INVESTIGATION OF ANTICANCER PROPERTIES OF *PHILENOPTERA VIOLACEA (KLOTZSCH) SCHRIRE AND XANTHOCERCIS ZAMBESIACA (BAKER) DUMAZ-LE-GRAND*

by

POLO-MA-ABIELE HILDAH NTSOELINYANE

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Promoters: Professor Samson Mashele (PhD)

: Doctor Idah Manduna (PhD)

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PH Ntsoelinyane 31 October 2014

PLAGIARISM AND DECLARATION OF INDEPENDENT WORK

I ___P.H. NTSOELINYANE_____ (student number: _ _____)
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Title of the project:

Investigation of anticancer properties of *Philenoptera violacea (Klotzsch) Schrire* and *Xanthocercis zambesiaca (Baker) Dumaz-le-Grand*

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TABLE OF CONTENTS

CHAPTER 5 "Antioxidant activity screening of *P.violacea* and *X.zambesiaca*"

CHAPTER 6 "Phytochemical analysis of *P.violacea* and *X.zambesiaca*"

Investigation of anticancer properties of *Philenoptera violacea (Klotzsch) Schrire and Xanthocercis zambesiaca (Baker) Dumaz-le-Grand*

ABSTRACT

The aim of this study was to assess *Philenoptera violacea (Klotzsch) Schrire* and *Xanthocercis zambesiaca (Baker) Dumaz-le-Grand* as anticancer agents, to determine the mutagenic and anti-mutagenic properties, anticancer activity, antibacterial and antioxidant activity of these plant extracts, radioprotective effects of *X.zambesiaca* and phytochemical screening of both plant extracts in order for them to be used in developing novel anticancer drugs. The extracts of medicinal plants were prepared using the maceration with methanol as solvent. Methanol extracts were tested for their growth inhibitory effects *in vitro* using the Sulforhodamine B assay. Mutagenic properties were determined by the Ames test, using Salmonella typhimurium strains TA98 and TA100 without S9. Antibacterial activity was determined using *p*-Iodonitrotetrazolium chloride assay. The antioxidant activity was determined using 2, 2- diphenyl-1-picrylhydrazyl assay. *X.zambesiaca* and *P.violacea* were classified as inactive (TGI >50 µg/ml) against breast (MCF7), colon (HCT116), renal (UACC-62), melanoma (TK-10) and prostate (PC3) cancer cell lines. Results of test for the mutagenic properties of *P.violacea* and *X.zambesiaca* plant extracts revealed that neither plant has mutagenic effects. Antibacterial activity results revealed that both plants' extracts had no antimicrobial activity against *Staphylococcus aureus*, *S. epidermidis*, *S. saprohyticus*, *Bacillus subtilis, Enterobacter cloacae*, *Escherichia coli or Pseudomonas aeruginosa*. *P.violacea* plant extract showed low antioxidant activity, while *X.zambesiaca* extract exhibited moderate dose-dependent antioxidant activity and can be considered a weak source of antioxidant. In view of these antioxidant results, the free radical scavenging ability of *X.zambesiaca* was confirmed by determining the radioprotective effects of this extract on human prostate cancer cells (DU145), which were irradiated to 2 Gy. Results showed a significant radiation protection effect that can lead to over 50% reduction in cell death. Qualitative phytochemical analysis of these plant extracts confirmed the presence of tannins, flavonoids, steroids, terpenoids, alkaloids and

cardiac glycosides from *P.violacea* extract, while *X.zambesiaca* extract showed the presence of flavonoids, saponins, terpenoids and glycosides. The gallic acid equivalents (GAEs) of the estimated phenolic concentrations ranged from 0.152 ± 0.0269 to 0.0895 ± 0.006 mg/GAE. Liquid chromatography-mass spectrometry analysis of these plant extracts showed the presence of nine compounds (including two unknown compounds) from *P.violacea,* and seven compounds from *X.zambesiaca.* The results thus indicate the possibility of using *X.zambesiaca* (methanol mixture of leaf, twig and flower) extracts as a radioprotector and an antioxidant agent to counteract and prevent cell damage by free radicals. These results were encouraging, despite the need for clinical studies to determine the real effectiveness and potential toxic effects of this extract *in vivo*. Our results led us to raise questions about the effect of *P.violacea* (methanol mixture of leaf, twig and flower) extracts, which proved to possess a number of active compounds that were somehow inactive, regardless of how many times we re-analyzed them. Finally, results revealed the importance of *X.zambesiaca* as a possible anticancer drug.

CHAPTER 1

"General background"

1.1 INTRODUCTION

Historically, plants have been used for a variety of purposes in all human cultures: food, culinary spices, medicine, cosmetics, spiritual symbols or aids and ornamental plants and are still being used for these reasons. Various but specific plants have been used as medicinal agents to treat illnesses since ancient times. Traditional medicine, with medicinal plants as the most important component, are sold in marketplaces or prescribed by traditional healers in their homes (Von Maydell, 1996), particularly in the rural areas of South Africa where herbal medicine is the main resource of the health care system. South Africa's rich plant biodiversity, with over 20 000 different species, is a great source of interest to the scientific community (Cherry, 2005). Although there is an abundance of plants used as medicine, there are specific traditional ways of processing and applying plant-based medications and these have been handed down from generation to generation.

According to Pavithra *et al.* (2010), since 1990s there has been a growing shift in interest towards plants as significant sources of new pharmaceuticals and herbalism has become mainstream throughout the world. It is estimated that three quarters of the world population rely on herbal and traditional medicine as a basis for primary health care (Efferth and Kaina, 2011). It was discovered that between 12 and 15 million South Africans still depend on traditional herbal medicines from as many as 700 indigenous plant species (Meyer *et al*., 1996). Up to 60% of the South African population consult one of an estimated 200 000 traditional healers, especially in rural areas where traditional healers are more numerous and accessible than Western doctors (Liu, 2011). Because of this strong dependence on plants as medicines, studies are conducted to determine their safety and their efficacy and on the other hand to discover new active principles in plants.

In South Africa and Africa as a whole, the evaluation and recognition of traditional medicine aims to improve its availability and wider application at low cost. The

introduction of modern medicines and practices (including standardization of doses), as well as other socio-economic development processes (such as medical records and access to medical aid schemes), can improve the application of traditional healing. Plant-derived natural products provide an interesting source for isolating and screening potent molecules to combat inflammatory diseases, hypertension and cancer. Modern isolation and screening technologies have enhanced the search for new lead molecules and increased interest in folk medicinal plant extracts in drug companies (Balunas and Kinghorn, 2005).

Pharmaceutical companies show interest in plant-derived drugs mainly owing to the current widespread belief that 'green medicine' is safe, with fewer side effects (Pavithra *et al*., 2010). There are still challenging tasks for scientists working in drug research to investigate the efficacy of herbal medicine, to distinguish favorable from adverse effects, to identify active principles in medicinal plants, to ban poisonous plants or contaminations from herbal mixtures (Efferth and Kaina, 2011) and to assess preparations for safety or toxicity to the tissue or organs of mammalian recipients. According to Cragg and Newman (2000), over 50% of the drugs in clinical trials for anticancer activity were isolated from natural sources. Cardoso *et al*. (2006) demonstrated that some plants used for traditional medicine have *in vitro* mutagenic activity. This has increased awareness about the potential mutagenic hazards resulting from long-term use of traditional medicinal plants (Elgorashi *et al*., 2003).

Many conventional drugs also induce genetic damage that itself can be carcinogenic. As a result, a section of the research community is focusing on identifying novel chemotherapeutic agents in plants that do not induce the destructive effects of conventional cytotoxic therapeutic agents. As some of the active ingredients are potentially toxic, there is a need to evaluate the safety of plant preparations. Therefore, traditional plants must be considered potentially unsafe and further testing before their continued use, is recommended. The following study is aimed at investigating *P.violacea* and *X.zambesiaca* as anticancer agents, determining the mutagenic and anti-mutagenic properties of the extracts, anticancer activity, antibacterial and antioxidant activity of plant extracts and phytochemicals studies of the plant extracts to allow them to be used in developing novel anticancer drugs.

1.2 LITERATURE REVIEW

1.2.1 Usage of natural medicinal plants

Cancer is a highly complex, ubiquitous and devastating disease causing 10 million new diagnoses worldwide per annum. Of these, 6.7 million will succumb. At present there are 24.6 million cancer patients living with cancer and hoping to survive (WHO, 2005). It is projected that deaths from cancer will continue to escalate, with an estimate of nine million deaths from cancer in 2015 and 11.4 million deaths by 2030 (WHO, 2005). It is a widely accepted view that cancer starts in just one of the body's billions of cells and progressively transforms to malignancy in multiple stages through sequential acquisition of additional mutations. The immune system of the body then fails to respond properly by destroying the cancerous cells. The cancerous cells lose their normal controls of cell division and continue to proliferate. The cells then divide to form abnormal cells, without normal genetic controls.

Currently, interest in alternative therapies is increasing as dissatisfaction with traditional therapies grows, thus there is marked scientific and commercial interest in the continuing discovery of new anticancer agents from natural product sources. The search for natural products to be used in cancer therapy represents an area of great interest in which the plant kingdom is the most important source, which provides many anti-tumor agents with novel structures and unique mechanisms of action (Chang *et al*., 1999). Chemotherapy treatment uses medicine to weaken and destroy cancer cells in the body, including cells at the original cancer site. These cells develop resistance and are restricted by dose-limiting side effects. The absence of markedly improved treatments despite decades of research, the toxicity of chemotherapy, cells developing resistance and the lack of significant improvement in cure rates for the major cancers contribute to dissatisfaction (DeVita *et al*., 1991). This has led to increasing interest even within the traditional medical community in finding alternative cancer treatments.

Several medicinal plants are traditionally used in the treatment of a variety of diseases, including cancer, by the population of South Africa and neighboring countries. Since ancient times, herbal medicine has always been one of the main components of the health care system. Many conventional drugs induce genetic

damage that itself can be carcinogenic. As some of the active ingredients are potentially toxic, there is a need to evaluate the safety of plant preparations. A segment of the research community is thus focusing on identifying novel chemotherapeutic agents in plants that do not induce the destructive effects of conventional cytotoxic therapeutic agents. Compounds that are derived from plants have contributed significantly in the development of some useful anticancer drugs such as taxol, etoposide, vinblastine and vincristine. Plants are potential sources of natural antioxidants and produce various antioxidative compounds to counteract reactive oxygen species, including free radicals such as superoxide anion radicals and hydroxyl radicals (Huda-Faujan *et al*., 2009). Thus, investigation of traditionally used medicinal plants is valuable as a source of potential chemotherapeutic agents and to assess the safety of the continuous use of medicinal plants.

1.2.2 Background of the plants under study

The flowering plant family *Fabaceae* includes approximately 18 000 species and 400 genera of flowering, leguminous plants (Brummitt *et al*., 2007). Trees, herbs, vines and shrubs of this plant family are native to all regions of the world and are commonly cultivated. *Fabaceae* is the most common family found in tropical rainforests and in dry forests in the Americas and Africa. The genus *Xanthocercis* belongs to the family of Fabaceae and comprises three species,namely *X. madagascariensis, X. rabiensis, X. zambesiaca*. These three species are large, evergreen trees that have not been scientifically assessed yet for either efficacy or safety to human tissues or organs.

Figure 1.1: *X.zambesiaca* tree (on the left) and flowers, leaf and twig (on the right)

X.zambesiaca is a protected plant in South Africa, grows on alluvial soils and is only found in Limpopo and Mpumalanga. This species is known as mmchetuchetu/musharo in Shona, nyala berry in English, hoenderspoor or njalaboom in Afrikaans, mhota/moladi/motlha in Setswana and mutshato in Vhenda. In this study, *X.zambesiaca* will sometimes be referred to as *X.zambesiaca.*

Figure 1.2: *P.violacea* tree (on the left) and flowers, leaf and twig (on the right)

P.violacea usually grows near water at low to medium altitudes but not in evergreen forests (Isman, 2008). It prefers well-drained soil and the plant is very sensitive to fire (Isman, 2008). Fruit is produced from May to October (Palmer and Pitman 1972). Medicinally the roots of this plant are used to treat stomach disorders, hookworms and coughs. Rotenone, used in the insecticide Derris, is extracted from the roots (Isman, 2008). The plants' wood is used for making grain mortars and tool handles, as it is strong, fairly hard and heavy. Many Africans are superstitious about *P.violacea,* as it is one of the rain trees. In Tanzania the leaves are put into the luggage of travelers for protection during a journey. The tree is also known to be a reliable indicator of ground water.

Philenoptera is a genus of 12 species belonging to the Fabaceae family and Papilionoideae subfamily (Drummond, 1981; Brummitt *et al*., 2007). *P.violacea* is known also as apple leaf or rain tree in English, appelblaar in Afrikaans, mphata in Sotho, mohata in Tsonga and isihomohomo in Zulu. It is a plant species in the

legume family (Fabaceae), found in the Democratic Republic of Congo, Tanzania, Zambia, northern Namibia, Zimbabwe, South Africa and Swaziland. *P.violacea* is a protected plant in South Africa, mainly found in the northern part of Limpopo and Kwazulu-Natal province and in the warmer parts of Mpumalanga and the Eastern Cape Province (Schrire, 2000). This species has been used in traditional remedies in South Africa. Roots have been used for gastro-intestinal problems, powdered root-bark for colds and snakebite treatment, root infusions as hookworm remedy and most parts of the plant as treatment for diarrhea (Bruschi *et al*., 2011). According to the literature, *X.zambesiaca* has demonstrated anti-tuberculosis activity and antihyperglycemic activity against diabetic mice. Isoflavones have also been identified from this plant. However, its anticancer activity has never been investigated. The efficacy, safety, toxicity and correct administration dose of the plant has not yet been determined, as these plants have never been investigated for pharmaceutical use before. In this study *P.violacea* and *X.zambesiaca* extracts will be analyzed to determine whether these plants can be used to develop novel anticancer drugs.

1.3 RESEARCH OBJECTIVES AND OUTCOMES

Problem statement: Current anticancer drugs yield dissatisfactory results because of their toxic side effects and dose dependency. There is a continuous need for cost-effective cancer therapy protocols that can be approved by national health authorities.

1.3.1 The aim of the study

The aim of the present research is to investigate the anticancer activity of *P.violacea* and *X.zambesiaca* plant extracts.

1.3.2 The objectives of this project are as follows:

1. To determine the anticancer activity of *P.violacea* and *X.zambesiaca* against human cell lines.
2. To determine the mutagenic and anti-mutagenic properties of *P.violacea* and *X.zambesiaca* extracts.
3. To determine the antibacterial activity of *P.violacea* and *X.zambesiaca* extracts.

4. To determine the antioxidant activity of *P.violacea* and *X.zambesiaca* extracts.
5. To determine the active phytochemicals of *P.violacea* and *X.zambesiaca* extracts.
6. To isolate and identify the bioactive compound from *P.violacea* and *X.zambesiaca* extracts.

1.3.3 Outcomes

(a) Null hypothesis

(I) P.violacea and *X.zambesiaca* plant extracts have anticancer activity.

(II) P.violacea and *X.zambesiaca* extracts have anti-mutagenic properties.

(III) P.violacea and *X.zambesiaca* plant extracts have antibacterial activity.

(IV) P.violacea and *X.zambesiaca* plant extracts have antioxidant activity.

(V) *P.violacea* and *X.zambesiaca* plant extracts contain phytochemicals.

(VI) *P.violacea* and *X.zambesiaca* plant extracts contain bioactive compounds.

(b) Alternative hypothesis

(I) P.violacea and *X.zambesiaca* plant extracts do not have anticancer activity.

(II) P.violacea and *X.zambesiaca* plant extracts do not have anti-mutagenic properties.

(III) P.violacea and *X.zambesiaca* plant extracts do not have antibacterial activity.

(IV) P.violacea and *X.zambesiaca* plant extracts do not have antioxidant activity.

(V) *P.violacea* and *X.zambesiaca* plant extracts do not contain phytochemicals.

(VI) *P.violacea* and *X.zambesiaca* plant extracts do not contain bioactive compounds.

1.4 METHODOLOGY

1.4.1 Plant material selection

The family *Fabaceae* contains species such as *Phaseolus vulgaris L, Glycine max L, Vigna unguiculata* (Bennink, 2002), *Glycyrrhiza glabra* (Kainsa *et al*., 2012) etc., which have been reported as useful anticancer agents. These publications resulted from a drive to find 60 African plants belonging to the *Fabaceae* family, in the hope

of discovering novel anticancer agents. *P.violacea* and *X.zambesiaca* extracts were randomly selected from the 60 plants.

1.4.2 Plant material collection

The plant materials *P.violacea* and *X.zambesiaca* were authenticated by scientists at the National Botanical Gardens in Bloemfontein, South Africa. These two plants were chosen from 60 plants belonging to the *Fabaceae* family. Twigs, leaves and flowers of *P.violacea* and *X.zambesiaca* were collected and mixed respectively. The collected material was dried at room temperature, pulverized by mechanical mills and weighed. It was then stored in a cool place until analysis.

1.4.3 Extraction method

The extraction was done using the maceration. Plant material (10 g of the dried twigs, leaves and flowers of each plant) was weighed, pulverized and soaked in methanol for 72 hours, with occasional stirring. The extracts were filtered and new solvent was added again for more extraction until the solvent remained clear. The methanol extract was concentrated by using a rotator evaporator.

1.4.4 Study layout

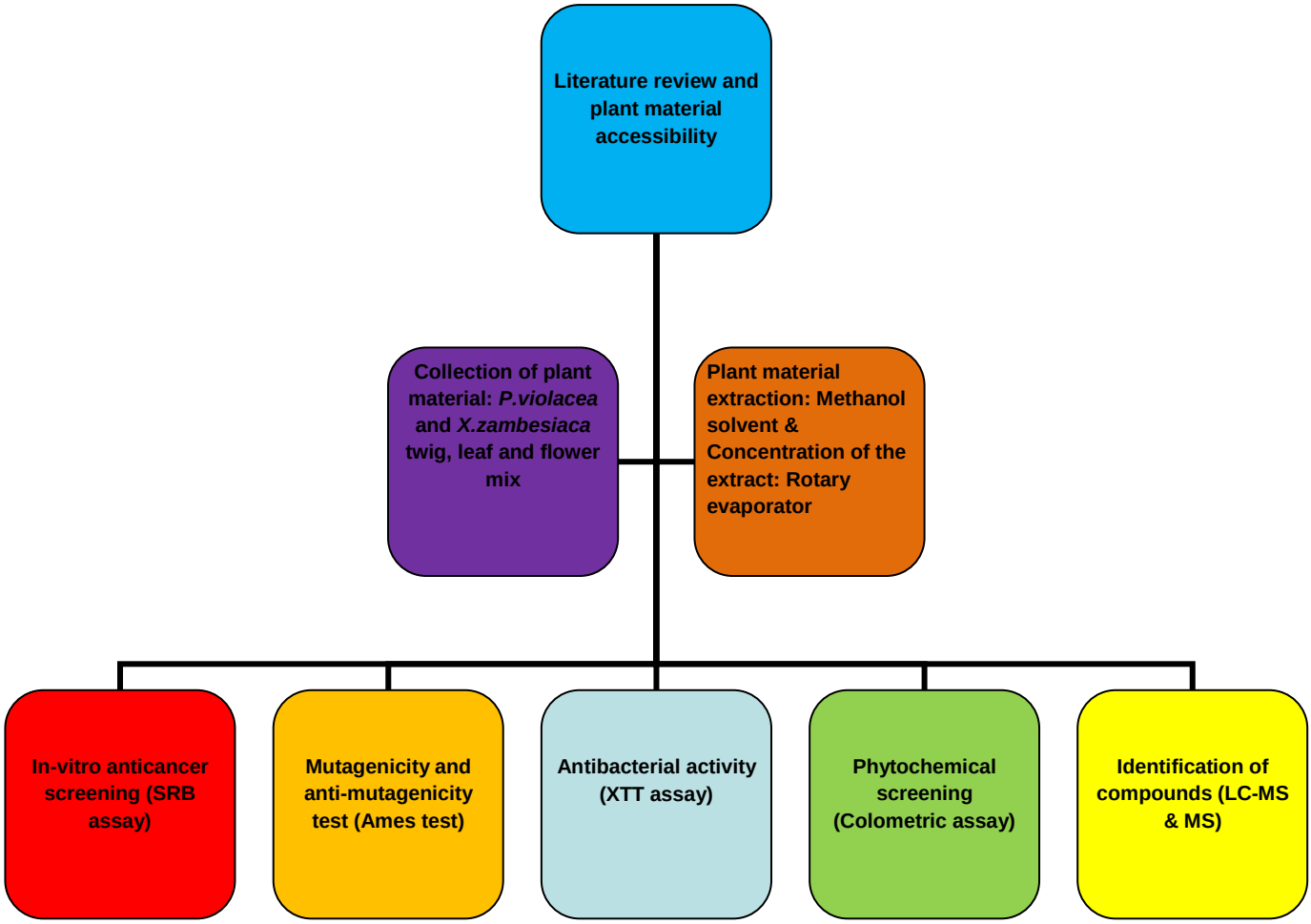

Figure 1.3: A diagrammatic summary of steps in the study of the investigation of anticancer activity of *P.violacea* and *X.zambesiaca* plant extract.

1.5 STATISTICAL ANALYSIS

Tests were carried out in triplicate and data reported as mean ± SD. The data obtained from the study were analyzed statistically by analysis of variance (ANOVA) using Graph pad prism version 5.0 software. Data from the test groups were compared with controls and $P<0.05$ was accepted and considered statistically significant.

1.6 STUDY OVERVIEW

Chapter 1 demonstrates the importance of medicinal plants in developing countries. The background of the plants investigated in this dissertation is discussed thoroughly in this section. The evidence of astounding dependence on traditional medicine has already led to the development of drugs derived purely from plants. The chosen plants are traditionally used for different alignments/diseases, but their effect against cancer has never been explored before. The study objectives are set out to report the activity of these plants as anticancer and chemopreventative agents in **Chapters 2-8**. **Chapter 2** presents the evaluation of the *in vitro* anticancer activity of both plants' extracts. **Chapter's 3-5** reports on studies that evaluate the *in vitro* toxicity of the plant extracts and the antibacterial and antioxidant properties of these plant extracts. **Chapters 6** and **7** focused on the evaluation of active compounds present in these methanol plants extracts. **Chapter 8** reports on the evaluation of the radioprotective effect of one plant that proved to have potential as an antioxidant agent. Based on the overall study findings, **Chapter 9** and **Chapter 10** provide a conclusion and recommendations, respectively.

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CHAPTER 2

"Anticancer activity of *P.violacea* and *X.zambesiaca*"

ABSTRACT

There is marked scientific interest in the continuing discovery of new anticancer agents from natural product sources, as current anticancer drugs have many negative side effects. Chronic inflammation is a major cause of cancer formation, and *P.violacea* is traditionally used to treat inflammation-related disorders, while *X.zambesiaca* has been demonstrated to have anti-tuberculosis properties. The aim of the present study was therefore to determine the anticancer activity of *P.violacea* and *X.zambesiaca* leaf, twig and flower extracts. Methanol extracts were tested for their growth inhibitory effects *in vitro* against breast (MCF7), colon (HCT116), renal (UACC-62), melanoma (TK-10) and prostate (PC3) cancer cell lines using the Sulforhodamine B assay. Based on total growth inhibition (TGI) of the cell lines, extracts were classified into four categories: inactive, weak, moderate and potent. Etoposide and parthenolide were used as positive controls in this assessment. Plant twig, leaf and flower mixtures of *X.zambesiaca* and *P.violacea* were classified as inactive (TGI >50 µg/ml). Results showed that these extracts do not have anticancer activity against selected human cancer cell lines, but this does not exclude their activity against other cancer cell lines. These plant extracts will be tested further for their anticancer activity against other cancer cell lines in the future.

2.1 INTRODUCTION

Traditional medicine is widely practiced, especially in African developing countries. This is a result of primary health care facilities being unable to manage the number of patients requiring aid, as well as the high cost of Western pharmaceuticals and health care. It is estimated that between 12 and 15 million South Africans still depend on traditional herbal medicines from as many as 700 indigenous plant species (Meyer *et al*., 1996). This great dependence and immense usage of traditional plants as the preferred form of health care is aided by the fact that most of these plants are widely available and affordable. Natural products have been used for the treatment of various diseases since ancient times. Currently, interest in alternative therapies is increasing as dissatisfaction with traditional therapies grows, thus there is marked scientific and commercial interest in the continuing discovery of new anti-cancer agents from natural product sources.

The search for natural products that provide many anti-tumor agents with novel structures and unique mechanisms of action is vital (Chang *et al.,* 1999), as the current chemotherapy treatments weaken and destroy cancer cells in the body, including cells at the original cancer site. However, some cancerous cells develop resistance, as the current treatment is restricted by dose-limiting and produces unpleasant side effects. The absence of markedly improved treatments despite decades of research, the toxicity of chemotherapy, cells developing resistance and the lack of significant improvement in cure rates for the major cancers contribute to dissatisfaction with the current cancer treatments (DeVita *et al*., 1991). This has led to increasing interest, even in the traditional medical community, in alternative cancer treatments. Therefore, this study was conducted to investigate the anticancer activity of *X.zambesiaca* and *P.violacea* plant extract.

2.2 LITERATURE REVIEW

Cancer can develop in any human tissue subsequent to the loss of normal cell-cycle control due to various factors that lead to cellular malfunction. These abnormal cells or undifferentiated cells with unregulated growth can proliferate and suppress the

growth of normal cells. Studies have revealed that certain cancers are more common in people of certain cultures than others. In South Africa, the cancer incidence rates among blacks are high for breast, prostate, skin and colon cancer, while cervix, esophageal, prostate and breast cancer incidence rates are high among Caucasians (Albrecht, 2003). In 1998, 60 172 new cancer cases were reported and 60 343 new cases in 1999, of which the ethnic group at highest risk was determined as South African Caucasians (Mqoqi *et al*., 2004). There is a need to discover and develop anticancer agents to combat the cancer crisis the world is facing.

Chemoprevention is a process that delays or prevents carcinogenesis in humans through the ingestion of dietary or pharmaceutical agents. Most traditional medicines used by South Africans are ingested. The searches for anticancer agents from plant sources dates back to the time of our forefathers and plant products have been proven to be an important source of anticancer drugs (Cragg and Newman, 2005). This is because of their biologically and chemically diverse nature, which allows for the discovery of completely new chemical classes of compounds. The discovery and development of plant-derived compounds with cytotoxic agents that are effective against a range of cancer cell lines, while being less active or non-toxic against the normal cell population, may lead to the first cures of human cancer. Shishodia and Aggarwal (2004) determined that approximately 74% of 90 prescribed anti-cancer drugs were developed in response to an investigation into the claims made by folkloric tradition. Thus the screening of anticancer activity from plants is determined in this study.

2.3 METHODOLOGY

2.3.1 Plant material

The plant materials *P.violacea* and *X.zambesiaca* were authenticated by scientists at the National Botanical Gardens in Bloemfontein, South African (MAS002). The collected material was dried at room temperature, pulverized by mechanical mills and weighed. It was then stored in a cool place until analysis.

2.3.2 Extraction method

The extraction was done using the maceration. Plant material (10 g of the dried twigs, leaves and flowers of each plant) was weighed, pulverized and soaked in methanol for 72 hours, with occasional stirring. The extracts were filtered and new solvent was added again for more extraction until the solvent remained clear. The methanol extract was concentrated using a rotator evaporator.

2.3.3 Anticancer screening

The human cell lines TK10, UACC62 and MCF7 were obtained from the National Cancer Institute (NCI) in the framework of a collaborative research programme between the Council for Scientific and Industrial Research (CSIR) and the NCI. The extracts and compounds were assayed in the three-cell line panel consisting of TK10 (renal), MCF7 (breast), and UACC62 (melanoma) cancer. Cell lines were routinely maintained as monolayer cell cultures at 37°C, 5% CO2 and 100% relative humidity in RPMI containing 5% fetal bovine serum, 2 mM L-glutamate and 50 µg/ml gentamicin. The primary anticancer assay was performed at the CSIR in accordance with the protocol of the Drug Evaluation Branch, NCI (Kuo *et al*., 1993; Leteurtre *et al*., 1994). The extracts or compounds were tested at a single concentration (100 ppm) and the culture was incubated for 48 hours. End point determinations were made with a protein-binding dye, Sulforhodamine B (SRB). The growth percentage was evaluated spectrophotometrically versus controls not treated with test agents. Results for each extract were reported as the growth percentage of the treated cells, compared to that of the untreated control cells. All the extracts that reduced the growth of two of the cell lines by 75% or more were tested further at 1/2 log serial dilutions of five concentrations ranging from 6.25-100 ppm. Cells to which no drugs were added served as controls. The blanks contained complete medium without cells. *Etoposide* and *Parthenolide* were used as a standard. Results of five dose screenings were reported as TGI (Total growth inhibition). The biological activities were separated into four categories: inactive (TGI >50 ppm), weak activity (15 ppm< TGI <50 ppm), moderate activity (6.25 ppm< TGI <15 ppm) and potent activity (TGI <6.25 ppm).

2.4 RESULTS

(I) 50% inhibition concentration

IC_{50}	MCF7	HCT116	TK-10	UACC-62	PC3
P.violacea	>100	>100	>100	>100	>100
X.zambesiaca	>100	>100	>100	>100	>100

Table 2.1. IC_{50} of methanol extract of *P.violacea* and *X.zambesiaca* on five human cell lines: MCF7, HCT116, TK-10, UACC-62 and PC3.

Table 2.1 shows the growth inhibitory effects of P. Violacea and X.zambesiaca. Total growth inhibition occurred at concentrations higher than 100 µg/ml.

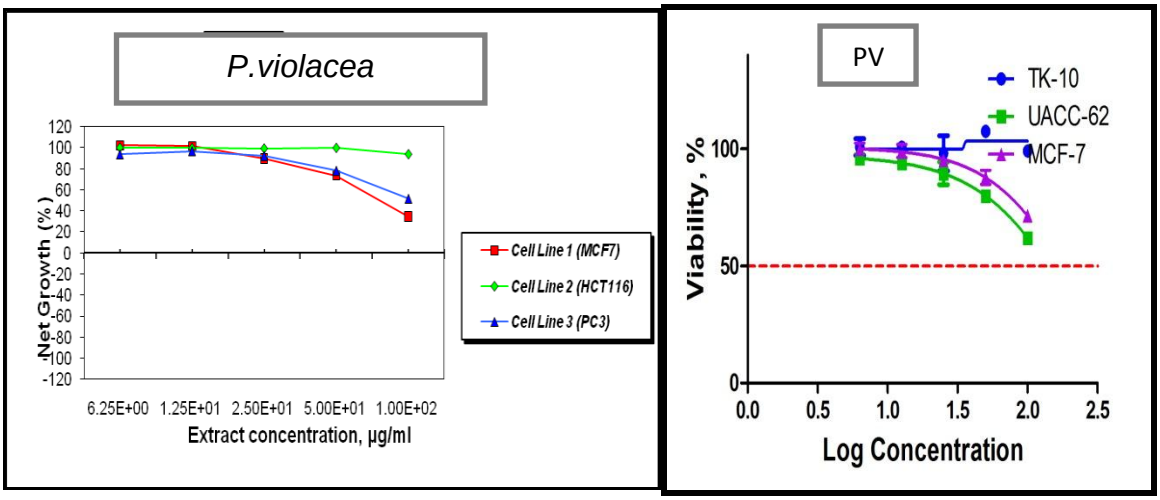

Figure 2.1. Growth inhibitory effect of ethanol extracts of *P.violacea* on five human cell lines: MCF7, HCT116, TK-10, UACC-62 and PC3.

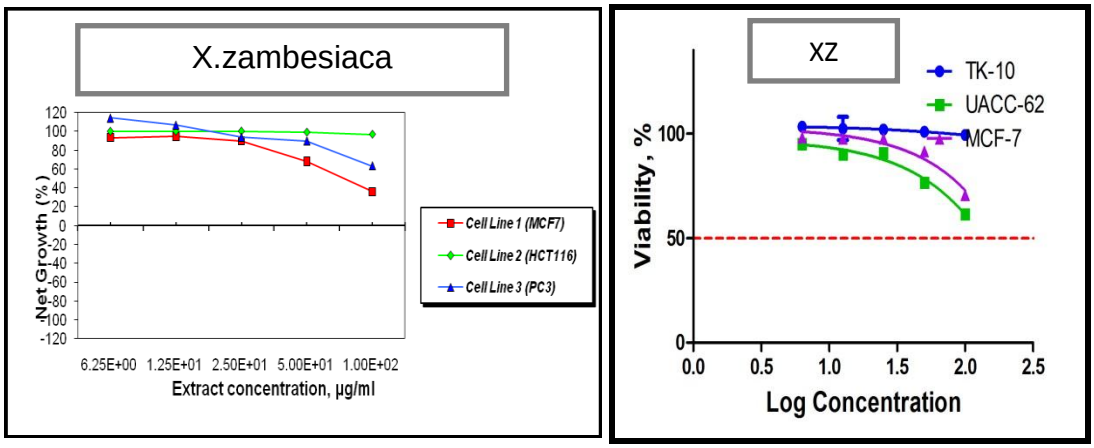

Figure 2.2. Growth inhibitory effect of ethanol extracts of *X.zambesiaca* on five human cell lines: MCF7, HCT116, TK-10, UACC-62 and PC3.

IC_{50}	MCF7	HCT116	PC3	TK-10	UACC-62
Etoposide	<6.25	19.96	34.64	-	-
Parthenolide	<6.25	-	-	<6.25	<6.25

Table 2.2. IC_{50} of Etoposide on three human cell lines (MCF7, HCT116 and PC3) and Parthenolide on human cell lines MCF7, TK-10 and UACC-62.

IC_{50},µg/ml	Status
> 100 µg/ml	**Inactive**
< 100 µg/ml>15 µg/ml	**Weak Activity**
< 15 µg/ml> 6.25 µg/ml	**Moderate Activity**
< 6.25 µg/ml	**Potent Activity**

Table 2.3. CSIR criteria

(II) Total growth inhibition

Activities	*P.violacea*	*X.zambesiaca*	*P.violacea and X.zambesiaca*	
	MCF7		HCT116	PC3
GI50	80.27	78.35	>100	>100
TGI	>100	>100	>100	>100
LC50	>100	>100	>100	>100
LC100	>100	>100	>100	>100

Table 2.4. GI50, TGI, LC50 and LC100 of methanol extract of *P.violacea* and *X.zambesiaca* on three human cancer cell lines: MCF7, HCT116 and PC3**.**

TGI, 50 µg/ml	Status
> 50 µg/ml	Inactive
< 50 µg/ml>15 µg/ml	Weak Activity
< 15 µg/ml> 6.25 µg/ml	Moderate Activity
< 6.25 µg/ml	Potent Activity

Table 2.5. CSIR criteria

2.5 DISCUSSION

The sulforhodamine B (SRB) assay is a colorimetric assay that indirectly estimates the number of viable cells by staining total cellular proteins. The evaluation of the cytotoxicity of *P.violacea and X.zambesiaca* was investigated using the SRB assay to assess the growth inhibition of cells. The ability of *P.violacea and X.zambesiaca* plant extracts to inhibit the *in vitro* growth of five human cancer cell lines, namely the breast (MCF7), colon (HCT116), renal (UACC-62), melanoma (TK-10) and prostate (PC3) cancer cell lines, was evaluated. The results were expressed as percentage cell growth inhibition at 100 µg/ml, while those indicating a percentage inhibition of greater than 80% at 100 µg/ml were expressed as IC_{50} values. This value is defined as the concentration causing 50% cell growth inhibition.

The results of five-dose screening (Table 2.1) were reported as 50% of cell growth inhibition (IC_{50}). The biological activities were separated into four categories: inactive (IC_{50} >100 µg/ml), weak activity (15 µg/ml < IC_{50} <100 µg/ml), moderate activity (6.25 µg/ml < IC_{50} <15 µg/ml) and potent activity (IC_{50} <6.25 µg/ml). According to the CSIR criterion (Table 2.3), both samples were considered inactive if parameter IC_{50} for two or all five cell lines was higher than 100 µg/ml. Therefore both samples can be estimated as inactive against the five cell lines, as the parameter IC_{50} was >100 µg/ml. Both positive controls in Table 2.2 showed anticancer activity (weak-potent).

The results of five-dose screening were reported as TGI in Table 2.4. The biological activities were separated into four categories: inactive (TGI >50 µg/ml), weak activity (15 µg/ml < TGI <50 µg/ml), moderate activity (6.25 µg/ml < TGI <15 µg/ml) and potent activity (TGI <6.25 µg/ml). For each tested extract, four response parameters were calculated: GI50 (50% growth inhibition signifying the growth inhibitory power of the test agent), TGI (which is the drug concentration resulting in total growth inhibition and signifies the cytostatic effect of the test agent), LC50 (50% lethal concentration, signifying the cytotoxic effect of the test agent), LC100 (100% lethal concentration, signifying the cytotoxic effect of the test agent). According to the CSIR criterion (Table 2.5), both samples were considered inactive if the parameter TGI was greater than 50 µg/ml for two or all five cell lines. Therefore both samples can be estimated as inactive against five cell lines, as the TGI was >100 µg/ml.

Both *P.violacea and X.zambesiaca* extracts can be estimated as inactive (Figures 2.1 and 2.2) against five selected cell lines. *X.zambesiaca* has been proven to have isoflavones (Harper *et al*., 1976). This compound regulates estrogen levels. As it is known that estrogen reduces the risks of ovarian and endometrial cancer (Narod *et al.,* 1998; Greer *et al.,* 2005), anticancer screening on these cell lines might show some positive results. Results showed that these extracts do not have anticancer activity against selected human cancer cell lines; however, this does not exclude their activity against other cancer cell lines such as ovarian and endometrial cancer. The results obtained have stimulated us to carry on working to determine their anticancer activity against other cell lines.

2.6 CONCLUSION

Both *P.violacea* and *X.zambesiaca* extracts showed no anticancer activity against the human cell lines MCF7, HCT116 and PC3. Further testing using other cell lines is required, also to identify the active compounds of both *P.violacea* and *X.zambesiaca* plant extract.

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CHAPTER 3

"Anti-mutagenic and mutagenic screening of *P.violacea* and *X.zambesiaca*"

ABSTRACT

P.violacea is traditionally used to treat tuberculosis and other inflammation-related disorders, while *X.zambesiaca* is traditionally used to treat diabetes, but these plants' safety and efficacy have not been investigated. Most plants used as traditional medicine have *in vitro* mutagenic properties, therefore it is important to screen for their mutagenic potency. Plants showing clear mutagenic properties are considered potentially unsafe and further testing before their continued use is recommended. The aim of this study was to investigate the mutagenic and anti-mutagenic properties of *P.violacea* and *X.zambesiaca* leaves, flower and twig extracts. Mutagenic properties were determined by the Ames test using *Salmonella typhimurium* strains TA98 and TA100 without S9. The positive control used was 4-nitroquinoline-1-oxide. Results of tests to determine the mutagenic properties of *P.violacea* and *X.zambesiaca* plant extracts revealed that neither plant has mutagenic effects. Based on these results, we therefore concluded that the mutagenicity and antimutagenicity effects of *P.violacea* and *X.zambesiaca* were observed without the presence of metabolic activation and with no direct carcinogenic potential to human cells.

3.1 INTRODUCTION

Numerous medicinal plants are traditionally used in the treatment of a variety of diseases, including cancer or even diseases that contribute to carcinogenesis in the African population. Since ancient times, herbal medicine has been one of the main components of the health care system (WHO, 2005). Many conventional drugs also induce genetic damage that itself can be carcinogenic. As some of the active ingredients are potentially toxic, there is a need to evaluate the safety of plant preparations. A segment of the research community is thus focusing on identifying

novel chemotherapeutic agents in plants that do not induce the destructive effects of conventional cytotoxic therapeutic agents. Compounds that are derived from plants have contributed significantly to the development of some useful anticancer drugs (Newman and Cragg, 2007). These include taxol, etoposide, vinblastine and vincristine. Plants are potential sources of novel anticancer drugs; nevertheless, they may sometimes contain toxic and mutagenic compounds.

Several medicinal plants are known to act as mutagenic, co-carcinogenic and/or carcinogenic agents. Prevention of cancer and other related diseases can be pursued by avoiding exposure to these agents. Previous studies on African traditional medicine showed that about 64 plant species used in African traditional medicine are potentially mutagenic/carcinogenic, based on their ability to induce genetic changes (Schimmer *et al*., 1988, 1994; Higashimoto *et al*., 1993; Kassie *et al*., 1996; De Sã Ferrira and Ferrão Vargas, 1999). Although plants used medicinally are widely assumed to be safe by their users, the issue of quality control may be addressed. *P.violacea* has traditionally been used to treat gastro-intestinal problems. Powdered root-bark has been used for colds and snakebite treatment, root infusions as hookworm remedy and most parts of the plant to treat diarrhea (Bruschi *et al*., 2011). *X.zambesiaca* has traditionally been used to treat diabetes mellitus and tuberculosis. These plant extracts should consequently be examined carefully for their safety perspective and they should be used with caution. This chapter aims to report the mutagenic and anti-mutagenic properties of *P.violacea* and *X.zambesiaca* (leaves, flower and twig mixture) extracts using the *in vitro* Ames test.

3.2 LITERATURE REVIEW

People have been relying on traditional medicine since ancient times, but as some plants are toxic and can be carcinogenic through a process of mutation, there is a need to determine the safety of these traditional medicines. Traditional medicine is prepared using different parts of the plant, a mixture of plants (infusions); sometimes traditional healers just take a bit of this and that without specific measurements. Traditional medicine can be described as unsafe until proven safe. People can use traditional medicine for years without knowing the toxicity or the damage the medicine is doing to them. Some plants may be carcinogenic through three

manifestations; (1) Directly mutagenic, (2) Promutagenic and (3) Comutagenic (Edenharder *et al*., 1993; Beudot *et al*., 1998). As it is well known that cancer cells are not formed overnight after a single process of mutation, many traditional medicine users can be diagnosed with cancer after prolonged usage (Verschaeve *et al*., 2004; Cavalcanti *et al*., 2006) of traditional medicine.

Cancer is a multistage process that can arise when a normal stem cell acquires two or more heritable genetic alterations. Genetic alterations that play an important role in human cancers include chromosomal translocation, gene amplification and point mutation (Moolgavkar *et al*., 1999). Within this basic framework, the kinetics of cell division and death play a major role in determining the risk of promutagenic lesions, spontaneous induction of DNA lesions, genomic rearrangement or loss and uncontrolled proliferation of undifferentiated cells. To elaborate further on the risks mentioned, fixation of promutagenic lesions occurs when DNA synthesis precedes DNA repair, while spontaneous induction of DNA lesions results from errors in DNA replication and genomic rearrangement or loss can occur during cell division (Hakem, 2008). Moreover, increased cell division can increase critical target populations, unless compensated for by increased differentiation and apoptosis.

Inhibition of apoptosis can influence the process by decreasing the rate of elimination of altered, “initiated’ cells. According to Moolgavkar *et al.* (1999), there is no universally accepted definition of the terms “genotoxic” and “nongenotoxic”. They further state that chemicals that increase the rate of genetic alterations by some means other than by increasing the rate of cell proliferation, differentiation, or death will be considered genotoxic. Chemicals that alter DNA through direct interactions, producing point mutation and chromosome damage are therefore treated as genotoxic, along with those that, for example, alter DNA repair.

There are good examples of the traditional way to illustrate the initiation stage of carcinogenesis (Schins and Hei, 2007), where genotoxic events lead to irreversible changes in DNA. Several DNA repair pathways exist but lack of repair or erroneous repair may occur especially in a milieu of increased proliferation. DNA damage can

also cause cell cycle arrest until the damage is properly repaired. However, DNA repair is not flawless and repair may cause mutations. DNA damage may sometimes trigger a pathway that drives that cell towards apoptosis, therefore such events may lead to mutations and ultimately to cell transformation (Figure 3.1).

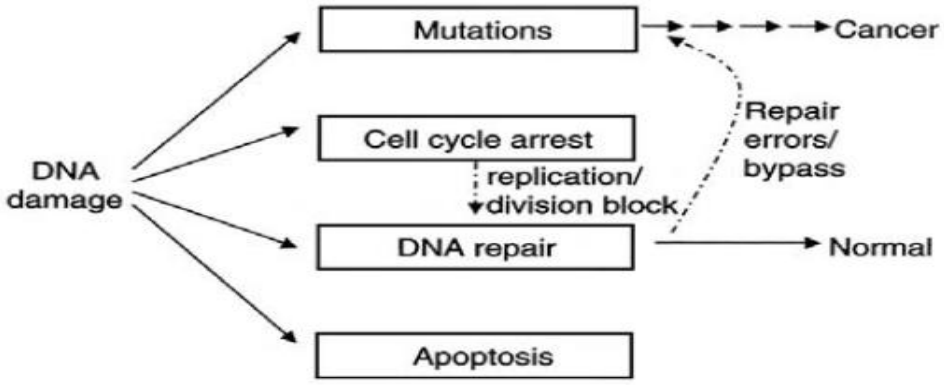

Figure 3.1: DNA damage may cause mutations that may lead to the development of cancer (Schins and Hei, 2007)

Compounds from secondary plant metabolism can be beneficial to human beings, but some are toxic and have a mutagenic effect (Barnes, 2003) when used by human beings. Gómez-Arroyo *et al.* (2007) demonstrated a high correlation between mutagenicity and carcinogenicity. In genetics, a mutagen is a physical or chemical agent that changes the genetic material, usually DNA, of an organism and thus increases the frequency of mutations above the natural background level. However, not all mutations are caused by mutagens: so-called “spontaneous mutations” occur owing to spontaneous hydrolysis, errors in DNA replication (Kunkel, 2004; Albertson and Preston, 2006), repair and recombination. Some mutagens do not increase the frequency of mutation copiously, therefore not all mutagens are carcinogens. The Ames mutagenicity test is a short-term bacterial reverse mutation assay specifically designed to detect a wide range of chemical materials that can produce genetic damage leadng to gene mutations (Griffith *et al*., 1996). The salmonella test can play a central role in a programme of prevention to identify mutagenic chemicals and to aid in the development of non-mutagenic products to prevent future human exposure (Cann and Ames, 1976).

Plants have their own mechanisms of producing secondary metabolites in small to large amounts and concentrations vary. Many of these secondary metabolites are highly toxic and are often stored in specific vesicles or in vacuoles. Several studies undertaken by Kinghorn *et al.* (2003) and Newman and Cragg (2007) indicate that this kind of storage functions on the one hand as a detoxification of the plant itself and, on the other hand, it protects the plant against pathogens that are present in the soil. The importance of these compounds to plants at large is usually of an ecological nature, as they are used as defenses against predators, parasites and diseases. Phenolics are used as anti-mutagenic and anti-inflammatory agents because of their strong antioxidant properties (Li *et al*., 2008). Plants with anti-mutagenic potential can be considered interesting for therapeutic use and worth further in-depth investigation of their pharmacological properties.

Pharmaceutical companies show interest in plant-derived drugs mainly because of the current widespread belief that 'green medicine' is safe, with fewer side effects (Pavithra *et al*., 2010). There are still challenging tasks for scientists working in drug research to investigate the efficacy of herbal medicine, to distinguish favorable from adverse effects, to identify active principles in medicinal plants, to ban poisonous plants or contaminations from herbal mixtures (Efferth and Kaina, 2011) and to assess plant-derived products for safety or toxicity to the tissue or organs of mammalian recipients. According to Cragg and Newman (2000), over 50% of the drugs in clinical trials for anticancer activity were isolated from natural sources. Most plants used as traditional medicine have in vitro mutagenic properties (Cardoso *et al*., 2006; Mohd-Fuat *et al*., 2007; De´ ciga-Campos *et al*., 2007), as a result, it is important to screen for their mutagenic potency. Therefore plants with these properties must be considered potentially unsafe and further testing before their continued use is recommended. This study is aimed at investigating the mutagenic and anti-mutagenic properties of *P.violacea* and *X.zambesiaca.*

3.3 METHODOLOGY

3.3.1 Plant material

The plant materials *P.violacea* and *X.zambesiaca* were authenticated by scientists at the National Botanical Gardens in Bloemfontein, South Africa. The collected material was dried at room temperature, pulverized by mechanical mills and weighed. It was then stored in a cool place until analysis.

3.3.2 Sterilisation

Autoclavable materials such as agar and broth were aseptically sterilized in an autoclave at 121^0C for 15 minutes. Petri dishes, beakers, McCartney bottles, pipettes, test tubes, filter papers and other metal apparatus such as spatulas and forceps were sterilized using a hot air oven at a temperature of 160^0C for one hour. The wire loops were sterilized by heating them on the blue flame of the Bunsen burner until red-hot and allowing them to cool and 70% alcohol was used to swab/clean the work bench area to prevent contamination. The process was carried out aseptically.

3.3.3 Extraction method

The extraction was done using the maceration. Plant material (10 g of the dried twigs, leaves and flowers of each plant) was weighed, pulverized and soaked in methanol for 72 hours, with occasional stirring. The extracts were filtered and new solvent was added again for more extraction until the solvent remained clear. The methanol solvent was removed completely using a rotator evaporator.

3.3.4 Preparation of culture media

All culture media were prepared according to the manufacturers' instructions. Agar was dissolved by boiling in the microwave and autoclaved at 121^0C for 15 minutes.

3.3.5 Organisms

The bacterial strains used for mutagenicity testing were the histidine-requiring *Salmonella typhimurium* tester strains TA98 (detects frameshift mutagens) and TA100 (detects mutagens that cause base-pair substitution) without metabolic activation.

3.3.6 Anti-mutagenic and mutagenic screening

The sample residues were dissolved in DMSO to a known concentration (5000, 500 and 50 μg/ml) prior to biological activity testing. The potential mutagenic effects of the investigated extracts were detected using the Ames test. The Ames assay was performed with *Salmonella typhimurium* strains TA98 and TA100 using the plate incorporation procedure described by Maron and Ames (1983). Briefly, 100 µL of bacterial stock was incubated in 20 ml of Oxoid nutrient for 16 hours at 37^0C on an orbital shaker. The overnight culture (0.1 ml) was added to 2 ml top agar (containing traces of biotin and histidine), together with 0.1 ml test solution (test extract, solvent control or positive control) and 0.5 ml phosphate buffer (for exposure without metabolic activation). The top agar mixture was poured over the surface of the agar plate and incubated for 48 hours at 37 °C. After incubation, the number of revertant colonies (mutants) was counted. All cultures were made in triplicate (except the solvent control where up to five replicates were made) for each assay. The positive control used was 4-nitroquinoline-1-oxide (4-NQO) at a concentration of 2 µg/ml.

Figure 3.2: A demonstration of the procedure to count number of revertant colonies (mutants) (Gómez-Arroyo *et al.,* 2007)

3.4 RESULTS

Test Sample	TA98			TA100		
	Number of colonies (revertants)			Number of colonies (revertants)		
	Concentration (μg/ml)			Concentration (μg/ml)		
	5000	500	50	5000	500	50
P.violacea	34.3±4.9	43.5±3.5	33.7±4.0	211.7±21.6	143.7±33.8	152.0±10.4
X.zambesiaca	36.3±4.0	36.7±6.8	40.0±2.6	158.3±8.1	159.7±12.5	160.3±1.5
4NQO (1)	157.3.±22.0			355.0±20.8		
Solvent control	26.6±2.7			155.6±8.3		

Table 3.1: No. of his+ revertants in *Salmonella typhimurium* strains TA98 and TA100 produced by *P.violacea* and *X.zambesiaca* plant extract.

3.5 DISCUSSION

The Ames assay is commonly used to detect mutagenic and anti-mutagenic activities and is a widely accepted method for identifying different chemicals and drugs that can cause bacterial reverse mutations. These gene mutations act as hot spots for mutagens that cause DNA damage via different mechanisms. When the *Salmonella* tester strains are grown on a minimal media agar plate containing a trace of histidine, only those bacteria that revert to histidine independence are able to form colonies (Mortelmans and Zeiger, 2000). In this study, we investigated the mutagenic and anti-mutagenic activities of *P.violacea* and *X.zambesiaca* by the *S. typhimurium*/microsome assay, without addition of an extrinsic metabolic activation system (Aicha *et al.,* 2008). The colonies were counted to determine the mutagenic and anti-mutagenic potencies of these two plant extracts.

The Ames test is a well-known bacterial mutagenicity test (Weisburger *et al.,* 1996; Mortelmans and Zeiger, 2000). In this test, reverse His− →His+ mutations are visualized by plating *Salmonella typhimurium* bacteria in histidine-poor growth

mutants that are able to form medium. In this medium only, His+ will able to form visible colonies. Different bacterial strains are used to identify different types of mutations. In this study, bacterial strains used for mutagenicity testing are the histidine-requiring *Salmonella typhimurium* tester strains and TA100 without metabolic activation. Strain TA98 gives an indication of frame-shift mutations, while strain TA100 indicates base-pair substitution. For a substance to be considered genotoxic in the Ames test, the number of revertant colonies on the plates containing the test compounds should be more than twice the number of colonies produced on the solvent control plates. In addition, a dose-response should be evident for the various concentrations of the mutagen tested.

In the present research, the anti-mutagenicity effect was seen against *S. typhimurium* TA100 and TA98, without the presence of metabolic activation. Based on results in chapter 6, the anti-mutagenicity activity of *P.violacea* and *X.zambesiaca* extracts may be due to the presence of phenolic compounds in these plant extracts. Results obtained from the mutagenicity test of *P.violacea* and *X.zambesiaca* plants are expressed as mean ± S.E.M (Table 3.1) and are based on the number of induced revertant colonies. Substances are considered active or mutagenic if the number of induced revertant colonies is twice the revertant colonies of the negative control (Maron and Ames, 1983). The mutagenic effects of any compound/extract are manifested in three ways (Snijman, 2007): (1) Directly mutagenic: Not requiring metabolic activation, (2) Promutagenic: Requiring metabolic activation, (3) Comutagenic: Enhancing the mutagenic response of a known mutagen irrespective of being mutagenic or not. According to our results, neither *P.violacea* nor *X.zambesiaca* was mutagenic in the salmonella/microsome tester strain TA 100 and TA98. However, the risk assessment of these extracts will not be complete unless they are tested for promutagenic and comutagenic effects. This can be done as a future study.

3.6 CONCLUSION

Most plants used as traditional medicine have *in vitro* mutagenic properties, therefore it is important to screen for their mutagenic potency. *P.violacea* and

X.zambesiaca have been used by Africans for the treatment of various diseases for centuries. Neither *P.violacea* nor *X.zambesiaca* extracts showed any mutagenic effect in the *salmonella*/microsome tester strain TA 100 and TA98 without metabolic activation. These plants can be therefore be considered potentially safe, but further confirmatory tests are required, such as the determination of cellular toxicity and a complete risk assessment of these extracts for promutagenic and comutagenic effects.

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CHAPTER 4

"Antimicrobial activity screening of *P.violacea* and *X.zambesiaca*"

ABSTRACT

Microorganisms' involvement in cancer has been identified for over a century, and different types of bacteria have been associated with carcinogenesis. Gram-negative pathogens, such as *Escherichia coli*, have been identified as being responsible for inflammatory response induction through toxins they secrete. Inflammation is considered a new domain in basic and clinical research in patients with prostate cancer and benign prostatic hyperplasia. Chronic inflammation is a major cause of cancer formation and *P.violacea* is traditionally used to treat tuberculosis and other inflammation-related disorders. The aim of this study was to investigate the antimicrobial activity of *P.violacea* and *X.zambesiaca* (mixture of leaves, flowers and twigs). Antibacterial activity was determined using the *p*-Iodonitrotetrazolium chloride assay on gram-positive bacteria: *Staphylococcus aureus*, *S. epidermidis*, *S. saprohyticus*, *Bacillus subtilis,* and gram-negative bacteria: *Enterobacter cloacae*, *Escherichia coli, Pseudomonas aeruginosa*. Results of tests for antibacterial activity revealed that *P.violacea* and *X.zambesiaca* plant extracts had no antimicrobial activity against selected micro-organisms.

4.1 INTRODUCTION

Traditional medicinal practices are used to treat a variety of diseases, including skin disorders, tuberculosis, urinary tract infections and gastrointestinal disorders etc. (Van Wyk, 2002). Gram-negative pathogens, such as *Escherichia coli,* have been identified in the prostate, and their toxins are responsible for the inflammatory response. Inflammation should be considered a new domain in basic and clinical research in patients with prostate cancer and benign prostatic hyperplasia (BPH). Bacterial infections, urine reflux, dietary factors, hormones and autoimmune response have been considered to cause inflammation in the prostate (De Nunzio *et*

al., 2011). From a pathophysiologic point of view, tissue damage associated with inflammatory response and subsequent chronic tissue healing may result in the development of BPH nodules and proliferative inflammatory atrophy. BPH benefits from antibacterial and anti-inflammatory treatment. *Staphylococcus aureus* could be regarded as the first bacterium to be described as a cancer-producing agent, and some authors have attempted to associate it with breast cancer (Velázquez *et al*., 2010).

African populations suffer chronic diseases whose treatment and follow-up create a major economic problem for them (Konkon *et al*., 2008). Of all the alternative modalities, herbal medicine is probably the most popular and the most ubiquitous (Akerele, 1993) as it is easily accessible and less expensive. The World Health Organization has described traditional herbal medicine as one of the surest means to achieve total health care coverage of the world's population (Okunlola *et al*., 2007). In traditional herbal practice in Africa, indigenous medicinal plants have been employed in the treatment of several important infections (Taylor *et al*., 2001). Interest in medicinal plant research has escalated, with the aim of identifying alternative antimicrobial therapies to overcome resistance (Aiyegoro and Okoh, 2009). Therefore this study was performed with the aim of determining the antimicrobial activity of *P.violacea* and *X.zambesiaca* plant extracts.

4.2 LITERATURE REVIEW

Microorganism involvement in cancer has been identified for over a century, and different types of bacteria have been associated with carcinogenesis. *Staphylococcus aureus,* together with type 16 papillomavirus (HPV-16), has also been found in the genome of different bacteria isolated from cervical cancer. According to Ma *et al*. (2009), HPV-16 induces cervical infection that leads to cervical cancer and *Staphylococcus aureus* is used as a vector by this virus. Moreover, the inflammatory microenvironment favors the survival and proliferation of neoplastic cells (Protti and De, 2012; Li *et al*., 2012), indicating that the modulation of factors fuelling chronic inflammation may have anticancer effects.

The ideal chemotherapeutic agent has a high therapeutic index with selective toxicity to cause damage to pathogens without causing similar harmful effects to its eukaryotic host. This lethal damage to pathogens might be through the inhibition of cell wall synthesis, protein synthesis or nucleic acid synthesis, as well as through the disruption of the cell membrane and the inhibition of certain essential enzymes. This results in selective disruption of the specific structure and/or function essential to bacterial growth and survival (Prescott *et al*., 1996). The efficacy of an antimicrobial agent can be estimated through the determination of the minimum inhibitory concentration (MIC), being the minimum concentration at which no microbial growth occurs after a specified exposure time to the antimicrobial agent (Prescott *et al*., 1996). However, there are factors that can influence the efficacy of an antimicrobial agent, such as the ability of the drug to reach the target site of infection and the susceptibility of the pathogen to the particular chemotherapeutic agent.

Natural products have played a vital role in the discovery of antimicrobial drugs, with the drug either being completely derived from the natural product, or serving as a lead for novel drug discovery. Plants synthesise a diverse array of compounds (secondary metabolites), which play a key role in the natural defence mechanism. These aromatic compounds have been found to be useful antimicrobial phytochemicals and, as a result, these compounds are now divided into different chemical categories: phenolics, terpenoids and essential oils, alkaloids, lectins and polypeptides, as well as polyacetylenes (Cowan, 1999). Fluoroquinolones were presented as a totally synthetic, significant class of antibiotics in the 1990s (Walsch, 2000). Some examples of naturally occurring antimicrobials that are currently used include drug classes such as the penicillins and cephalosporins (β-lactam being the empirically active component). An increase in the isolation and identification of antimicrobial compounds may contribute greatly to success in antibiotic discovery.

There are numerous methods for the screening of biological extracts for potential antimicrobial activity. The microbroth dilution susceptibility method in 96-well microtiter plates has become the preferred method for drug susceptibility testing because it has small sample requirements, is cost-effective and has a high throughput rate (Pauli *et al*., 2005). The p-Iodonitrotetrazolium chloride (INT) assay is a microplate assay that determines the MIC of biological extracts using INT dye.

This dye acts as an electron acceptor and is reduced by viable bacteria to produce a colored product. The yellow tetrazolium dye is reduced by viable microorganisms to a pink/purple color. The MIC, for the INT assay, is defined as the lowest extract concentration that exhibits complete bacterial growth inhibition and prevents the dye from changing color (Eloff, 1998).

Plants have been used in the traditional treatment of microbial infections for centuries and this knowledge has been passed on from generation to generation. The knowledge of indigenous people about plants and their products plays a vital role in the health care of a great proportion of the population (Iwu *et al*., 1999), especially in developing countries. One of the most serious threats to the success of antimicrobial agents' development is the challenge of drug-resistant pathogens, resulting from the disproportionate use of antimicrobial agents. Resistance is defined as the ability of a microorganism to remain viable and multiply actively under conditions that would normally have proved to be inhibitory (Prescott *et al*., 1996). Increased drug resistance may be a result of limited drug diffusion due to the lack of or through the overexpression of certain membrane proteins (Cloete, 2003). This phenomenon of increased drug resistance, combined with the multiplicity of side effects caused by existing agents, makes the search for new antimicrobial agents a highly relevant and important subject for research. Hence this study was done to determine the antimicrobial activity of *P.violacea* and *X.zambesiaca* plant extracts.

4.3 METHODOLOGY

4.3.1 Plant material

The plant materials *P.violacea* and *X.zambesiaca* were authenticated by scientists at the National Botanical Gardens in Bloemfontein, South Africa. The collected material was dried at room temperature, pulverized by mechanical mills and weighed. It was then stored in a cool place until analysis.

4.3.2 Sterilization

Autoclavable materials such as agar and broth were aseptically sterilized in an autoclave at 121^{0}C for 15 minutes. Petri dishes, beakers, McCartney bottles, pipettes, test tubes, filter papers and other metal apparatus such as spatulas and forceps were sterilized using a hot air oven at a temperature of 160^{0}C for one hour. The wire loops were sterilized by heating them on the blue flame of the Bunsen burner until red-hot and allowing them to cool and 70% alcohol was used to swab/clean the work bench area to prevent contamination. The process was carried out aseptically.

4.3.3 Extraction method

Plant extracts were dissolved in DMSO at stock concentrations of 100 mg/mL. Working concentrations of 4 mg/mL were prepared in Mueller-Hinton (MH) broth.

4.3.4 Microorganisms, growth conditions and media

Staphylococcus aureus, *S. epidermidis*, *S. saprohyticus*, *Bacillus subtilis*, *Enterobacter cloacae*, *Escherichia coli* and *Pseudomonas aeruginosa* (clinical strains) were grown in MH broth (Merck). One microorganism colony was inoculated in the broth and allowed to grow for 16 hours (log growth phase) at 37^{0}C in an orbital shaker (150 rpm).

Control drugs: Imipenem monohydrate (Sigma) and ampicillin sodium salt (Calbiochem)/doxycycline hyclate (Sigma) were used as positive controls against Gram-negative bacteria and Gram-positive bacteria, respectively. Antibiotics were dissolved in MH broth at stock concentrations of 2 mg/mL and filter-sterilized (0.2 µM

filter). Working concentrations of 64 µg/mL imipenem and 16 µg/mL ampicillin and doxycyline were prepared in MH broth.

4.3.5 Microbroth dilution method

MH broth (50 µL) was added to all test wells (i.e. plant extracts and antibiotics), except for the highest plant extract and antibiotic concentration wells, to which 100 µL of the working concentrations were added. Serial dilutions were prepared for the plant extracts (2 mg/mL to 125 µg/mL) and antibiotic (32-0.125 µg/mL and 8-0.016 µg/mL for imipenem and ampicillin/doxycycline, respectively). The cultures were assessed and adjusted to a 0.5 McFarland standard (absorbance at 600 nm = 0.08-0.1; equivalent to ~$1.5x10^8$ cells/mL) and 50 µL added to each test well. The following controls were prepared: (i) antibiotic/medium control (50 µL MH broth + 50 µL of highest antibiotic/fluconazole concentration); (ii) plant extract color control (50 µL MH broth + 50 µL of highest plant extract concentration); (iii) 2% DMSO control (50 µL MH broth + 50 µL 4% DMSO); and (iv) microorganism control (50 µL MH broth + 50 µL microorganism). Plates were sealed with microplate sealing tape and incubated at 37^0C for 24 h.

P-Iodonitrotetrazolium chloride assay

After the incubation period, the absorbance of wells was read at 600 nm. 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT, Sigma) was prepared at a working concentration of 0.2 mg/mL in distilled water and filtered (0.2 µM filter). INT (20 µL) was added to each well and the plates were further incubated for 30-60 min at 37^0C until there was a color change. The absorbance of wells was read at 600 nm. Viable cells reduced the yellow dye to a pink/purple color, whereas no color change indicated inhibition of bacterial growth.

4.4 RESULTS

	1	2	3	4	5	6	7	8	9	10	11	12
A	control 1	control 3	B2	B1	B0.5	B0.25	B0.125	C2	C1	C0.5	C0.25	C0.125
B												
C												
D												
E	control 2	control 4	control 5	AB32	AB16	AB8	AB4	AB2	AB1	AB0.5	AB0.25	AB0.125
F												
G												
H												

Table 4.1: Microplates template

control 1 = 50 uL medium and 50 uL 32 ug/mL antibiotic, control 2 = 50 uL medium and 50 uL 2 mg/mL extract B; no microorganism (color control), control 3 = 50 uL medium and 50 uL 2mg/mL extract C; no microorganism (color control), control 4 = 50 uL 2% DMSO and 50 uL microorganism, control 5 = 50 uL medium and 50 uL microorganism. AB = antibiotic (imipenem); B = *P.violacea* plant extracts*;* C= *X.zambesiaca* plant extracts

OD600 nm before INT was added

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.04	0.098	0.409	0.428	0.454	0.415	0.275	0.403	0.438	0.372	0.44	0.371
B	0.039	0.099	0.408	0.338	0.409	0.512	0.344	0.404	0.387	0.341	0.315	0.403
C	0.04	0.097	0.46	0.285	0.343	0.328	0.35	0.41	0.362	0.524	0.235	0.33
D	0.04	0.099	0.427	0.439	0.378	0.402	0.302	0.429	0.365	0.381	0.373	0.326
E	0.067	0.381	0.389	0.045	0.049	0.047	0.046	0.048	0.047	0.041	0.043	0.042
F	0.071	0.286	0.346	0.046	0.046	0.047	0.047	0.047	0.046	0.042	0.041	0.049
G	0.071	0.337	0.363	0.047	0.05	0.046	0.05	0.051	0.047	0.043	0.043	0.042
H	0.457	0.321	0.405	0.046	0.047	0.047	0.048	0.049	0.048	0.043	0.044	0.042

OD600nm after INT was added (~45 min)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.04	0.115	0.728	0.747	0.902	0.77	0.601	0.721	0.83	0.715	0.84	0.64
B	0.04	0.113	0.709	0.635	0.711	1.034	0.525	0.71	0.733	0.726	0.781	0.732
C	0.042	0.111	0.735	0.683	0.746	0.511	0.589	0.673	0.699	0.763	0.637	0.702
D	0.041	0.112	0.723	0.605	0.617	0.442	0.429	0.734	0.572	0.585	0.612	0.666
E	0.076	0.436	0.645	0.048	0.047	0.048	0.054	0.045	0.069	0.042	0.048	0.042
F	0.078	0.661	0.771	0.047	0.047	0.049	0.059	0.047	0.072	0.065	0.042	0.153
G	0.082	0.712	0.807	0.05	0.046	0.045	0.06	0.076	0.08	0.048	0.043	0.045
H	0.494	0.727	0.753	0.048	0.047	0.205	0.106	0.214	0.137	0.06	0.044	0.26

Table 4.2: OD600 nm readings

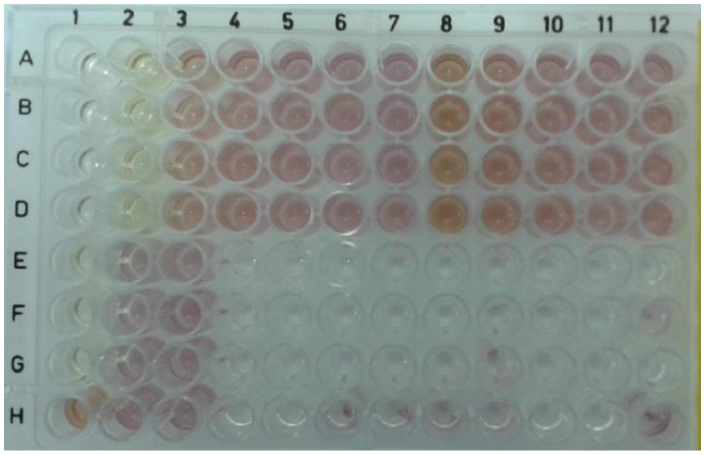

Figure 4.1: Photos of the INT plates

Microorganism	Positive control	MIC
Bacillus subtilis	Imipenem	4-8 µg/mL
Enterobacter cloacae	Imipenem	16 µg/mL
Escherichia coli	Imipenem	16-32 µg/mL
Pseudomonas aeruginosa	Imipenem	32 µg/mL
Staphylococcus aureus	Ampicillin/doxycycline	0.5-1 µg/mL
Staphylococcus epidermidis	Ampicillin/doxycycline	0.125-2 µg/mL
Staphylococcus saprophyticus	Ampicillin/doxycycline	1-2 µg/mL
Bacillus subtilis	Imipenem	4-8 µg/mL

Table 4.3. MIC values of antibiotics used

Absorbance readings of 96-well plates were taken at a wavelength of 600 nm before and after INT addition. The percentage inhibition for the plant extracts was not calculated, as there was no observable (Figure 4.1) inhibition of microbial growth. For the Gram-positive bacteria (i.e. *S. aureus*, *S. epidermidis* and *S. saprophyticus*) imipenem was initially used as a positive control, but it caused complete inhibition even at the lowest concentration (0.125 µg/mL). Ampicillin and doxycyline were used to determine MIC values for the Gram-positive bacteria.

4.5 DISCUSSION

It is known that nonpolar or lipophilic extracts do not easily diffuse into agar. The differences in solubility, volatility and diffusion characteristics are among the factors that affect the antimicrobial potency of medicinal plant extracts (Cos *et al*., 2006). The microdilution method of quantifying MICs has become the preferred technique for evaluating the antibacterial potency of plant extracts or compounds (Cos *et al*., 2006). Weak microbial inhibitors are classified as those agents with MIC values greater than the ranges listed in Table 1. The antimicrobial activity of *P.violacea* and *X.zambesiaca* plant extracts was determined using INT assay against selected Gram positive and Gram negative bacteria that contribute to carcinogenesis. The sensitivity of *Staphylococcus aureus*, *S. epidermidis*, *S. saprohyticus*, *Bacillus subtilis*, *Enterobacter cloacae*, *Escherichia coli* and *Pseudomonas aeruginosa* with respect to *P.violacea* and *X.zambesiaca* plant extracts was similar, indicating poor antimicrobial activity.

X.zambesiaca is traditionally used to treat diabetes mellitus and has been scientifically proven to have anti-hyperglycemic effects (Nojima *et al*., 1998).This plant is also used by traditional healers in Limpopo (South Africa) for the treatment of symptoms such as wounds, boils, purulent sores and diarrhea (Masoko, 2013). Masoko (2013) further reported good antimicrobial activity of leaves of *X.zambesiaca* with methanol, using a microplate serial dilution technique with *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Enterococcus faecalis* as test organisms. *P.violacea* is reported to be used in traditional remedies to treat gastro-intestinal problems; most parts of the plant have been used to treat diarrhea (Mnxati, 2009). Its powdered root-bark is used for colds and snakebite treatment and root infusions as hookworm remedy. According to results, neither plant extract showed any antimicrobial activity against *S. epidermidis*, *S. saprohyticus*, *Bacillus subtilis or Enterobacter cloacae.* In tests conducted by Masoko (2013) against bacteria such as *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli* it did not show such activity either.

However, in our study we mixed different parts of the plant: leaves, twigs and flowers. There is a possibility that other compounds from twig and flowers might

suppress the activity of active antimicrobial compounds from the leaves of our plant extracts instead of having a synergetic effect. A number of factors might contribute to these outcomes, such as the area where the plants are collected and climate changes. Another factor might be the preparation of extracts by traditional healers, as they sometimes prepare extracts using more than one plant and there is no systematic approach in doing that. The solvent used to prepare the extract also affected our results, as traditional healers use other solvents, such as water, and different extraction methods, such as boiling.

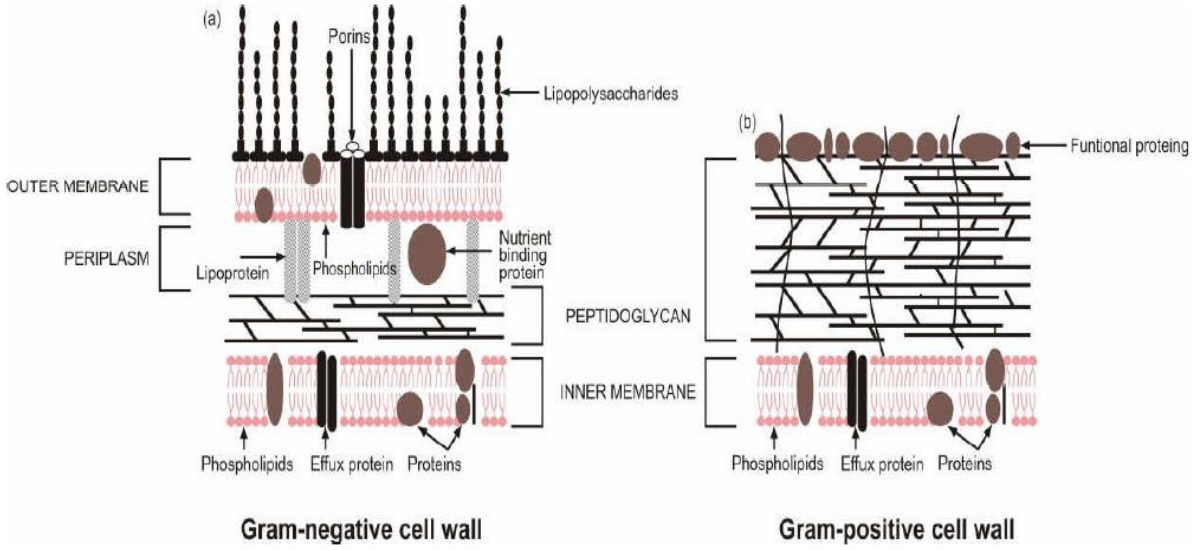

Figure 4.2: The comparative structural complexity of the outer membranes and cell walls of Gram-negative and Gram-positive bacteria (Denyer and Maillard, 2002)

Resistance may also be strengthened by additional resistance mechanisms used by the microorganism, such as decreased accumulation of the antimicrobial agent within the cell (Denyer and Maillard, 2002). Many harmful agents, including antibiotics, are either hydrophobic or relatively large hydrophilic compounds and are thus hardly able to penetrate the outer membrane, which must also have an effect. This is as a result of the structure of the bacterial cell wall (Figure 4.2), with the addition of higher lipid content for Gram negative bacteria (Linfield *et al*., 1982). Interactions of lipophilic compounds with hydrophilic parts of the membrane will bring about a more toxic effect against the micro-organism (Sikkema *et al*., 1995).

It has also been suggested that the polysaccharide constituents of the outer membrane aid the bacterial cell in evasion of phagocytosis and protect the deeper parts of the outer membrane from complement and antibody binding (Vaara, 1992).

In comparison, Gram-positive bacteria possess a much thicker peptidoglycan layer, which does not act as an effective barrier to permeation, and inhibitors are thus able to pass through more easily (Scherrer and Gerhardt, 1971). However, neither Gram-negative nor Gram-positive bacteria displayed any sensitivity to the extracts, while controls showed positive results. These results were not expected, as it had been reported previously that *X.zambesiaca leaves* had antimicrobial activity against Gram-negative and Gram-positive bacteria (Masoko, 2013).

4.6 CONCLUSION

P.violacea and *X.zambesiaca* showed no antimicrobial activity against *S. epidermidis, S. saprohyticus, B. subtilis, E. cloacae, P. aeruginosa, S. aureus* and *E. coli*. From our findings, we could not support the ethnobotanical uses of these plants as reported by traditional healers. However, factors that might have contributed to these results are not excluded.

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CHAPTER 5

"Antioxidant activity screening of *P.violacea* and *X.zambesiaca*"

ABSTRACT

Free radicals are important mediators that provoke inflammatory processes and are neutralized by antioxidants, which exert an anti-inflammatory effect. Free radical scavenging molecules such as flavonoids, tannins, alkaloids and other metabolites possess anti-inflammatory, anti-carcinogenic, antibacterial and antiviral properties. The aim of this study was to investigate the antioxidant activities of *P.violacea and X.zambesiaca* leaf, flower and twig extracts. These two plants were chosen from 60 plants found in Africa belonging to the *Fabaceae* family. The antioxidant activity was determined using 2, 2- diphenyl-1-picrylhydrazyl assay. The scavenging activity of the *X.zambesiaca* extract was compared with that of *P.violacea* and standard antioxidant (ascorbic acid). *X.zambesiaca* showed moderate (50%) free radical scavenging activity, while *P.violacea* extract had low free radical scavenging activity at a concentration of 2.5 mg/ml when compared to ascorbic acid. Our results indicate that *P.violacea* plant extract has low antioxidant activity, while *X.zambesiaca* extract exhibits moderate dose-dependent antioxidant activity and can be considered as a weak source of antioxidant.

5.1 INTRODUCTION

Oxygen is important for life processes to occur, but an excess of oxygen could result in oxidative damage, which may even lead to cell DNA damage. The damage is not due to the presence of oxygen, but rather to its role in the reduction of certain products to toxic free radicals. These free radicals are produced in living cells and are therefore part of the cell's normal metabolic processes, including detoxification processes and immune system defenses when reactive oxygen species (ROS) living independently within the body disrupt normal cell structure and alter the DNA of the

cell, which leads to cell malfunction (Kerr *et al*., 1994). This cellular malfunction may lead to uncontrolled proliferation of abnormal cells.

The excessive generation of free radicals, ROS, such as superoxide anions, hydroxyl radicals and hydrogen peroxide, contributes to the development of various diseases. Diseases such as cancer, rheumatoid arthritis, certain neurodegenerative diseases and tissue damage will result and ageing will take place, especially if free radical production exceeds the capacity of tissues to remove these ROS radicals (Larkins, 1999). It is also known that in a situation of oxidative stress, ROS radicals can stimulate the release of cytokines and chemokines that drive recruitment and activation of additional inflammatory cells, including activated neutrophils and macrophages, and these play an important role in the pathogenesis of cancer (Auroma, 1998; Michael *et al*., 2006).

People have been relying on plants as a source of treatment for various diseases and that happens even today, especially in rural areas where traditional healers outnumber western doctors. Most medicine used by western doctors is also derived from plants. Free radicals are important mediators that provoke inflammatory processes and are neutralized by antioxidants that exert an anti-inflammatory effect (Filomena *et al*., 2008). Free radical scavenging molecules such as flavonoids, tannins, alkaloids, quinones, amines, vitamins and other metabolites possess anti-inflammatory, anti-carcinogenic, antibacterial and antiviral activities (Sala *et al*., 2002). Plants with antioxidant properties are used for minimizing the severity of inflammation-related diseases and a health-promoting effect of antioxidants from plants is thought to arise from their protective effects by counteracting ROS (Wong *et al*., 2006). Thus the aim of the study was to determine the antioxidant activity of *P.violacea and X.zambesiaca* plant extract.

5.2 LITERATURE REVIEW

The defense system against free radicals is provided by free radical scavengers that act as antioxidants. Free radical scavengers function by donating an electron to the free radical, which pairs with the unpaired electron and thereby stabilizes it. These

antioxidants may remove catalytic metal ions, remove ROS such as O^{2-}, scavenge initiating radicals such as OH^{-} and rearrange electrons that produce oxygen very rapidly (Gutteridge, 1994). Antioxidant defense involves both enzymatic mechanisms, which utilise specific enzymes such as superoxide dismutase, catalase and glutathione peroxidase, and non-enzymatic mechanisms, which utilise nutrients and minerals (Aggarwal *et al*., 2005).

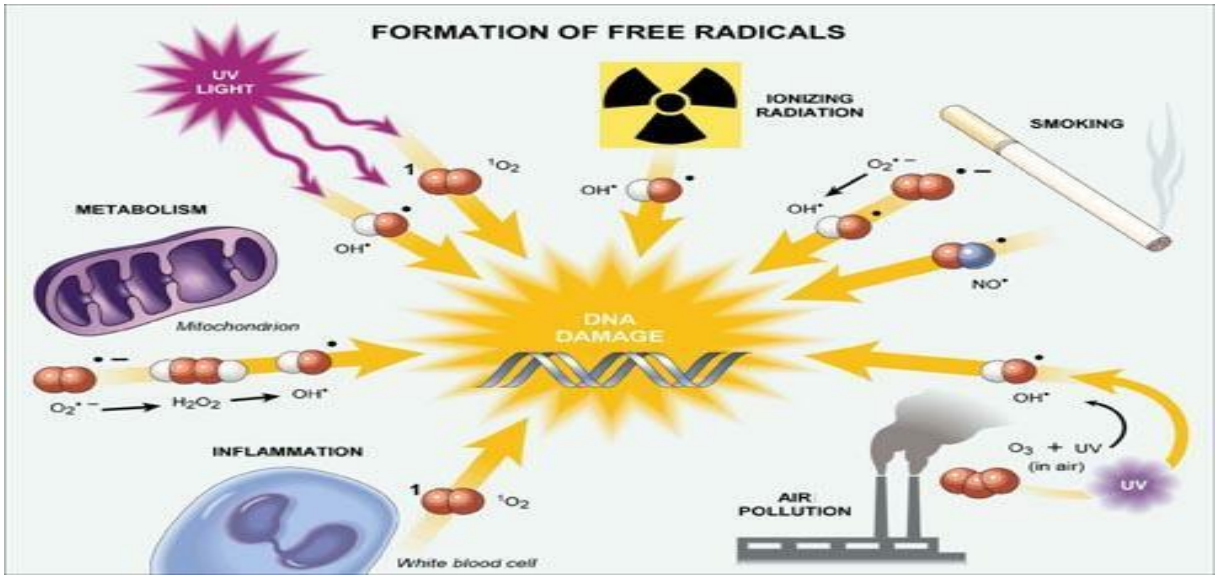

Figure 5.1: Diagram illustrating the formation of free radicals from the environment (Zander, 2013)

An antioxidant defense mechanism is crucial to survival and can operate at different levels within the cells through the prevention of radical formation, interception of formed radicals and recognition of excessively damaged molecules. These errors are not repaired but rather eliminated to prevent mutations from occurring during replication (Kerr *et al.,* 1994). Non-enzymatic antioxidants are classified as either water-soluble or lipid-soluble, depending on whether they act primarily in the aqueous phase or in the lipophilic region of the cell membranes. The hydrophilic antioxidants include vitamin C (ascorbic acid) and certain polyphenol flavonoid groups, while the lipophilic antioxidants include ubiquinols, retinoids, carotenoids, apocynin, procyanidins, certain polyphenol flavonoid groups and tocopherols (Middleton *et al*., 2000).

Antioxidant properties produced by plant species have a full range of applications in human healthcare, as they protect against free radicals. Knowledge of the potential antioxidant compounds present in a plant species does not necessarily indicate its antioxidant capacity, as the total antioxidant effect may be greater than the individual antioxidant activity of one compound, owing to synergism between different antioxidant compounds in the same plant. In search of sources of novel antioxidants with low toxicity, medicinal plants have over the past few years been studied extensively for their radical scavenging activity (Molyneux, 2004). As plants produce a large number of antioxidants to control the oxidative stress caused by sunbeams and oxygen, it is clear that plants may represent a source of new compounds with antioxidant activity (Scartezzini and Speroni, 2000). Therefore, in this study, the antioxidant activity of *P.violacea and X.zambesiaca* plant extracts were determined.

5.3 METHODOLOGY

5.3.1 Plant material

The plant materials *P.violacea* and *X.zambesiaca* were authenticated by scientists at the National Botanical Gardens in Bloemfontein, South Africa (MAS002). The collected material was dried at room temperature, pulverized by mechanical mills and weighed. It was then stored in a cool place until analysis.

5.3.2 Extraction method

The extraction was done using the maceration. Plant material (10 g of the dried twigs, leaves and flowers of each plant) was weighed, pulverized and soaked in methanol for 72 hours with occasional stirring. The extracts were filtered and new solvent was added again for more extraction until the solvent remained clear. The methanol extract was concentrated using a rotator evaporator.

5.3.3 Antioxidant activity screening

Scavenging ability towards 1, 1-Diphenyl Picrylhydrazyl (DPPH) radical

The DPPH assay will be performed as described by Shirwaikar *et al*. (2006) to estimate the free radical scavenging properties of different plant extracts. This

method depends on the reduction of purple DPPH radicals to yellow-colored diphenylpicrylhydrazine and the remaining DPPH radicals, which show maximum absorption at 517 nm, will be measured. An amount of 100 µl of various concentrations of each sample will be added to 2 ml solution of 0.1 mM DPPH. As control, 100 µl of methanol and 2 ml DPPH will be used. After 60 min of incubation at 25^0C in the dark, the absorbance will be recorded at 517 nm. The experiment will be performed in triplicate. The DPPH radical scavenging activity will be calculated according to the following equation:

% DPPH radical scavenging activity = 1 - [Asample/Acontrol] x 100

where Asample and Acontrol are absorbance of the sample and control. Ascorbic acid will be used as a standard. The SC_{50} (concentration of sample required to scavenge 50% of DPPH radicals) values will be determined. The decrease of absorbance of DPPH solution indicates an increase in the DPPH radical scavenging activity.

5.4 RESULTS

Concentration in mg/ml	*P.violacea*		*X.zambesiaca*		Ascorbic acid	
	% scavenging	Δ Absorbance @517	% scavenging	Δ Absorbance @517	% scavenging	Δ Absorbance @517
2.5	19.8	0.762±0.012	50.0	0.475±0.002	97.6	0.013±0.001
1.25	11.8	0.838±0.010	45.3	0.52±0.001	97.2	0.016±0.001
0.625	7.4	0.880±0.006	44.1	0.531±0.001	96.6	0.019±0.003
0.313	4.6	0.907±0.021	43.0	0.542±0.007	68.5	0.173±0.007
0.156	1.2	0.939±0.004	42.8	0.544±0.005	19.2	0.444±0.006

Table 5.1: *In vitro* anti-oxidant activity (mg/ml) of *P.violacea, X.zambesiaca extracts* as shown by the DPPH assay. Results are represented as mean ± standard deviation, n=3.

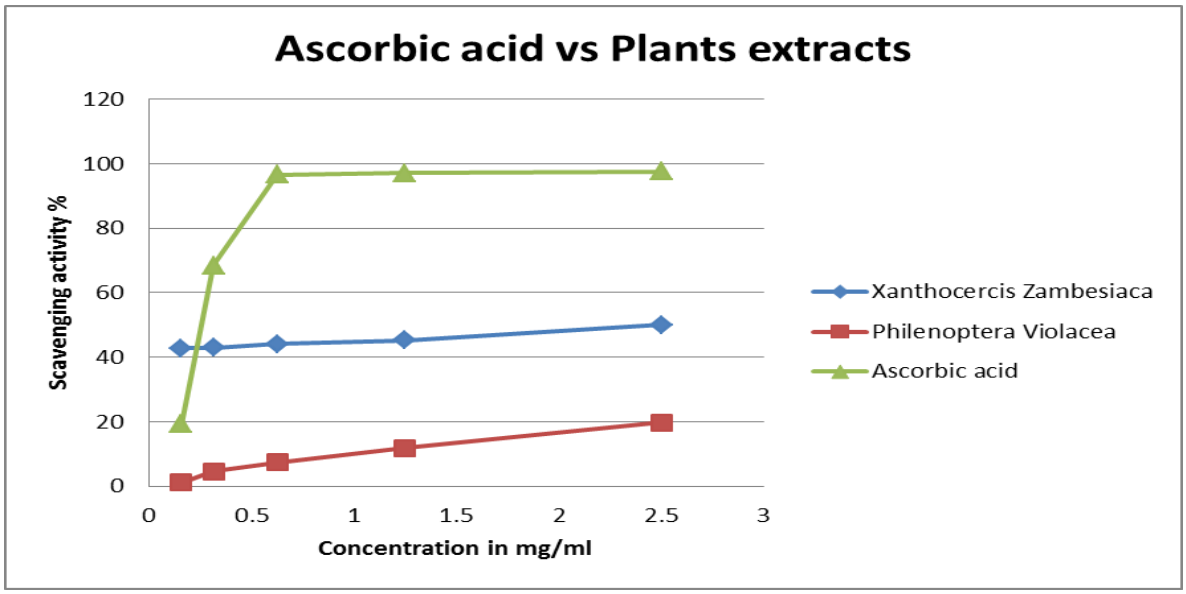

Figure 5.2: % Scavenging activity *P.violacea* and *X.zambesiaca extracts* versus their concentrations in mg/ml

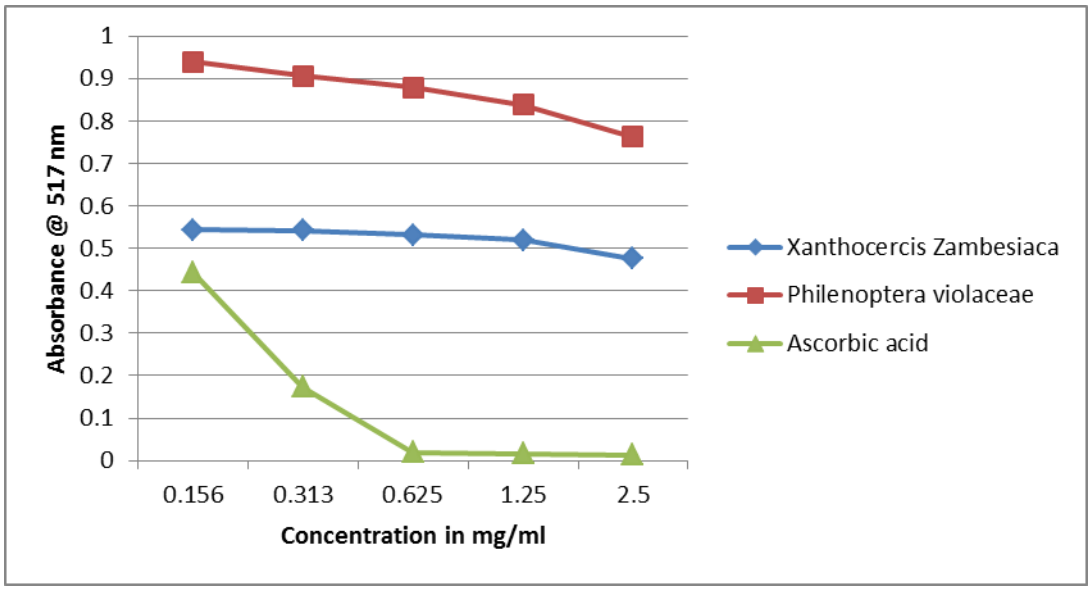

Figure 5.3: Δ Absorbance @ 517 nm wavelength of *P.violacea* and *X.zambesiaca extracts* versus their concentrations in mg/ml.

5.5 DISCUSSION

In normal conditions, the human body possesses many defense mechanisms against oxidative stress, including antioxidant enzymes and non-enzymatic compounds. The natural antioxidant mammalian mechanism sometimes becomes insufficient and then the excess of free radicals can damage both the structure and

function of a cell membrane in a chain reaction, leading to many degenerative diseases (Atta-ur-Rahman and Choudhary, 2001). Antioxidants reduce oxidative stress in cells and are therefore useful in the treatment of many human diseases, including cancer, cardiovascular diseases and inflammatory diseases. Plants are a cheap source for the extraction of antioxidant compounds, thus having an important economic advantage.

The principle of the DPPH assay was based on the hydrogen-donating capacity of the test sample. In the DPPH test, the antioxidants reduce the DPPH radical to a yellow-colored compound, diphenylpicrylhydrazine, and the extent of the reaction depends on the hydrogen-donating ability of the antioxidants (Figure 5.4). DPPH was reduced by the donated hydrogen, changing color from deep purple to yellow by the standard. The absorption of the deep violet DPPH solution was measured at 517 nm, after which absorption decreased because of decolorisation to a yellow color, in the event of reduction. This decrease in absorption was stoichiometric according to the degree of reduction (Figure 5.3). The remaining DPPH absorbance was measured at a time interval of 60 min after the addition of the DPPH, which corresponded inversely with the radical scavenging activity of the sample extract or antioxidant activity.

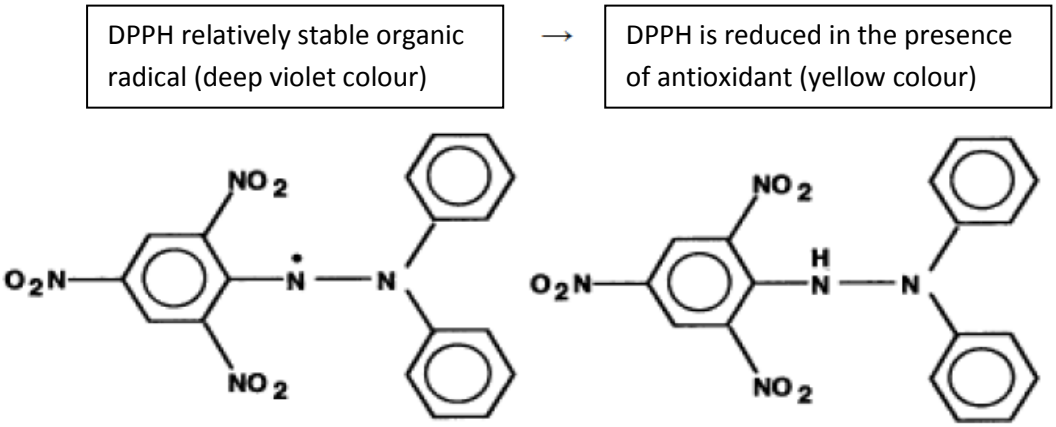

Figure 5.4: Diagrammatic representation of chemical reaction of the reduction of DPPH in the presence of an electron-donating anti-oxidant (Shirwaikar *et al*., 2006).

The methanol extract of both *P.violacea* and *X.zambesiaca* demonstrated concentration-dependent scavenging activity by quenching DPPH radicals (Figure 5.1). The free radical scavenging activities of *P.violacea* and *X.zambesiaca* extracts were observed and compared with the scavenging activity of ascorbic acid in table 5.1. Ascorbic acid showed high activity with SC_{50} (68.5%) from a concentration of

0.313 mg/ml. The free radical scavenging activity of *P.violacea* (19.8%) showed scavenging activity lower than 50% at a high concentration of 2.5 mg/ml. *X.zambesiaca* extract's scavenging activity of 50% was at a concentration of 2.5 mg/ml. P values were determined using the two-tailed test, and for both extracts were $p < 0.05$, thus statistically significant in *P.violacea* ($p= 0.0010$) and *X.zambesiaca* ($p= 0.0011$). Our results indicate that *P.violacea* plant extract has low antioxidant activity, while *X.zambesiaca* extract exhibits moderate dose-dependent antioxidant activity and can be considered a weak source of antioxidant.

5.6 CONCLUSION

P.violacea and *X.zambesiaca* demonstrated dose-dependent free radical scavenging activity. The former has low antioxidant potential and the latter has moderate antioxidant activity potential.

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CHAPTER 6

"Phytochemical analysis of *P.violacea* and *X.zambesiaca*"

ABSTRACT

Most medicine used by western doctors is derived from plants. Phytochemicals or secondary metabolites are chemical compounds formed during the plants' normal metabolic processes and plants use them to protect themselves. Most phytochemicals have properties that can protect human cells against oxidative damage. The aim of this study was to investigate the phytochemical constituents and total phenolic content of *P.violacea and X.zambesiaca* leaf, flower and twig extracts with colometric assays. The estimated total phenol content values of *P.violacea* and *X.zambesiaca* extracts were analyzed at 1 mg/ml against gallic acid using Folin and Ciocalteu's phenol reagent. *P.violacea* extracts showed more polyphenols than *X.zambesiaca*. Qualitative phytochemical analysis of these plant extracts confirmed the presence of tannins, flavonoids, steroids, terpenoids, alkaloids and cardiac glycosides from *P.violacea* extract, while *X.zambesiaca* extract showed the presence of flavonoids, saponins, terpenoids and glycosides. The gallic acid equivalents (GAEs) of the estimated phenolic concentrations ranged from 0.152 ± 0.0269 to 0.0895 ± 0.006 mg/GAE. Our results indicate that *P.violacea* extract has a number of phytochemical compounds when compared to *X.zambesiaca*. Future study will be done to isolate and identify the active compounds of both plant extracts.

6.1 INTRODUCTION

Phytochemicals are bioactive chemicals of plant origin that are formed during the plants' normal metabolic processes and plants use them to protect themselves (Watson *et al.,* 2001; Ning *et al*., 2009). They are regarded as secondary metabolites because the plants that manufacture them may have little need for them. They are naturally synthesized in all parts of the plant body: bark, leaves, stem, roots, flowers, fruits and seeds, therefore any part of the plant body may contain active components

(Tiwari *et al.,* 2011). The quantity and quality of phytochemicals present in plant parts may differ from one part to another. In fact, there is still a lack of information on the distribution of the biological activity in different plant parts essentially related to the difference in distribution of active compounds (or active principles), which are more common in some plant parts than in others (Lahlou, 2004).

Medicinal plants are of great importance to the health of individuals and communities in general. The value of medicinal plants lies in chemical substances that produce a certain physiological action on the human body. Many indigenous medicinal plants are added to food meant for pregnant women and nursing mothers for medicinal purposes, as reported by Okwu (1999, 2001) and Hill (1952). The most important bioactive constituents of plants beneficial to humankind are alkaloids, tannins, flavonoids and phenolic compounds. There is therefore a need to look inwards to search for herbal medicinal plants with the aim of identifying, isolating and characterizing compounds that will be added to the potential list of drugs. This study was aimed at identifying biological compounds from *P.violacea* and *X.zambesiaca* plant extract.

6.2 LITERATURE REVIEW

It is known that natural products are an excellent source of compounds with a wide variety of biological activities. A number of mechanisms exist by which phytochemicals aid in the prevention of cancer. These mechanisms of phytochemical chemo-preventive agents are categorized into two basic groups, namely blocking agents and suppressing agents (Ugbogu *et al*., 2013). The preventative action most probably results from the additive or synergistic effects of a number of phytochemicals present in a particular part of the plant. Since cancer is a multi-step process, phytochemicals' chemo-preventive mechanism may include anti-oxidant and free radical scavenging activity, antiproliferative activity, cell-cycle arresting activity, induction of apoptosis, activity as enzyme cofactors; (vi) enzyme inhibition; (vii) gene regulation; (viii) activity as hepatic phase I enzyme inducers and (ix) activity as hepatic phase II enzyme inducers (Manson *et al*., 2000; Juge *et al*., 2007; Surh, 2003).

Compounds such as flavonoids are found in a wide variety of plant extracts, fruit and vegetables, beverages and herbs. Flavonoids are a group of polyphenolic secondary metabolites with a large number of biochemical and pharmacological properties, including cancer-preventative effects (Williams and Grayer, 2004). Biochemical interferences produced by flavonoids are associated with their capacity to control cell growth. A number of mechanisms by which flavonoids are able to prevent carcinogenesis have been reported. These mechanisms include their free radical scavenging ability, the modification of enzymes to activate or detoxify carcinogens and the inhibition of the induction of the transcription factor activator protein activity by tumor promoters (Canivence-Lavier *et al*., 1996; Shih *et al*., 2000; Moon *et al.*, 2006). Flavonoids have the ability to interfere with the different steps of the formation of benign tumor cells that may progress to malignant tumors, as illustrated in Figure 6.1

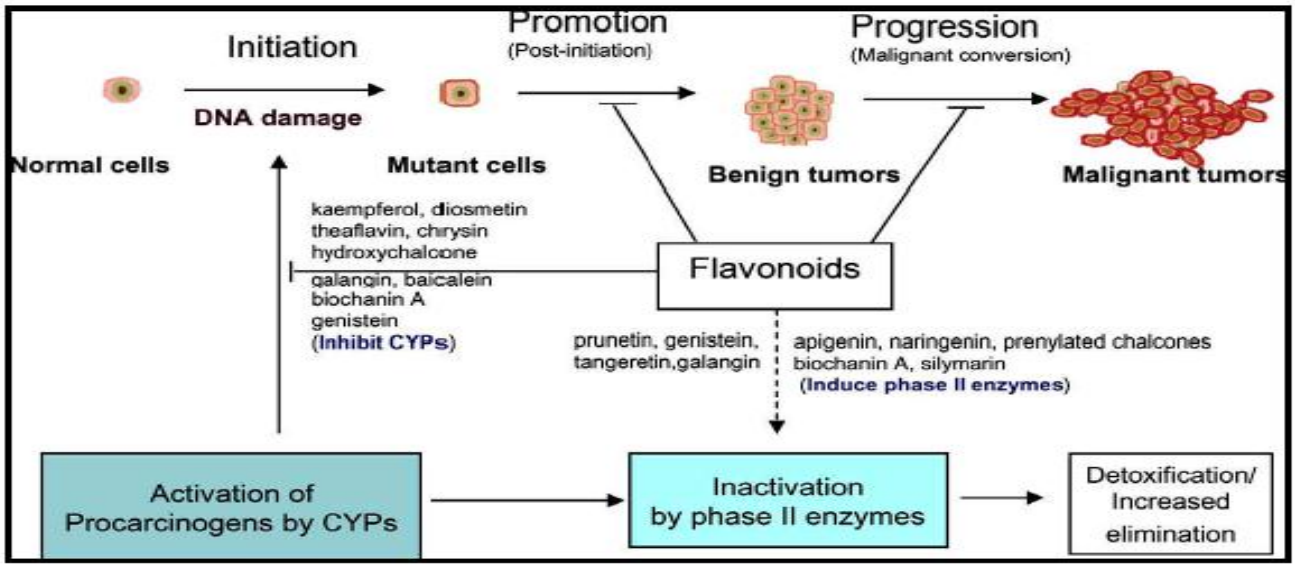

Figure 6.1: Flavonoids blocking and suppressing multi-stage carcinogenesis. (Moon *et al.,* 2006)

Bioactive compounds in medicinal plants

Plants have their own mechanisms of producing secondary metabolites in small to large amounts and concentrations vary. Van Wyk and Wink (2004) reported that there are generally three major groups of secondary metabolites, namely nitrogen-

containing compounds (e.g. alkaloids, and terpenoids), phenolics (e.g. flavonoids and tannins) and glycosides.

Alkaloids are well known for potent pharmacological activities in analgesics, anti-malarial medication, anti-spasmodics and products for the treatment of hypertension, mental disorders and tumors (Rajnikant, 2005). Humans have found numerous uses for plant alkaloids, from medicinal (pain relievers, tranquilizers, stimulants, muscle paralyzers) to agricultural (pesticides and herbicides). Some common examples of plant alkaloids include caffeine and cocaine (Robins, 1994). Previous studies indicate that alkaloids have antitumor potential and among other characteristics have shown *in vivo* activity against various human viruses (Duri *et al*., 1994; Hutchings *et al*., 1996). Fig.6.2 shows the chemical structure of a typical alkaloid with the basic unit of nitrogen from the amino acid.

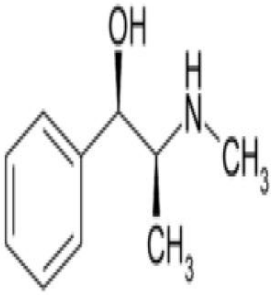

Figure 6.2. The chemical structure of ephedrine, a phenethylamine alkaloid (Wikipedia, 2014< http://en.wikipedia.org/wiki/Phenols>).

Terpenoids are toxins and feeding deterrents to many plant-feeding insects and mammals (Taiz and Zeiger, 2002). Many terpenoids play important roles as plant hormones and in the chemical defenses of plants against microbial diseases and insect herbivores (Croteau, 1998). They are reported to have medicinal properties such as anti-carcinogenic, anti-malaria, anti-ulcer, antimicrobial and diuretic activity (Aharoni *et al*., 2005). Previous studies reported that plants with terpenes possessed strong antimicrobial activity (Marin *et al*., 2001; Ahmed *et al*., 2005). Figure 6.3 shows the chemical structure of a typical terpenoid with the basic five-carbon skeleton.

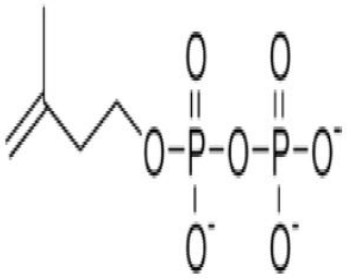

Figure 6.3. Chemical structure of the isopentenyl pyrophosphate, a terpenoid (Wikipedia, 2014< http://en.wikipedia.org/wiki/Phenols>).

Flavonoids are naturally occurring polyphenolic compounds containing two benzene rings linked together with a pyrone ring in the case of flavones or a dihydropyrone ring in the case of flavanones (Giuseppe *et al*., 2007)**.** Flavonoids are normal constituents of the human diet and are responsible for a variety of biological activities. Some of these act as enzyme inhibitors and antioxidants, and have been reported to have anti-inflammatory properties (Middleton *et al*., 2000). However, the molecular mechanisms explaining how flavonoids suppress the inflammatory response are still being elucidated (Havsteen, 2002). A few studies have shown that certain flavonoids down-regulate NO_2 mediators of inflammation production in response to inflammatory stimuli (Liang *et al*., 1999; Kim *et al*., 2001) but no more precise mechanisms of action are known yet. Flavonoids have long been recognized as possessing antiallergenic, anti-inflammatory, antiviral, antiproliferative and antioxidative activities (Robards *et al*., 1999).

Flavonoids are water-soluble phenolic molecules containing 15 carbon atoms and are a group of low molecular weight chemical compounds, among others the phenylbenzopyrones, found in all vascular plants. They are common constituents of fruit, vegetables, nuts, seeds, stems, flowers, tea, wine and honey (Grange and Davey, 1990). The physiologically active constituents have been used to treat human diseases (Cushnie and Lamb, 2005). These constituents have been reported to possess many useful medicinal properties, including anti-inflammatory activity, estrogenic activity, enzyme inhibition, antimicrobial activity (Havsteen, 1983), anti-allergic activity (Harborne and Baxter, 1999), antioxidant activity (Middleton and Chithan, 1993), vascular activity and cytotoxic antitumor activity (Harborne and Williams, 2000). Some of the recognized activities of flavonoids include anti-allergic, anti-cancer, antioxidant, anti-inflammatory, anti-viral and many health-promoting

effects (Harborne, 1996). Figure 6.4 shows the chemical structure of a typical flavonoid with the basic unit of a ketone.

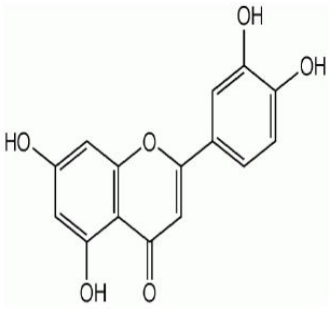

Figure 6.4. The chemical structure of luteolin, a flavonoid (Wikipedia, 2014< http://en.wikipedia.org/wiki/Phenols>).

Tannins are a complex group of plant secondary metabolites that are soluble in polar solutions and are distinguished from other polyphenolic compounds by their ability to precipitate proteins (Silanikove *et al*., 2001). The amount and type of tannins synthesized by plants vary considerably, depending on plant species, cultivars, tissues, stage of development and environmental conditions (Cornell, 2000). Plant parts containing tannins include the bark, woody part, fruit, fruit pods, leaves, roots and plant galls. These secondary metabolites are evenly distributed in all leaf tissues. Plants containing more than 10% tannins may have potentially adverse effects on humans, including upset stomachs, renal damage, hepatic necrosis and increased risk of esophageal and nasal cancer (Kemper, 1999). Figure 6.5 shows the chemical structure of a typical tannin with the basic unit of phenol groups.

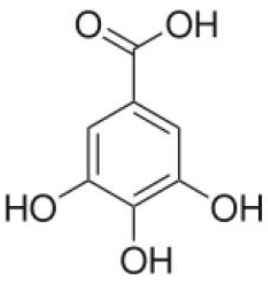

Figure 6.5. Chemical structure of gallic acid, a tannin (Wikipedia, 2014< http://en.wikipedia.org/wiki/Phenols>).

Phenolic acids are a large and heterogeneous group of biologically active non-nutrients. They are present in plants as hydroxylated derivatives of benzoic and cinnamic acids (Havsteen, 1983; Shahidi and Naczk, 1995). Phenolic compounds

are important in the defense mechanisms of plants under different environmental stress conditions such as wounding, infection and excessive light or ultraviolet irradiation (Dixon and Paiva, 1995). Phenolics are not only unsavory or poisonous, but also of possible pharmacological value (Strack, 1997). Hydroxybenzoic acids have a general structure derived directly from benzoic acid and variations in the structures of individual hydroxybenzoic acids lie in the hydroxylations and methylations of the aromatic ring (Macheix *et al*., 1990). Phytochemicals are inexpensive, effective, readily applicable and accessible bioactive compounds that neutralize free radicals causing cell damage. The inherent potential of these phytochemicals in the chemoprevention of cancer cannot be overemphasized, especially considering their robust safety records when compared with conventional anti-cancer therapies. Phytochemicals are directly responsible for different activities such as antioxidant, antimicrobial, antifungal and anticancer ones (Kokate, 1997; Harborne, 1998; Hossain and Nagooru, 2011). This has extended the field of research for potential anticancer compounds, as some derived anticancer compounds are already extensively used, such as etoposide, teniposide, vinblastine and vincristine (Lee, 1999; Mans *et al*., 2000). Thus, the aim of this study was to identify biological compounds present in *P.violacea* and *X.zambesiaca* plant extract.

6.3 METHODOLOGY

6.3.1 Plant material

The plant materials *P.violacea* and *X.zambesiaca* were authenticated by scientists at the National Botanical Gardens in Bloemfontein, South Africa (MAS002). The collected material was dried at room temperature, pulverized by mechanical mills and weighed. It was then stored in a cool place until analysis.

6.3.2 Extraction method

The extraction was done using the maceration. Plant material (10 g of the dried twigs, leaves and flowers of each plant) was weighed, pulverized and soaked in methanol for 72 hours, with occasional stirring. The extracts were filtered and new

solvent was added again for more extraction until the solvent remained clear. The methanol extract was concentrated using a rotator evaporator.

6.3.3 Phytochemical analysis

The extracts were subjected to preliminary phytochemical screening according to Okwu (2005).

I. **Test for tannis**

0.2 ml of extract was added to a few drops of 1% $FeCl_3$. A resulting green/blue-black color indicated the presence of tannins.

II. **Test for flavonoids**

0.1 ml extract was added to 5 ml ammonium hydroxide solution and 3 drops of H_2SO_4. The formation of a yellow color solution and a white precipitate indicated the presence of flavonoids.

III. **Test for saponins**

0.1 ml extract was shaken vigorously in 3 ml of water. Foam formation indicated the presence of saponins.

IV. **Test for steroids**

To 2 ml of the extract, 2 ml of chloroform and 2 ml of concentrated H_2SO_4 were added and then the solution was shaken well. The presence of steroids is indicated by the chloroform (upper) layer turning reddish and the acid layer showing a yellow color with greenish fluorescence.

V. **Test for terpenoids**

1 ml of extract was mixed with 2 ml of chloroform and 2 ml H_2SO_4 was carefully added to form a layer. A yellow layer at the interface indicated presence of terpenoids.

VI. **Test for alkaloids**

Exactly 0.1 ml extract was added to 1 ml of Mayer's reagent. The solution turning creamy was indicative of the presence of alkaloids.

VII. **Test for cardiac glycosides**

In a test tube, 5 ml of the extract was mixed with 2 ml glacial acetic acid, 1 drop of 5% $FeCl_3$ and 1 ml concentrated H_2SO_4. Formation of a brown ring at the interface indicated the de-oxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form gradually throughout the thin layer.

VIII. **Determination of total phenolic content**

Total phenols content in the extracts obtained were estimated by a colorimetric assay based on procedures described by Singleton and Rossi (1965) with some modifications. Briefly, 1 mL of sample was mixed with 1 mL of folin and Ciocalteu's phenol reagent. After 3 min, 1 mL of saturated sodium carbonate solution was added to the mixture and adjusted to 10 mL with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm wavelength. Gallic acid was used for constructing the standard curve. The results were expressed as mg of GAEs/g of extract.

6.4 RESULTS

Phytochemical test	A	B
Tannins	**+**	**--**
Flavonoids	**+**	**+**
Saponins	**--**	**+**
Steroids	**+**	**--**
Terpenoids	**+**	**+**
Alkaloids	**+**	**--**
Cardiac glycosides	**+**	**+**

Table 6.4.1: Phytochemical screening of *P.violacea (A) and X.zambesiaca (B) extracts.* **+** means present and **–** means absent.

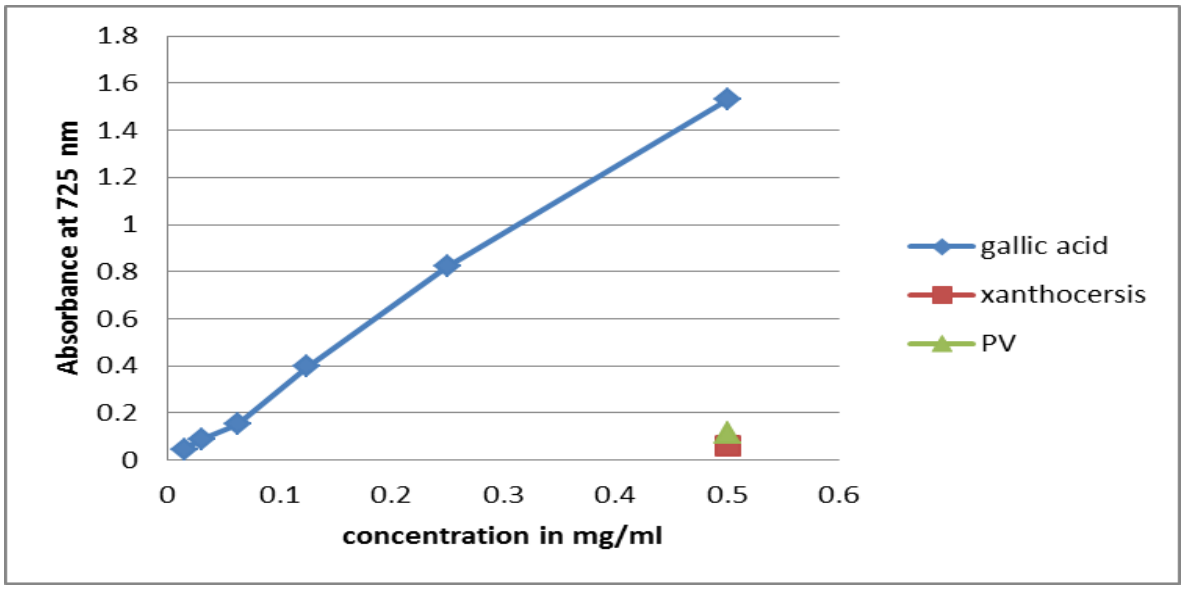

Figure 6.2: Gallic acid calibration curve for the estimation of *P.violacea and X.zambesiaca* phenol content.

Sample	Absorbance Mean	Polyphenol concentration (mg/GAE)
***P.violacea* (1 mg/ml)**	**0.114**	**0.152 ± 0.0269**
***X.zambesiaca* (1 mg/ml)**	**0.063**	**0.0895 ± 0.006**

Table 6.4.2: Estimated total phenolic content of *P.violacea and X.zambesiaca extracts*

6.5 DISCUSSION

Phytochemicals are inexpensive, effective, readily applicable and accessible bioactive compounds (Okwu, 1999). The present study carried out on *P.violacea* and *X.zambesiaca* plant extracts revealed the presence of medicinally active constituents. The phytochemically active compounds of *P.violacea* and *X.zambesiaca* were qualitatively analyzed in a mixture of twigs, leaves and flowers of each plant. The phytochemical screening results in Table 6.4.1 revealed the presence of tannins, flavonoids, steroids, terpenoids, alkaloids and cardiac glycosides from *P.violacea* (leaves, flowers and twigs) extract, while *X.zambesiaca* (leaves, flowers and twigs) extract showed the presence of flavonoids, saponins, terpenoids and glycosides**.**

The medicinal value of plants lies in some chemical substances such as alkaloids, tannins, flavonoids and other phenolic compounds, which have a definite physiological action on the human body (Hill, 1952). Different phytochemicals have been found to possess a wide range of activities, which may help in protection against chronic diseases. For example, alkaloids protect against chronic diseases, saponins protect against hypercholesterolemia and antibiotic properties, steroids and triterpenoids have analgesic properties and steroids and saponins are responsible for central nervous system activities. Phytochemical screening of *P.violacea* and *X.zambesiaca* plant extracts was investigated to study the presence of alkaloids, flavonoids, steroids, saponins, tannins, terpenoids and cardiac glycosides.

The beneficial health effects of plants are attributed to flavonoids, a class of secondary metabolites that protect the plant against ultraviolet light and even herbivores (Harbone and Williams, 1992). The protective effects of flavonoids are due to their capacity to transfer electrons to free radicals. Alkaloids and their synthetic derivatives are used as basic medicinal agents for their antispasmodic and bactericidal effects (Stray, 1998; Okwu, 2004). Alkaloids in *X.zambesiaca* may be responsible for its antibacterial effect (Masoko, 2013). Tannins in *P.violacea* have astringent properties and hasten the healing of wounds (snakebite) and inflamed mucous membranes (due to tuberculosis/colds) (Mnxati, 2009). *X.zambesiaca* have been proven to have isoflavones (Harper *et al*., 1976). The isoflavones bind to estrogen receptors in the body, thereby blocking the cancer-promoting effects of estrogen (Reddy *et al.,* 2003). This compound might be beneficial for the prevention of ovarian cancer, but this can only be verified by anticancer screening of this extract on the ovarian cell line.

Phenolic compounds are commonly found in the plant kingdom and have been reported to have multiple biological effects, including antioxidant activity (Kähkönen *et al.,* 1999). Polyphenols are the most abundant antioxidants in our diet. Foods and beverages rich in polyphenols may have great potential with respect to prevention of diseases. Estimated total phenolic content in this study was measured using the Folin-Ciocalteau assay and a phenolic acid (such as gallic acid) to set up a calibration curve (Figure 6.2). Total phenolic content was estimated using the gallic standard curve and expressed as GAE in mg/g extract (Singleton and Rossi, 1965).

Table 6.4.2 displays the estimated total phenol content values of *P.violacea* and *X.zambesiaca* extracts, which were analyzed at 1 mg/ml against gallic acid.

Polyphenolic compounds are effective in the prevention of oxidative stress related diseases. Both plant extracts showed the presence of flavonoids. Flavonoids are a group of polyphenolic compounds with diverse characteristics and chemical structures. The therapeutic potential of these flavonoids has been determined and they are known to have a number of pharmacological and biochemical properties, namely antibacterial, antiviral, anti-allergic, vasodilatory and anti-inflammatory ones, exhibiting activity against the enzymes cyclo-oxygenase and lipoxygenase. (Middleton *et al*., 2000). *P.violacea* extracts showed more polyphenols than *X.zambesiaca*. The GAEs of the estimated phenolic concentrations ranged from 0.152 ± 0.0269 to 0.0895 ± 0.006 mg/GAE. The total phenolic content of *X.zambesiaca* extracts showed a correlation with the antioxidant activity determined in Chapter 5. However, there was no correlation on total phenol content values of *P.violacea* and active biological compounds it comprises. *P.violacea* showed the presence of flavonoids and alkaloids, which are known for their effects as antioxidant and anticancer agents, but at the same time they had no antioxidant and anticancer properties, as discussed in Chapter 2 and Chapter 5. Intensive research is still needed to comprehend the pharmacological effects and the identification of flavonoids and alkaloid categories present in this part of the plant.

6.6 CONCLUSION

The plants screened for phytochemical constituents seemed to have the potential to act as a source of useful drugs and also to improve the health status of the consumers as a result of the presence of various compounds that are vital for good health.

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CHAPTER 7

"Identification of active compounds from *P.violacea* and *X.zambesiaca*"

ABSTRACT

Nature has been a good supply of various medicinal agents for thousands of years and more than 50% of modern drugs have been isolated from natural sources such as plants. Both *P.violacea* and *X.zambesiaca* species have been used in traditional remedies by South Africans for years. *P.violacea* is traditionally used to treat inflammation-related disorders and diarrhea, while *X.zambesiaca* is used to treat diabetes mellitus. It was previously demonstrated that the latter has anti-tuberculosis activity and antihyperglycemic activity in diabetic mice. The aim of this study was to determine the chemical composition of methanol crude extracts (mixture of twigs, leaves and flowers) of *P.violacea* and *X.zambesiaca*. Liquid chromatography-mass spectrometry (LC-MS) analysis of these plant extracts showed the presence of nine compounds: 4-Acetoxy-3,5-dimethoxybenzoic acid, 2″-O- *β –D-Apriofuranosyl, 6″*-O-α-L-*rhamnopyranosyl*, Quercetin-3-*O*-[α-L- rhamnopyranosyl(1→6)]-*β -D*-galactofuranoside, 3-O- *β –D-Galactopyranosyloxy-3' .4' .5.7-tetrahydroxyflavone, Quercetin 3-galactoside, 7-O-[α-L-Rhamnopyranosyl-(1→6)- β-D-glucopyranoside] or 6,8-Bis(C-β-glucosyl)-apigenin, 3-O-[α-L-Rhamnopyranosyl-(1→2)-β-D-glucopyranoside], 20-O-α-L-rhamnopyranoside* and two unknown compounds from *P.violacea,* and seven compounds, *Apigenun-6-C-α-L-arabinopyranosyl-8-C- β -L-arabinopyranoside*, *Metaplexigenin-3-O- β-D-oleandropyranosyl-(1→4)- β-D-digitoxopyranosyl -(1→4)- β-D-digitoxopyranoside*, *Arvenin I, 3-O- β-D-Glucuronopyranoside: (Cloversaponin II), Apigenin-4'-O- β –D-xylofuranodyl(-(1→4)-O- β-D-glucopyranoside and 6-Me ether, 7-O- β –D-galactopyranoside: (Chamaejasmoside)* from *X.zambesiaca.* Identified compounds correlate with the reported total phenolic content results shown in Chapter 6 of this study, as it was demonstrated that *P.violacea* has more phenols that *X.zambesiaca*.

7.1 INTRODUCTION

Plants have an almost limitless ability to synthesize chemical substances, mainly secondary metabolites, of which numerous compounds have been isolated (Rafael, 2008). Chemical analysis of *P.violacea* showed the presence of tannins, flavonoids, steroids, terpenoids, alkaloids and cardiac glycosides and *X.zambesiaca* showed the presence of flavonoids, saponins, terpenoids and glycosides, reported in Chapter 6. The existence of such chemical compounds was expected, as their derivatives are found in most plants. Alkaloids, flavonoids, terpenoids, tannins, phenolics and many others are very important for both humans and plants (Rafael, 2008). They act as cell wall support materials (Wallace, 1994) and as colorful attractants for birds and insects, helping seed dispersal and pollination (Harborne, 1994). Flavonoids and phenolic acids have also been reported as having antioxidative (Robards *et al.*, 1999) and anticarcinogenic effects (Stavric, 1994).

It is estimated that about 61% of the 877 small molecule new chemical entities introduced as drugs worldwide in the past 30 years were developed from natural products (Satyajit and Lutfun 2007). It was also estimated that about 74% of anticancer drug candidates are natural products or structural analogues of natural products. Approximately 60% of all drugs in clinical trials for a multiplicity of cancers are of natural origin. In the past, the identification of compounds was labor-intensive, time-consuming and required too much sample. However, modern drug discovery approaches apply full automation and robotics where hundreds of molecules can be screened, using several assays within a short time and very small amounts of compounds. A number of techniques are used for the identification and analysis of plants chemical constituents such as liquid chromatography–mass spectrometry detector (LC-MS) detector and liquid chromatography–nuclear magnetic resonance spectroscopy. Therefore, the aim of this study was to determine the chemical composition of methanol crude extract (mixture of twigs, leaves and flowers) of *P.violacea* and *X.zambesiaca* crude extracts.

7.2 LITERATURE REVIEW

LC-MS is a chromatographic technique used to separate a mixture of compounds with the purpose of quantifying and identifying the specific components of the mixture. High performance liquid chromatography (HPLC) was revolutionized in 2004 by Waters into a new sophisticated, fast, high-resolution separation instrument with sensitivity of analysis called ultra-performance liquid chromatography (UPLC) (Grumbach, 2011). This UPLC fractionates and isolates chemical compounds in short periods of time with little solvent consumption and minute quantities of sample (Stephen and Tchelitcheff, 2006). Kang (2011) determined the robustness, fast speed and good resolution of UPLC and its ability to consume less solvent than HPLC. HPLC uses more solvent and solution to be tested, is time-consuming and generates high back-pressure that influences the analytical column. The original HPLC method takes about 35 minutes to two hours per analysis, while UPLC takes about five minutes (Grumbach, 2011). HPLC generates high column back-pressure when smaller size particles are used because of the short column used but this does not cause loss of efficiency.

The principle of UPLC is governed by the van Deemter equation: H= A + B/v + Cv (Grumbach, 2011). Where A is a constant velocity representing the Eddy dispersion, B (axial dispersion) is the diffusion of molecules, C is the kinetic resistance (high temperature, small particles and core-shell particle), and v is the flow rate. This equation describes the relationship between flow rate and high equivalent of theoretical plate (HETP)/column efficiency, which is dependent on the diameter of particles packed into the analytical column (Swartz, 2005). Column packing particle size is inversely proportional to efficiency and resolution. As particle size decreases below 2,5 µm, efficiency increases; this leads to high speed and peak capacity (Swartz and Murphy, 2004) resulting in a rapid increase in back-pressure. UPLC is designed to use low volume injections with minimal carryover to achieve high sensitivity, accuracy and precision (Lucie *et al.,* 2006). UPLC provides resolution, speed and sensitivity with no compromises. This instrument allows short analysis time, uses very small particle packed analytical columns and performs under very high pressures without influencing the analytical column or other components of the

chromatographic system (Swartz, 2005). High back-pressure improves chromatographic resolution. UPLC increases productivity because it is fast.

There are two classes of compounds from plants, namely primary and secondary metabolites. Primary metabolites are required for the sustenance of the plant, while secondary metabolites are not a necessity for the plant's survival (Harborne and Baxter, 1999). The latter are mostly compounds that are active for treatment and prevention of diseases in humans and animals and are thus termed bioactive compounds. Bioactive compounds are generally unique to individual plant species and are thus species-specific, but some may be found in several or many plant species of a genus, in several related genera, or even families (Kinghorn *et al.*, 2003). The crucial factor for definitive accomplishment of isolating bioactive plant constituents is the selection of the "right" plant and the relevant part of the plant, which contains the active compounds. As distillate, certain secondary metabolites in specific organs and variation in bioactivity are often encountered in different parts of the same plant (O'Neill and Lewis, 1993).

Previous investigations of *X.zambesiaca* revealed the presence of 7,8-dioxy-isoflavonoids, 2-benzylbenzo[*b*]furan-3(2H)-ones (Bezuidenhout *et al*., 1987), along with the isolation of novel isoflavones, 7,8,3'-trihydroxy-4'-methoxyisoflavone and α,3,4,4'-tetrahydroxy-2'-methoxydihydrochalone (Bezuidenhout *et al*., 1988). However, these were isolated from the heartwood of the plant and it is known that different parts of the plant may contain dissimilar compounds. *X.zambesiaca* leaf extract was demonstrated to have five compounds, namely fagomine, 3-*epi*-fagomine, 3,4-di-*epi*-fagomine, 3-*O*-β-d-glucopyranosylfagomine and 4-*O*-β-d-glucopyranosylfagomine (Kato *et al*., 1997). The two compounds mentioned last were identified as new natural products. Fagomine is a good inhibitor of isomaltase and certain α- and β-galactosidases, whereas 3-*epi*-fagomine is a more potent inhibitor of isomaltase and β-galactosidases than fagomine, but does not inhibit α-galactosidase. Compound 3,4-di-*epi*-fagomine exhibited no significant inhibition against the glycosidases used (Kato *et al*., 1997). *X.zambesiaca* was also identified as having diarylpropanes such as diarylalkanes and/or diarylalkanols, which have

the capability of inhibiting binuclear enzyme function, particularly tyrosinase, which prevents melanin overproduction. When one considers that a single plant may contain up to thousands of phytoconstituents, the possibilities of making new discoveries become self-evident. Our phytochemical investigation of *X.zambesiaca* and *P.violacea* (mixture of leaves, twigs and flowers) has resulted in the identification of other compounds of which the presence has not been recorded before from these specific parts of the plants.

7.3 METHODOLOGY

7.3.1 Plant material

The plant materials *P.violacea* and *X.zambesiaca* were authenticated by scientists at the National Botanical Gardens in Bloemfontein, South Africa (MAS002). The collected material was dried at room temperature, pulverized by mechanical mills and weighed. It was then stored in a cool place until analysis.

7.3.2 Extraction method

The extraction was done using the maceration. Plant material (10 g of the dried twigs, leaves and flowers of each plant) was weighed, pulverized and soaked in methanol for 72 hours with occasional stirring. The extracts were filtered and new solvent was added again for more extraction until the solvent remained clear. The methanol extract was concentrated using a rotator evaporator.

7.3.3 Identification of active compounds with LC-MS

LC–MS/MS analysis for the identification of active compounds from *P.violacea* and *X.zambesiaca* methanol crude extracts was carried out using the Waters Synapt G2 instrument. This is an Agilent 1100 LC system consisting of degasser, binary pump, auto sampler and column heater. The column outlet was coupled to an Agilent MSD Ion Trap XCT mass spectrometer equipped with an ESI ion source. Data acquisition and mass spectrometric evaluation were carried out on a personal computer with Acquity binary solvent manager instrument system. For the chromatographic separation, Waters UPLC on a Waters BEH C18, 2.1x100 mm column was used. The column was held at 95% solvent A (0.1% formic acid in water) and 5% solvent B

(0.1% formic acid in acetonitrile) for 1 min, followed by an 11 min step gradient from 5% B to 100% B, then it was kept for 4 min with 100% B; finally, the elution was achieved with a linear gradient from 100% B to 5% B for 2 min. The flow rate was 0.4 ml/min and the injection volume was 0.01 ml. The following parameters were used throughout all MS experiments: for electrospray ionization with negative ion polarity the capillary voltage was set to 3 kV, the drying temperature to 350^{0}C and cone voltage of 15 V, the maximum nebulizer pressure to 15000 psi and the seal wash was 5 min. The total run time was 15 minutes, the scan speed was 26 000 m/z/s (ultra-scan mode) and the lock mass was Leucine enkaphelin. The phenolics were identified using a combination of HPLC with diode array detection and liquid chromatography with atmospheric pressure chemical ionization mass spectrometry (ESI-LC/MS/MS) on the basis of their ultraviolet spectra, mass spectra and by comparison of the spectra with those of available authentic standards.

7.4 RESULTS

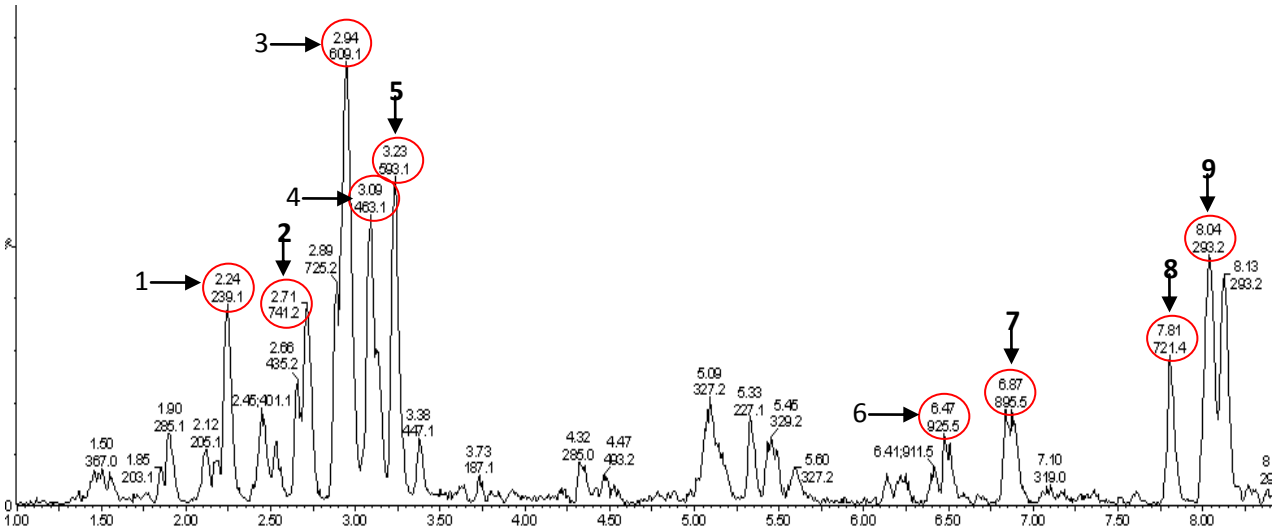
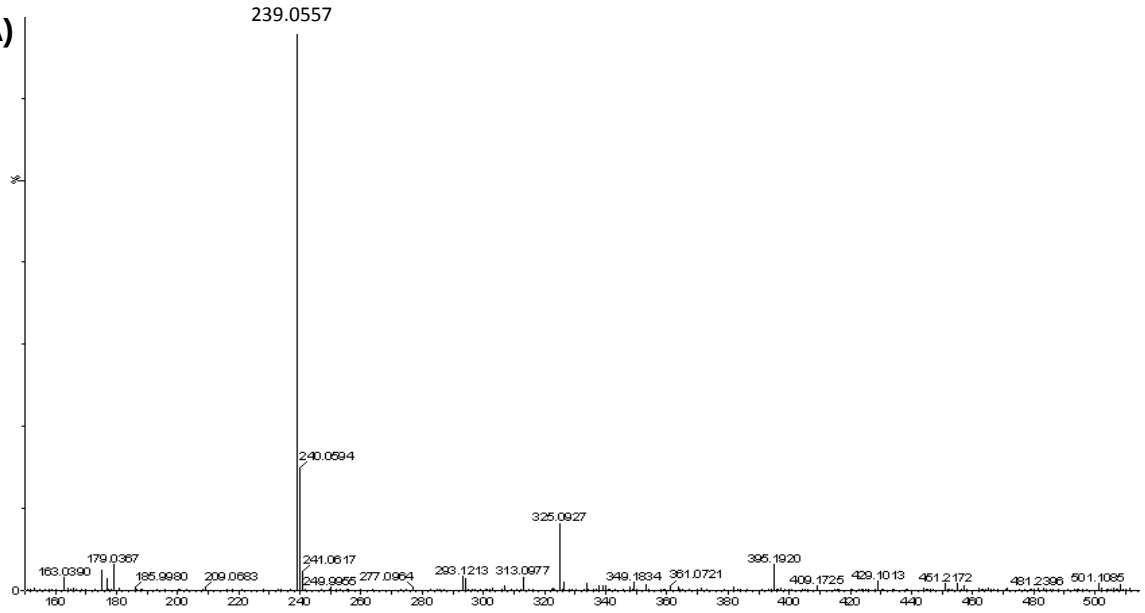


Figure 7.1: LC/MS chromatograms of *P.violacea* extract (ESI negative). Nine phenolic components were identified.

(A)



(B)

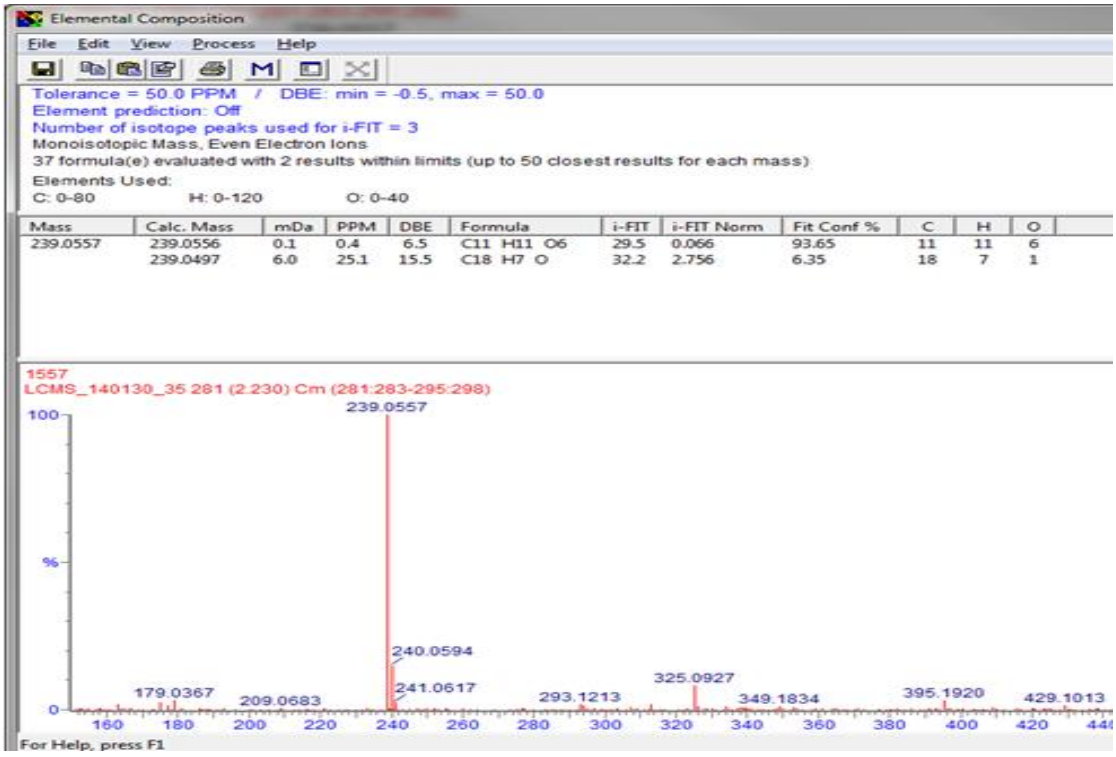


Figure 7.2: (A) mass spectrum (peak at 2.2 min) of identified compound numbered 1 from *P.violacea.* (B) Elemental composition predictions of identified compound numbered 1 from *P.violacea.*

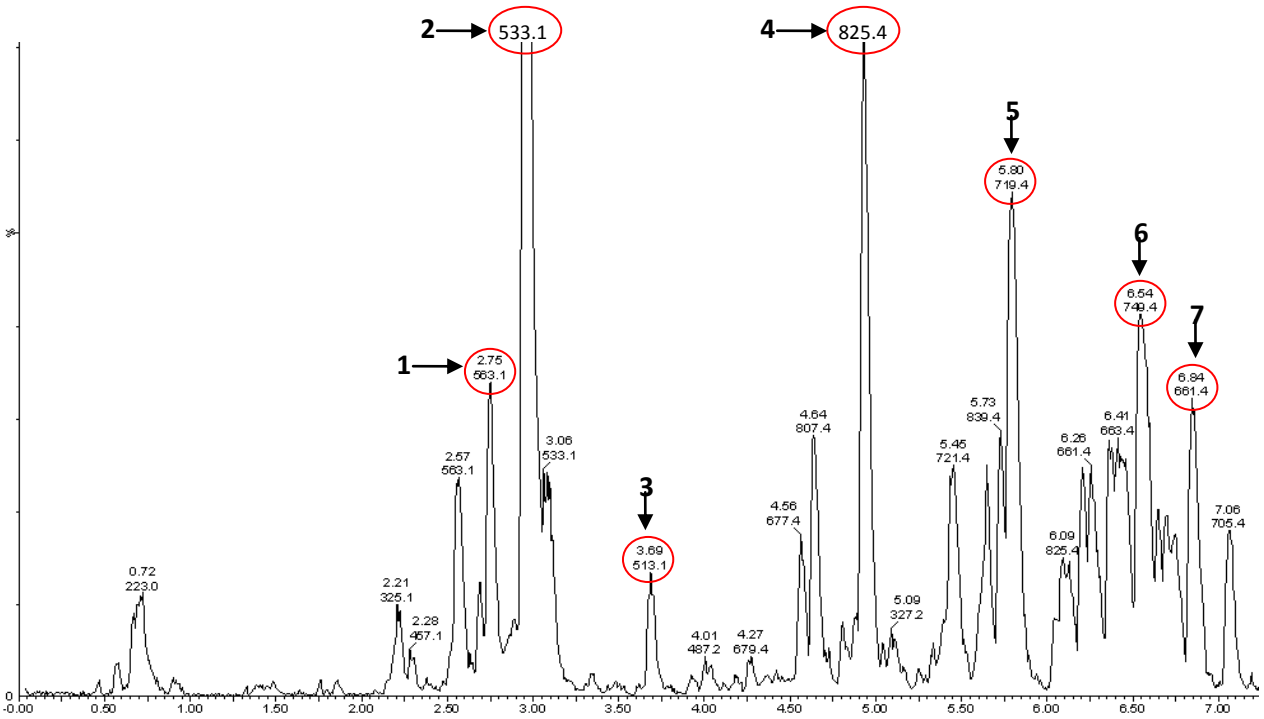


Figure 7.2: LC/MS chromatograms of *X.zambesiaca* (ESI negative). Seven phenolic components were identified.

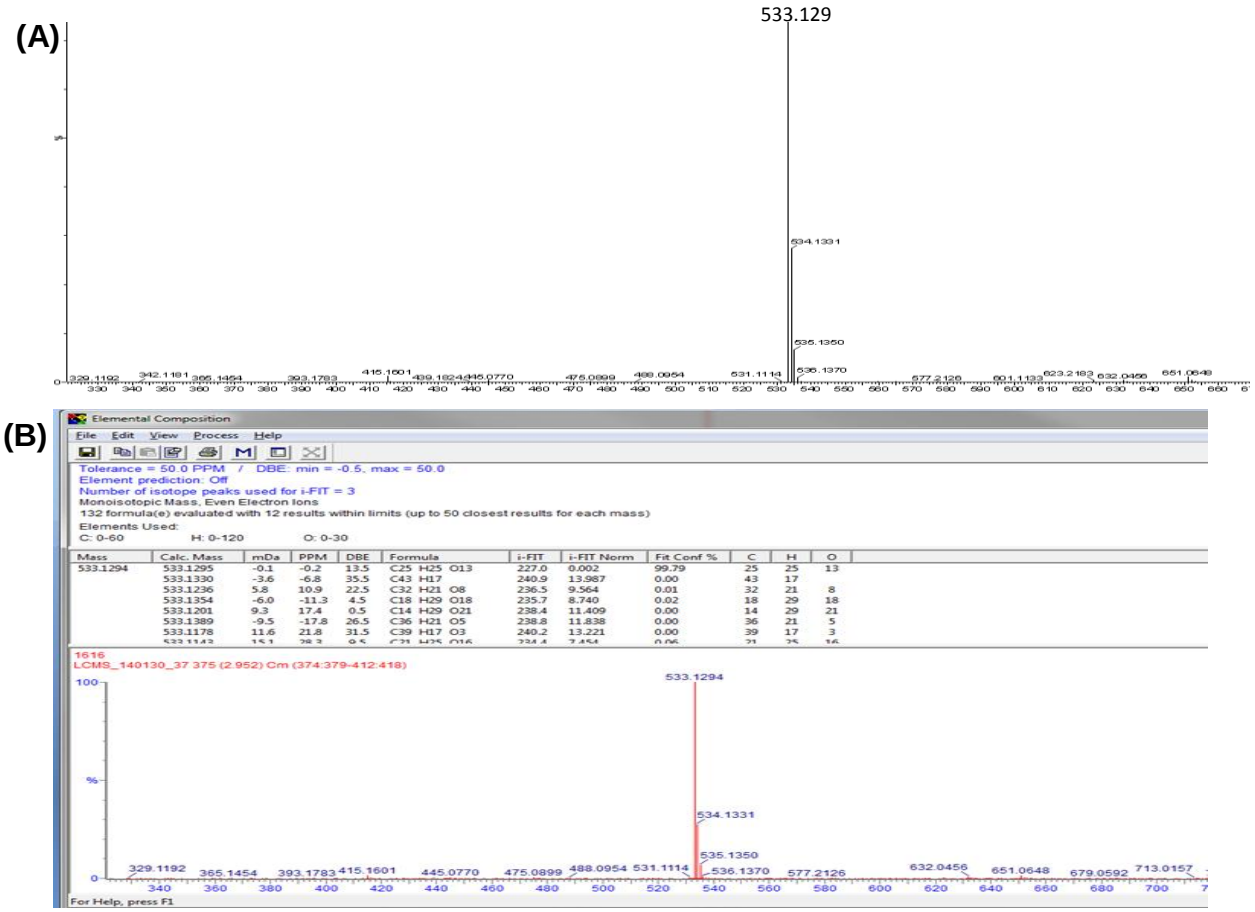


Figure 7.2: (A) mass spectrum (peak at 2.2 min) of identified compound numbered 1 from *Xanthorcercis zambesiaca.* (B) Elemental composition predictions of identified compound numbered 1 from *Xanthorcercis zambesiaca.*

Table 7.1: Identification of phenolic compounds in *P.violacea* methanol extract using their mass unit and retention times.

Peak	(m/z)	Rt (min)	Tentative ID	References
1	239.05	2.24	*4-Acetoxy-3,5-dimethoxybenzoic acid*	(All chemical compounds, 2012)
2	741.18	2.71	*2"-O- β –D-Apriofuranosyl, 6"-O-α-L-rhamnopyranosyl*	(Yannai, 2004)
3	609.14	2.94	*Quercetin-3-O-[α-L-rhamnopyranosyl(1→6)]-β -D-galactofuranoside*	(Zhou *et al*., 2011)
4	463.08	3.09	*3-O- β –D-Galactopyranosyloxy-3' .4' .5.7-tetrahydroxyflavone. Quercetin 3-galactoside*	(Yannai, 2004)
5	593.14	3.32	*7-O-[α-L-Rhamnopyranosyl-(1→6)- β-D-glucopyranoside]* *or 6,8-Bis(C-β-glucosyl)-apigenin*	(Zhou *et al*., 2011)
6	925.51	6.47	*3-O-[α-L-Rhamnopyranosyl-(1→2)-β-D-glucopyranoside], 20-O-α-L-rhamnopyranoside*	(Yannai, 2004)
7	895.50	6.87	*Unknown*	*
8	721.36	7.81	*2-oxepanone; 2-propenoic acid [3-hydroxy-2-[[3-hydroxy-2,2 bis(hydroxymethyl)propoxy]methyl]-2-hydroxymethyl)propyl] ester; 2-propenoic acid 1-[2-[2-(1-oxoprop-2 enoxy)propoxy]propoxy]propan-2-yl ester*	(Yannai, 2004)

9	293.21	8.04	*Unknown*	*

Identification was aided by comparison with previous literature reports.

(m/z) = Molecular mass, Rt = retention time, * = no literature

Table 7.2: Identification of phenolic compounds in *X.zambesiaca* methanol extract using their mass unit and retention times.

Peak	(m/z)	Rt (min)	Tentative ID	References
1	563.14	2.75	*Apigenin-4'-O- β –D-xylofuranodyl(-(1→4)-O- β-D-glucopyranoside and 6-Me ether*	(Zhou *et al*., 2011)
2	533.1	2.29	*Apigenun-6-C-α-L-arabinopyranosyl-8-C- β -L-arabinopyranoside*,	(Zhou *et al*., 2011)
3	513.1	3.69	*7-O- β –D-galactopyranoside: (Chamaejasmoside)*	(Yannai, 2004)
4	825.4	4.93	*Metaplexigenin-3-O- β-D-oleandropyranosyl-(1→4)- β-D-digitoxopyranosyl -(1→4)- β-D-digitoxopyranoside*	(Yannai, 2004)
5	719.3	5.80	*23,24-Dihydro, 25-Ac, 2-O-b-D-glucopyranoside:*	(Yannai, 2004)
6	749.37	6.54	*5E,8Z,11Z,14Z)-eicosa-5,8,11,14-tetraenoic acid [1-acetyloxy-3-[[(3R,4R,5S)-3,4,5-triacetyloxy-6-(acetyloxymethyl)-2-oxanyl]oxy]propan-2-yl] ester*	(All chemical compounds, 2012)
7	661.35	6.84	*3-O- β-D-Glucuronopyranoside: (Cloversaponin II)*	(Yannai, 2004)

Identification was aided by comparison with previous literature reports

(m/z) = Molecular mass, Rt = retention time

7.5 DISCUSSION

In this study, LCMS techniques have been employed to investigate the phenolic content of *P.violacea* and *X.zambesiaca* methanol plant extracts. This was aimed at identifying only compounds present in these plant extracts, but not to isolate and re-analyze the activity of the isolated active compounds. Fragmentation of the compounds by the mass spectrum was performed to obtain the molecular chemical formula of the compounds that formed significant peaks. The relative retention times and mass spectra of the extract components were compared with those of authentic samples and with mass spectra from a data library. As shown in Figure 7.1, LC-MS analysis of the *P.violacea* methanol extract resulted in the identification of nine compounds, with more than 90% similarity with the standard mass spectra in the library. The LC-MS analysis of *X.zambesiaca* extract shown in Fig 7.2 resulted in the identification of seven compounds, also with more than 90% similarity with the standard mass spectra in the library.

In short, for *P.violacea* LC-MS analysis, the first five components with the greatest peak area to be eluted were at 2.24 min till 3.32 min (peak 1 to peak 5), which was in the more hydrophilic region (short retention time). The other four main components eluted at 6.47 min (peak 6), 7.81 min (peak 7), 6.87 min (peak 8) and 8.04 min (peak 9) in the more hydrophobic region (longer retention time). Then, for *X.zambesiaca* LC-MS analysis, the first three components were eluted in the more hydrophilic region, with the greatest peak area at 2.75 min till 3.69 (peak 1 to peak 3) and the other four main components eluted in the more hydrophobic region at 4.93 min till 6.84 min (peak 4 to peak 7). The deprotonated molecule mass (m/z) measured by the MS in ESI negative mode was used to determine the molecular formula of the compounds extracted. The tentative names of these identified compounds were determined using the molecular formula and the molecule mass, using the literature (Yannai, 2004).

The following compounds were identified: 4-Acetoxy-3,5-dimethoxybenzoic acid, 2″-O- *β –D-Apriofuranosyl, 6″*-O-α-L-*rhamnopyranosyl*, Quercetin-3-*O*-[α-L-rhamnopyranosyl(1→6)]-*β –D*-galactofuranoside, 3-O- *β –D-Galactopyranosyloxy-3' .4' .5.7-tetrahydroxyflavone. Quercetin 3-galactoside, 7-O-[α-L-Rhamnopyranosyl-(1→6)- β-D-glucopyranoside] or 6,8-Bis(C-β-glucosyl)-apigenin, 3-O-[α-L-Rhamnopyranosyl-(1→2)-β-D-glucopyranoside], 20-O-α-L-rhamnopyranoside* and 2 unknown compounds from *P.violacea,* and 7 compounds: *Apigenun-6-C-α-L-arabinopyranosyl-8-C- β –L-arabinopyranoside*, *Metaplexigenin-3-O- β-D-oleandropyranosyl-(1→4)- β-D-digitoxopyranosyl –(1→4)- β-D-digitoxopyranoside*, *Arvenin I, 3-O- β-D-Glucuronopyranoside: (Cloversaponin II), Apigenin-4'-O- β –D-xylofuranodyl(-(1→4)-O- β-D-glucopyranoside and 6-Me ether, 7-O- β –D-galactopyranoside: (Chamaejasmoside)* from *X.zambesiaca*. There was no literature on the identification of these compounds from species belonging to the Fabacea family. Therefore, this is the first report of the presence of these compounds from methanol extract (leaf, twig and flower mixture) from *P.violacea* and *X.zambesiaca* belonging to the Fabacea family.

X.zambesiaca was found to contain compounds with a molecule structure named 'pyranoside' and 'pyranosyl' in abundance. Pyranoside has been reported to possess neuroprotective properties (Chen *et al.,* 2014; Yu *et al.,* 2014) and pyranosyl has been reported as an iron-chelating agent. Furthermore, control of iron in the body by chelating drugs may have a significant beneficial effect on tissue damage caused by free radicals (Lauffer, 1992). Therefore, the presence of this molecule in *X.zambesiaca* may support its activity against free radicals, as demonstrated in Chapter 5 of this study.

P.violacea has also been found to contain pyranoside pyranosyl and two unknown compounds; in addition to that, flavonoids were present in abundance. Flavonoids are classified into various classes (Singh *et al.,* 2014), such as flavonols (quercetin), flavones (apigenin), flavanones (hesperetin, naringenin), flavonoid glycosides (astragalin, rutin), flavonolignans (silibinin), flavans (catechin, epicatechin) to name a few. Flavones are reported as possible antioxidant, anti-proliferative, anti-tumor, anti-microbial, estrogenic, acetyl cholinesterase, anti-inflammatory agents and are also used to treat cancer, cardiovascular disease and neurodegenerative disorders

(Middleton and Chithan, 1993; Cushnie and Lamb, 2005). Despite the presence of abundant flavones in this plant extract, tests for its anticancer, antioxidant and antimicrobial activity yielded negative results. Therefore there was no correlation with the identified active compounds and pharmacological activities of this plant and this was not expected. This might be due to the concentration of these compounds; they are present, but not in large quantities. To elucidate this, each part of this plant must be analyzed separately and a quantitative analysis to determine the amount of active flavonoids present must be done in the future.

7.6 CONCLUSION

Nature has been a good supply of various medicinal agents for thousands of years and more that 50% of modern drugs have been isolated from plants. LC-MS analysis of *P.violacea* and *X.zambesiaca* demonstrated the presence of respectively nine and seven compounds from these methanol plant extracts. These identified compounds have pharmacological properties that may be beneficial to human beings as chemopreventative agents. However, this can only occur after the isolation of these active compounds. Two unknown compounds identified from *P.violacea* need further study in order to identify them, as they are nowhere to be found in the current literature.

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CHAPTER 8

"Radioprotective effect of *X.zambesiaca*"

ABSTRACT

The science of radiation protection is a fundamental result of peaceful and fully equipped applications of ionizing radiation. Several chemical agents/synthetic radioprotectors have been tried against the hazardous effects of ionizing radiation in experimental studies with success and satisfactory outcomes. In the previous investigations *X.zambesiaca* was demonstrated as having antioxidant activity. The aim of this study was to confirm the free radical scavenging features of *X.zambesiaca* methanol extract. Human prostate cancer cells (DU145) were irradiated to 2 Gy in the presence of the extract at a concentration of 0.1 mg/ml and the cell surviving fraction (SF2 - with *X.zambesiaca*) was determined using the colony-forming assay. Based on clonogenic cell survival, *X.zambesiaca* showed significant radiation protection with a radioprotective factor value of 1.53, indicating that *X.zambesiaca* can lead to over 50% reduction in cell death. This confirms the free radical scavenging features of the extract and suggests that it might be useful as a radioprotector.

8.1 INTRODUCTION

The most injurious effects on the viability of cells and human health are oxidative changes in nucleic acids, which cause mutagenic processes and cancer (Ferrari and Torres, 2003). According to the WHO (2012), cancer is the second leading cause of death worldwide. Therefore, prevention of cancer development and its treatment remains a serious challenge for scientists worldwide. One of the most popular and important tools used to cure cancer in current radiation therapy is X-radiation (Ertekin *et al*., 2004). Although radiation is capable of damaging many cellular components, the main target is nuclear DNA. Radiation energy results in both direct and indirect effects, e.g. the production of free radicals, such as superoxide anion in human lymphocytes (Belloni *et al.,* 2008). Previous studies have shown that known

antioxidants, such vitamins C and E and b-carotene, were protective against genetic damage induced by exposure to gamma radiation (Konopacka *et al*., 1998).

Plant polyphenols are important components of human diet and a number of them are considered to possess chemopreventive and therapeutic properties against cancer (Sebastià *et al.,* 2014). They are recognized as naturally occurring antioxidants but also as pro-oxidant, pro-apoptotic or chromosomal aberration inducers (Sweeney, 1979; Weiss and Landauer, 2003). Polyphenolic compounds possess a wide range of pharmacological properties and their mechanisms have been the subject of considerable interest. The science of radiation protection is a fundamental result of peaceful and fully equipped applications of ionizing radiation. Several chemical agents/synthetic radioprotectors have been tried with success and satisfactory outcomes against the hazardous effects of ionizing radiation in experimental studies (Pallavi *et al.,* 2012). However, the practical applicability of the majority of these synthetic compounds remains limited, owing to their high toxicity at their optimum protective doses (Sweeney, 1979).

Natural medicinal drugs offer an alternative to synthetic compounds and are considered either nontoxic or less toxic than their synthetic counterparts. A large number of plants containing antioxidant phytochemicals are reported to be radioprotective in various model systems (Pallavi *et al.,* 2012). Most plants with antioxidant properties have shown anticarcinogenic activities in several *in vitro* and *in vivo* assays (Signorelli and Ghidoni, 2005; Shishodia *et al*., 2007; Epstein *et al*., 2010; Koide *et al*., 2011). They have also shown the capacity to modulate radiation-induced damage by means of various biological endpoints, by radiosensitizing tumor cells while radioprotecting non-cancerous cells (Sebastià *et al.,* 2014). This suggests that, besides the anticarcinogenic effect, another possible application of the antioxidant capacity of polyphenols from plants could be based on their radioprotective activity. *P.violacea* showed less potential to scavenge free radicals (see Chapter 5), thus it was excluded in the determination of radioprotective effect. The present study aimed at evaluating the radioprotective effect of methanolic extract of *X.zambesiaca* on radiation-induced human prostate cancer cell lines at a dose of 2 Gy.

8.2 LITERATURE REVIEW

8.2.1 Effects of ionizing radiation on the immune system of organisms

Radiation protection, also known as radiological protection, is the science and practise of protecting people and the environment from the harmful effects of ionizing radiation. It is commonly applied to cancerous tumors because of its ability to control cell growth. Ionizing radiation works by damaging the DNA of cancerous tissue, leading to cellular death. To spare normal tissues (such as skin or organs, which radiation must pass through to treat the tumor), shaped radiation beams are aimed from several angles of exposure to intersect at the tumor, providing a much larger absorbed dose at the target area than in the surrounding, healthy tissue. The amount of radiation used in photon radiation therapy is measured in gray (Gy), and varies depending on the type and stage of cancer being treated. A significant radiation dose-dependent effect was shown to be from 1 to 7.5 Gy (Manda *et al*., 2014) and in this study a moderate dose of 2 Gy gamma-radiation was used.

Any radiation whose interaction with the environment leads to the formation of the electric charges of opposite signs (positive and negative) is considered ionizing radiation. This includes various types of micro-particles and physical fields capable of ionizing any substance. In turn, the ultraviolet radiation and visible light range are not ionizing but in some cases they may be the same (Russian, 2009). The effects of any doses of ionizing radiation on any living organism result in changes in morphology and functional activity, both at the cellular and system levels, causing an increase or suppression of immune reactivity whose final manifestation is immunodeficiency. The consequence of immunodeficiency is auto-sensitization of organisms, the development of opportunistic infections caused by pathogenic micro-flora, oncological diseases and other complications (Kusunoki and Hayashi, 2008). A safe compound that would prevent the ablation of immune system function and other radiation-induced oxidative tissue damage would provide an effective medical countermeasure against nuclear or radiological attack.

8.2.2 Biological damage by radiation

Radiation therapy leads to the DNA damage of cancerous cells. DNA damage can be caused by one of two types of energy, photon or charged particle. The damage is either direct or indirect ionization of the atoms that make up the DNA chain. Indirect ionization occurs as a result of the ionization of water, forming free radicals, notably hydroxyl radicals, which then damage the DNA (Butler *et al., 1984)*. In photon therapy, most of the radiation effect is through free radicals (Hadi *et al.,* 2007). Cells have mechanisms for repairing single-strand DNA damage and double-stranded DNA damage. However, double-stranded DNA breaks are much more difficult to repair and can lead to dramatic chromosomal abnormalities and genetic deletions. Targeting double-stranded breaks upsurges the probability that cells will undergo cell death.

Cancer cells are generally less differentiated and more stem cell-like; they reproduce more than most healthy differentiated cells and have a diminished ability to repair sub-lethal damage. Single-strand DNA damage is then passed on through cell division; damage to the cancer cells' DNA accumulates, causing them to die or reproduce more slowly. Radiation is understood to effect cancer treatment by destroying the proliferative capacity of tumor cells and is effective in controlling local disease. Recent evidence suggests that the intrinsic radiosensitivities of cells derived from various cancers correlate with the amount of radiation dose required in controlling those cancers (Perron *et al.,* 2011). Several physical, chemical, biological and physiological factors that influence cellular radiation response have been well characterized and are important in understanding the radio-responsiveness of individual tumors. Recently, research into radioprotection from plants has escalated in the hope of developing improved cancer treatment that will eventually result in improved local control and additional cures of this disease.

8.2.3 Mechanisms of radiation protection

A variety of radioprotective mechanisms have been proposed to elucidate the prophylactic and therapeutic effects of a large number of agents (Maisin, 1998; Nair *et al*., 2001; Weiss and Landauer, 2003). Naturally occurring antioxidants are only one class of radioprotectors. Although a large number of potential radioprotectors,

especially those containing sulfhydryl groups, can be considered antioxidants, as they are clear protectors that work mainly through receptor-mediated mechanisms, e.g. bioactive lipids, cytokines and growth or through receptor-mediation, but produce similar results even though the pathways are different. Sulfhydryl compounds may protect cellular DNA by a combination of mechanisms, including free radical scavenging, hydrogen donation and modulation of repair processes (Murray, 1998), whereas a cytokine may induce cellular antioxidant activity, such as superoxide dismutase and metallothionein, via receptor-mediated mechanisms (Neta, 1997).

The radioprotectors can elicit their action by various mechanisms, such as: 1) by suppressing the formation of reactive species, 2) detoxification of radiation-induced species, 3) target stabilization and 4) enhancing the repair and recovery processes (Nair *et al.,* 2001). Pharmacological agents were found to be radioprotective by interfering with the delivery of oxygen into irradiated tissues. The chemical or biochemical consumption of oxygen can bring about hypoxia in cells and tissues. This may be one of the mechanisms by which sulphydryl compounds (RSH), which can undergo an oxidation reaction with molecular oxygen, manifest radioprotection (Halliwell and Gutteridge, 1984). Several free-radical scavengers are known to interact with aqueous free radicals and to prevent the radiation-induced lethality of cells. The radioprotectors may also react with water radicals or radicals of bio-molecules (X•) by donating hydrogen atoms to repair the radical species:

$$2\ X\bullet + 2\ RSH \Rightarrow 2\ XH + RSSR$$

$$2\ RSH + 2OH \Rightarrow RSSR + 2H2O$$

Radioprotectors can also interact with cellular targets, like DNA, by forming mixed disulfides and prevent radiation damage by stabilizing the target. Several amino thiol radioprotectors, such as cysteamine, guanidoethyldisulfide and glutathione disulfide, bind to DNA and their DNA binding parallels their radioprotective potency (Nair *et al.,* 2003). The radioprotective activity of a number of thiol compounds (RSH, R'SH *etc.*) has been correlated with the ratio and extent of mixed disulfide (RSSR') formation (Nair *et al.,* 2001). The regeneration of native proteins can be achieved by a thiol disulfide exchange with glutathione, possibly catalyzed by thiol transferase, and

subsequent action of glutathione redox system coupled with glutathione reductase and NADPH. However, the mixed disulfide hypothesis is limited to the protection of enzymes and proteins and fails to explain the radioprotection of nucleic acids, because the SH group is restricted to proteins only.

8.2.4 Radioprotection from plants

Phenolic compounds can reduce reactive oxygen species (ROS) levels in the cellular environment and play an important role in modulating oxidative stress (Lee & Park, 2003). These compounds are known as anti-carcinogenic food components owing to their antioxidant activity (Page *et al.,* 2011). The relationship between antioxidant structures and their activity has been investigated in many studies, and the structural arrangements exerting the greatest antioxidant activity of the compound have been identified. Among these structures, the most widely investigated group of compounds are flavonoids (Burda and Oleszek, 2001; Rice-Evans *et al.*, 1996). Most studies have been performed using aglycones and flavonoid glycosides, which occur naturally in plant tissues (Wolfe and Liu, 2008; Kweon *et al*., 2001). Plant species have been examined according to the content of bioactive phenolic compounds in relation to the antioxidant activity (Kweon *et al*., 2001; Ferreres *et al*., 2008) and correlation was observed. Results from these studies further confirmed the potential of these compounds as antioxidant agents.

Naturally occurring polyphenols comprise a wide variety of compounds divided into several classes that occur in fruit and vegetables, wine and tea, chocolate and other cocoa products (Manach *et al*., 2004). The beneficial effects of phytophenols are mainly attributed to their antioxidant properties, since they can act as chain breakers or radical scavengers, depending on their chemical structures (Rice-Evans, 2001). Gallic acid is a polyhydroxy-phenolic compound (Figure 8.1) and is nontoxic to mammals at pharmacological doses (Okuda *et al*., 1993; Hatano, 1995; Takana, 1999). Gallic acid is known to have anti-inflammatory, antioxidant, free radical scavenging, and radioprotective activity (Kroes *et al*., 1992; Gandhi and Nair, 2005; Lo Scalzo, 2010). It is employed as an antioxidant in food, in cosmetics and in the pharmaceutical industry (Kähkönen *et al.,* 1999)

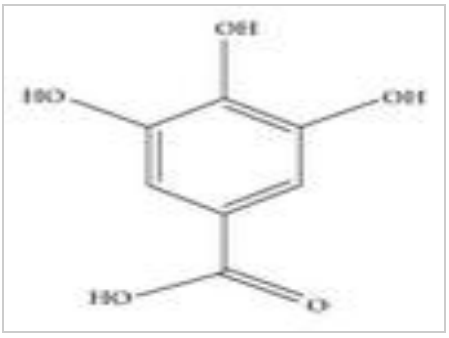

Figure 8.1: Gallic acid (GA) [3,4,5-trihydroxybenzoic acid] (Hatano, 1995).

On the other hand, some polyphenols from plants were demonstrated as radiosensitizers by means of their pro-oxidant activities or as inducers of chromosomal aberrations leading to cell death or apoptosis (Sandeep and Nair, 2011; Ramachandran and Nair, 2011; Manach *et al*., 2004; Rice-Evans, 2001). Since ionizing radiation and chromosomal aberrations have been associated with cancer, such chemically induced chromosomal radiosensitization may escalate the risk of carcinogenesis (Hatano, 1995). Cell cycle interference related to repair processes of radiation-induced damage and the activation of checkpoints is, therefore, avoided. The effect of these polyphenols on the induction and repair of radiation-induced chromosomal aberrations in cycling cells has also been investigated in other studies.

Protection of biological systems from ionizing radiation is of supreme significance in planned as well as unplanned, accidental exposure to radiation (Arora *et* al., 2005; Jagetia 2007), and the development of new and effective agents to combat radiation damage using nontoxic radioprotectors is of considerable interest in health care, particularly in radiodiagnostics and therapy. Many synthetic as well as natural compounds have been investigated in the recent past for their efficacy to protect biological systems against the harmful effects of radiation. These include sulfhydryl compounds, antioxidants, plant extracts, immunomodulators and other agents (Maisin, 1998; Nair *et al*., 2001). However, the inherent toxicity and side effects of the synthetic agents at the effective radioprotective concentration warrants a further search for safer and more effective radioprotectors. Extracts of various plants with free radical mediated conditions in humans can be beneficial, as they contain compounds having antioxidant activity, which can prevent damage induced by ROS

and reactive nitrogen species (Arora *et al*., 2006). Therefore, in the present study, the radioprotective effect of *X.zambesiaca* was investigated.

8.3 METHODOLOGY

8.3.1 Plant material

The plant material *X.zambesiaca* was authenticated by scientists at the National Botanical Gardens in Bloemfontein, South Africa. The collected material was dried at room temperature, pulverized by mechanical mills and weighed. It was then stored in a cool place until analysis.

8.3.2 Extraction method

The extraction was done using the maceration. Plant material (10 g of the dried twigs, leaves and flowers of each plant) was weighed, pulverized and soaked in methanol for 72 hours with occasional stirring. The extracts were filtered and new solvent was added again for more extraction until the solvent remained clear. The methanol solvent was removed completely using a rotator evaporator.

8.3.3 Cell culture

Human prostate cancer cells (DU145) were obtained from Professor P Bouic (Synexa Life Sciences, Montague Gardens, South Africa), and grown in Minimum Essential Medium (Sigma-Aldrich, Germany) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, UK), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Lonza, Belgium). Cultures in exponential growth were trypsinised to give single-cell suspensions, plated in triplicate (400–1500 cells per flask) into 25 cm^2 tissue culture flasks (Greiner Bio-One, Frickenhausen, Germany) and incubated for 3 hours to allow the cells to attach.

8.3.4 Clonogenic assay

To confirm the free radical scavenging features of extract X.zambesiaca, cell cultures were irradiated to 2 Gy in the presence of the extract at a concentration of 0.1 mg/ml, and were subsequently incubated at 37°C in a humidified atmosphere (95% air and 5% CO_2) for 10 days to allow for colony formation. The cells were

irradiated using a ^{60}Co γ-irradiation source at a dose rate of 0.827 Gy/min. Colonies were fixed in glacial acetic acid:methanol:water (1:1:8, by volume), stained with 0.01% amido black in fixative, counted, and the cell surviving fraction [SF2 (with X.zambesiaca extract)] was determined. Another batch of cell cultures was irradiated to 2 Gy in the absence of extract and the corresponding surviving fraction [SF2 (no *X.zambesiaca* extract)] was obtained. To evaluate the radioprotective capacity of the extract, an RPF was derived as:

$$\text{RPF} = \frac{SF_2 \text{ (with } X.zambesiaca\text{)}}{SF_2 \text{ (without } X.zambesiaca\text{)}}$$

8.4 RESULTS

Based on the clonogenic assay, the cell surviving fractions for DU145 at 2 Gy with and without *X.zambesiaca* were found to be 0.23 ± 0.13 and 0.15 ± 0.10, respectively. The corresponding RPF value emerged as 1.53 ± 0.57, indicating that an extract of *X.zambesiaca* can lead to over 50% reduction in cell death. This confirms the free radical scavenging features of the extract and suggests that it might be useful as a radioprotector.

8.5 DISCUSSION

Traditionally, radioprotectors are defined as agents that are administered before radiation exposure, whereas therapeutic agents are administered after exposure. Many naturally occurring antioxidants exhibit a long window of protection, including post-irradiation protection against lethality and mutagenesis (Weiss and Landauer, 2003). In Chapter 5 we illustrated the ability of *X.zambesiaca* as a free radical scavenging agent by determining its antioxidant activity. To prove the radioprotective capacity of these antioxidants further, we applied the extract to radiated cancer cells. *X.zambesiaca* plant extract studied in this project showed the capability of offering 50% protective effect but was less toxic (Chapter 3 of this dissertation) than synthetic radioprotectors, such as phosphorothioates. The potential application of this plant as a protective agent shows promise - either prophylactic benefits for anticipated

exposure in emergency situations or therapeutic benefits after radiation accidents/incidents.

Nuclear and radiological accidents can result in moderate to severe radiation injuries and many casualties. The harmful impact of γ-radiation on biological systems is well researched and documented. Aglycones, which are compounds remaining after the glycosyl group on a glycoside is replaced by a hydrogen atom, were proven to have high radioprotective capacity. The observed radioprotective effect of this compound was explained by Konopacka *et al*. (1998) to result from its antioxidant properties. In Chapter 7 of this dissertation, aglycones were found in abundance in *X.zambesiaca,* therefore its radioprotective activity compared relatively well to aglycones' radioprotective capacity. The results obtained suggested that the free radical scavenging activity agents present in this plant extract may be used as compounds with promising radioprotective potential.

We have previously reported the free radical scavenging activity of *X.zambesiaca*. In continuation, the present *in vitro* studies were undertaken to elucidate its precise role in the modulation of radiation-induced damage in human prostate cancer cell line. DU145 cells were treated with radiation using a ^{60}Co γ-irradiation source at a dose rate of 0.827 Gy/min in order to determine radiation-induced damage. Radiation-induced DNA clustered lesions and apoptosis are the major likely mechanisms implicated in clonogenic cell death. Radiation exposure (2 Gy) triggered a loss of clonogenicity in DU145 cells. Cell growth is hindered by alteration of membrane permeability, damage to DNA and proteins responsible for cell cycle progression (Maddika *et al*., 2007). Cell count studies and cell surviving fraction values were determined, and the RPF value obtained indicated that *X.zambesiaca* plant extract can lead to over 50% reduction in cell death.

These results could be influenced by the increase in cell number and competition among growing cells for the available, but limited supply of nutrients and growth factors. Such argumentation in cell growth or proliferation rate against radiation damage could be the result of several factors, such as inhibition of free radical generation, repair of damaged DNA, protection of cell membrane and an increase in

the producing ability of cells. Furthermore, the predominant mechanism of radiation injury, both in tumors and in normal tissues, is by induction of apoptosis or clonogenic cell death by free radical-mediated DNA damage (Karran, 2000). Thus, screening potent free radical scavengers as radioprotectors is one of the important strategies of drug development.

8.6 CONCLUSION

Based on the clonogenic assay, the radioprotective factor value emerged as 1.53 ± 0.57, indicating that an extract of *X.zambesiaca* can lead to over 50% reduction in cell death. This confirms the free radical scavenging features of this plant extract and suggests that it might be useful as a radioprotector.

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CHAPTER 9

"General conclusion"

ABSTRACT

The objectives of the study were:

1. To determine the anticancer activity of *P.violacea* and *X.zambesiaca* against human cell lines.
2. To determine the mutagenic and anti-mutagenic properties of *P.violacea* and *X.zambesiaca* extracts.
3. To determine the antibacterial activity of *P.violacea* and *X.zambesiaca* extracts.
4. To determine the antioxidant activity of *P.violacea* and *X.zambesiaca* extracts.
5. To determine the active phytochemicals of *P.violacea* and *X.zambesiaca* extracts.
6. To isolate and identify the bioactive compound from *P.violacea* and *X.zambesiaca* extracts.

The following appropriate conclusions were drawn on the basis of experimental data: the null hypothesis was accepted for objectives number 2: *P.violacea* and *X.zambesiaca* extracts have anti-mutagenic properties, 4: *X.zambesiaca* plant extract does not have antibacterial activity, 5: *P.violacea* and *X.zambesiaca* plant extracts have phytochemicals, and 6: *P.violacea* and *X.zambesiaca* plant does not have bioactive compound. The null hypothesis was rejected for objectives number 1: *P.violacea* and *X.zambesiaca* plant extracts do not have anticancer activity, 3: *P.violacea* and *X.zambesiaca* plant extracts have antibacterial activity and 4: *P.violacea* plant extract does not have antioxidant activity.

9.1 INTRODUCTION

Cancer is one of the most prominent diseases in humans. Plants remain a prime source of drugs for the treatment of cancer and can provide leads for the development of novel anticancer agents. Bacterial infections and autoimmune response leading to chronic inflammation have been associated with carcinogenesis (De Nunzio *et al*., 2011). One of the most important contributions to the development of cancer is oxidative damage to DNA (Fang *et al*., 2012). Permanent genetic alterations may occur in cells where DNA is damaged and where division of this DNA occurs before it can be repaired. These cells may begin to divide more rapidly and result in carcinogenesis (Reddy *et al*., 2003). The radical scavenging properties of antioxidants are known to eliminate and prevent the generation of free radicals. These properties have been contributing directly or indirectly to the prevention of pathogenesis and carcinogenesis (Ferrari and Torres, 2003), whereas the ability of a plant extract to kill or inhibit the growth of microorganisms is of interest for the development of antimicrobial agents. A deleterious effect of radiation is the production of reactive oxygen species, which include superoxide anion (O_2^-, a free radical), hydroxyl radical (•OH), and hydrogen peroxide (H_2O_2) (Fang, 1991). These reactive species may contribute to radiation-induced cytotoxicity.

The pace of research in the continuing discovery of new anticancer agents from natural product sources has been staggering lately (Kinghorn *et al.,* 2003). Along with determination of the pharmacological properties of plant extracts, studies on the toxicity properties of plant extracts are indispensable to assure the safety of the extract (Deciga-Campos *et al.,* 2007). In frequently reported studies, polyphenols have been suggested as responsible for potent biological activities of extracts prepared using methanol and ethanol (Michielin *et al*., 2009). The quantification of phenolic content by folin ciocalteu reagent and characterization of the extract by LC-MS were carried out in this study. This study was intended to demonstrate the pharmacological activity of *P.violacea* and *X.zambesiaca* against cancer-inducing factors in order to determine the ability of these plant extracts as possible novel anticancer drugs.

9.2 SUMMARY OF RESULTS

Analysis	*P.violacea*	*X.zambesiaca*
Anticancer activity	Negative results	Negative results
Antimutagenicity activity	Not mutagenic	Not mutagenic
Antibacterial activity	Negative results	Negative results
Antioxidant activity	Low	50% scavenging acitivity
Phytochemical screening	Tannins, flavonoids, steroids, terpenoids, alkaloids and cardiac glycoside	Flavonoids, saponins, terpenoids and cardiac glycoside
Fingerprinting	9 compounds identified	7 compounds identified
Radioprotection effect	----	It is a radioprotector

Table 9.1: Summary of all experimental results obtained from this research project.

9.3 HOLISTIC DISCUSSION

P.violacea and *X.zambesiaca* extracts have been used as traditional medicine for a variety of diseases and their safety needs to be determined. For mutagenicity testing, the bacterial strains used were the histidine-requiring *Salmonella typhimurium* tester strains and TA100 without metabolic activation. Strain TA98 gives an indication of frame-shift mutations, while strain TA100 indicates base-pair substitution. According to our results, neither *P.violacea* nor *X.zambesiaca* was mutagenic in the salmonella/ microsome tester strain TA 100 and TA98. Therefore at this moment we can say both plants do not induce mutation, but this test must also be performed with metabolic activation to declare these extracts 100% safe and not toxic.

It was demonstrated that *in vitro* antimicrobial activity is dependent upon various intrinsic and extrinsic factors, such as molecular weight, degree of deacetylation, viscosity, solvent, pH, test strains, temperature (Raafat & Sahl, 2009). Other

antimicrobial activity analysis might produce different results because the polarity of the natural compounds can affect the diffusion of compounds onto the culture medium. Compounds with less polarity diffused more slowly than more polar ones (Moreno *et al.*, 2006). The advantage of broth microdilution when compared with the agar-based method is that broth microdilution can decrease much labor and time. However, limitations of the broth microdilution method are primarily associated with the lack of or poor growth of many anaerobic microorganisms. Testing some fastidious anaerobes gives inconsistent and unreliable results because of poor growth of strains due to excessive exposure to oxygen during the set-up procedure (CLSI, 2009).

The ability of *P.violacea* and *X.zambesiaca* extracts to inhibit the growth of selected bacterial strains was evaluated by using the broth microdilution method. In the broth microdilution method, microbial growth was indicated by the presence of reddish-pink color after the addition of tetrazolium salt. The lowest concentration of extract inhibiting growth of test inhibition concentration (MIC) was determined. The MIC value indicates the antimicrobial property of an extract. Extract with MIC < 1 mg/ml is regarded as exhibiting activity (Seyoum *et al.,* 2006). It was found that methanolic extracts of both *P.violacea* and *X.zambesiaca* are weak inhibitors of the selected bacterial strains, as all micro-organisms managed to grow in the presence of these extracts at a concentration above 1.0 mg/ml. Thus the extracts were regarded as not active against those bacteria. It is known that in most instances, gram positive bacteria are more susceptible to plant extracts than gram negative ones; however, this was not the case, as both gram positive and gram negative bacteria were resistant.

Antioxidants are the compounds that terminate the attack of reactive species such as free radicals and prevent ageing and different diseases associated with oxidative damage inside the body system (Rice-Evans *et al.,* 1996). These free radicals can oxidize biomolecules, i.e. nucleic acids, proteins, lipids and DNA, cause tissue damage and initiate degenerative diseases. Oxidative damage plays a significantly pathological role in human diseases such as cancer, emphysema, cirrhosis, atherosclerosis and arthritis etc. (Niki *et al.,* 1994). Almost all organisms are protected to some extent from free radical (peroxide, hydro-peroxide or lipid peroxyl)

damage. Antioxidant supplements or dietary antioxidants may be sources of protection that the body needs to protect it against the damaging effects of free radicals. Advantages of the DPPH assay are that the test is simple and rapid and needs only a spectrophotometer and a dark room to perform, which probably explains its widespread use in antioxidant screening. DPPH is a stable nitrogen radical that bears no similarity to the highly reactive and transient peroxyl radicals involved in lipid peroxidation. Many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to DPPH owing to steric inaccessibility. DPPH is decolorized by reducing agents as well as H transfer, which also contributes to inaccurate interpretations of antioxidant capacity.

Various researchers have used the scavenging effect of a chemical on DPPH radical as a quick and reliable parameter to assess the *in vitro* antioxidant activity. From the reaction of the extracts with DPPH radical, it was observed that the scavenging activity of both extracts was concentration-dependent (Chapter 5). The concentration of *X.zambesiaca* required to scavenge 50% of DPPH radical present in the reaction mixture (SC_{50}) was higher compared to *P.violacea,* but lower when compared to ascorbic acid. Methanolic extract of *P.violacea* scavenges 50% of DPPH radical at a concentration of greater than 2.5 mg/ml, while methanolic extract of *X.zambesiaca* scavenges 50% of DPPH radical at a concentration of 2.5 mg/ml. In comparison with ascorbic acid, which showed high activity with SC_{50} (68.5%) from a concentration of 0.313 mg/ml, both extracts' activity was found to be low.

Studies have shown that dietary phytochemical antioxidants are capable of removing free radicals. Among them, phenolic and polyphenolic compounds, such as flavonoids and catechin in edible plants, exhibit potent antioxidant activities (Decker, 1995; Fang 2002). The phytochemical screening results in Chapter 6 revealed the presence of tannins, flavonoids, steroids, terpenoids, alkaloids and cardiac glycosides from *P.violacea* (leaves, flowers and twigs) extract, while *X.zambesiaca* (leaves, flowers and twigs) extract showed the presence of flavonoids, saponins, terpenoids and glycosides. Recently, intensive research has been focused on developing tumor therapies from saponins. *X.zambesiaca* extract contained saponins and glycosides. Saponins exhibit potent anticancer activity in several human cancer cells through apoptosis-inducing pathways (Kaskiw *et al.,* 2009) and

glycosides are compounds that strongly influence the anticancer activity of the plant extracts (Yan *et al.,* 2009). However, even though *X.zambesiaca* contained saponins, it was unsuccessful at abolishing selected cancer cells. These results did not make sense, unless there was a compound present in the extract that suppressed the activity of saponins against cancer cell lines. Further investigations are required in future to elucidate this occurrence.

The objective of the identification of phenolic compound present in these plant extracts was to have an idea of what they comprise so that one can fractionate and obtain pure compounds in amounts sufficient for further analysis in future studies. LC-MS results confirmed the total phenolic content of both these plants, as we identified a correlation between the estimated total phenolic content and a number of compounds identified. *X.zambesiaca* had seven active compounds identified using LC-MS and less estimated total phenolic content when compared to *P.violacea,* which had a high estimated total phemolic content and nine active compounds identified. The presence of flavonoids detected in *P.violacea* was expected, because this class of secondary metabolite is a conspicuous constituent of species belonging to the Fabaceae family (Hertog *et al*., 1993; Yen *et al*. 1996), which proved that methanol is the most suitable solvent for extraction of phenolic compounds.

Antioxidant polyphenols have radiomodulatory properties that are radioprotecting in respect of non-cancerous cells while radiosensitizing tumor cells. This dual action may be the result of their radical scavenging properties and their effects on cell-cycle checkpoints that are activated in response to radiation-induced chromosomal damage. It could also be caused by their effect on regulatory pathways, affecting detoxification enzymes, the up-regulation of endogenous protective systems and cell-cycle-dependent processes of DNA damage. This part of the work was aimed at elucidating the free radical scavenging features of *X.zambesiaca* as a radioprotector. *P.violacea* was not assessed for its radioprotective effect, as it had low free radical scavenging potential. Based on the clonogenic assay, the cell surviving fractions for DU145 at 2 Gy with and without *X.zambesiaca* were found to be 0.23 ± 0.13 and 0.15 ± 0.10, respectively. The corresponding RPF value emerged as 1.53 ± 0.57, indicating that *X.zambesiaca* extract can lead to over 50% reduction in cell death.

This confirmed the free radical scavenging features of the extract and suggested that it might be useful as a radioprotector.

9.4 HOLISTIC CONCLUSION

Both *P.violacea* and *X.zambesiaca* can be considered safe to be used in view of the evidence obtained through antimutagenic activity with no metabolic activation analysis. The methanolic leaf, twig and flower mixture of these plants extracts was for the first time reported to contain tannins, flavonoids, steroids, terpenoids, alkaloids and cardiac glycoside. Nevertheless, both extracts did not have *in vitro* anticancer activity against breast (MCF7), colon (HCT116), renal (UACC-62), melanoma (TK-10) and prostate (PC3) cancer cell lines and also did not have the potential to inhibit the growth of *Staphylococcus aureus*, *S. epidermidis*, *S. saprohyticus*, *Bacillus subtilis*, *Enterobacter cloacae*, *Escherichia coli* and *Pseudomonas aeruginosa* (clinical strains). *X.zambesiaca* showed moderate antioxidant activity and over 50% reduction in cell death of prostate cancer cells when exposed to radiation. These results confirmed its ability to scavenge free radicals and the radioprotective effect of this plant extract. It was also shown that *P.violacea* has low antioxidant potential (ability to scavenge free radicals), thus, the radioprotective effect of this plant extract was not explored, as there was no need to do this. A number of phytochemicals and the estimated total phenolic content were higher than in *X.zambesiaca*, with nine compounds identified, of which two are unknown. Seven compounds were identified in *X.zambesiaca* and this correlated with the estimated total phenolic content.

9.5 LIMITATIONS OF THE STUDY

- It was not possible to do this study using human subjects, as the test plants have not been used in animal models and clinical trials yet.
- It was not possible to test the plant extracts against a larger number of tumor cell lines and bacterial strains at this stage owing to limited resources and time.

- It was not possible to analyze the activity of each and every part of the plant, as the extract was already mixed as per ethnobotanical instructions.
- It was financially challenging to obtain plant extracts from different geographical areas, as this requires transport and the correct timing of these plants' blooming period.

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CHAPTER 10

"Recommendations for further research"

This study was intended to contribute to the knowledge base of the anticancer therapeutic potential of *P.violacea and X.zambesiaca*. It should be noted that plant material used in this study was received from the University of the Free State and their preparation (mixture of twigs, flowers and leaves) was based on ethnobotanical claims made by cancer-treating traditional healers they consulted. Our chosen plants were randomly selected from 60 plants belonging to the Fabaceae family. Since other compounds are extracted better with different solvents because of the differences in polarity, extraction using a variety of solvents could have demonstrated a vast difference as far as the experimental results are concerned. Furthermore, to account for possible geographical and chemotypic variations, plant material should be studied from several populations in the future.

I. Anticancer

- This study has shown that *P.violacea and X.zambesiaca* do not have potential anticancer activity *in vitro* against breast (MCF7), colon (HCT116), renal (UACC-62), melanoma (TK-10) and prostate (PC3) cancer cell lines using the Sulforhodamine B (SRB) assay. Previous studies have shown the presence of isoflavones from *X.zambesiaca* and we have also identified the existence of this compound.

- Firstly, we will recommend the analysis of anticancer activity of these extracts on other cancer cell lines, such as the ovarian cancer cell line.

- Secondly, activity-guided fractionation, isolation and identification of identified compounds are imperative, as these may lead to the development of novel treatments in the global struggle against cancer and cancer-related ailments.

II. Mutagenicity

- The crude *P.violacea and X.zambesiaca* extracts were observed to be non-toxic. However, the risk assessment of these extracts will not be complete unless they are tested for promutagenic and comutagenic effects.

- It is important to note that in order to establish a toxicity profile, other cell lines should be investigated, such as liver HepG2 cells (for metabolic activation), as well as *in vivo* studies.

III. Antimicrobial activity

- In this study, it was apparent that *P.violacea and X.zambesiaca* did not have the potential to inhibit the growth of *Staphylococcus aureus*, *S. epidermidis*, *S. saprohyticus*, *Bacillus subtilis*, *Enterobacter cloacae*, *Escherichia coli* and *Pseudomonas aeruginosa* through a variety of concentrations when using broth microdilution assay.

- The concentration of compounds within each of the species must be determined, as well as whether synergistic, antagonistic or additive effects occur.

- Other types of antimicrobial activity assay can be undertaken, such as disk diffusion, as some limitations of the broth microdilution method are primarily associated with the lack of or poor growth of many anaerobic microorganisms. Some micro-organisms used were facultative anaerobes.

IV. Anti-oxidant activity

- No single testing method is capable of providing a comprehensive profile of the antioxidant capacity of a plant species, because of the complexity of oxidation-anti-oxidation processes, and therefore different methods are to be used to determine the anti-oxidant potential.

V. Phytochemical investigation

- It is recommended that a thorough geographical variation study be performed on each species to explain the diversity between individuals within a population and between populations.

- Quantitative results obtained about the presence of compounds from these plants warrant further study, such as purification and then LC-MS, as the LC-MS on crude extract gave a tentative identification of molecules only.

- After purification, further analysis must be conducted using different detectors and chromatographic techniques in order to obtain the quantity of the compounds.

VI. Isolation and identification of compounds

- Isolation and structural elucidation of the compound/s responsible for the antioxidant activity of *X.zambesiaca* should be thoroughly investigated.

- The presence of these active compounds can be determined by isolating and repeating the compounds' identification process, but now with the pure and targeted compounds, with the guidance of compounds identified under phytochemical screening analysis.

VII. Radioprotection

- *X.zambesiaca* was found to protect prostate cancer cell lines against radiation, thereby contributing to the overall radioprotective activity. However, isolation of the active principle(s) responsible for the observed radioprotection, correlation of pharmacokinetics with dynamics and the evaluation of mechanisms of radioprotection will need to be pursued further.

- The antioxidant activity of the investigated compounds in this study must be compared with their radioprotective properties to demonstrate a correlation between the radioprotective activity of the phenolic glycosides and their antiradical activity, using a non-enzymatic method.