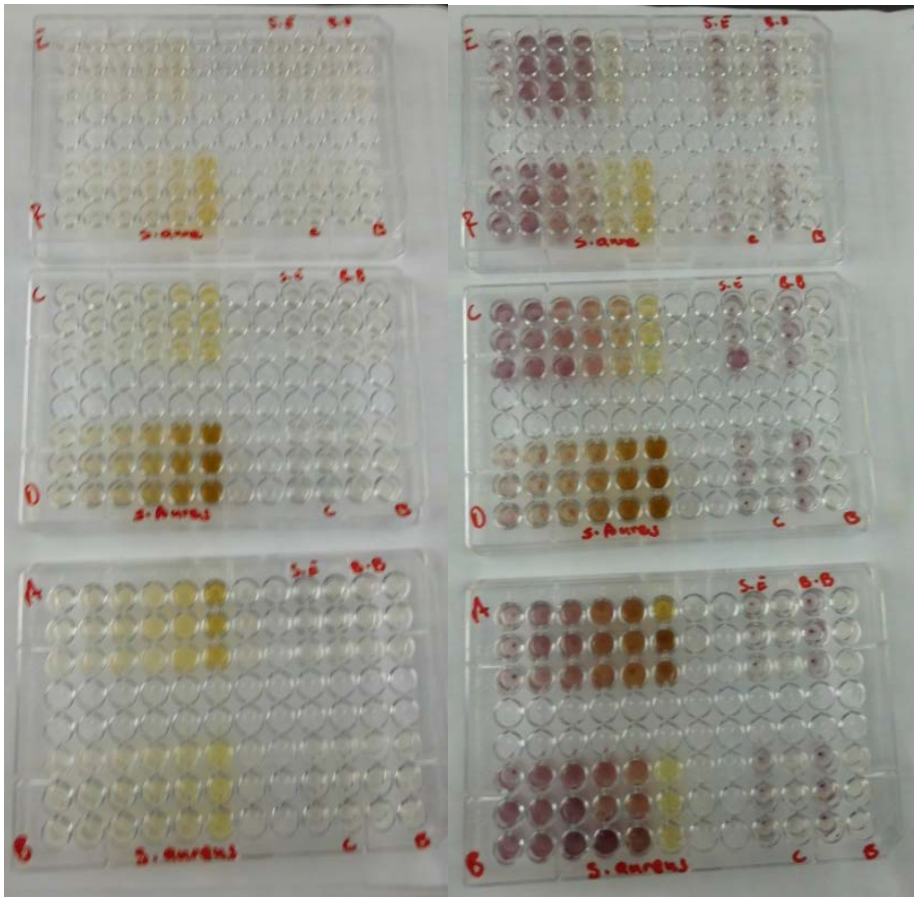

Fig .4.1: 96 well microtiter plates inoculated with *S. aureus*, *G. polycephala* (A, B, C), *S. serratuloides* (D, E, F) and controls before addition of INT (left) and after addition of INT (right)

The lack of bactericidal activity of extracts from *Gnidia polycephala* and *Senecio serratuloides* against the bacteria used in this study may be explained by the findings of Aderogba *et al*., (2012) who postulate various mechanisms of action by plant extracts in treating microbial infections. Plant extracts can either have a direct or an indirect effect. A direct effect implicates the action of the active agents; whereas the indirect effect implicates the stimulation of the host's immune system to overcome the effects of microorganism. Sakarkar and Deshmukh (2011) suggest that certain medicinal plants by themselves may not be potent enough to achieve the desired therapeutic effects, but may help improve the body's own immunity towards diseases, and in this way be useful in the treatment and management of infectious diseases. In addition, it may be necessary to transform

compounds within medicinal plants with metabolic systems of the body to activate therapeutic components within them. This has been observed in several traditional medicinal plants such as *Senna alexandrina* (Senna) which has laxative effects. In this case, the intestinal flora hydrolyses the glycosides to release the aglycones which in turn cause alterations in the water permeability of the walls of the gastrointestinal tract and enhances peristalsis, and in this way the plant imposes its laxative effects (Houghton *et al*., 2007).

Furthermore, the ethnomedical uses of these medicinal plants in the traditional medicine systems can also provide a possible explanation for the lack of bactericidal activity found in our laboratory investigations. For instance, in traditional medicine, *G. polycephala* is used to stabilize heart conditions, treat tuberculosis, tonsillitis, and wounds (Munkombwe *et al*., 2003). The medicinal effects of *G. polycephala* in these instances, may not be attributed to its ability to inhibit the investigated bacteria. Moreover, the inactivity of the investigated plant extracts may be attributed to the plant parts investigated as opposed to the parts used in traditional medicine. According to van Wyk *et al.,* (2009) the leaves of *S. serratuloides* are applied over cuts, swellings, burns and sores to encourage healing. The leaves are also dried then crushed into fine powder and snuffed to treat headaches. Small dosages of the plant teas are drank to purify blood for skin eruptions or swollen gums and chest pains, however, this study screened stem extracts and not the leaves of the plant.

The use of the microdilution method to determine the minimum inhibitory concentration (MIC) of the plant extracts against the selected bacteria may have been a methodological shortcoming in this study. The MIC was determined visually as the lowest concentration that led to growth inhibition. Perhaps, this approach is

better suited to determine the minimum bactericidal concentration (MBC) instead of the MIC, which could most accurately be determined calorimetrically by using a multiwell spectrophotometer.

Finally, because chloramphenicol was active against all tested bacterial species and the tested crude plant extracts were inactive in our experimental setup, we recommend further antimicrobial investigations from fractions of the plant extracts instead of crude preparations. This is also because active compounds isolated in their pure form have proven to have higher activities than the crude compounds (Magama *et al.,* 2003; Suleiman *et al.,* 2012; Yessoufou *et al*., 2015).

4.4. Conclusion

The investigated plant extracts were inactive against most of the tested bacteria except for the acetone and methanol extracts of both *G. polycephala* and *S. serratuloides* against *S. aureus*. The results of this study do not validate the use of *G. polycephala* and *S. serratuloides* as direct antimicrobial agents, and do not support the use of these plants as possible leads for new antimicrobial drug development.

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CHAPTER 5

PHYTOCHEMICAL PROPERTIES OF EXTRACTS FROM *GNIDIA POLYCEPHALA AND SENECIO SERRATULOIDES*

5.1. Introduction

Nature has provided generations of humans with medicinal agents for centuries (Dadsena *et al*., 2013). Medicinal plants have since remained the principal constituent of traditional medical systems and still serve as an inexhaustible source of alternative medicines for the treatment and management of various human ailments (Fuku *et al*., 2013). It is estimated that 80% of the total world population relies on traditional medicine which comprises primarily of compounds of plant origin (Alabri *et al*., 2014).

Plants have phytochemical constituents that participate in all biochemical processes, such as growth, development, and reproduction as well as in the interactions of the plant with both its biotic and abiotic environments (Islam *et al*., 2015). Phytochemicals are grouped into two groups based on their roles in plant metabolism, that are primary and secondary metabolites (Bhumi and Savithramma, 2014; Dias *et al*., 2015). Primary metabolites include amino acids, carbohydrates and proteins, whereas secondary metabolites comprise of the alkaloids, flavonoids, saponins, steroids, tannins amongst others (Bhumi and Savithramma, 2014; Rehana Banu and Nagarajan, 2014). Plants produce secondary metabolites mainly to aid their defense against predation by microorganisms, herbivores and insects. Furthermore, terpenoids are involved in plant odor, tannins and quinines are involved in pigmentation, whereas capsacins are involved in flavour. They also function in pollination by attracting pollinators and symbionts, and also respond to abiotic stresses (Dadsena *et al*., 2013; Fuku *et al*.,

2013). The therapeutic potential of medicinal plants lies in the phytochemical constituents that cause certain medicinal and pharmacological actions in human beings (Behere and Boru 2014; Rehana Banu and Nagarajan, 2014; Islam *et al*., 2015).

In recent years, alternative medicine has increasingly become popular, and it is estimated that 44% of all newly developed drugs are derived from natural products (Suleiman *et al.,* 2010) and play a crucial role in the pharmaceutical industry with regards to drug development programmes (De *et al*., 2010). In the discovery and consequent development of any novel plant-derived drug with pharmacological significance, preliminary information is generally provided by qualitative phytochemical screening. Qualitative phytochemical screening of plant extracts gives information regarding the presence of clinically important constituents. In the event that the plant contains important bioactive constituents, these may be isolated from the mixture of compounds by using chromatographic techniques best suited for this purpose (Jayashree, 2013). A number of phytochemicals with therapeutic effects have been isolated from medicinal plants. These include vincristine, vinblastine and curcumin which are anticancer phytochemicals (Oh *et al*., 2016), roseoside, glycyrrhetinic acid and strictinin which have anti-diabetic properties (Ogundele *et al*., 2017) as well as thymol, eugonol and carvacrol which are antimicrobials (El-Zawahry *et al*., 2013).

It is against this background that this chapter focused on determining the phytochemical composition of the extracts from *Gnidia polycephala* and *Senecio serratuloides*.

5.2. Methods

5.2.1. Qualitative phytochemical screening of extracts from *Gnidia polycephala* and *Senecio serratuloides*

The phytochemical analysis of *Gnidia polycephala* and *Senecio serratuloides* was carried out using different qualitative methods adapted from Iqbal *et al*., (2015) and Sharma *et al*., (2012). Specifically, the plant material was screened for the presence of alkaloids, flavonoids, saponins and tannins because of their importance for the current study. Flavonoids and tannins are associated with anticancer properties, alkaloids, flavonoids and saponins have antidiabetic activities and alkaloids, saponins, and tannins are antimicrobials (Aremu *et al*., 2010; Bahmani *et al*., 2014; Elekofehinti *et al*., 2015; Iqbal *et al*., 2015).

5.2.1.1. Alkaloids (Dragendorff's test)

About 15mg of plant material was separately boiled with 6ml of 1% HCl on a water bath for five minutes and filtered. To 1 ml of the filtrate, two drops of the Dragendorff's reagent (Potassium bismuth iodide solution) were added. The presence of alkaloids is detected by the reddish brown precipitate (Sharma *et al*., 2012; Iqbal *et al*., 2015).

5.2.1.2. Flavonoids

To determine the presence of flavonoids, 200mg of the plant material was dissolved in diluted NaOH and then HCl was added to it, then observed for a colour change (Sharma *et al*., 2012).

5.2.1.3. Saponins

The presence of saponins from the plant material was detected by boiling 100mg in 5mL dH_2O and shaking vigorously 1ml of the filtrate and observed for froth formation. The presence of saponins is indicated by froth which persists on warming in a water bath for five minutes (Sharma *et al*., 2012; Iqbal *et al*., 2015).

5.2.1.4. Tannins

To detect the presence of tannins, 200mg powdered plant material was boiled in 10mL dH_2O, filtered and 2ml of the filtrate treated with 2mL of $FeCl_3$ solution and observed for a colour change. The presence of tannins is indicated by formation of a precipitate or black or blue-green colour change (Sharma *et al*., 2012; Iqbal *et al*., 2015).

5.2.2. Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS analysis was carried out at the Central Analytical Facilities (CAF) at the Stellenbosch University, Stellenbosch. Five milligrams of dried plant extracts were re-suspended in 2ml ethyl acetate with vortexing and sonication to assist dissolution of the active compounds. The samples were then centrifuged and analyzed by GCMS analysis using an Agilent 6890N GC linked to a 5975B Mass detector.

The identification of compounds producing peaks in the total ion chromatograms (TIC) was done using both the Wiley and AMDIS databases, as well as data published in the literature. Specifically, the Chemstation software linked to the GCMS was used to acquire the TIC and their associated spectra. The resulting

peaks and their associated spectra were then searched using the Wiley 375 Mass Spectra database. Due to the complexity of the chromatograms, there is a certain degree of the spectral overlap and to overcome this, the Automated Mass Spectral Deconvolution and Identification Software (AMDIS) was used to separate multiple overlapping spectra lying within single chromatographic peaks. The spectra of the separated components were then compared with the spectra of National Institute of Standard and Technology (NIST) 05 Mass Spectral databases which has about 215 339 stored spectra. The identity of the spectra above 95% was needed for the identification of components

5.3. Results and discussion

5.3.1. Qualitative phytochemical screening

The phytochemical screening of the *Gnidia polycephala* and *Senecio serratuloides* extracts revealed the presence of secondary metabolites such as hydrolysable tannins in both the *Gnidia polycephala* and *Senecio serratuloides* plant material. Flavonoids were also determined in *Senecio serratuloides* plant material. Alkaloid and saponins were not determined in both plants.

The absence of alkaloids from both *Gnidia polycephala* and *Senecio serratuloides* may explain the poor antimicrobial activities of the plants demonstrated in Chapter four of this study. Alkaloids have been described as powerful poisons and numerous alkaloids derived from medicinal plants exhibit biological activities such as antimicrobial, anti-inflammatory, antimalarial, cytotoxicity, antispasmodic and pharmacological effects (Iqbal *et al*., 2015). Previous phytochemical screening identified the presence of alkaloids in *S. serratuloides* (Gould *et al*., 2015). The

absence of alkaloids from *S. serratuloides* stems in the present study; could be the result of different geographical locations in which soil minerals and environmental factors greatly influence the phytochemical contents of a plant (Khanam *et al*., 2015).

5.3.2. Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-MS analysis of the *Gnidia polycephala* extracts led to the identification of the different compounds listed in Table 5.1.

Table 5.1: Phytochemical profiles of *Gnidia polycephala* extracts by GC-MS

Retention Time:	Area %:	Name of Compound:	Formula:	Match Quality %:
Aqueous Extract:				
5.759	2.946	Benzoic acid	$C_7H_6O_2$	96
6.12	5.4755	1,2-Benzenediol	$C_6H_6O_2$	86
7.241	1.1338	Phenol,2,6-dimethoxy-	$C_8H_{10}O_3$	97
10.008	9.2697	4-Methoxy-3-propoxybenzaldehyde	$C_{11}H_{14}O_3$	74
10.065	6.6863	Phenol,2,6-dimethoxy-4-(2-propenyl)-	$C_{11}H_{14}O_3$	50
10.389	7.4402	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	$C_{10}H_{12}O_3$	94
Acetone extract:				
5.781	1.7051	Benzoic acid	$C_7H_6O_2$	95
6.904	0.7659	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	55
7.701	0.2486	Vanillin	$C_8H_8O_3$	93
10.493	0.8685	Tetradecanoic acid	$C_{14}H_{28}O_2$	98
11.002	0.2051	2-Pentadecanone,6,10,14-trimethyl-	$C_{18}H_{36}O$	74
11.884	7.8957	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	99
12.922	5.2287	Linoleic acid	$C_{18}H_{32}O_2$	98
13.064	1.085	Octadecanoic acid	$C_{18}H_{36}O_2$	99
15.539	10.8604	1,2-Benzedicarboxylic acid	$C_8H_6O_4$	91
Methanol Extract				
4.719	0.9804	Thymine	$C_5H_6N_2O_2$	47
5.232	0.768	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl	$C_6H_8O_4$	76
6.107	0.3575	Benzofuran, 2,3-dihydro-	C_8H_8O	81
7.245	0.6356	Phenol,2,6-dimethoxy	$C_8H_{10}O_3$	91

7.701	0.1637	Vanillin	$C_8H_8O_3$	83
8.096	0.1235	Phenol,2-methoxy-4-(1-propenyl)-, (E)-	$C_{10}H_{12}O_2$	91
10.071	1.66	Phenol,2,6-dimethoxy-4-(2-propenyl)	$C_{11}H_{14}O_3$	90
10.485	1.4437	Tetradecanoic acid	$C_{14}H_{28}O_2$	93
11.8		n-Hexadecanoic acid	$C_{16}H_{32}O_2$	
12.94	12.9008	9,12,15-Octadecatrienoic acid (Z, Z, Z)-	$C_{18}H_{30}O_2$	96
13.054	0.8549	Octadecanoic acid	$C_{18}H_{36}O_2$	96

The compound 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol (Fig 5.1), was identified from the aqueous extract of *Gnidia polycephala*. This phenolic compound is known to have antimicrobial activities (Ravikumar *et al*., 2012). Surprisingly, the aqueous extract of *Gnidia polycephala* did not display any antimicrobial activity against the tested bacteria may be because this compound was present in insignificant quantities. This can be attributed to the fact that water is not known as the best solvent of extraction for antimicrobial activity (Dzoyem *et al.,* 2016).

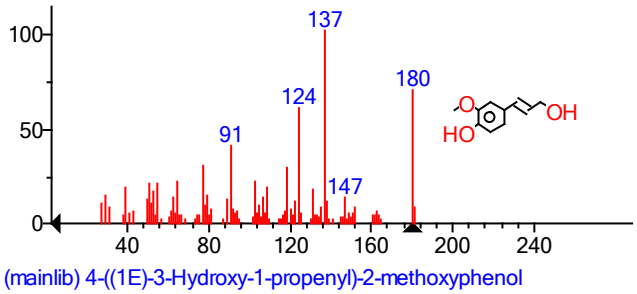

Fig 5.1: MS spectrum of 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol

Tetradecanoic acid (Fig 5.2), was present in both the methanol and acetone extracts of *Gnidia polycephala*. This compound has been reported to have significant larvicidal and repellent activities against mosquitoes (Sivakumar *et al*., 2011). It is possible that the presence of this compound can explain the toxic and

irritating effects of *G. polycephala* that have been reported (Bhandurge *et al*., 2013).

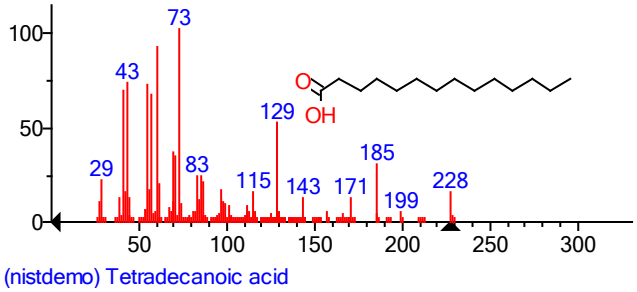

Fig 5.2: MS spectrum of tetradecanoic acid

The acetone extract of *G. polycephala* contained the compound 2-Methoxy-4-vinylphenol (Fig 5.3), which has antimicrobial, antioxidant and anti-inflammatory properties (Ravikumar *et al*., 2012). This compound may have contributed to the antimicrobial activity against *S. aureus* (section 4.3).

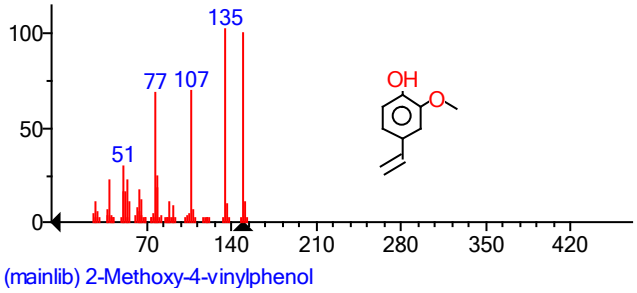

Fig 5.3: MS spectrum of 2-Methoxy-4-vinylphenol

The GC-MS analysis of the aqueous and acetone of *Senecio serratuloides* extracts led to the identification of 12 compounds listed in Table 5.2.

Table 5.2: Phytochemical profiles of *Senecio serratuloides* extracts by GC-MS

Retention Time:	Area %:	Name of Compound:	Formula:	Match Quality %:
Aqueous extract				
4.476	4.476	Phenol, 2-methoxy-	$C_7H_8O_2$	94
6.2123	4.5338	1,2-Benzenediol	$C_6H_6O_2$	91
6.8967	8.3153	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	90
7.2424	3.7713	Phenol,2,6-dimethoxy	$C_8H_{10}O_3$	97
10.3396	1.6534	Ethanone,1-(4-hydroxy-3,5-dimethoxyphenyl)-	$C_{10}H_{12}O_4$	90
11.532	1.1821	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	95
11.8142	2.3578	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	90
12.2798	3.9323	9-Acetyl-S-octahydrophenanthrene	$C_{16}H_{20}O$	49
Acetone extract				
11.5322	0.2122	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	90
12.915	0.8783	Linoleic acid	$C_{18}H_{32}O_2$	99
Methanol extract				
11.8567	4.1462	Hexadecanoic acid	$C_{16}H_{32}O_2$	99
13.0632	1.8932	Octadecanoic acid	$C_{18}H_{36}O_2$	91

The compound 2-Methoxy-4-vinylphenol (Fig 5.4), identified from the aqueous extract of *Senecio serratuloides* and is reported to have antimicrobial, antioxidant and anti-inflammatory (Ravikumar *et al*., 2012).

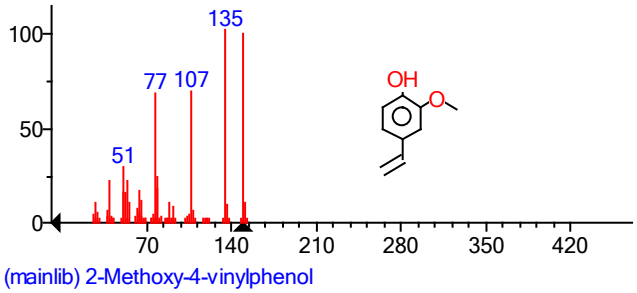

Fig 5.4: MS spectrum of 2-Methoxy-4-vinylphenol

Previous studies have isolated Daphnetin-8-β-D-glucoside, Umbelliferone,2-O-α-D-glucosyloxy-4-methoxybenzenepropanoicacid, Methyl2-O-β-D-glucosyloxy-4-methoxybenzenepropanoate, 7-O-beta-D-Apiofuranosyl-(1-6)-beta-D-glucopyranosyl-umbelliferone (adicardin) and 4-((1E)-3-Hydroxyprop-1-en-1-yl)-2,6-dimethoxyphenyl-β-D-glucopyranoside from *Gnidia polycephala* (Bhandurge *et al*.,2013; Munkombwe *et al*., 2003) but these were not found in this study. A crucial trait of medicinal plants is that they do not consistently produce similar chemicals in the same quantities. The concentration of the produced phytochemicals depends on the growth conditions, storage, and processing of the plant material (Boost *et al*., 2016).

5.4. Conclusion

Gnidia polycephala and *Senecio serratuloides* are commonly used as traditional medicinal plants. This study is the first record of the anticancer and cytotoxic, antidiabetic and antimicrobial properties of these medicinal plants. In the search for novel alternative medicines from medicinal plants, it is required to explore and identify potential compounds with medicinal importance. The results of this study revealed the presence of various compounds with known medicinal importance. Although these compounds were present in insignificant quantities, we

recommend further phytochemical investigations on fractions from these medicinal plants.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

6.1. Introduction

Medicinal plants are considered the best source of novel drug leads in developing alternative medicines (Chintamunnee and Mahomoodally, 2012) because of their availability and cultural acceptance (Verschaeve *et al*., 2004). *Gnidia polycephala* and *Senecio serratuloides* are commonly used as traditional medicinal plants to provide relief and therapy to various diseases (Sections 1.6 and 1.7). To date, little research has been carried out to evaluate their bioactivity. It is for this reason that the bioactivity of the acetone, methanol and aqueous extracts from *G. polycephala* and *S. serratuloides* was evaluated. Table 6.1 presents a summary of the results.

Table 6.1. Bioactivity of extracts from *Gnidia polycephala* and *Senecio serratuloides*

	Results			
	G. polycephala		*S. serratuloides*	
Anticancer activity	dH_2O[1] and MeOH[2]: inactive Acetone: weak		dH_2O: inactive MeOH and acetone: weak	
Cytotoxicity	dH_2O: weak hazard MeOH and acetone: low hazard		dH_2O and MeOH: low hazard Acetone: weak hazard	
Antidiabetic activity	α-amylase inhibition dH_2O: -11.79% MeOH: 10.81 ± 1.32% Acetone: 66.34 ± 0.84%	α-glucosidase inhibition dH_2O: 54.84 ± 0.65% MeOH: 45.43 ± 0.56% Acetone: 81.75± 0.86%	α-amylase inhibition dH_2O: -0.60% MeOH: 6.59± 0.010% Acetone: 9.71 ± 0.019%	α-glucosidase inhibition dH_2O: 28.44 ± 1.98% MeOH: 22.95 ± 1.50% Acetone: 78.86 ± 1.10%
Antimicrobial activity	MeOH: MIC of 10 mg/ml against *S. aureus* Acetone: MIC of 10 mg/ml against *S. aureus*		MeOH: MIC of 5 mg/ml against *S. aureus* Acetone: MIC of 5 mg/ml against *S. aureus*	
Phytochemical classes detected	Hydrolysable tannins		Hydrolysable tannins and flavonoids.	
Phytochemical compounds identified	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol, tetradecanoic acid, and 2-Methoxy-4-vinylphenol.		2-Methoxy-4-vinylphenol.	

[1]: Aqueous extract, [2]: Methanol extracts

6.2. Discussion

Our studies are in contradiction with the report by Gould *et al*., (2015) that the South African National Biodiversity Institute (SANBI) has classified *S. serratuloides* as a poisonous plant. There are also reports in literature that the Genus *Gnidia* possesses toxic diterpene esters (Franke *et al*., 2002). Specifically, Gnidia *polycephala* has been reported to be toxic when consumed by livestock (Munkombwe *et al*., 2003). Selectivity of the anticancer activity of *G. polycephala* and *S. serratuloides* extracts was determined by comparing the anticancer activity (IC_{50}) of each plant extract against the cancer cell lines with that of the normal human fetal lung fibroblast cell line, the acetone extract of *G. polycephala* demonstrated higher selectivity for the TK10 and UACC62 cell lines. Likewise, the acetone extract of *S. serratuloides* demonstrated higher selectivity for UACC62 cells, whereas the methanol extract of *S. serratuloides* demonstrated higher selectivity for all cell lines used in this study (Section 2.3). The high selectivity index indicates that the extracts offer the potential of safer therapy (Valdẻs *et al*., 2010).

Hydrolysable tannins were found in both the *Gnidia polycephala* and *Senecio serratuloides* extracts, as well as flavonoids in *Senecio serratuloides* (Table 6.1). Literature attributes the antimicrobial activity of most medicinal plants to the presence of alkaloids (Iqbal *et al*., 2015), which were lacking in this study. There is also no direct association between tannin content and antibacterial activity (Ahmed *et al.,* 2012). This confers with the weak or lack of anticancer (section 2.3) and antimicrobial activities (section 4.3) displayed by the tested plant extracts. The absence of the other classes of phytochemical compounds could be as a result of the difference in geographical locations where our plant samples and the plant samples reported in literature to possess these phytochemical classes were

collected. This is because soil minerals and environmental factors greatly influence the phytochemical contents of a plant (Khanam *et al*., 2015). Additionally, medicinal plants are known not to consistently produce similar chemicals in the same quantities. The concentration of the produced phytochemicals depends on the biochemical factors within the plant as well as external factors. These include the plant part tested, storage and processing of the plant material and climatic conditions as well as the type of soil the plant is growing in (Boost *et al*., 2016; Javadi *et al*., 2015). For instance, this study investigated *Gnidia polycephala* stem, leaves and flower extracts which were prepared as a mixture. On the contrary, glycosides, syringin and adicardin were detected from the stem and root extracts of the plant in previous studies (Munkombwe *et al*., 2003). Similarly, the period during which the plant is harvested may also have an effect on the composition of secondary metabolites of the same plant species and ultimately lead to variations in the activity of the same plant species collected at different periods (Buwa and Van Staden, 2007). Therefore, the inconsistency or differences of the results of our study in comparison to those in literature may be attributed to the possible differences in the season in which the plants were harvested, geographic location, soil types as well as climatic conditions.

Another contributing factor may be the age of the investigated plant samples. In their study, Hu *et* al., (2003) found the three-year-old *Aloe vera* plant they tested to possess significantly higher contents of polysaccharide and flavonoids than those they found in two- and four- year old *Aloe vera* plants.

Alternatively, the investigated plant extracts may not by themselves induce direct pharmacological properties but, may enhance the body's own healing process. For instance, previous studies have found *S. serratuloides* to heal skin wounds in a pig

model two days earlier than untreated wounds. This was attributed to the effect of the plant on early phases of wound healing because *S. serratuloides* lowered the wound pH and a lower pH is known to promote cellular migration and proliferation. An appropriate pH is required for the overall integrity of intact skin in order to maintain homeostasis and the correct balance of cellular debris as well the modulation of growth factors, cytokines and cytoplasmic receptor domains (Gould *et al*., 2015). Similarly, some anticancer drugs are known to exhibit their antitumor activity *in vivo* but have no *in vitro* cytotoxic activity. This is reportedly so because of immune modulation by the compound which might lead to anticancer activity *in vivo* (Kamahabwa *et al*., 2000).

6.3. Conclusions and recommendations

The current study documents the *in vitro* bioactivity of *Gnidia polycephala* and *Senecio serratuloides* and further confirm the importance of medicinal plants in the search for the discovery and development of alternative drug leads. The *Gnidia polycephala* extracts proved to have the potential to be developed as anti-diabetic drugs. We recommend further *in vitro* experiments to establish the mechanism of action from the extracts with high α-amylase and α-glucosidase inhibitory activities as well as to determine if the inhibition is as a result of the compounds acting synergistically or individually.

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