Seasonal Pharmacological and Phytochemical Properties of Medicinal Bulbs

By

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Submitted in fulfilment of the academic requirements for the degree of Master of Science

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Seasonal Pharmacological and Phytochemical Properties of Medicinal Bulbs

I, Bhekumthetho Ncube, student number: 209522727 declare that:

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We hereby declare that we acted as Supervisors for this MSc student:

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Regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our ability and approved the final document for submission to the Faculty of Science and Agriculture Higher Degrees Office for examination by the University appointed Examiners.

SUPERVISOR: PROFESSOR J VAN STADEN

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Publication 1:

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ABSTRACT

Medicinal bulbs form part of the diversified flora in South Africa. The plants are used extensively in South African traditional medicine in the treatment of various ailments. Due to the ever-increasing demand and the unrestricted collection of medicinal plants from the wild, many of these slow growing bulbous plant species are driven into over-exploitation and extinction. The main parts collected for use are the underground bulbs, leading to the destructive harvesting of the whole plant. This form of plant harvesting poses threats to the long term sustainability of these plant resources from their natural habitats. Sustainable harvesting of these plants should be within the limits of their capacity for self-renewal. However, this seldom occurs with the often inconsiderate medicinal plant gatherers. Conservation of these plants is therefore necessary. A strategy that would take into consideration the sustainable harvesting and perhaps simultaneously provide similar medicinal benefits, would be the substitution of bulbs with leaves of the same plant. This study was aimed at evaluating the seasonal pharmacological and phytochemical properties in bulbs/corms and leaves of medicinal bulbs with a view of promoting the substitution of bulbs with leaves in traditional medicinal use.

Four medicinal bulbous plants, *Tulbaghia violacea*, *Hypoxis hemerocallidea*, *Drimia robusta* and *Merwilla plumbea* were evaluated for the pharmacological and phytochemical properties in their bulbs/corms and leaves in spring, summer, autumn and winter seasons, with a view of promoting the use of leaves as a conservation strategy. Dried plant materials were sequentially extracted with petroleum ether (PE), dichloromethane (DCM), 80% ethanol (EtOH) and water in each season. The extracts were tested for activities against Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*), Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) bacteria and the fungus *Candida albicans* using the *in vitro* microdilution assays to obtain minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC). The four plant species were also evaluated for their ability to inhibit cyclooxygenase (COX-1 and COX-2) enzymes. Spectrophotometric methods were used to evaluate saponin and phenolic contents of samples from the four plant species in each season.

Antibacterial activity was fairly comparable between bulbs/corms and leaves of *H. hemerocallidea, T. violacea,* and *M. plumbea*, with at least one extract showing some good activity (MIC < 1 mg/ml) in most of the seasons. Bulb extracts of *D. robusta* did not show good antibacterial activity while the leaf extracts showed good activity (0.78 mg/ml) against *B. subtilis* in spring, summer, and autumn and *S. aureus* (0.78 mg/ml) in autumn. The best antibacterial activity was recorded in winter, with MIC values as low as 0.195 mg/ml from the DCM bulb extracts of *T. violacea* against *K. pneumoniae* and *S. aureus* and PE corm extracts of *H. hemerocallidea* (0.195 mg/ml) against *B. subtilis*. Good antibacterial activity from water extracts were only recorded from corm extracts of *H. hemerocallidea* in summer, autumn and winter, *H. hemerocallidea* leaf extracts in autumn and winter, and *M. plumbea* bulb extracts in autumn. The leaf extracts of all the screened plant species demonstrated good fungicidal activity in autumn, with *H. hemerocallidea* corm water extracts recording an MFC value as low as 0.39 mg/ml. The leaf extracts of *H. hemerocallidea* (water), *D. robusta* (DCM) and *M. plumbea* (DCM) had good MFC values of 0.78 mg/ml each, in spring. The DCM leaf extracts of *T. violacea* also showed good fungicidal activity (0.78 mg/ml) in summer, while corm water extracts of *H. hemerocallidea* had an MFC value of 0.39 mg/ml in winter. There were no fungicidal activities recorded from all the bulb extracts in all the seasons.

All the PE and DCM extracts in all the tested plant samples recorded between moderate (40-70%) and high (> 70%) COX-1 and COX-2 inhibition levels across all seasons. The EtOH corm extracts of *H. hemerocallidea* also demonstrated moderate to high inhibitory activity against COX-1 enzyme across all seasons. Bulb and leaf extracts of *T. violacea* showed selective inhibitory activity for COX-2 enzyme in all the seasons. The highest COX inhibitory levels were recorded in COX-2 from the PE leaf (spring) and bulb (autumn) extracts of *T. violacea*, with both recording 100% inhibitory activity.

Phytochemical analysis revealed higher total phenolic compounds in bulbs/corms and leaves of all the analysed plant species, to be either higher in spring or winter. Plant material collected in autumn had the least levels of total phenolics. An almost similar trend to that of total phenolics was observed for flavonoids, gallotannins and condensed tannins in most plant samples, with higher levels either in spring or

winter. Total saponins were consistently higher in winter than in the other seasons in all the screened plant species. There were in some cases, relationships between the peaks in the levels of some phytochemical compounds and the observed levels of bioactivity in different assays.

The results obtained from this study demonstrate that the leaves of the screened plant species may substitute or complement bulbs in the treatment of certain ailments in traditional medicine. Thus, plant part substitution can be sustainably utilised in the conservation of these plant species while retaining the same medicinal benefits.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ABC	ATP Binding Cassette
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
ATM	African Traditional Medicine
ATP	Adenosine Triphosphate
CBN	Carbon/Nitrogen Balance
COX	Cyclooxygenase
CTE	Catechin equivalents
CTM	Chinese Traditional Medicine
DCM	Dichloromethane
DPM	Disintegrations per minute
EtOH	80% Ethanol
GAE	Gallic Acid Equivalents
HIV	Human immunodeficiency virus
INT	*p*-Iodonitrotetrazolium chloride
LCE	Leucocyanidin equivalents
LPS	Lipopolysaccharides
LSD	Least Significant Difference
MATE	Multidrug and toxin extrusion
MDR	Multidrug resistance
MH	Mueller-Hinton
MFC	Minimum Fungicidal Concentration
MIC	Minimum inhibitory concentration
NRF	National Research Foundation
NSAIDs	Nonsteroidal anti-inflammatory drugs
NU	Bews Herbarium
PAL	Phenylalanine ammonia-lyase
PCM	Protein Competition Model
PE	Petroleum ether
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGI_2	Prostaglandin I_2
PAL_2	Phospholipase A_2
PGH_2	Prostaglandin H_2
RCPGD	Research Centre for Plant Growth and Development
ROS	Reactive oxygen species
SAAB	South African Association of Botanists
WHO	World Health Organisation
YM	Yeast malt

CHAPTER 1

Introduction and Literature Review

1.1. Introduction

Medicinal plants are used by billions of people in most developing countries as a source of medicine. This is because of the frequently inadequate provision of modern medicine, their low costs, effectiveness, as well as cultural beliefs and preferences (**SHELDON *et al*., 1997; SHANLEY and LUZ, 2003**). The scale and intensity of human interactions with plants have led to the progressive and wide-scale habitat loss, degradation and fragmentation, with subsequent loss of plant species. The ever increasing human population places a heavy demand on land for agriculture and urbanisation and hence continues to reduce the natural habitats in which these plants survive. As a result, most of these plants are at the risk of over-exploitation and extinction, and their conservation needs renewed attention. Africa and in particular South Africa is endowed with a much diversified flora and an abundance of bulbous medicinal plants are part of this rich biodiversity.

1.2. African traditional medicine

Traditional Medicine (TM) has been defined by **WHO** (**2001**), as the sum total of all the knowledge and practical experience used in the diagnosis, prevention and elimination of physical, mental or social imbalances. In contrast with western medicine, which is technically and analytically based, African Traditional Medicine (ATM) takes a holistic approach. Good health, disease, success or misfortune are not seen as chance occurrences but are believed to arise from the actions of individuals and ancestral spirits according to the balance or imbalance between the individual and the social environment (**STAUGARD, 1985; NGUBANE, 1987**). It is through this complex perception that the treatment of various human ailments follows rather diverse forms that sometimes vary between cultures and regions. The philosophy is however the same in most of the southern African communities.

Most African countries continue to rely heavily on the use of traditional medicines as their primary source of health care (**CUNNINGHAM, 1993**). **WHO** (**2008**) estimates

this to 70% of the total African population. ATM is a well-established system of medicine, parallel to the western or orthodox medicinal system, actively utilised by both urban and rural communities (**TABUTI *et al*., 2003**) and is used in the treatment of an extensive range of ailments. ATM is relatively accessible, more affordable; more closely corresponds to the patient's ideology, and is believed to be effective (**SHELDON *et al*., 1997; WHO, 2001**)

ATM employs a holistic approach that integrates spiritual, plant and animal components. Although animals and plants form the integral components of the holistic approach to the treatments of a diverse of ailments in ATM (**CUNNINGHAM, 1993; BODEKER, 2004**), plants are regarded as the root of this medical practice (**GURIB-FAKIM, 2006**). The treatment or prescription may at times call for animal sacrifice but will almost always call for the use of herbal medicines (**DU TOIT, 1980**). In many African cultures, traditional healers form important links in the chain of health personnel by providing health care and prescribing medicinal plants as remedies. Traditional healers were also exclusively responsible for medicinal plant collection in the past (**NGUBANE, 1987**).

Because of the extensive use and over-reliance on ATM, medicinal plants provide a significant source of income for rural people through the commercial trade of wild-harvested material. This often indiscriminate and unsustainable harvesting from the wild has led to the progressive depletion of natural environments and in more severe cases has led to species extinction due to over-exploitation (**MANDER, 1997; WILLIAMS *et al*., 2000**). This has resulted in the loss of biodiversity. This loss of biodiversity not only leads to diminishing biological resources, but also a shift in cultural practices (**WIERSUM *et al*., 2006**). The potential loss of cultural value which has a strong link to medicinal plant biodiversity supports the initiative for conservation of medicinal plant biodiversity in Africa (**ANYINAM, 1995; WIERSUM *et al*., 2006**)

Despite its long history, ATM is not acceptable in many countries due to the lack of proper documentation of indigenous knowledge and techniques. The profound knowledge of herbal remedies in traditional cultures developed through trial and error over many centuries, has been carefully passed on verbally from one generation to

another (**GURIB-FAKIM, 2006**). This largely oral rather than written system of knowledge, accentuates the fragility of this type of indigenous knowledge. In an attempt to bridge this knowledge gap, WHO has, over the past two decades, supported the use of traditional medicine, especially in developing countries by promoting the incorporation of its useful elements into national health care systems (**WHO, 2001**).

1.3. Traditional medicine in South Africa

The demand for traditional medicine and services in South Africa is considerable relative to the western health care services. **MANDER** (**1998**) estimates that 27 million of the country's population depends on traditional medicine for their primary health care needs. The heavy reliance of such a significant portion of the population on traditional medicine is due largely to the remarkable plant and cultural diversity in South Africa. The low socio-economic standing of the large, predominantly rural population makes for the great majority to use traditional methods of health care. With a medical doctor to patients ratio in South Africa of 1:17 400 (**PRETORIUS *et al*., 1993**), there is no doubt that ATM plays an important role in the nation's health care system. Furthermore, the use of traditional medicine is not confined to rural, low income groups but is also used in urban areas and is regarded as a basic requirement for treating certain conditions irrespective of education and income levels (**MARSHALL, 1998; COCKS and DOLD, 2000**).

The great range of climatic zones presents South Africa with a rich and diversified flora consisting of an estimated 24 000 taxa **(LOW and REBELO, 1996)**. It is estimated that approximately 3 000 plant species are used for medicinal purposes by an estimated 200 000 indigenous traditional South African healers (**VAN WYK *et al*., 1997)**.

The high demand on ATM has been attributed to the increase in HIV/AIDS epidemic and other common infectious diseases in South Africa **(MANDER, 1997)**. Together, these factors have resulted in the commercial exploitation of economically valuable plants by commercial gatherers to obtain an income (**CUNNINGHAM, 1988**).

In recognition of the value of traditional medicine to the general population, **BODEKER (2004)** suggested the need for national governments to invest more research on traditional medicinal plants and the development of good policies, regulations and trade standards. In this regard, the South African government through the National Research Foundation (NRF) has promoted a greater interest into the research of the country's natural resources (**LIGHT *et al*., 2005**). As a result, research in ethnobotany and ethnopharmacology has increased. Several outstanding books have been published on South African medicinal plants (**WATT and BREYER-BRANDWIJK, 1962; HUTCHINGS *et al*., 1996; VAN WYK *et al*., 2009**). There has also been a significant increase in the number of publications on the healing and pharmaceutical values of these plants.

1.4. Plants as sources of drugs

The use of plants as medicines represents by far the biggest human use of the natural resources. Plants provide the predominant ingredients of medicines in most medical traditions (**HAMILTON, 2004**). Despite the recent advances in drug development through molecular modelling, combinatorial chemistry and synthetic chemistry, natural plant-derived compounds are still proving to be an invaluable source of medicines for humans (**SALIM *et al*., 2008**). The economic value of both current plant based pharmaceuticals, and those as yet undiscovered is considerable (**PRINCIPE, 1989**).

On numerous occasions, the folklore records of many different cultures have provided leads to plants with useful medicinal properties (**SWAIN, 1972; GOLDSTEIN *et al*., 1974; LEWIS and ELVIN-LEWIS, 1977**). The chemical investigation and purification of extracts from plants purported to have medicinal properties have yielded numerous purified compounds which have proven to be indispensable in the practice of modern medicine (**GOLDSTEIN *et al*., 1974, TYLER *et al*., 1988**). Although there is no reliable figure for the total number of medicinal plants, it is estimated that about 53 000 plant species are used for medicinal purposes worldwide (**SCHIPPMANN *et al*., 2002**). The number of plant species that

provide ingredients for drugs used in Western Medicine is very few relative to the total of medicinally used plants. **FARNSWORTH and SOEJARTO** (**1991**) reported that in 1991, there were 121 drugs in use in the United States of America (USA) derived from plants, with 95 species acting as sources. Despite the small number of source species, drugs derived from plants are of immense importance in terms of numbers of patients treated. Reports estimate that approximately 25% of all prescriptions dispensed from community pharmacies in the USA between 1959 and 1980 contained one or more ingredients derived from higher plants (**FARNSWORTH and SOEJARTO, 1991**). Moreover, in developing countries, medicinal plants continue to be the main source of medication. In China alone, 7 295 plant species are utilised as medicinal agents (**MAHIDOL *et al*., 1998**).

The botanical medicine industry is experiencing rapid growth worldwide (**LAIRD, 1999**). A study of the 25 best-selling pharmaceutical drugs in 1997 found that 11 of them (44%) were either biologicals, natural products or entities derived from natural products, with a total value of US$ 17.5 billion (**HAMILTON, 2004**) The world market for herbal remedies in 1999 was calculated to be worth US$ 19.4 billion, with Europe in the lead (US$ 6.7 billion), followed by Asia (US$ 5.1 billion), North America (US$ 4.0 billion), Japan (US$ 2.2 billion), and then the rest of the world (US$ 1.4 billion) (**LAIRD and PIERCE, 2002**). Plant-derived drugs thus represent stable markets upon which both medical practitioners and patients rely. Worldwide markets in plant-derived drugs are difficult to estimate, but undoubtedly amount to many additional billions of dollars (**PRINCIPE, 1989; BALANDRIN *et al*., 1993**).

The potential of higher plants as sources of new drugs is still largely underutilised (**LAIRD, 1999**). It has been estimated that only an approximate 5 to 15% of the estimated 250 000 existing species of higher plants have been evaluated for the presence of biologically active compounds (**BALANDRIN *et al*., 1993**). Moreover, it is often the case that even plants that are considered to have been investigated, have been screened for only a single type (or, at best a few types) of biological activity (**BALANDRIN *et al*., 1993**). Thus, since at least 85% of the world's higher plant species remain to be investigated, and because many plant secondary metabolites are genus- and/or species-specific, chances are therefore excellent that many other plant constituents with potentially useful biological properties remain

undiscovered and undeveloped. Due to their fundamental contributions to human health, the value of medicinal plants to human livelihoods is essentially infinite. The plant kingdom therefore remains an important resource of potentially useful bioactive compounds that needs to be conserved

1.4.1. The role of plant-derived natural products in modern medicine

Despite the large number of drugs derived from total synthesis, plant-derived natural products still contribute to the overall total number of new chemical entities that continue to be launched onto the market. Some drugs, having botanical origins, are still extracted directly from plants, others are made through transformation of chemicals found within them, while others are today synthesised from inorganic materials, but have their historical origins in research into the active compounds found in plants. There are undoubtedly many more secrets still hidden in the world of plants (**MENDELSOHN and BALICK, 1995**), which provides further evidence for the importance of natural products.

Of the 252 drugs considered as basic and essential by the WHO, 11% are exclusively of plant origin and a significant number are synthetic drugs obtained from natural precursors (**RATES, 2001)**. It is estimated that 60% of anti-tumour and anti-infectious drugs already on the market or under clinical trial are of natural origin (**SHU, 1998**). Plants have proven to be an irreplaceable important component of modern medicine.

The isolation of morphine from *Papaver somniferum* by the German pharmacist Friedrich Sertürner in 1805 marked the beginning of the isolation of natural compounds from plants **(KINGHORN, 2001)**. This subsequently opened doors for the isolation of a large number of plant-derived drugs, the majority of which are still in use today. Galantamine is one of the natural products discovered through an ethnobotanical lead and was first isolated from *Galanthus woronowii* in Russia in the early 1950s (**HEINRICH and TEOH, 2004**). Arteether is a potent antimalarial drug and is derived from artemisinin, a sesquiterpene lactone isolated from *Artemisia annua*, a plant used in Chinese Traditional Medicine (CTM) (**GRAUL, 2001**).

Artemisinin has an advantage over most other antimalarial drugs in that it acts very rapidly (**VAN AGTMAEL *et al*., 1999**). Taxol is an anticancer taxane diterpenoid derived from the Pacific or western yew tree, *Taxus brevifolia* (**BALANDRIN *et al*., 1993**). Diosgenin, a steroidal sapogenin obtained from the tubers of various *Dioscorea* species that grow in Mexico and Central America, can be converted chemically into progesterone, a hormone that can be used as a female oral contraceptive (**SALIM *et al*., 2008**). Some examples of other important drugs obtained from plants include, digoxin from *Digitalis* species, quinine and quinidine from *Cinchona* species, vincristine and vinblastine from *Catharanthus roseus*, atropine from *Atropa belladonna* and morphine and codeine from *Papaver somniferum* (**BALUNAS and KINGHORN, 2005**). It is for this enormous and valuable plant-derived drug output, that plants will remain an essential part of the search for novel medicines against human diseases.

In view of the significant role plants play in human health, their conservation is undoubtedly necessary. A combination of botanical and ethnobotanical knowledge is essential for the conservation of medicinal plants, particularly with the growing problems of over-harvesting and the loss of natural habitats caused by over-population, resulting in the loss of potentially useful plants.

1.5. Medicinal plant trade in South Africa

With the continued importance placed on TMs, cities have become concentrated centres of demand, drawing in medicinal plants from outlying rural areas and across national boundaries. The trend towards increased commercialisation of medicinal plants in South Africa has resulted in overharvesting and, in some cases, near-extinction of some valued indigenous plant species. The harvesting of medicinal plants was formerly the domain of trained traditional medical practitioners, renowned for their skills as herbalists and diviners and had a limited effect on plant resources (**CUNNINGHAM, 1991**). Strict customary conservation practices were respected, which regulated plant collection times and quantities. With the advent of urbanization and the consequent commercialization of traditional health care, the demand for medicinal herbs has increased significantly (**WILLIAMS *et al*., 2000**). As a result,

harvesting has become the domain of untrained, and often indiscriminate, commercial gatherers with no other sources of income. Harvesting and the provision of medicinal plants to meet the urban demand has thus become an environmentally destructive activity that can result in depletion of favoured but slow growing species. Consequently, the trade in traditional medicines is now greater than at any time in the past and is certainly the most complex resource management issue facing conservation agencies, healthcare professionals and resource users in South Africa today (**CUNNINGHAM, 1997**).

Traditional medicine traders in South Africa can be classified into two sectors, namely: formal businesses and informal markets (**MANDER and MCKENZIE, 2005**). The marketing however, is largely informal, dominated by simple technologies and interactions, and driven primarily by economics. This enables a wide range of community members to engage in various aspects of the trade. Rural people, especially women, are the major players involved in the harvesting and trading of medicinal plants in this informal sector **(MANDER, 1998; WIERSUM *et al.*, 2006)**. The formal sector is represented by herb traders, including traditional healers, trading from fixed licensed premises called herbal (*muthi*) shops.

Most households in South Africa spend between 4 to 8% of their annual income on TM and services **(MANDER, 1998)**. Consumer surveys from South Africa suggest that between 35 000 and 70 000 tonnes of plant material are consumed per year, with a market value of between US$75-150 million (**MANDER and MCKENZIE, 2005**). An estimate of over half a million people in both rural and urban areas are involved in the herbal trade, resulting in 20 000 tonnes of plant materials with an approximate value of US$60 million being traded annually **(MANDER, 1998)**. **MANDER** (**1998)**, estimates that over 4 000 tonnes of plant material is traded in KwaZulu-Natal to over 6 million indigenous medicine consumers. In the Eastern Cape Province, approximately 525 tonnes of plant material, comprising at least 166 taxa and valued at approximately US$6 million per annum, is traded annually (**DOLD and COCKS, 2002**). The supply of plant products is not only critical for the welfare of approximately 27 million South African consumers, but it is also critical for the welfare of people who earn their income from the industry. An estimated population of between 20 000 and 30 000 people in KwaZulu-Natal alone, derive their income

from trading indigenous plants in some form (**MANDER, 1998**). **WILLIAMS *et al*. (2000)** reported that some material traded in Gauteng markets originates from the Eastern Cape and KwaZulu-Natal provinces, thus revealing that the trade is not only local but also inter-provincial.

The actual economic value of the medicinal plant trade may be higher than estimated by the different researchers because the market is informal and the volume of sales are not easily quantified due to lack of records and the often illicit nature of the trade (**BOTHA *et al.*, 2004**).

1.5.1. Bulbs and the medicinal plant trade

Demand for certain species in the medicinal plant trade, particularly those believed to have high healing properties, exceeds supply. This has resulted in some plant species being so greatly exploited that they are seldom found in unprotected areas and are thus highly priced. Bulbs are ranked as one of the most popular plant groups sold at most of the traditional medicinal markets in South Africa (**MANDER, 1997**). Their use in herbal medicine involves destructive uprooting of the whole plant.

Harvesting of whole plants, roots and bulbs account for approximately 50% of the more than 500 medicinal plant species sold in the traditional medicine market in Johannesburg (**WILLIAMS, 2003**). **MANDER,** (**1998**) reported that of the most important bulbs traded in the markets of KwaZulu-Natal, approximately 28% had the whole plant harvested. In a survey by **DOLD and COCKS** (**2002**), it was found that of the 60 most frequently traded plant species in the Eastern Cape province, 63% comprised bulbs, tubers and roots. Of the most commonly available species in at least two third of the *muthi* shops in Witwatersrand, 31% were bulbs (**WILLIAMS *et al*., 2000**).

The species-specific demand has seen most of the bulbs being highly priced (**MANDER, 1998**) and this exerts significant pressure on the population survival in their natural growing areas. This is so because an estimated 99% of the more than 500 species sold for use in traditional medicine in South Africa originate from wild

sources (**WILLIAMS, 1996**). Of the top 10 most frequently sold plant species in Eastern Cape, the greatest quantity for a single species was that of *Hypoxis hemerocallidea*, a plant whose corm is harvested for medicinal use, with 11 000 kg/year valued at R322 500 (**DOLD and COCKS, 2002**). *Bowiea volubilis, Boophone disticha, Bulbine latifolia, Drimia robusta, Eucomis comosa, Hypoxis hemerocallidea, Merwilla plumbea* and *Tulbaghia alliacea*, all of which are bulbous plants, are reported to be heavily traded, unsustainably harvested and with high market values in South African medicinal markets (**MARSHALL, 1998; DOLD and COCKS, 2001**). This non-sustainable harvesting not only threatens the survival of these valuable medicinal plant species, but also the livelihoods of people that depend on them.

1.6. Conservation of medicinal plants

Sustainability of harvesting of medicinal plants is challenged by many factors, from both social and ecological perspectives. It is generally accepted that for any resource, a relationship exists between resource stock, population size and sustainable rate of harvesting. Low stocks are likely to produce small unsustainable yields, particularly if the target species is slow growing and slow reproducing (**CUNNINGHAM, 1988)**.

CBD (**1992**) defined sustainable use as „*the use of components of biological diversity in a way and at a rate that does not lead to the long-term decline of biological diversity, thereby maintaining its potential to meet the needs and aspirations of present and future generations*'. Sustainable use in its strictest sense is conservation. According to **WALTER and GILLETT** (**1998**), 34 000 species or 8% of the world's flora are threatened with extinction. If this is applied to the estimate that 53 000 plant species are used medicinally (**SCHIPPMANN *et al*., 2002**), it leads us to estimate that 4 240 medicinal plant species are threatened. There are growing concerns about loss of biological diversity and these concerns exist, for a large part, because most species of medicinal plants are collected from the wild (**WILLIAMS *et al*., 2000**).

The basic idea behind sustainable harvesting is that a biological resource should be harvested within the limits of its capacity for self-renewal. **OLMSTED and**

ALVAREZ- BUYLLA (**1995**) and later **TICKTIN** (**2004**) reported a clear relationship between the plant parts harvested, harvesting methods and the impact on plant demography. **JÄGER and VAN STADEN** (**2000**) attributed the increased use of the non-renewable plant materials such as roots, bulbs and bark as the major contributing factor to the decline of most species in the wild. **NAULT and GAGNON** (**1993**), for example, showed that harvest rates of between 5% and 15% resulted in population declines in *Allium tricoccum*, a species whose underground bulbs are utilised in traditional medicine. **ROCK *et al.*** (**2004**) found this species to have limited population recovery over time. The rates of growth and demographic responses to harvest may also vary significantly over climatic and soil gradients (**SIEBERT, 2000; SVENNING and MACIA, 2002**). Importantly, the use of bulbous plants in traditional medicine involves the destructive harvesting of the whole plant. The species often require several years to reproduce. Further to this, medicinal plant users believe that the most potent principles are located in the underground plant parts (**MANDER, 1998**), thereby encouraging non-sustainable harvesting from the wild. This pressure exerts ecological instability on the resource base, hence the need for conservation.

About 99% of the plant species sold for use in traditional medicine in South Africa are drawn from wild sources and bulbs form a significant portion of this total (**WILLIAMS, 1996**). Given their quantities on the South African medicinal markets, and the reports on their population decline (**CUNNINGHAM, 1993; MANDER, 1998; DOLD and COCKS, 2002; WILLIAMS, 2003**), bulbs, are a group of plants that require conservation priority. A number of possible conservation strategies for medicinal plants have been proposed and some implemented with some degree of success (**CUNNINGHAM, 1991; CBD, 1992; HAMILTON, 2004**). Establishment of conservation areas and enforcement of laws against plant collection and domestication and cultivation of medicinal plant species, are among the most viable options. South Africa however, has a low success rate in the use of law enforcement for biodiversity conservation (**WIERSUM *et al.*, 2006**). **JÄGER and VAN STADEN** (**2000**), highlighted domestication as a strategy to create opportunities for alternative/additional income and help to relieve pressure on the wild population. Another strategy which until now has not received much research attention is to encourage healers to collect and use alternative plant parts such as leaves and flowers instead of bulbs or roots (**ZSCHOCKE *et al.*, 2000**). The value of selective

harvesting as an important strategy of ensuring survival of medicinal plants, lies in the fact that the structural parts (roots, bulbs, barks) are not removed during collection.

Given the demand for a continuous and uniform supply of medicinal plants and the accelerating depletion of wild resources, increasing the number of medicinal plant species through cultivation would appear to be an important strategy for meeting a growing demand (**UNIYAL *et al.*, 2000**). Cultivation of medicinal plants however, has not been widely adopted as a conservation strategy in most cultures. The total number of species of medicinal plants cultivated on any scale is few (**HAMILTON, 2004**). China is probably the country with the greatest acreage of medicinal plants under cultivation, but, even so, only 100-250 species are cultivated (**SCHIPPMANN *et al*., 2002**). One explanation may be found in the observation that cultivated plants are sometimes considered qualitatively inferior when compared with wild gathered specimens (**SCHIPPMANN *et al*., 2002**). The reason is primarily cultural. In Botswana for example, traditional medicinal practitioners cited that cultivated material was unacceptable, as cultivated plants did not have the power of material collected from the wild (**CUNNINGHAM, 1994**).

The limitations of cultivation as an alternative to wild harvest have been examined in several case studies (**CBD, 1992; SHELDON *et al*., 1997; HAMILTON, 2004**). The conclusion is therefore shared that, notwithstanding the level of interest in cultivation as a means for enhanced production, most medicinal plant species will continue to be harvested from the wild to some extent. The need to recognize and strengthen the role of plant part substitution cannot therefore, be overlooked. The success of this will depend on the necessary research undertaken to validate the pharmacological similarities and/or differences between various parts of the same plant. Without conservation measures being taken, it is feared that valuable bulbs will not survive the onslaught of modern living.

1.7. Plant secondary metabolites

While plants are sessile organisms, their metabolic capabilities extend far beyond their borders. Plants produce an enormous wealth of structurally diverse, organic compounds that are not directly involved in the normal growth, development, or reproduction and are collectively referred to as secondary metabolites (**WINK, 2003**). Plant secondary metabolites are involved in the adaptation of plants with their environment. They mostly contain more than one functional group and therefore often exhibit multiple functionalities and bioactivities (**DEY and HARBORNE, 1989**), which include therapeutic properties such as antimicrobial, antioxidant, anti-inflammatory, anti-carcinogenic inhibitory activities (**CROZIER *et al*., 2006; GURIB-FAKIM, 2006**). The wide chemical diversity of secondary metabolites throughout the plant kingdom represents an extremely rich biogenic resource for the discovery of novel and innovative drugs (**GURIB-FAKIM, 2006**). Although they are structurally diverse, secondary metabolites derive their synthesis from limited products of primary metabolism (**CROZIER *at al*., 2006**). Phenolic compounds, alkaloids and saponins are amongst the most commonly occurring secondary metabolites in the plant kingdom.

1.7.1. Phenolic compounds

Phenolic compounds or polyphenols are characterised by at least one aromatic ring bearing one or more hydroxyl substituent, including functional derivatives (esters, methyl esters, glycosides) (**DEY and HARBORNE, 1989**). They are a diverse group of higher secondary metabolites, derived from the shikimate pathway and phenylpropanoid metabolism, with phenylalanine as a precursor (**DIXON and PAIVA, 1995; RYAN *et al*., 1999**). Their synthesis depends on numerous enzymes involved in different metabolic pathways (**JAY-ALLEMAND *et al*., 2001**) and their metabolism is completely integrated into morphological and biochemical regulatory patterns of plants (**BARZ *et al*., 1985**). The basic building compound is phenol (**Figure 1.1**). Polyphenols are ubiquitous in the plant kingdom but the type of compound present varies considerably according to the genus and species under consideration (**CROZIER *at al*., 2006**). Phenolic compounds form an integral part of the cell wall structure in plants, mainly in the form of polymeric materials such as

lignins which serve as mechanical support and barriers against microbial invasion (**WALLACE and FRY, 1994**). The compounds exhibit a considerable free radical scavenging activity, which is determined by their reactivity as hydrogen- or electron donating agents and the stability of the resulting antioxidant-derived radical, which prevent the oxidation of various food ingredients, particularly fatty acids and oils (**RICE-EVANS *et al*., 1997; SUBBA RAO and MURALIKRISHNA, 2002**). Flavonoids and tannins are amongst the broad groups of phenolic compounds.

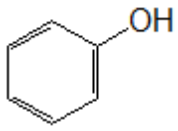


Figure 1.1. Basic compound phenol in which different phenolic compounds derive their skeletal structure

1.7.1.1. Flavonoids: Biosynthesis and biological activity

Flavonoids which are built upon a flavone skeleton are the most widespread and diverse class of low molecular weight phenolic compounds (**HEIM *et al*., 2002**). Biosynthetically they are derived from a combination of the shikimic acid and the acetate pathways (**WATERMAN and MOLE, 1994**). They are constituents of plant leaves, fruits, seeds, vegetables, flowers, and roots, with over 4 000 different compounds identified in plants (**HEIM *et al*., 2002; CROZIER *at al*., 2006**). They are found in high concentrations in the epidermis of leaves and the peels of fruits (**HRAZDINA, 1992; CROZIER *at al*., 2006**). Small differences in basic substitution patterns give rise to several subgroups, with the main subclasses being flavonols, flavones, flava-3-ols, isoflavones, flavanones and anthocyanidins (**HEIM *et al*., 2002**). The generic flavonoid structure is as shown in **Figure 1.2**.

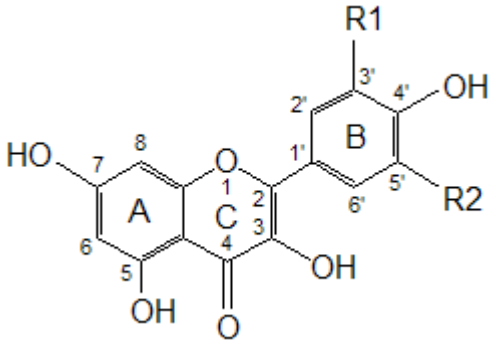


Figure 1.2. Generic structure of a flavonoid in which rings A, B and C and the numbering system are shown. Kaempferol, R1=H, R2=H; quercetin, R1=OH, R2=H; myricetin, R1=OH, R2=OH

Structural and chemical diversity of flavonoids is related to their diverse properties and roles in plants. In plants, these compounds afford protection against ultraviolet radiation, pathogens, and herbivores (**HARBORNE and WILLIAMS, 2000**). **HRAZDINA** (**1992**), working with a *Pisum sativum* cv. *argenteum* mutant, reported that the greatest accumulation of both anthocyanin and flavonol glycosides occurs in the upper epidermal layers of leaf peels, which were directly exposed to visible and ultraviolet (UV) light irradiation, confirming a UV protection role for these compounds. In view of this, environmental factors seem to have a significant contribution to the content of flavonoids and phenolic acids in plants. The anthocyanin copigments in flowers serve to attract pollinating insects (**HARBORNE and WILLIAMS, 2000**) and are responsible for the characteristic red and blue colours of berries, wines, and certain vegetables all of which are major sources of flavonoids in the human diet (**CARANDO *et al*., 1999; STEWART *et al*., 2000; LOPEZ *et al*., 2001**).

One of the undisputed functions of flavonoids is their role in protecting plants against microbial invasion (**WATERMAN and MOLE, 1994; HARBORNE and WILLIAMS, 2000**). This is achieved through their presence in plants as constitutive agents and also their accumulation as phytoalexins in response to microbial attack (**GRAYER *et al*., 1994; HARBORNE, 1999**). The ability of flavonoids to inhibit spore germination of pathogens has been explored and exploited in both ancient and modern human medicine for the treatment of human pathogenic diseases. This is a associated with the long history use of plants as medicinal remedies.

Flavonoids have attracted a lot of research interest in the pharmacological and pharmaceutical spheres, and this has seen characterisation of various plant flavonoids as antifungal, antibacterial, antiviral, anti-inflammatory, antioxidant, antitumor, anti-hepatotoxic, anti-lipolytic, vasodilator, immunostimulant and, antiallergic agents (**IINUMA *et al*., 1994; ALIAS *et al*., 1995; WILLIAMS *et al*., 1999; BURDA and OLESZEK, 2001; GURIB-FAKIM, 2006**). Several consistent lines of evidence support the role of flavonoids in radical scavenging, chelating and oxidant activities against various reactive oxygen species (ROS) in animal cells. Consistent with most polyphenolic antioxidants, both the configuration and total number of hydroxyl groups, in addition to the flavan backbone, substantially influence several mechanisms of antioxidant activity and thus the free radical scavenging capacity of flavonoids can primarily be attributed to this characteristic (**HEIM *et al*., 2002; MIDDLETON *et al*., 2008**).

The HIV epidemic has stimulated an ever increasing interest in plant flavonoids as potential sources of drug agents for controlling the immunodeficiency virus which is the causative agent of AIDS (**HARBORNE and WILLIAMS, 2000**). It is clear that plant flavonoids has played and will continue to play, a vital role in human health systems and the drug discovery process.

1.7.1.2. Tannins: Biosynthesis and biological activity

Tannins are phenolic compounds that exhibit complex and highly variable chemical structures. They are broadly categorised into hydrolysable and condensed tannins, based on whether acids or enzymes can hydrolyse the components or whether they condense the components to polymers (**GURIB-FAKIM, 2006**). Both these classes of tannins are rich in highly reactive hydroxyl groups which emanate from each of the benzene rings, and form complexes with proteins, including enzymes (**WALLACE and FRY, 1994**), and polymers such as cellulose and hemicellulose (**HASLAM, 1989**).

Hydrolysable tannins are based upon the fundamental structural unit of gallic acid (3, 4, 5-trihydroxy benzoic acid) and are almost invariably found as multiple esters with *D*-glucose to form gallotannins (**Figure 1.3**) (**HASLAM, 1989**). Derivatives of hexa-hydroxydiphenic acid (ellagitannins) are derived from oxidative coupling of adjacent galloyl ester groups in a polygalloyl D-glucose ester (**MAAS *et al*., 1991**). Gallic acid and its metabolites are widely distributed in plants and particularly herbaceous dicotyledons (**GROSS, 1992**). In particular plant tissues, e.g. plant galls, metabolites of gallic acid often accumulate in substantial quantities and constitute a reagent of considerable antiquity used in the analysis of mineral water and as a component of invisible ink (**HASLAM, 1989**).

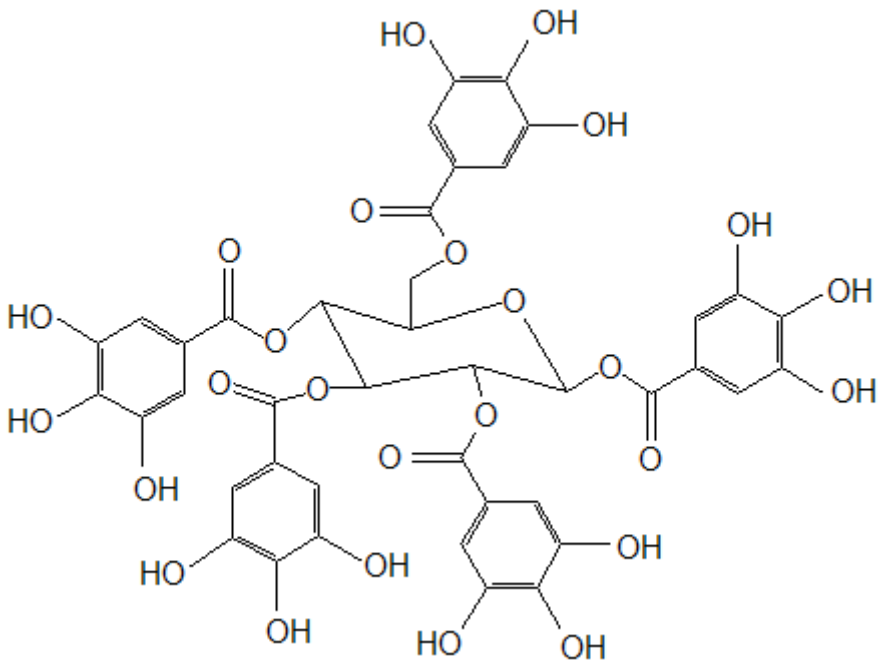


Figure 1.3. Chemical structure of a gallotannin

Condensed tannins, commonly referred to as proanthocyanidins, are oligomers of 3-flavanols (catechins) and 3,4-flavan-diols (leucoanthocyanidins) linked together by carbon to carbon bonds (**HAGERMAN, 2002**). The flavan-3-ol units are linked principally through the 4 and the 8 positions. The term proanthocyanidin is derived from the acid-catalysed oxidation reaction that produces red anthocyanidins upon heating in an acidic alcohol solution (**PORTER *et al*., 1985**), a reaction that forms the basis of the butanol-HCl assay for proanthocyanidins. They are the most widespread

polyphenols in plants after lignins and can be found in leaves, fruits, woods, barks, or roots, often in high concentrations (**MATTHEWS *et al*., 1997**). In plants, condensed tannins may act as feeding deterrents in reproductive tissues and developing fruit and also impart astringency to fresh fruit, fruit juices and wine (**HASLAM, 1989**). **FEENY** (**1970**), surmised that tannins, are characteristic of the chemical defence of plants and act as quantitative-dosage dependent-barriers to predators that may feed on them. The relevant physiological effects of tannins upon predatory is assumed to be derived from their ability to complex with proteinaceous materials.

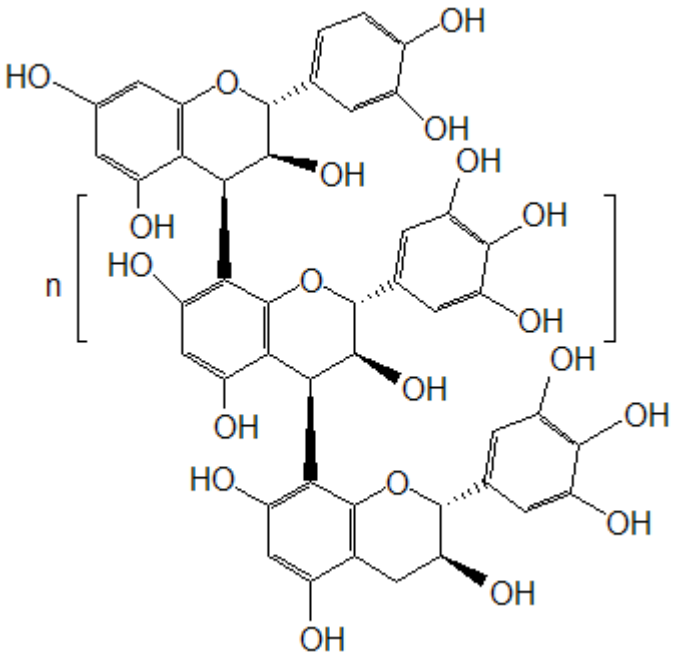


Figure 1.4. Chemical structure of a proanthocyanidin consisting of the catechin and epicatechin polymeric units, where „n' is any number that makes up the polymer

Because tannins have been shown to have antibiotic, antifeedant, or biostatic effects on a variety of organisms that consume them (**HASLAM, 1989**), the chemical properties of both condensed and hydrolysable tannins have been exploited in the discovery of versatile medicinal agents. Tannins are thus, reported to possess numerous medicinal properties such as antibacterial, antifungal, antiviral, anti-diarrhoeal, free radical scavenging, immunomodulatory, anti-inflammatory, anti-tumour and antidote activities (**OKUDA *et al*., 1992; HASLAM, 1996; GURIB-**

FAKIM, 2006). Some, and certainly most of the beneficial effects which tannins exert as constituents of drugs and herbal remedies may well follow from their interaction with enzymes (proteins) within cell systems (**SCHULTZ *et al*., 1992; HASLAM, 1996**). **VANDEN BERGHE *et al.*** (**1985**) evaluated the claims for antiviral activity made for natural products, including various tannins, derived from over 900 plant species, and concluded that tannins act principally by binding to the virus and/or protein of the host cell membrane and thus arrest adsorption of the virus. Similarly, bacterial and fungal enzymes and toxic proteins may be bound by tannins and inactivated (**SCHULTZ *et al*., 1992**).

1.7.2. Saponins

Saponins are plant secondary metabolites that contain an aglycone of either tetracyclic steroidal or a pentacyclic triterpenoid nature (sapogenin) linked to one or more oligosaccharide moities (glycosides) through a glycosidic bond (**ALEXANDER *et al*., 2009**). Saponins are a diverse group of compounds that are widely distributed in the plant kingdom. They have been reported to be present in more than 100 plant families (**SPARG *et al.*, 2004**). Saponins can form stable foam in aqueous solutions, a characteristic which the froth test is based on. The carbohydrate side chain consists of pentoses, hexoses and uronic acids. The presence of both polar (sugar) and non-polar (steroid or triterpene) groups provides this group of compounds with strong surface-active properties (**MAKKAR *et al*., 2007**) that are responsible for many of its adverse and beneficial effects.

In triterpenoid saponins, the aglycone part can either be a pentacyclic triterpenoid or a tetracyclic triterpenoid, both of which contain 30 carbon atoms. Steroidal saponins are modified triterpenoids with a tetracyclic structure containing 27 carbon atoms. In plants, steroidal saponins are drived from cycloartenol, a cyclic triterpenoid (**REES *et al.*, 1968**). The steroidal form is almost exclusively found in monocotyledonous plant families (**SPARG *et al.*, 2004**). The number and length of saccharide chains attached to the sapogenin/aglycone core in both steroidal and triterpenoid saponins can vary giving rise to monodesmosidic, bidesmosidic or tridesmodic saponins

(**HOSTETTMANN and MARSTON, 1995**). Monodesmodic saponins have a single sugar chain while bi- and tridesmodic have two and three respectively.

In plants, saponins are found in tissues that are most vulnerable to fungal or bacterial attack or insect predation (**WINA *et al*., 2005**). It is believed, therefore, that one of their natural roles in plants is to act as a chemical barrier against attack by potential pathogens (**OSBOURN, 1996**) and hence their reported antimicrobial activities. They have been shown to have a range of biological activities and potential health benefits that include hypocholesterolemic, anti-coagulant, anticarcinogenic, hepato-protective, hypoglycemic, immunomodulatory, neuroprotective, anti-inflammatory, anti-oxidant activity and platelet aggregation (**JUST *et al*., 1998; FRANCIS *et al*., 2002**). Saponins have also been observed to kill protozoans and molluscs, and act as antifungal, antibacterial and antiviral agents (**SPARG *et al.*, 2004; TREYVAUD *et al*., 2000**).

1.8. Seasonal dynamics of secondary metabolites in plants

Plants cannot escape from the environmental extremes of light, temperature, and drought, nor move to regions with better nutritional conditions, and have thus evolved highly complex mechanisms to integrate physiology and metabolism in order to adapt to the conditions to which they are exposed. Secondary metabolites form an integral component of these adaptive mechanisms. External factors quantitatively affect secondary metabolism processes through their effects on plant development, growth rates, dry matter partitioning and partitioning of metabolites to the secondary metabolite of interest and these factors can also trigger abrupt activation of qualitative changes in secondary metabolite production (**LAUGHLIN, 1993; LOMMEN *et al*., 2008**). Climatic (abiotic) factors often have an especially large influence on the biosynthesis and levels of secondary metabolites in plants (**COLEY, 1987; WATERMAN and MOLE, 1994; NARVAEZ *et al*., 2010**).

Under natural conditions, plants rarely experience single abiotic factors one by one, but are much more likely to be exposed to multiple stresses simultaneously. Seasonal climatic changes bring about a variety of different stress combination

factors and hence the response by plants is not always predictable and very complex (**HOLOPAINEN and GERSHENZON, 2010**). An exponential increase, for example, in a variety of volatile organic compounds, with a linear increase in temperature has been described in a range of plant species (**PARKER, 1977; SHARKEY and LORETO, 1993; SHARKEY and YEH, 2001**). Among other abiotic stresses, high light intensity and water stress have also been reported to increase plant secondary metabolites (**SHARKEY and YEH, 2001; MIRDEHGHAN and RAHEMI, 2007; DERITA *et al*., 2009**). More often, these factors are investigated individually. **GOUINGUENE and TURLINGS** (**2002**) highlighted that when two or more factors co-occur, their effects are sometimes additive, while in other cases the influence of one factor has priority. In maize, the combination of high temperature and simulated lepidopteran herbivory resulted in greater metabolite accumulation than when either stress was applied alone (**GOUINGUENE and TURLINGS, 2002**). The responses are, therefore, very diverse. However, very few studies have attempted to investigate plant secondary metabolites under multiple stresses. Such research is essential in that the response of plants to multiple stress combinations cannot always be extrapolated from responses to individual stress factors (**MITTLER, 2006**).

Knowledge of how multiple stresses affect secondary metabolite accumulation in plants will provide more information to evaluate the biological roles of these metabolites in mitigating stress and provide criteria for describing their optimum yields and quality. A quantitative insight into the potential amount and seasonal dynamics of secondary metabolites (phytochemicals) in medicinal plant use is basic to the understanding of when to collect plants. Coinciding medicinal plant harvesting with maximum biological activity of a particular plant species would serve as a logical way for the effective utilisation of plant-based medicine.

1.8.1. Influence of other stress factors

In addition to climatic factors, plants are subjected to a multitude of biotic stress factors such as herbivore and pathogenic attacks (**DIXON and PAIVA, 1995; HOLOPAINEN and GERSHENZON, 2010**). Herbivore damage to vegetative parts has been repeatedly shown to cause the increased release of inducible secondary

compounds (**HAGERMAN and BUTLER, 1991; BERNAYS and CHAPMAN, 2000**). Levels of secondary metabolites in plant tissues have also been reported to vary with resource availability (**COLEY *et al*., 1985**). Levels of proanthocyanidins increase following nutritional stress such as limitation in available phosphate (**KOUKI and MANETAS, 2002**). Low iron levels were shown to stimulate increased biosynthesis of phenolic compounds (**DIXON and PAIVA, 1995**). In general, source-sink relations are expected to constrain the levels of secondary metabolites accumulated by the growing plants (**PRICE *et al*., 1989**) but their dynamics warrants further exploration.

1.8.2. Organ and tissue specific biosynthesis and transport

The organs forming the plant body consist of several different cell types that are organized in relation to each other and that confer specific functions to the resulting organ. In addition to morphogenesis, developmental processes result in biochemical specialization of cells for the biosynthesis and/or accumulation of secondary metabolites (**IBRAHIM *et al*., 1987; HASHIMOTO and YAMADA, 1994**). The sites of certain plant secondary metabolite biosynthesis, storage and final function often differ at the subcellular, cell, and even tissue and organ levels (**FACCHINI and DE LUCA, 1995; GROTEWOLD, 2001; ZHAO and DIXON, 2009**). Nicotine, for example, an alkaloid synthesised in the roots of *Nicotiana tabaccum* has its site of action in the leaves (**WINK and ROBERTS, 1998**). Efficient transport systems for metabolites across endomembranes and the plasma membrane are therefore required.

Phenolic compounds are found in all parts of the plant but their nature and concentration varies greatly between the various tissues (**SOLER-RIVAS *et al*., 2000; RYAN *et al*., 2002**). Differential cell-specific expression and intercellular transport of metabolites have been suggested for the phenylpropanoid pathway (**REINOLD and HAHLBROCK, 1997**). Secondary metabolites are often transported from source cells to neighbouring cells, or even further to other tissues or remote organs (**HASHIMOTO and YAMADA, 2003; YAZAKI, 2006; YAZAKI *et al.,* 2008**). This intracellular, intercellular, intratissue and interorgan translocation requires membrane transport events to be involved.

The mechanism for the long-distance transport of alkaloids is well elucidated in the Solanaceae family (**HASHIMOTO and YAMADA, 2003**). Nicotine has been shown to be translocated from the roots (site of synthesis) of tobacco plants via the xylem to the leaves (**WINK and ROBERTS, 1998; ST-PIERRE *et al.,* 1999**). Several ATP-binding cassette (ABC) transporters, multidrug and toxin extrusion (MATE) transporters and others for secondary transport, which use electrochemical gradients of protons or sodium ions, have been identified to be involved in the intercellular and intracellular transport activities of alkaloids and other secondary metabolites in different plant species (**WINK and ROBERTS, 1998; ST-PIERRE *et al.,* 1999; YAZAKI *et al.,* 2008; ZHAO and DIXON, 2009**).

Flavonoids have been extensively studied, especially their transport. Most flavonoids exist in their glycosylated forms in plants and glycosylation is thought to be a prerequisite for their transport into vacuoles (**KLEIN *et al.,* 1996; ZHAO and DIXON, 2009**). The involvement of primary transporters that transport anthocyanins in and across vacuolar membranes was first suggested in the *bronze-2 (bz-2)* mutants of maize (**MARRS *et al.,* 1995**). Accumulation and related membrane transport of quinine compounds suggest that vesicle trafficking-like mechanisms are responsible (**RYAN *et al*., 2002**)

Secondary metabolites are usually end products of energy demanding biosynthetic pathways (**HASLAM, 1989**) and therefore represent a significant metabolic cost for plants. Since plants have limited resources to support their growth, this tends to result in trade-offs between primary and secondary metabolism (**McKEY, 1979**). Along with the selection of certain compounds, there would also be selection to minimise metabolic cost by retrieving metabolites from senescing tissues, transporting compounds from one plant part to another (**McKEY, 1979**). More often with deciduous plants, leaves are shed during particular times of the year and sometimes these plants go dormant during certain seasons and when stressful environmental conditions are encountered. There is a possibility however, that secondary metabolites during such changes, might be translocated to become more concentrated in certain plant parts. If this happens, active principles may be accumulated at high levels in such organs and confers them with high biological activity.

1.9. Significance and aims of the study

The unrestricted collection of medicinal plants from the wild has put many of the slow growing bulbous plant species at the risk of over-exploitation and extinction in South Africa. Conservation of medicinal plant species is important for the sustainability of the plant-based traditional medicine systems as well as maintenance of biodiversity. In the case of slow growing medicinal plants whose underground parts are harvested, a conservation strategy that would take into consideration the sustainable harvesting and perhaps simultaneously provide similar medicinal benefits would be the substitution of underground parts with leaves.

This research was aimed at evaluating the seasonal pharmacological and phytochemical properties of bulbs and the renewable plant parts (leaves) in medicinal bulbs of South Africa using *in vitro* bioassays. The findings of the research are expected to provide information that will help provide a basis for the substitution of bulbs with leaves in the traditional medicinal use of bulbous plants as a conservation strategy. The research will also help further better understanding of the physiological response of these plants and as such provide information on which plant part to collect in each season for best biological activity.

CHAPTER 2
Plant Collection and Extraction

2.1. Introduction

Plant cells are a rich biogenic source of bioactive compounds referred to as plant secondary metabolites. Plant species contain varying levels of different classes of these secondary metabolites with a diverse range of chemical properties. Exploitation of plant material resources for pharmacological evaluation and pharmaceutical development relies on the effective extraction of all the different classes of these secondary compounds.

The aim of an extraction process should be to provide the maximum yield of compounds that are of high quality. In light of these considerations, the extraction process should always aim to optimise factors that affect both quality and quantity of extracts. Extraction efficiency is commonly a function of process conditions. Temperature, contact time, chemical solubility parameters and solvent-to-solid ratio are some of the variables that affect the extraction process (**HERODEŽ *et al.*, 2003; PINELO *et al*., 2005**). Generally, an increase in temperature has a positive effect on extraction yields and rates, however, chemical transformations occur with thermolabile compounds and hence compromise the quality of extracts (**JEONG *et al*., 2004**). Reduction of particle size of the plant material increases the superficial area available for mass transfer by the extracting solvent and increases extraction yield with a shortened extraction time (**CACACE and MAZZA, 2003**). The effect of the solvent-sample ratio has been investigated by a number of workers (**SCHWARTZBERG and CHAO, 1982; HERODEŽ *et al*., 2003; PINELO, *et al*., 2005**) and it has been established that, according to mass transfer principles, the higher the ratio, the higher the total amount of compounds obtained, regardless of the solvent used. The final composition of the extracts, therefore, depends on the amount of the solvent used per gram of dry matter.

Extraction of phytochemical compounds from plant materials using solvents constitutes an important step in the investigation of a plant's pharmacological properties. The extraction process utilises the biological concepts of permeability of

the plant cells. The chemical characteristics of the extraction solvents and the diverse structure and polarity of phytochemical compounds necessitates the use of different extraction solvents with different polarities to ensure all compounds are extracted from the plant material.

2.2. Materials and methods

2.2.1. South African seasons

South Africa has a weather pattern with four distinct seasons. Winter is the coldest and driest season in most parts of the country and covers the period May to July. Most vegetation shed leaves during this dry winter period. However, in regions such as the Western Cape, they get most of their rains during winter. Following winter is the hot, windy and dry spring season which is characterised by flowering in most of the plant species and occurs from mid-August to late-October. Summer succeeds spring and is the longest of the seasons stretching from November to February. Summer is the rainy season and is characterised by high temperatures. Most of the vegetation is green in summer with a predominantly rich geophyte component. The Western Cape has a Mediterranean climate, so this is the exception. Autumn marks the transition from summer into winter and usually falls between March and April. Plant leaves begin senescing during this season.

2.2.2. Plant selection and collection

Based on indigenous ethnopharmacological records, four medicinal bulbous plants used by different South African tribes in the treatment of various ailments (**Table 2.1**), were selected for this research study. The bulbs were selected on the basis of how extensively they are utilised in traditional medicine as well as their demands and volumes on the traditional medicine markets, through literature review. Plants were collected in summer (December), autumn (March), winter (June) and spring (September) from the University of KwaZulu-Natal Botanical Garden, Pietermaritzburg, South Africa and separated into bulbs and leaves. Voucher specimens were identified and deposited at Bews Herbarium (NU) in the University of KwaZulu-Natal, Pietermaritzburg. The samples were dried in an oven at a

constant temperature of 50 °C, ground into a fine powder using an Ultra Centrifugal Mill (ZM 200, Retsch®, Germany) and stored in airtight containers in the dark at room temperature.

2.2.3. Extract preparation

The ground samples were sequentially extracted with 20 ml/g of petroleum ether (PE), dichloromethane (DCM), 80% ethanol (EtOH) and water in a sonication bath (Julabo GMBH, Germany) containing ice for 1 h. The crude extracts were then filtered under vacuum through Whatman's No. 1 filter paper. Organic extracts were concentrated *in vacuo* at 35 °C using a rotary evaporator (Rotavapor-R, Büchi, Switzerland) while water extracts were collected into pre-weighed glass jars and freeze dried. The concentrated organic extracts were put into pre-weighed glass sample vials and subsequently dried under a stream of cold air. The percentage yields of the extracts were determined with reference to the mass of the initial ground material. The dried extracts were kept in airtight glass sample vials at 10 °C in the dark until required for the different assays.

2.2.4. Percentage yield of extracts

The percentage yield of extracts from each extracting solvent in each season was calculated as the ratio of the mass of the dried extract to the mass of the ground plant sample.

2.2.5. Statistical analysis

All extractions were done in triplicate. Data on percentage extract yields for each extracting solvent in four different seasons were arcsine transformed and subjected to one-way analysis of variance (ANOVA) using GenStat 12th edition (VSN International, UK). Significantly different means were separated using Least Significant Difference (LSD) technique ($P \leq 0.05$).

2.3. Results and discussion

Table 2.1. Medicinal plants used in this study and their ethnobotanical uses

Family	Species name **Voucher number**	Ethnobotanical uses
Alliaceae	*Tulbaghia violacea* Harv. **NCUBE 04 NU**	Bulbs and leaves used for the treatment of gastro-intestinal ailments, asthma, tuberculosis, high blood pressure, colds and fever, malaria (**HUTCHINGS *et al*., 1996; VAN WYK *et al*., 2009**)
Hypoxidaceae	*Hypoxis hemerocallidea* Fisch.& C.A. Mey **NCUBE 01 NU**	Plant decoctions have purging effects, taken as tonics against wasting diseases, including tuberculosis and cancer, treatment of urinary tract infections and prostate cancer (**WATT and BREYER-BRANDWIJK, 1962; VAN WYK and GERICKE, 2000**)
Hyacinthaceae	*Drimia robusta* Bak **NCUBE 03 NU**	Bulbs used to treat urinary infections, diseases of the uterus (**HUTCHINGS *et al*., 1996; VAN WYK *et al*., 2009**)
Hyacinthaceae	*Merwilla plumbea* (Lindl.) Speta **NCUBE 02 NU**	Bulbs are used for wound healing, treatment of gastro-intestinal ailments which includes stomach aches, constipation, intestinal worms, diarrhoea, dysentery, nausea and indigestion (**HUTCHINGS, 1989; VAN WYK *et al*., 2009**),

NU = Bews Herbarium, University of KwaZulu-Natal, Pietermaritzburg.

2.3.1. Percentage yield of extracts

The percentage yields of the extracts obtained with different extracting solvents for all the seasons revealed that ethanol extracted the largest quantities of compounds (**Table 2.2**) except for *T. violacea* bulbs, where water extracted the highest quantities. Petroleum ether and DCM extracted relatively low amounts of extracts in all the screened plant species, with DCM having the lowest yield. The solubility of plant compounds in different extracting solvents depends on the chemical characteristics of both the solvent and compounds. Alcohol is regarded as a universal solvent that tends to extract compounds with a wide range of polarities (**EVANS, 1996; PINELO *et al*., 2005**) and hence provides a broader range of compounds. The high percentage extract yields obtained with ethanol extraction could be due to this property and its particularly strong affinity for polar compounds which are abundantly produced in plant cells (**MARSH *et al*., 1999**). Most plant species produce small amounts of lipophilic compounds, and thus PE and DCM solvents had lower percentage yield of extracts.

The type and class of the occurring compounds and their overall quantity in different plant species could vary largely as a function of the plant part considered. In *T. violacea* bulb, high extract yields were obtained with water extraction while leaves yielded high extracts from ethanol extraction. Although the trend in the extract yields of different extracting solvents followed a somewhat similar pattern in all the other plant species (**Table 2.2**), the quantities differed remarkably between the bulbs/corms and leaves. These remarkable differences demonstrate the variation in the chemical constituents and quantities of compounds accumulated by these different organs of a plant.

There were significant differences in the extract yields of each extracting solvent between seasons. Environmental factors, together with the plant's morphological characteristics at different developmental stages usually play a major role in determining the quantity and quality of the plant's chemical constituents (**RICHARDSON *et al.*, 2004; VILJOEN *et al*., 2005**). These factors, however, change markedly with changing seasons and could therefore account for the differences in the extract yields.

Table 2.2. Extract yields (% of dry matter) of investigated medicinal plant species. Values represent the means ± standard error (*n* = 3).

Plant species	Plant part	Extract	Yield (%)			
			Spring	Summer	Autumn	Winter
Tulbaghia violacea	Bulb	PE	0.34 ± 0.01^{a}	0.33 ± 00^{a}	0.57 ± 0.01^{c}	0.45 ± 0.03^{b}
		DCM	0.43 ± 0.02^{b}	0.25 ± 0.02^{a}	0.27 ± 0.01^{a}	0.71 ± 0.02^{c}
		EtOH	8.20 ± 0.13^{b}	11.2 ± 0.28^{c}	7.30 ± 0.14^{a}	11.6 ± 0.28^{c}
		Water	27.9 ± 0.35^{a}	33.5 ± 0.28^{b}	36.5 ± 0.50^{c}	41.5 ± 0.91^{d}
	Leaf	PE	0.95 ± 0.01^{a}	1.60 ± 0.28^{b}	1.50 ± 0.14^{b}	1.30 ± 0.13^{ab}
		DCM	0.61 ± 0.03^{a}	0.96 ± 0.06^{b}	0.98 ± 0.02^{b}	1.40 ± 0.07^{c}
		EtOH	25.9 ± 0.07^{b}	26.2 ± 0.41^{b}	24.0 ± 0.21^{a}	23.9 ± 0.07^{a}
		Water	17.3 ± 0.21^{a}	18.3 ± 0.35^{b}	16.9 ± 0.00^{a}	16.9 ± 0.41^{a}
Hypoxis hemerocallidea	Corm	PE	0.23 ± 0.01^{a}	0.35 ± 0.01^{c}	0.29 ± 0.01^{b}	0.23 ± 0.00^{a}
		DCM	0.16 ± 0.01^{a}	0.17 ± 0.01^{a}	0.16 ± 0.02^{a}	0.26 ± 0.02^{b}
		EtOH	22.7 ± 0.40^{b}	23.1 ± 0.28^{b}	21.5 ± 0.42^{a}	29.8 ± 0.07^{c}
		Water	13.3 ± 0.26^{d}	9.90 ± 0.14^{c}	9.10 ± 0.12^{b}	6.30 ± 0.35^{a}
	Leaf	PE	0.77 ± 0.03^{a}	0.97 ± 0.04^{b}	0.80 ± 0.02^{a}	0.75 ± 0.03^{a}
		DCM	0.53 ± 0.04^{a}	0.67 ± 0.04^{b}	0.83 ± 0.04^{c}	0.66 ± 0.02^{b}
		EtOH	10.4 ± 0.35^{b}	13.8 ± 0.41^{d}	11.4 ± 0.21^{c}	8.80 ± 0.29^{a}
		Water	8.90 ± 0.34^{c}	8.00 ± 0.21^{b}	9.40 ± 0.28^{c}	6.70 ± 0.14^{a}
Drimia robusta	Bulb	PE	0.40 ± 0.02^{a}	0.47 ± 0.03^{b}	0.52 ± 0.01^{b}	0.38 ± 0.01^{a}
		DCM	0.31 ± 0.03^{a}	0.32 ± 0.01^{a}	0.30 ± 0.01^{a}	0.38 ± 0.00^{b}
		EtOH	30.5 ± 2.12^{b}	25.0 ± 0.35^{a}	28.9 ± 0.014^{b}	29.9 ± 0.28^{b}
		Water	24.9 ± 0.21^{d}	20.8 ± 0.29^{a}	22.9 ± 0.27^{b}	24.1 ± 0.14^{c}

Plant species	Plant part	Extract	Yield (%)			
			Spring	Summer	Autumn	Winter
Drimia robusta	Leaf	PE	1.20 ± 0.12^{a}	1.00 ± 0.00^{a}	2.10 ± 0.07^{b}	-
		DCM	0.75 ± 0.05^{ab}	0.52 ± 0.02^{a}	1.00 ± 0.14^{b}	-
		EtOH	10.7 ± 0.36^{a}	18.5 ± 0.29^{c}	17.2 ± 0.21^{b}	-
		Water	15.5 ± 0.28^{c}	11.4 ±0.35^{b}	7.20 ± 0.14^{a}	-
Merwilla plumbea	Bulb	PE	0.46 ± 0.01^{bc}	0.41 ± 0.02^{b}	0.53 ± 0.01^{c}	0.39 ± 0.00^{a}
		DCM	0.46 ± 0.02^{a}	0.54 ± 0.00^{b}	0.60 ± 0.01^{c}	0.54 ± 0.03^{b}
		EtOH	24.2 ± 0.42^{b}	22.5 ± 0.14^{b}	17.3 ± 0.28^{a}	31.3 ± 2.48^{c}
		Water	7.40 ± 0.28^{b}	5.60 ± 0.42^{a}	13.6 ± 0.14^{c}	12.3 ± 0.42^{c}
	Leaf	PE	1.30 ± 0.07^{a}	1.30 ± 0.00^{a}	1.30 ± 0.00^{a}	1.40 ± 0.07^{a}
		DCM	1.10 ± 0.14^{a}	1.74 ± 0.06^{b}	1.30 ± 0.07^{a}	0.99 ± 0.08^{a}
		EtOH	17.1 ± 0.21^{c}	16.0 ± 0.14^{b}	16.4 ± 0.35^{bc}	15.2 ± 0.14^{a}
		Water	14.5 ± 0.56^{b}	14.6 ± 0.14^{b}	15.5 ± 0.28^{b}	10.8 ± 0.28^{a}

PE = Petroleum ether; DCM = Dichloromethane; EtOH = 80% ethanol.

Values in a row with different letters are significantly different at $P \leq 0.05$

There were no leaf samples for *D. robusta* in winter.

2.4. Conclusions

The type of solvent used in the extraction of active principles from plant materials plays a major determining role on the class and amount of compounds extracted. Plant materials seem to contain mostly polar compounds as evidenced by the high extract yields obtained from ethanol and water extraction. Lipophilic compounds, on the other hand constitute a lesser percentage of the plant's dry matter and as such lesser polar solvents (PE and DCM) yielded less extract quantities. As expected, the order of decreasing extract yield in terms of percentage was found to be EtOH > water > DCM > PE in all plant species except *T. violacea* bulb which was: water > EtOH > DCM > PE. Differential extraction of pharmacological compounds using solvents with different polarities helps in the understanding of chemical properties of the active compounds and simplifies the isolation process.

CHAPTER 3

Antibacterial and Antifungal Properties

3.1. Introduction

The challenge of disease and death caused by infectious diseases is strikingly apparent, in both developed and developing nations around the world (**HAMBURG, 2008**), with developing countries being the most affected. As infectious diseases evolve and pathogens develop resistance to existing pharmaceuticals, the search for new novel leads, possibly with different modes of action against fungal, parasitic, bacterial, and viral diseases has intensified over the recent years (**GIBBONS, 2004; AVILA *et al*., 2008**). Antibiotics are used as antibacterial agents for the treatment of infectious diseases. However, this important resource is often scarce in most developing countries. It is for this reason that infectious diseases are still a significant cause of mortality and morbidity in developing countries (**HART and KARIUKI, 1998**). To these populations, plants offer a unique resource that provides a diverse array of natural products, which they exploit in the treatment of a diverse range of ailments.

To fully exploit the potential medical value of plants both in traditional and modern medicine, robust and multiple pharmacological screening for biological activities is essential. A single plant species may show good bioactivity on a number of bioassay tests and hence, if not toxic, may be used to treat such ailments in traditional medicine.

3.1.1. Bacterial infections

Bacteria forms one of the most abundant groups of unicellular organisms capable of adapting in a diverse range of environments (**SLEIGH and TIMBURY, 1998**). The bacterial group consists of microorganisms that are both useful and harmful to humans. Although not all bacterial species are harmful to humans, bacterial infections are widespread and ranked as one of the major causes of death worldwide (**SLEIGH and TIMBURY, 1998; ROTUN *et al*., 1999; AVILA *et al*., 2008**).

Bacteria are prokaryotes characterised by the presence of a cell wall that confers rigidity and protects against osmotic damage (**SLEIGH and TIMBURY, 1998**). Bacteria are broadly differentiated into two classes based on their cell wall structure. Gram-negative bacteria are characterised by the presence of an outer membrane, which contains lipopolysaccharides (LPS). It contains specific proteins, which include specific pore-forming proteins through which hydrophilic molecules are transported. The lipid is embedded in the outer membrane, whereas the polysaccharides which are anchored to the lipid, projects from the cell surface. The periplasmic space separates the peptidoglycan layer from the cytoplasmic membrane (**SLEIGH and TIMBURY, 1998**). In Gram-positive bacteria, the peptidoglycan layer is closely associated with the cytoplasmic membrane and is much thicker. They also lack a periplasmic space. Teichoic acids are part of the cell wall of Gram-positive bacteria and serves to maintain the level of divalent cations outside the cytoplasmic membrane. The Gram-staining technique is used to separate bacteria into these two classes (**DAVIES *et al*., 1983**).

Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae and *Staphylococcus aureus* are among the numerous medically important bacteria responsible for a number of infectious diseases. *Staphylococcus aureus* is a Gram-positive commensal bacterium that is commonly cited as being a major hospital-acquired pathogen (**ROTUN *et al*., 1999; RUBIN *et al*., 1999**). The bacterium is a causative agent for such infections as, septic arthritis, boils, conjunctivitis, endocarditis, pneumonia, skin infections, meningitis, wound infections and food poisoning (**SLEIGH and TIMBURY, 1998; RUBIN *et al*., 1999**). The ability of the strains of *S. aureus* to acquire resistance to practically all useful antibiotics (**GIBBONS, 2004**) is a cause for considerable concern in medical circles. *Bacillus subtilis* is a soil saprophyte, endospore-forming Gram-positive bacterium. It causes food poisoning and occasionally, some opportunistic infections such as conjunctivitis (**MURRAY *et al.*, 1998; RYAN and RAY, 2004**). *Escherichia coli*, a Gram-negative bacterium is present in mammals as part of the normal flora of the gastrointestinal tract. The bacterium is also a causative agent of infections such as, urinary tract and wound infections, bacteriaemia and pneumonia (**SLEIGH and TIMBURY, 1998**). The non-motile Gram-negative *K. pneumoniae* is responsible for urinary tract infections, septicaemia, meningitis and pneumonia in humans (**EINSTEIN, 2000**).

3.1.2. Fungal infections

Besides bacterial infections, humans are also battling with the challenges of fungal infections. The number of deaths due to fungi largely surpasses that by parasites in developed countries (**LATGÉ and CALDERONE, 2002**). As opposed to bacteria, very few fungal pathogens are causative agents of infectious diseases. *Candida albicans*, forms part of the normal flora of the respiratory, gastrointestinal and female genital tracts in humans and is responsible for up to 90% of fungal infections (**CHAITOW, 1996; SLEIGH and TIMBURY, 1998**). Immunosuppression is the key factor that triggers the establishment and dissemination of fungal infections. The establishment of an infection by a fungal pathogen and its invasion and growth in host tissues requires that the fungus is aggressive at a time when the host's immune response is debilitated (**LATGÉ and CALDERONE, 2002**). The opportunistic pathogens are normally eradicated by the innate immunity of the immunocompetent host. Up to 90% of all HIV/AIDS patients suffer fungal infections at some point during the course of the disease, with up to 20% of them dying as a direct consequence of such infections (**DIAMOND, 1991; CHAITOW, 1996**).

Responding to environmental cues, the usual unicellular yeast-like form of *C. albicans* in humans transforms into an invasive, multicellular filamentous form and becomes infectious to the host tissue (**ZACCHINO *et al*., 2003**). In HIV/AIDS patients, *C. albicans* infection initially leads to development of oral candidiasis, which may extend to the oesophagus and prevent intake of adequate oral nutrition, leading to increased morbidity and mortality (**KLEIN *et al*., 1984; REICHART, 2003**). Although *C. albicans* remains the most common opportunistic yeast pathogen in HIV/AIDS and other immunocompromised patients, other *Candida* species less susceptible to fluconazole are becoming more common (**ABI-SAID *et al*., 1997**). These include species such as, *C. dubliniensis*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. krusei*, and *C. guilliermondii* (**BARCHIESI *et al*., 1993; MEIS *et al*., 1999**). The role of *C. dubliniensis* as a pathogen has been limited to oral candidiasis.

In Africa, HIV/AIDS has become a devastating epidemic. In fact, widespread HIV infection in Africa has provided fertile ground for the transmission of infectious diseases (**WHO, 2007**). South Africa has the fastest growing HIV epidemic in the

world, with more than 5.7 million people being infected by 2007 (**UNAIDS, 2008**). The most affected population group are the poor. Owing to this high prevalence and the inadequate access and provision of health care systems for the vulnerable resource poor populations of South Africa, alternative management of candidiasis and other fungal infections, among HIV/AIDS patients, is therefore a priority.

3.1.3. Treatment of infectious diseases

The foremost figure in the modern fight against infectious disease was Alexander Fleming, the discoverer of penicillin, the first antibiotic, in 1929 (**BROWN, 2004**). Antibiotics have, since then, remained a pillar in the treatment of microbial infections in both humans and animals (**AVILA *et al*., 2008**). Bacterial species vary tremendously in their susceptibility to an antibiotic, for example, most strains of *Streptococcus pneumoniae* are inhibited by 0.01 µg/ml of benzylpenicillin (the minimum inhibitory concentration) whereas for *E. coli*, 32-64 µg/ml are required to inhibit growth, a level which cannot be achieved in the body (**HAWKEY, 1998**). Broad spectrum antibiotic agents such as cephalosporins and fluoroquinolones have been developed for the blanket treatment of a number of bacterial infections. The widespread use of broad spectrum agents together with misuse and various other factors have tilted the delicate balance between humans and bacteria. As a result, pathogens have developed resistance to antimicrobials. The number of efficacious drugs available clinically for the treatment of fungal infections is very limited. Antimicrobial resistance is also a major challenge in the treatment of fungal infections (**RUHNKE *et al*., 1994**). New alternatives to treating fungal infections are required to circumvent the often poorly efficient but expensive fungal treatments. Resistance to antibiotics constitutes a major threat to public health and has become a global concern. The search for alternative agents is therefore inevitable.

Resistance modifying agents are compounds which potentiate the activity of an antibiotic against a resistant strain (**GIBBONS, 2004**). These compounds may for example, specifically target a resistance mechanism, such as the inhibition of multidrug resistance (MDR). Pressure to find novel antimicrobial agents with new modes of action will drive exploitation of plant sources as antimicrobials. The choice

is logical given the ecological rationale that plants produce natural products as a chemical defence against microbes in their environment.

In order to assess the seasonal bioavailability of antimicrobial compounds between leaves and bulbs of frequently used traditional bulbs, antibacterial and antifungal evaluation was carried out using microdilution techniques against two Gram-positive (*B. subtilis* and *S. aureus*) and two Gram-negative (*E. coli* and *K. pneumoniae*) bacterial strains and a fungus *C. albicans*.

3.2. Materials and methods

3.2.1. Extract preparation

The extracts were prepared as described in **Section 2.2.3** (Chapter 2). The dried organic extracts were resuspended in 70% ethanol and water extracts in water to a concentration of 50 mg/ml.

3.2.2. Preparation of microbial stock cultures

Bacterial stock strains used for the antibacterial bioassays were cultured in Mueller-Hinton (MH) agar (Merck, Germany). Sterile (25 ml) MH was poured into plastic Petri dishes and allowed to gel. The plates were allowed to cool overnight at 4 °C after which stock strains of the bacteria were streaked and sub-cultured. The bacteria-inoculated plates were incubated at 37 °C for 24 h to allow bacterial colonies to develop. The plates were then stored at 4 °C to prevent further bacterial growth. The stocks were sub-cultured following the same procedure every 30 days in order to maintain viability. A similar procedure was followed for the maintenance of *C. albicans* with Yeast Malt (YM) agar (Becton Dickinson, USA) used in place of MH agar.

3.2.3. *In vitro* antibacterial bioassay

Minimum inhibitory concentration (MIC) values for the antibacterial activity of the plant extracts were determined using the microplate dilution technique (**ELOFF, 1998**). One hundred microlitres of each resuspended extract (50 mg/ml) were two-fold serially diluted with sterile distilled water, in duplicate, down the 96-well microtitre plate (Greiner Bio-one GmbH, Germany) for each of the four bacteria strains used. A similar two-fold serial dilution of neomycin (Sigma-Aldrich, Germany) (0.1 mg/ml) was used as a positive control for each bacterium. Water and 70% ethanol were included as negative/solvent controls. Overnight MH (Oxoid, England) broth cultures (incubated at 37 °C in a water bath with an orbital shaker) of four bacterial strains: two Gram-positive (*B. subtilis* American type culture collection (ATCC) 6051 and *S. aureus* ATCC 12600) and two Gram-negative (*E. coli* ATCC 11775 and *K. pneumoniae* ATCC 13883) were diluted with sterile MH broth to give a final inoculum of approximately 10^6 cfu/ml (colony forming units). One hundred microlitres of each bacterial culture were added to each well. The plates were covered with parafilm and incubated overnight at 37 °C. Bacterial growth was indicated by adding 50 µl of 0.2 mg/ml *p*-iodonitrotetrazolium chloride (INT) (Sigma-Aldrich, Germany) and a further incubation at 37 °C for 1 h. Since the colourless tetrazolium salt is biologically reduced to a red product due to the presence of active organisms, the MIC values were recorded as the concentrations in the last wells in which no colour change was observed after adding the INT indicator. Bacterial growth in the wells was indicated by a reddish-pink colour. The assay was repeated twice.

3.2.4. *In vitro* antifungal bioassay

A microdilution method as described by **ELOFF** (**1998**) and modified for fungi (**MASOKO *et al*., 2007**) was used to determine the antifungal activity of the extracts against *C. albicans* (ATCC 10231). An overnight fungal culture was prepared in YM (Oxoid, England) broth. Four hundred microlitres of the overnight culture were added to 4 ml of sterile saline (0.85% NaCl) and the absorbance was read at 530 nm using a UV-visible spectrophotometer (Varian Cary 50, Australia). The absorbance was adjusted with sterile saline to match that of a 0.5 M McFarland standard solution of

between 0.25-0.28 absorbance units. From this standardised fungal stock, a 1:1000 dilution with sterile YM broth was prepared giving a final inoculum of approximately 10^6 cfu/ml.

One hundred microlitres of each resuspended extract (50 mg/ml) were serially diluted two-fold with sterile water in a 96-well microtitre plate. A similar two-fold dilution of Amphotericin B (Sigma-Aldrich, Germany) (2.5 mg/ml) was used as the positive control while water, fungal free YM broth and 70% ethanol were used as negative and solvent controls respectively. One hundred microlitres of the dilute fungal culture were added to each well. The plates were covered with parafilm and incubated at 37 °C for 24 h after which 50 µl (0.2 mg/ml) INT were added and incubated for a further 24 h at 37 °C. The wells remained clear where there was inhibition of fungal growth. MIC values were recorded as the lowest concentrations that inhibited fungal growth after 48 h. To determine the fungicidal activity, 50 µl of sterile YM broth was added to all the clear wells, covered with parafilm and further incubated at 37 °C for 24 h after which the minimum fungicidal concentrations (MFC) were recorded as the last clear wells. The assay was repeated twice with two replicates per assay.

3.3. Results and discussion

3.3.1. Antibacterial activity

The antibacterial MIC values for the different sample extracts are presented in **Table 3.1.** There were no leaf samples for *D. robusta* in winter as the plant shed all their leaves during this season (**BRYAN, 1989**). Antibacterial MIC values less than, or equal to 1 mg/ml were considered as good activity (**ALIGIANNIS *et al.,* 2001**). The extracts exhibited a wide range of MIC values that ranged from 0.195 mg/ml to over 12.5 mg/ml. With the exception of *H. hemerocallidea* in summer and *M. plumbea* in winter, at least one extract of the leaves of the screened plant species exhibited good antibacterial activity against at least one of the test bacteria in all seasons. Bulb extracts of *D. robusta* did not show good activity in any of the four seasons. This is surprising due to its widespread utilisation in traditional medicine for the treatment of urinary infections and diseases of the uterus (**HUTCHINGS *et al*., 1996**), which may

be caused by *K. pneumoniae* and *B. subtilis*. The other bulb extracts, except *H. hemerocallidea* (spring) and *M. plumbea* (summer), were active against at least one test bacterial strain in each season. The best antibacterial activities were recorded in the winter season from the DCM bulb extracts of *T. violacea* against *K. pneumoniae* and *S. aureus,* and PE corm extracts of *H. hemerocallidea* against *B. subtilis*, with all having an MIC value of 0.195 mg/ml. This good activity in winter particularly from the bulb/corm extracts, suggest that the plants could be minimising metabolic cost by retrieving metabolites from senescing leaf tissues and transporting compounds to the underground parts (**McKEY, 1979**). Plants accumulate metabolites in various cellular and tissue structures during growth and development (**JANAKIRAM *et al*., 2000**) and some secondary metabolites form integral structural components of some tissues, for example, the polymeric phenolic compounds which form part of the cell wall (lignin) structure (**WALLACE and FRY, 1994**). Since the winter season marks the end of the senescence stage of most of these plants, accumulation of such secondary metabolites could have reached their highest levels. This may possibly be put forward as one of the explanations for the observed low MIC values of the extracts in winter.

Relatively low antibacterial activity was observed in most of the water extracts. MIC values were greater than 1 mg/ml except for some of the corm and leaf extracts of *H. hemerocallidea*, for which MIC values ranged from 0.39 and 0.78 mg/ml in summer, autumn and winter and *M. plumbea* leaves in summer. It is interesting to note that in most traditional herbal preparations, water is used as the major extractant (**HOFFMANN, 1989**), yet most of the water extracts tested in this study revealed poor antibacterial and antifungal activity. The trend is however, consistent with most of the findings in other studies (**RABE and VAN STADEN, 1997; MCGAW *et al*., 2001; UDDIN *et al*., 2008**). This suggests that most of the active constituents in these plant species are non-polar since water extracts mainly polar compounds. However, the results indicate that at least some of the biologically active compounds in *H. hemerocallidea* and *M. plumbea* (bulb) are polar and exhibit greater activity in autumn and winter (**Tables 3.1** and **3.2**). In comparing the different extracting solvents used, the good antibacterial activities were obtained from PE and DCM extracts. The two solvents extract mainly non-polar (lipophilic) compounds. Despite the high yields from water and ethanol extracts (**Table 2.2**), their antibacterial activity

was very poor in almost all the tested plants species in all the seasons. However, ethanol extracts from other plant species have shown good antibacterial activity in other research studies (**JÄGER, 2003**). The low activity shown by water extracts is particularly surprising due to the fact that most of the herbal remedies are prepared using water as a solvent (**HUTCHINGS *et al*., 1996; VAN WYK *et al*., 2009**). Infusions are prepared by macerating the plant material for a certain period of time in cold or boiling water (**VAN WYK *et al*., 2009**). Dosage is, however, important with regard to which solvent is being used. If water is used, the dosage would be higher, whereas the same dosage using a lipophilic solvent may be harmful. To address these shortcomings and possibly improve the potency of the traditional herbal preparations, exhaustive extraction mechanisms have to be employed.

In contrast, corms of *H. hemerocallidea* did not show good activity in spring but the activity shifted from the leaf to the corm in summer. The shift in the activity between plant organs and different seasons suggests a possible corresponding shift in and/or accumulation of some compounds responsible for the activity (**KOPTUR, 1985**) since most of these phytochemicals are produced in response to external stimuli (**KUBO *et al*., 1976; DERITA *et al*., 2009**) such as light intensity, moisture stress and temperature amongst others. It is possible therefore, that, according to the season of the year, the concentration levels and presence of these bioactive compounds could vary in parallel with the presence or absence of the stimuli, resulting in changing antibacterial properties. In addition to the intrinsic morphological, physiological and biochemical differences between bulbs and leaves, the observed differences in the activity between leaves and bulbs of the same plant could also be explained by dynamics in the production of the active compounds in response to stimulation factors.

The low activity shown by most extracts against the Gram-negative bacteria (*E. coli* and *K. pneumoniae)* was not surprising since these bacteria are more tolerant than Gram-positive bacteria (**PAZ *et al*., 1995; RABE and VAN STADEN, 1997; CHARIANDY *et al*., 1999**) owing to them having lipopolysaccharides on the outer membrane which presents a barrier to various antimicrobial molecules (**SLEIGH and TIMBURY, 1998**). The trend is however, consistent with the previous research findings (**RABE and VAN STADEN, 1997; SHALE *et al*., 1999**). The MIC values for

the winter extracts of *T. violacea* bulb (DCM), *H. hemerocallidea* corm (PE and water), *H. hemerocallidea* leaf (water), and *M. plumbea* bulb (DCM) against *K. pneumoniae,* and the water extracts of *H. hemerocallidea* corm (summer and autumn) and leaf (autumn and winter) against *E. coli,* indicate the potential value of these extracts in the development of pharmaceutical agents against Gram-negative bacteria. *Escherichia coli* and *K. pneumoniae* are known causative agents for diseases such as, urinary tract and wound infections, bacteriaemia, pneumonia septicaemia and meningitis in humans (**SLEIGH and TIMBURY, 1998; EINSTEIN, 2000**)

Table 3.1. Antibacterial activity (MIC mg/ml) in bulb/corm and leaf extracts of *T. violacea*, *H. hemerocallidea, D. robusta* and *M. plumbea* in spring, summer, autumn and winter seasons.

Plant species	Part	Extract	Spring				Summer				Autumn				Winter			
			Bs	Ec	Kp	Sa	Bs	Ec	Kp	Sa	Bs	Ec	Kp	Sa	Bs	Ec	Kp	Sa
Tulbaghia violacea	Bulb	PE	**0.78**	3.125	1.56	**0.78**	3.125	3.125	1.56	3.125	3.125	3.125	3.125	3.125	**0.78**	3.125	1.56	**0.39**
		DCM	**0.78**	3.125	**0.78**	**0.78**	**0.78**	3.125	1.56	3.125	**0.78**	3.125	3.125	3.125	**0.39**	1.56	**0.195**	**0.195**
		EtOH	6.25	3.125	1.56	12.5	6.25	3.125	1.56	12.5	3.125	1.56	1.56	6.25	6.25	3.125	3.125	3.125
		Water	>12.5	>12.5	3.125	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	12.5	>12.5	12.5	12.5	6.25	12.5
	Leaf	PE	1.56	3.125	1.56	3.125	**0.78**	1.56	1.56	1.56	**0.78**	1.56	3.125	**0.78**	1.56	1.56	6.25	3.125
		DCM	**0.78**	3.125	1.56	**0.78**	**0.39**	1.56	1.56	3.125	**0.78**	1.56	1.56	3.125	**0.78**	1.56	3.125	3.125
		EtOH	6.25	3.125	12.5	12.5	6.25	1.56	1.56	6.25	6.25	3.125	1.56	6.25	3.125	3.125	3.125	12.5
		Water	>12.5	12.5	12.5	12.5	>12.5	12.5	6.25	12.5	>12.5	6.25	12.5	12.5	12.5	>12.5	6.25	12.5
Hypoxis hemerocallidea	Corm	PE	1.56	6.25	3.125	6.25	3.125	3.125	3.125	6.25	3.125	3.125	3.125	3.125	**0.195**	1.56	**0.78**	1.56
		DCM	1.56	3.125	3.125	3.125	3.125	3.125	3.125	3.125	1.56	3.125	3.125	3.125	1.56	3.125	1.56	1.56
		EtOH	3.125	3.125	1.56	3.125	**0.78**	1.56	1.56	3.125	3.125	3.125	1.56	3.125	3.125	3.125	1.56	3.125
		Water	>12.5	12.5	3.125	12.5	**0.78**	**0.78**	1.56	**0.39**	1.56	**0.78**	1.56	**0.78**	**0.78**	1.56	**0.39**	**0.78**
	Leaf	PE	**0.78**	3.125	3.125	3.125	3.125	3.125	3.125	6.25	3.125	3.125	3.125	3.125	3.125	3.125	3.125	6.25
		DCM	**0.39**	6.25	3.125	1.56	3.125	3.125	1.56	3.125	3.125	3.125	3.125	3.125	1.56	1.56	**0.78**	1.56
		EtOH	1.56	3.125	1.56	**0.78**	1.56	1.56	1.56	1.56	3.125	1.56	1.56	**0.78**	1.56	1.56	1.56	1.56
		Water	>12.5	>12.5	3.125	>12.5	3.125	3.125	3.125	1.56	1.56	**0.78**	**0.78**	**0.78**	**0.78**	**0.78**	**0.78**	**0.78**
Drimia robusta	Bulb	PE	3.125	3.125	3.125	6.25	3.125	3.125	3.125	6.25	3.125	3.125	3.125	3.125	3.125	3.125	3.125	6.25
		DCM	3.125	3.125	3.125	3.125	3.125	3.125	3.125	3.125	3.125	3.125	6.25	3.125	1.56	3.125	3.125	3.125
		EtOH	>12.5	3.125	1.56	1.56	6.25	3.125	3.125	12.5	6.25	3.125	3.125	12.5	6.25	6.25	6.25	12.5
		Water	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	12.5	>12.5	>12.5	>12.5	>12.5	12.5	6.25	12.5	12.5

Plant species	Part	Extract	Spring				Summer				Autumn				Winter			
			Bs	Ec	Kp	Sa	Bs	Ec	Kp	Sa	Bs	Ec	Kp	Sa	Bs	Ec	Kp	Sa
Drimia robusta	Leaf	PE	1.56	3.125	1.56	1.56	**0.78**	3.125	3.125	3.125	1.56	1.56	3.125	3.125	-	-	-	-
		DCM	**0.78**	3.125	3.125	3.125	1.56	1.56	3.125	3.125	**0.78**	1.56	1.56	**0.78**	-	-	-	-
		EtOH	3.125	3.125	1.56	3.125	3.125	1.56	3.125	3.125	6.25	1.56	1.56	1.56	-	-	-	-
		Water	>12.5	6.25	6.25	3.125	3.125	3.125	3.125	3.125	6.25	3.125	6.25	3.125	-	-	-	-
Merwilla plumbea	Bulb	PE	6.25	6.25	6.25	12.5	3.125	6.25	3.125	12.5	3.125	3.125	3.125	3.125	6.25	6.25	6.25	12.5
		DCM	**0.78**	3.125	3.125	12.5	3.125	3.125	3.125	3.125	**0.78**	1.56	3.125	3.125	**0.78**	3.125	**0.78**	**0.78**
		EtOH	3.125	3.125	3.125	1.56	12.5	6.25	6.25	12.5	12.5	6.25	6.25	3.125	6.25	6.25	6.25	12.5
		Water	>12.5	>12.5	>12.5	12.5	3.125	6.25	12.5	3.125	3.125	6.25	3.125	**0.78**	12.5	12.5	>12.5	>12.5
	Leaf	PE	6.25	6.25	3.125	6.25	1.56	3.125	3.125	3.125	3.125	3.125	3.125	3.125	3.125	3.125	3.125	3.125
		DCM	1.56	3.125	3.125	**0.78**	1.56	3.125	1.56	1.56	3.125	1.56	3.125	**0.78**	1.56	1.56	1.56	1.56
		EtOH	3.125	3.125	3.125	3.125	3.125	1.56	3.125	3.125	6.25	1.56	3.125	1.56	3.125	3.125	3.125	3.125
		Water	6.25	12.5	>12.5	12.5	3.125	3.125	3.125	**0.78**	3.125	6.25	3.125	6.25	1.56	1.56	3.125	3.125
Neomycin (µg/ml)			1.95	0.98	0.49	1.95	1.95	0.98	0.49	1.95	1.95	0.98	0.49	1.95	1.95	0.98	0.49	1.95

Bs = *Bacillus subtilis*, Ec = *Escherichia coli*, Kp = *Klebsiella pneumoniae*, Sa = *Staphylococcus aureus.*

Values boldly written are considered very active (MIC < 1 mg/ml)

PE = Petroleum ether, DCM = Dichloromethane, EtOH = 80% ethanol

3.3.2. Antifungal activity

At least one leaf extract in all of the four plant species analysed showed good antifungal activity (MIC < 1 mg/ml) (**Table 3.2**) against *C. albicans* in all the seasons except for *T. violacea* (winter) and *M. plumbea* (summer and winter). Very few bulb extracts showed good fungistatic (MIC) activities, with *M. plumbea* yielding no active extracts in any of the seasons. However, in determining the minimum fungicidal concentrations (MFC), some of the extracts with good MIC (< 1 mg/ml) values did not have a correspondingly good MFC, suggesting that such extracts have fungistatic effects. The fungistatic effect is of little application in the development of good antifungal drugs. An ideal antifungal drug should be composed of compound(s) that are fungicidal with a selective mechanism of action (**POLAK, 1999; ZACCHINO *et al*., 2003**). To understand the mechanisms of action of extracts which showed good fungicidal activity, further research on their mode of antifungal action would be necessary.

Extracts of plant material gathered in autumn showed better fungicidal activity (MFC) compared to the plant extracts gathered from the other seasons. This could be as a result of the accumulation of the active compounds during this season in response to favourable stimuli. The compounds, on the other hand could have accumulated during the preceding spring and summer seasons and reached high concentration levels in autumn. Autumn marks the beginning of the senescence phase of plant growth in most of the plant species and accumulation of dry matter and other compounds are expected to have reached their maximum at this stage (**BIDWELL, 1974; RIIPI *et al*., 2002**). The good fungicidal activity by the extracts in autumn was however, not reflected in the proceeding winter season. The co-occurring changes in physical foliage characteristics (senescence) and bioactivity of several plant extracts between the two seasons indicates a decline in foliage suitability for use as antifungal agents in winter. For the treatment of fungal ailments in TM, based on the findings from this study, the use of plant material collected in autumn appears to be ideal.

Although good MIC activity in the leaf extracts was maintained across seasons, there tended to be a shift in some cases in the activity from one solvent extract to another.

For example, with *H. hemerocallidea* leaf extracts, an MIC value of 0.78 mg/ml was recorded from the DCM, ethanol and water extracts in spring. In summer, this activity was lost from these three extracts and shifted to the PE extract. This sudden shift suggests that the activity cannot solely be attributed to a single compound but rather different compounds or combinations with the same and/or synergistic effects on the fungus. Particularly interesting is that only ethanol and water extracts of *H. hemerocallidea* corms showed good MFC activity in autumn and winter amongst all the bulb extracts. Lack of fungicidal activity (MFC > 1 mg/ml) from bulb extracts could be due to lack or low concentration of the active compound(s) in these extracts (**RABE and VAN STADEN, 1997; NACIF DE ABREU and MAZZAFERA, 2005**).

Table 3.2. Antifungal activity (MIC and MFC mg/ml) in bulb/corm and leaf extracts of *T. violacea*, *H. hemerocallidea, D. robusta* and *M. plumbea* in spring, summer, autumn and winter seasons.

Plant species	Part	Extract	Spring		Summer		Autumn		Winter	
			MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Tulbaghia violacea	Bulb	PE	6.25	6.25	3.13	6.25	**0.78**	1.56	1.56	3.13
		DCM	3.13	6.25	1.56	3.13	1.56	3.13	**0.78**	1.56
		EtOH	6.25	12.5	3.13	6.25	3.13	6.25	**0.78**	3.13
		Water	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	12.5	>12.5
	Leaf	PE	**0.78**	1.56	**0.39**	6.25	**0.78**	**0.78**	3.13	3.13
		DCM	1.56	1.56	**0.78**	**0.78**	**0.78**	**0.78**	1.56	1.56
		EtOH	3.13	6.25	3.13	6.25	3.13	3.13	3.13	3.13
		Water	12.5	12.5	12.5	12.5	12.5	12.5	12.5	>12.5
Hypoxis hemerocallidea	Corm	PE	6.25	6.25	6.25	6.25	3.13	6.25	3.13	6.25
		DCM	6.25	6.25	3.13	3.13	**0.78**	1.56	3.13	3.13
		EtOH	3.13	3.13	3.13	3.13	**0.78**	**0.78**	3.13	3.13
		Water	3.13	3.13	3.13	3.13	**0.39**	**0.39**	**0.39**	**0.39**
	Leaf	PE	6.25	6.25	**0.78**	6.25	3.13	3.13	3.13	6.25
		DCM	**0.78**	3.13	1.56	1.56	1.56	3.13	3.13	3.13
		EtOH	**0.78**	3.13	3.13	3.13	3.13	3.13	1.56	3.13
		Water	**0.78**	**0.78**	3.13	3.13	**0.39**	**0.78**	**0.78**	3.13
Drimia robusta	Bulb	PE	6.25	6.25	**0.78**	6.25	**0.78**	3.13	3.13	6.25
		DCM	6.25	6.25	6.25	6.25	3.13	3.13	3.13	6.25
		EtOH	**0.39**	3.13	6.25	6.25	6.25	6.25	3.13	3.13
		Water	>12.5	>12.5	>12.5	>12.5	12.5	12.5	12.5	12.5
	Leaf	PE	**0.39**	3.13	**0.78**	6.25	**0.78**	**0.78**	-	-
		DCM	**0.78**	**0.78**	1.56	1.56	**0.39**	**0.78**	-	-
		EtOH	6.25	6.25	**0.78**	3.13	1.56	3.13	-	-
		Water	3.13	3.13	3.13	6.25	3.13	3.13	-	-
Merwilla plumbea	Bulb	PE	6.25	6.25	6.25	6.25	3.13	3.13	3.13	6.25
		DCM	6.25	6.25	3.13	6.25	3.13	3.13	1.56	6.25
		EtOH	6.25	12.5	3.13	12.5	3.13	6.25	1.56	6.25
		Water	>12.5	>12.5	>12.5	>12.5	3.13	6.25	12.5	12.5

Plant species	Part	Extract	Spring		Summer		Autumn		Winter	
			MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
	Leaf	PE	3.13	6.25	1.56	6.25	1.56	1.56	3.13	3.13
Merwilla		DCM	**0.39**	**0.78**	3.13	3.13	**0.78**	**0.78**	3.13	3.13
plumbea		EtOH	3.13	6.25	3.13	3.13	**0.78**	**0.78**	3.13	6.25
		Water	6.25	6.25	6.25	12.5	3.13	3.13	6.25	6.25
Amphotericin B (µg/ml)			9.77	78.1	9.77	78.1	9.77	78.1	9.77	78.1

Values boldly written are considered very active (MIC and MFC < 1 mg/ml)

PE = Petroleum ether, DCM = Dichloromethane, EtOH = 80% ethanol

3.3.3. Total activity of plants extracts in different seasons

Relating the extract yields (%) (**Table 2.2**) with antibacterial and antifungal activity (mg/ml), the total activity (g/ml) of the plant extracts in different seasons were calculated. **Tables 3.3** and **3.4** present total activity of the plant extracts against the tested bacterial and fungal strains respectively. In comparing the total activity of the active extracts of each extracting solvent across seasons, it was noted that in some instances, the activity varied from one season to another. For example, the yield (0.61%: equivalent to 6.1 mg/g) and MIC value (0.78 mg/ml) of the DCM leaf extracts of *T. violacea* in spring against *B. subtilis* gives a total activity of 7.8 ml/g [(6.1 mg/g)/0.78 mg/ml] (**ELOFF, 2004**) compared to the total activity of 24.6 ml/g [(9.6 mg/g)/0.39 mg/ml] of the same extract against the same bacterium in summer (**Table 3.3**). Total activity (ml/g) indicates the degree to which the active compound(s) in one gram of plant material can be diluted and still inhibit the growth of the tested microorganism (**ELOFF, 2000**). This implies that *T. violacea* DCM leaf extracts prepared from 1 g of plant material in summer could be diluted up to a volume of 24.6 ml and still retains activity against *B. subtilis*, while only 7.8 ml would be required for the same extracts to retain activity against the same bacterium in spring. This, therefore, indicates that gram for gram, DCM leaf extracts of *T. violacea* plants had a more concentrated active compound(s) in summer than in spring.

A comparison between the DCM bulb and leaf extracts of *T. violacea* against *B. subtilis*, on a season to season basis, reveals that leaf extracts had more concentrated active compounds compared to bulb extracts. The maximum dilutions for the DCM leaf extracts of *T. violacea* were; 7.8 ml/g, 24.6 ml/g, 12.6 ml/g, and 17.9 ml/g in spring, summer, autumn and winter respectively, compared with 5.5 ml/g, 3.2 ml/g, 3.5 ml/g and 18.2 ml/g from the DCM bulb extracts in the same corresponding seasons. The leaf extracts of *T. violacea* demonstrates a high concentration of active compound(s) against *B. subtilis* in all the seasons except winter. Harvesting of *T. violacea* leaves for the treatment of diseases caused by *B. subtilis*, based on the results from this study, would be considered ideal in spring, summer and autumn, and bulbs should be in winter.

Among the extracts that showed good antibacterial activity against Gram-negative bacterial strains (*E. coli* and *K. pneumoniae*), corm water extracts of *H. hemerocallidea* showed a maximum dilution volume of up to 126.9 ml in summer against *E. coli*, while in autumn the same extract had a dilution volume of 116.7 ml against the same bacterial strain. Although the dilution volume dropped by 10.2 ml in autumn, total activity remained high, an indication that the active compound(s) in these extracts were produced in high quantities during the two seasons. In winter, however, *H. hemerocallidea* corm water extracts lost activity against this bacterium and instead, had a dilution volume of 161.5 ml against the other Gram-negative bacterium, *K. pneumoniae.* In comparison with the corm water extracts, leaf water extracts of *H. hemerocallidea* recorded had a dilution volume of 120.5 ml against both Gram-negative bacterial strains (*E. coli* and *K. pneumoniae*) in autumn and 85.9 ml in winter against the two strains. These results indicate that in winter and autumn, *H. hemerocallidea* leaves produced higher quantities of the active compound(s), although the concentrations indicated a slight decrease in winter. Considering the scarcity of plant extracts with good antibacterial activity against Gram-negative bacteria in most of the previous studies with different plant materials (**RABE and VAN STADEN, 1997; SHALE *et al*., 1999**), *H. hemerocallidea* offers good prospects for the treatment of diseases caused by these bacteria in traditional medicine. Based on the results from this study, a conservation strategy for *H. hemerocallidea*, that would take into consideration, sustainable harvesting and simultaneously provides medicinal benefits, would be the harvesting of leaves in spring, autumn and winter, and corms in summer. The seasons coincide with the good antibacterial activity exhibited by the corm and leaf extracts and where both extracts show good activity, (e.g. autumn and winter in the current study), harvesting of leaves would be considered more sustainable.

Seasonal variation was also observed in the DCM leaf extracts of *D. robusta*, which had a total activity of 9.6 ml/g against *B. subtilis* in spring and the dilution volume increased by 3.2 ml to give a total activity of 12.8 ml/g against the same bacterium in autumn. The same extracts however, recorded no good activity in summer. For the DCM bulb extracts of *M. plumbea*, a total activity of 5.9 ml/g was recorded against *B. subtilis* in spring while no activity was recorded in summer, and 7.7 ml/g and 6.9 ml/g against the same bacterium in autumn and winter respectively. The DCM extracts of

M. plumbea had a dilution volume of 14.1 ml against *S. aureus* in spring, with no activity in summer and winter, and the dilution volume was 16.7 ml against the same bacterium in autumn.

The total activity of the extracts against *C. albicans* is presented in **Table 3.4.** Of those extracts that showed good fungicidal activity (MFC < 1 mg/ml), the highest dilution volume of 433.3 ml each, were recorded in the corm water extracts of *H. hemerocallidea* in autumn and winter respectively. Plant material gathered in autumn, appeared to have good total fungicidal activity compared to extracts collected in the other seasons. At least one extract of all the tested plant species, exhibited good fungicidal activity in autumn. In light of the fact that *C. albicans* is resistant to most of the screened plant extracts (**HEISEY AND GORHAM, 1992; BUWA and VAN STADEN, 2006**), the high dilution volumes exhibited by corm extracts (EtOH and water) and leaf extracts (water) of *H. hemerocallidea* and leaf extracts (EtOH) of *M. plumbea* in autumn, demonstrate the high concentration of anticandidal compounds in these extracts. These, together with all the other extracts that showed good fungicidal activity, offer promising prospects for the treatment of candidiasis particularly for HIV/AIDS patients. Candidiasis is a common opportunistic infection among HIV/AIDS patients and is one of the major causes of death in developing countries (**REICHART, 2003**).

By considering not only the biological activity (MIC values) of the plant extracts but also the total quantity extracted from the plant material and determining the maximum dilution volume, more valuable information on the best sources of plant material for use in the traditional medicine can be made available. The information also comes in as a handy tool for the isolation of active compounds from plant materials. Results of the antimicrobial and total activity from the current study identify leaves as having better activity than bulbs and can thus complement or substitute bulbs/corms in traditional medicine as a conservation strategy.

Table 3.3. Total antibacterial activity (ml/g) in bulb/corm and leaf extracts of *T. violacea*, *H. hemerocallidea, D. robusta* and *M. plumbea* in spring, summer, autumn and winter seasons.

Plant species	Part	Extract	Total activity (ml/g)															
			Spring				Summer				Autumn				Winter			
			Bs	Ec	Kp	Sa	Bs	Ec	Kp	Sa	Bs	Ec	Kp	Sa	Bs	Ec	Kp	Sa
Tulbaghia violacea	Bulb	PE	**4.4**	1.1	2.2	**4.4**	1.1	1.1	2.1	1.1	1.8	1.8	1.8	1.8	**5.8**	1.4	2.9	**11.5**
		DCM	**5.5**	1.4	**5.5**	**5.5**	**3.2**	0.8	1.6	0.5	**3.5**	0.9	0.9	0.9	**18.2**	4.6	**36.4**	**36.4**
		EtOH	13.12	26.2	52.6	6.6	17.9	35.8	71.8	9.0	23.4	46.8	46.8	11.7	18.6	37.1	37.1	37.1
		Water	>22.3	>22.3	89.3	>22.3	>26.8	>26.8	>26.8	>26.8	>29.2	>29.2	29.2	>29.2	33.2	33.2	132.8	33.5
	Leaf	PE	6.1	3.0	6.1	3.0	**20.5**	10.3	10.3	10.3	**19.2**	9.6	4.8	**19.2**	8.3	8.3	2.1	4.2
		DCM	**7.8**	2.0	3.9	**7.8**	**24.6**	6.2	6.2	3.1	**12.6**	6.3	6.3	3.1	**17.9**	9.0	4.5	4.5
		EtOH	41.4	82.9	20.7	20.7	42.0	167.9	167.9	42.0	38.4	76.8	153.8	38.4	76.5	76.5	76.5	19.1
		Water	>13.8	13.8	13.8	13.8	>14.6	14.64	29.3	14.64	>13.5	27.0	13.5	13.5	13.5	>13.5	27.0	13.5
Hypoxis hemerocallidea	Corm	PE	1.5	0.4	0.7	0.4	1.1	1.1	1.1	0.6	0.9	0.9	0.9	0.9	**11.8**	1.5	**2.9**	1.5
		DCM	1.0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1.0	0.5	0.5	0.5	1.7	0.8	1.7	1.7
		EtOH	72.6	72.6	145.5	72.6	**296.2**	148.1	148.1	73.9	68.8	68.8	137.8	68.8	95.4	95.4	191.0	95.4
		Water	>10.6	10.6	42.6	10.6	**126.9**	**126.9**	63.5	**253.8**	58.3	**116.7**	58.3	**116.7**	**80.8**	40.4	**161.5**	**80.8**
	Leaf	PE	**9.9**	2.5	2.5	2.5	3.1	3.1	3.1	1.6	2.6	2.6	2.6	2.6	2.4	2.4	2.4	1.2
		DCM	**13.6**	0.8	1.7	3.4	2.1	2.1	4.3	2.1	2.7	2.7	2.7	2.7	4.2	4.2	**8.5**	4.2
		EtOH	66.7	33.3	66.7	**133.3**	88.5	88.5	88.5	88.5	36.5	73.1	73.1	**146.2**	56.4	56.4	56.4	56.4
		Water	>7.1	>7.1	28.5	>7.1	25.6	25.6	25.6	51.3	60.3	**120.5**	**120.5**	**120.5**	**85.9**	**85.9**	**85.9**	**85.9**
Drimia robusta	Bulb	PE	1.3	1.3	1.3	0.6	1.5	1.5	1.5	0.6	1.7	1.7	1.7	1.7	1.2	1.2	1.2	0.6
		DCM	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.5	1.0	2.4	1.2	1.2	1.2
		EtOH	>24.4	97.6	195.5	195.5	40.0	80.0	80.0	20.0	46.2	92.5	92.5	23.1	47.8	47.8	47.8	23.9
		Water	>20.0	>20.0	>20.0	>20.0	>16.6	>16.6	>16.6	16.6	>18.3	>18.3	>18.3	>18.3	19.3	38.6	19.3	19.3

Plant species	Part	Extract	Total activity (ml/g)															
			Spring				Summer				Autumn				Winter			
			Bs	Ec	Kp	Sa	Bs	Ec	Kp	Sa	Bs	Ec	Kp	Sa	Bs	Ec	Kp	Sa
Drimia robusta	Leaf	PE	7.7	3.8	7.7	7.7	**12.8**	3.2	3.2	3.2	13.5	13.5	6.7	6.7	-	-	-	-
		DCM	**9.6**	2.4	2.4	2.4	3.3	3.3	1.7	1.7	**12.8**	6.4	6.4	**12.8**	-	-	-	-
		EtOH	34.2	34.2	1.56	34.2	59.2	1.56	59.2	59.2	27.5	110.5	110.5	110.5	-	-	-	-
		Water	>12.4	24.8	24.8	49.6	36.5	36.5	36.5	36.5	11.5	23.0	11.5	23.0	-	-	-	-
Merwilla plumbea	Bulb	PE	0.7	0.7	0.7	0.4	1.3	0.66	1.3	0.3	1.7	1.7	1.7	1.7	0.6	0.6	0.6	0.3
		DCM	**5.9**	1.5	1.5	0.4	1.7	1.7	1.7	1.7	**7.7**	3.8	1.9	1.9	**6.9**	3.125	**6.9**	**6.9**
		EtOH	77.4	77.4	77.4	155.1	18.0	36.0	36.0	18.0	13.8	27.7	27.7	55.4	50.1	50.1	50.1	25.0
		Water	>5.9	>5.9	>5.9	5.9	17.9	9.0	4.5	17.9	43.5	21.8	43.5	**174.4**	9.8	9.8	>9.8	>9.8
	Leaf	PE	2.1	2.1	4.2	2.1	8.3	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.5	4.5	4.5	4.5
		DCM	7.1	3.5	3.5	**14.1**	11.2	5.6	11.2	11.2	4.2	8.3	4.2	**16.7**	6.3	6.3	6.3	6.3
		EtOH	54.7	54.7	54.7	54.7	51.2	102.6	51.2	51.2	26.2	105.1	52.5	105.1	48.6	48.6	48.6	48.6
		Water	23.2	11.6	>11.6	11.6	46.7	46.7	46.7	**187.2**	49.6	24.8	49.6	24.8	69.2	69.2	34.6	34.6

Bs = *Bacillus subtilis*, Ec = *Escherichia coli*, Kp = *Klebsiella pneumoniae*, Sa = *Staphylococcus aureus.*

Values boldly written represent total activity of extracts that were considered active in the antibacterial bioassay

PE = Petroleum ether, DCM = Dichloromethane, EtOH = 80% ethanol

Table 3.4. Total antifungal activity (ml/g) in bulb/corm and leaf extracts of *T. violacea*, *H. hemerocallidea, D. robusta* and *M. plumbea* in spring, summer, autumn and winter seasons.

Plant species	Part	Extract	Total activity (ml/g)							
			Spring		Summer		Autumn		Winter	
			FS	FC	FS	FC	FS	FC	FS	FC
Tulbaghia violacea	Bulb	PE	0.5	0.5	1.1	0.5	**7.3**	3.7	2.9	1.4
		DCM	1.4	0.7	1.6	0.8	1.7	0.9	**9.1**	4.6
		EtOH	13.1	6.6	35.8	17.9	23.3	11.7	**148.7**	37.1
		Water	>22.3	>22.3	>26.8	>26.8	>29.2	>29.2	33.2	>33.2
	Leaf	PE	**12.2**	6.1	**41.0**	2.56	**19.2**	**19.2**	11.5	11.5
		DCM	3.9	3.9	**12.3**	**12.3**	**12.7**	**12.7**	8.9	8.9
		EtOH	82.7	41.4	83.7	41.9	76.7	76.7	76.4	76.4
		Water	13.8	13.8	14.6	14.6	13.5	13.5	13.5	>13.5
Hypoxis hemerocallidea	Corm	PE	0.4	0.4	0.6	0.6	0.9	0.5	0.7	0.4
		DCM	0.3	0.3	0.5	0.5	**2.1**	1.0	0.8	0.8
		EtOH	72.5	72.5	73.8	73.8	**275.6**	**275.6**	95.2	95.2
		Water	55.3	55.3	58.5	58.5	**433.3**	**433.3**	**433.3**	**433.3**
	Leaf	PE	1.2	1.2	**12.4**	1.6	2.6	2.6	2.4	1.2
		DCM	**6.8**	1.7	4.3	4.3	5.3	2.7	2.1	2.1
		EtOH	**133.3**	33.2	44.1	44.1	36.4	36.4	56.4	28.1
		Water	**114.1**	114.1	25.5	25.5	**241.0**	**120.5**	**85.9**	21.4
Drimia robusta	Bulb	PE	0.6	0.6	**6.0**	0.75	**6.7**	1.7	1.2	0.6
		DCM	0.5	0.5	0.5	0.5	1.0	1.0	1.2	0.6
		EtOH	**782.1**	97.4	40.0	40.0	46.2	46.2	95.5	95.5
		Water	>19.2	>19.2	>16.6	>16.6	18.3	18.3	19.3	19.3
	Leaf	PE	**30.8**	3.8	**12.8**	1.6	**26.9**	**26.9**	-	-
		DCM	**9.6**	**9.6**	3.3	3.3	**25.6**	**12.8**	-	-
		EtOH	34.4	34.4	**237.2**	59.1	110.3	55.0	-	-
		Water	49.5	49.5	36.4	18.2	23.0	23.0	-	-
Merwilla plumbea	Bulb	PE	0.7	0.7	0.7	0.7	1.7	1.7	1.2	0.6
		DCM	0.7	0.7	1.7	0.9	1.9	1.9	3.5	0.9
		EtOH	38.7	19.4	71.9	18.0	55.3	27.7	200.6	50.1
		Water	>5.9	>5.9	>4.5	>4.5	43.5	21.8	9.8	9.8

Plant species	Part	Extract	Total activity (ml/g)							
			Spring		Summer		Autumn		Winter	
			FS	FC	FS	FC	FS	FC	FS	FC
	Leaf	PE	4.2	2.1	8.3	2.1	8.3	8.3	4.5	4.5
Merwilla		DCM	**28.2**	**14.1**	5.6	5.6	**16.7**	**16.7**	3.2	3.2
plumbea		EtOH	54.6	27.4	51.1	51.1	**210.3**	**210.3**	48.6	24.3
		Water	23.2	23.2	23.4	11.7	49.5	49.5	17.3	17.3

Values boldly written represent total activity of extracts that were active in the antifungal bioassay

PE = Petroleum ether, DCM = Dichloromethane, EtOH = 80% ethanol

FS = Fungistatic (MIC in the antifungal assay), FC = Fungicidal (MFC in the antifungal assay)

3.4. Conclusions

The results of the current study showed that leaves have comparable and in some cases better antimicrobial activity than bulbs. The leaves of these plant species could feasibly be harvested for medicinal use against fungal and bacterial infections to improve the sustainability of these medicinal plant resources. Harvesting of leaves inflict much less damage on the plant than harvesting bulbs/corms. However, the time of harvest will depend on the plant part to be used since it has been established that the level of bioactivity can vary in different plant parts in different seasons. Water extracts of *H. hemerocallidea* showed good activity against at least two test bacteria in autumn and winter seasons while in *T. violacea* bulb, DCM extracts had the best antibacterial activity with an MIC value of 0.195 mg/ml against *K. pneumoniae* and *S. aureus* in winter. The bulb extracts (DCM) of *M. plumbea* also demonstrated good antibacterial activity against three test bacteria, *B. subtilis, K. pneumoniae,* and *S. aureus* in winter. For antibacterial activity, winter seem to be an ideal season for collection.

Leaves demonstrated better antifungal activity across the four seasons compared to bulbs. Regarding, the marked differences in antifungal activities noticed between the leaves and bulbs, the use of leaves in the treatment of fungal infections would be more rational than the use of bulbs. Total activity results identify plant material gathered in autumn as comparably high levels of fungicidal compounds in most plant species. The use of these of plant material, gathered in autumn for the treatment of candidal infections, will most probably yield better results. What is needed to progress these leads, is further antimicrobial screening against other resistant, clinically relevant bacterial and fungal strains. Research aimed at identifying specific compounds responsible for the observed bioactivity and environmental conditions that enhance production of these and other compounds could have a positive impact on the economic prospects of these medicinal plants and their future role in public health. The high total activity shown by the water extracts of *H. hemerocallidea* warrants further investigation with the possibility of isolating the active compound(s).

CHAPTER 4

Cyclooxygenase Enzyme Inhibition

4.1. Introduction

The immune system of the body is composed of many interactive, specialized cell types that collectively protect the body from pathogenic infections and other sources of stimuli. Inflammation is one of the first responses of the immune system to infection involving the recruitment of immune cells to the site of injury. In addition, an inflammatory reaction serves to establish a physiological barrier against the spread of infection and to promote healing of any damaged tissue following the clearance of the stimuli (**COHN and LANGMAN, 1996**). Inflammation is therefore defined as a localized protective, non-specific immune response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants (**FERRERO-MILIANI *et al*., 2006**).

The complex process of inflammation begins with the mobilisation of arachidonic acid (AA) from the membrane phospholipids through the action of the enzyme phospholipase A_2 (PLA_2) following stimulation (**PATRIGNANI *et al*., 2005**). The liberated AA serves as the substrate to the rate limiting step in prostanoid biosynthesis, catalysed by cyclooxygenase (COX) enzymes. Prostanoids are lipid mediators that coordinate a wide variety of physiologic and pathologic processes via membrane receptors on the surface of target cells (**FITZPATRICK and SOBERMAN, 2001**). They produce a remarkably broad spectrum of effects that embraces practically every biological function as well as being of prime importance as mediators of pain, fever and swelling during inflammation. Under physiologic conditions, prostanoids play an important role in the cytoprotection of the gastric mucosa, haemostasis, and renal haemodynamics (**PATRIGNANI *et al*., 2005**). COX enzymes catalyse the transformation of AA into an unstable prostaglandin H_2 (PGH_2) from which all other prostanoids are metabolised through isomerases with different structures and exhibiting cell- and tissue-specific distribution (**PATRIGNANI *et al*., 2005**).

4.1.1. Cyclooxygenase (COX) enzymes in inflammation

Two COX enzymes, COX-1 and COX-2, are responsible for the production of prostaglandin H_2 (PGH_2). The expression of the two COX isozymes is differently regulated but the enzymes are moderately similar in their amino acid sequences (**FLETCHER *et al.*, 1992**). The constitutive enzyme, COX-1, encoded by a 2.8-kilobase transcript located on human chromosome 9, serves certain physiologic housekeeping functions, such as thrombogenic thromboxane A_2 generation by platelets, antithrombogenic PGI_2 production by endothelial cells, generation of cytoprotective prostanoids in the gastric mucosa, and regulation of renal blood flow by prostanoids (**FLETCHER *et al.*, 1992; MURAKAMI *et al.*, 1994; OSIRI and MORELAND, 1999; SMITH and LANGENBACH, 2001**). In contrast, expression of the inducible COX-2, encoded by a 4.4-kilobase transcript from human chromosome 1, is found almost exclusively in inflamed tissues responding to inflammatory stimuli such as proinflammatory cytokines, growth factors, mitogens, bacterial lipopolysaccharide, and tumour promoters (**FLETCHER *et al.*, 1992**).

The view that the two isoforms of the COX enzyme mediate physiologic and inflammatory processes, respectively, implies separate pathways of AA metabolism with different benefits to the organism. There are reports that the two enzyme isoforms use different AA pools in response to different cellular stimuli for prostaglandin synthesis (**MURAKAMI *et al.*, 1994; REDDY and HERSCHMAN, 1994**). The COX-2 gene has the structure typical of an “immediate-early” gene product that is rapidly up-regulated during inflammation and other pathologic processes (**CROFFORD, 1997; BOTTING, 2006**). COX-2 is thought to contribute to the generation of prostanoids at sites of inflammation (**CROFFORD *et al.*, 2000**). COX-1 is consistently expressed in a wide variety of tissues and produces prostanoids involved in the regulation of normal kidney and stomach functions as well as vascular homeostasis (**MORITA, 2002**). The concentration of the COX-1 enzyme largely remains stable, but small increases in expression occur in response to stimulation with hormones or growth factors (**BOTTING, 2006**). There are, however, no examples of separate stimulus-specific regulation of COX-1 and COX-2 in the same cell at a time when both enzymes are expressed and functioning.

4.1.2. Anti-inflammatory agents

Based on the compelling evidence that COX enzymes are involved in inflammatory processes, these enzymes became the research targets for drug development in the treatment of inflammation. Prostanoid biosynthesis is inhibited by nonsteroidal anti-inflammatory drugs (NSAIDs) that are widely prescribed as analgesics and anti-inflammatory agents. Treatment with NSAIDs inhibits the production of prostaglandins and down-regulates inflammation-related pathological symptoms such as pain and swelling. Their mechanism of action, particularly aspirin and ibuprofen includes inhibition of both the COX-1 and COX-2 isozymes thereby preventing the pathological synthesis of prostaglandins (**MORITA, 2002**). Inhibition of COX-1 may result in the removal of the cytoprotection function, leading to damaging side effects in the gastrointestinal tract (**GILROY *et al*., 1998**). The use of NSAIDs however, results in major changes to the pathophysiological functions of the body leading to some side effects such as gastric and renal ulceration, irritation and bleeding (**OSHIMA, 1996; BOTTING, 2006**). It was later elaborated that these side effects are associated with inhibition of COX-1 while the anti-inflammatory activity was due to inhibition of COX-2 (**COPELAND, 2005**).

The undesirable side effects associated with the use of NSAIDs has stimulated research interest in alternative forms of anti-inflammatory agents. Selective inhibition of COX-2 enzyme has been shown to significantly lower the development of gastro-toxicity and related side effects (**FERRERO-MILIANI *et al*., 2006**). Traditional medicine offers a vast biogenic resource base for exploitation in the discovery of innovative anti-inflammatory agents. Several natural plant-derived compounds such as flavonoids, saponins, tannins, alkaloids and essential oils possess some anti-inflammatory activities (**JUST *et al*., 1998; GURIB-FAKIM, 2006**). Plant-derived natural products are therefore important in the search for anti-inflammatory agents. The four medicinal bulbous plants were evaluated for their seasonal variation in the inhibition of COX-1 and 2 enzymes, which are involved in the biosynthesis of prostaglandins in inflammation processes.

4.2. Materials and methods

4.2.1. Enzyme and substrate preparation

COX-1 (isolated from ram seminal vesicles) and human recombinant COX-2 containing a six histidine sequence near the N-terminus isolated from a Baculovirus over expression system in *Sf* 21 cells (Sigma-Aldrich, USA) were used in the anti-inflammatory bioassays. The enzymes were diluted with Tris(hydromethyl)aminomethane (TRIS) storage buffer (pH 8.0) to obtain 50 µl aliquots, each with 75 units of enzyme concentration. The prepared COX enzymes (50 µl, 75 units) were stored in an ultra-freezer at -70 ºC until required for the assays.

The substrate ^{14}C-arachidonic acid (Sigma-Aldrich, Germany) was prepared by mixing radio-labelled and unlabelled arachidonic acid to obtain the final concentration (17 Ci/mol, 30 µM) required for the bioassay.

4.2.2. Preparation of plant extracts

The extracts were prepared as described in **Section 2.2.3** (Chapter 2). The stored dried sample extracts were resuspended in 70% ethanol for organic solvent extracts (PE, DCM and EtOH) and in water for aqueous extracts. The extracts were resuspended to a concentration of 10 mg/ml.

4.2.3. *In vitro* cyclooxygenase-1 (COX-1) inhibitory assay

The COX-1 inhibitory activity of plant extracts were evaluated as described by **WHITE and GLASSMAN** (**1974**) and modified by **JÄGER *et al*.,** (**1996**). For the organic extracts, 2.5 µl aliquots (10 mg/ml) were diluted with 17.5 µl distilled water in 1.5 ml Eppendorf tubes while 20 µl aliquots of water extracts were added directly to the Eppendorf tubes. Stock solution of COX-1 enzyme stored at -70 °C was activated with 1450 µl of co-factor solution (0.3 mg/ml L-epinephrine, 0.3 mg/ml reduced glutathione and 1 µM hematin in 0.1 M Tris buffer, at pH 8.0) and pre-incubated on ice for 5 min. The enzyme/co-factor solution (60 µl) were then added to each Eppendorf tube with sample extracts in duplicate and the mixture incubated at

room temperature for 5 min. The substrate, (20 µl) [^{14}C]-arachidonic acid (17 Ci/mol, 30 µM), was added t o each t ube t o initiate t he enzym atic reaction and t he preparations were i ncubated in a w ater bat h at 37 º C for 10 min. The f inal concentrations o f extract samples i n the bioassay were 250 µg/ml for organic extracts and 2 mg/ml f or aqueous ex tracts. A fter i ncubation, the r eaction m ixtures were pl aced on ice and the reaction t erminated by adding 2N H Cl (10 µl) in each reaction t ube. Four m icrolitres (0.2 m g/ml) of unlabeled pr ostaglandins (PGE_2: $PGF_{2\alpha}$; 1:1) (Sigma-Aldrich) w ere a dded t o each E ppendorf tube as a ca rrier solution.

Pasteur pipettes were packed with silica gel (silica gel 60, 0.063-0.200 mm, Merck) to a hei ght of 3 cm i n. Prostaglandins were se parated f rom un metabolised arachidonic acid by applying the test mixtures to the columns with 1 m l of eluent 1 [hexane: 1, 4 -dioxane: glacial a cetic a cid (70:30:0.2 v:v: v)]. Unm etabolised arachidonic acid was eluted first with 4 m l of eluent 1. The prostaglandin products were eluted with 3 ml of eluent 2 [ethyl acetate: methanol (85:15; v:v)] and collected into sc intillation vial s. To e ach v ial, 4 m l o f scintillation f luid (Beckman Cou lter^{TM}, USA) were added and t he disintegration per minute of the radioactive material was counted us ing a LS 6500 m ultipurpose sci ntillation co unter (Beckman C oulter^{TM}, USA). Controls for the assay, in duplicate, included a solvent blank, background and an indomethacin st andard. The s olvent b lank and background c onsisted of 70% ethanol (2.5 µl) instead of sample extracts. The background controls were kept on ice throughout the assay and the enzyme was inactivated using 2N HCl (10 µl) before adding [^{14}C]-labelled arachidonic acid. Indomethacin (2.5 µl) (positive control) was used at a co ncentration of 5 µM. The assa y was repeated t wice. The percentage of prostaglandin synthesis inhibition by the plant extracts were calculated by comparing the amount of radioactivity present in the sample to that in the solvent blank and background samples using the equation below:

$$\text{COX inhibition (\%)} = \left\{1 - \left(\frac{DPM_{extract} - DPM_{background}}{DPM_{solvent\ blank} - DPM_{background}}\right)\right\} \times 100$$

where DPMextract, DPMbackground and DPMsolvent blank represent the disintegrations per minute for sample extract, background and solvent blank respectively.

4.2.4. *In vitro* cyclooxygenase-2 (COX-2) inhibitory assay

The COX-2 inhibitory activity was assessed using a method described by **NOREEN *et al*.,** (**1998**) with slight modifications (**ZSCHOCKE and VAN STADEN, 2000**). The assay followed a similar experimental procedure as described for the COX-1 bioassay except that for the preparation of the co-factor solution, 0.6 mg/ml L-epinephrine was used in place of 0.3 mg/ml and 200 µM Indomethacin instead of 5 µM. The rest of the assay was as described for COX-1 (**Section 4.2.3**)

4.2.5. Statistical analysis

Data on percentage inhibition activity for each extracting solvent in four different seasons were arcsine transformed and subjected to one-way analysis of variance (ANOVA) using GenStat 12th edition (VSN International, UK). Significantly different means were separated using the Least Significant Difference (LSD) technique ($P \leq 0.05$) and results were presented as means ± standard errors.

4.3. Results and discussion

4.3.1. COX-1 inhibitory activity

The percentage inhibition of the COX-1 enzyme by four bulb and leaf extracts in four different seasons is presented in **Table 4.1**. The COX inhibitory activity was defined at four levels, with activity below 20% being considered insignificant, 20-40% low, 40-70% moderate, and 70-100% high (**TUNÓN *et al*., 1995**). Moderate to high levels were considered to be good activity. With the exception of the corm extracts of *H. hemerocallidea* and bulb extracts of *M. plumbea* in autumn, all the PE extracts showed high inhibition (%) levels (> 70%) in all seasons. Of the DCM extracts, only extracts from *T. violacea* bulbs and *D. robusta* leaves showed high inhibitory activity

across all the seasons. All the other DCM extracts displayed moderate to high (> 40%) inhibitory activity in all seasons. The highest COX-1 inhibitory activity (99.8 %) was recorded from the PE extracts of *T. violacea* bulbs in winter. In all the extracts that showed some level of inhibition, there was significant variation in this activity from season to season.

Although the inhibitory activity of PE and DCM extracts against COX-1 enzyme varied significantly from season to season, the activity fluctuated between moderate to high levels. As an adaptation for survival and growth within changing environmental conditions, plants have evolved different morphological and chemical traits (**WINK, 2003**). This has resulted in different types and quantities of chemical compounds being produced by plants in different seasons. The concentrations of active compound(s) responsible for the anti-inflammatory activity could therefore fluctuate with seasons. The differences in the inhibitory activity of plant extracts in different seasons could be explained by these dynamics. Interestingly, however, of all the extracts that showed good activity (moderate to high levels) in this study, the activity was maintained at these levels (> 40%) in all seasons. This trend therefore, justifies the collection and use of these extracts for treatment of pain related ailments in traditional medicine in any of the seasons.

Water extracts were tested at a higher concentration (2 mg/ml) than organic extracts (250 µg/ml) in this bioassay. However, all water and ethanol extracts, except for the ethanol extracts of *H. hemerocallidea* corms and *T. violacea* leaves, displayed insignificant to low COX-1 inhibitory activity. The extracts of *H. hemerocallidea* (corm) were the only ethanol extracts that showed moderate to high activity across all seasons. The moderate to high inhibitory activity of PE and DCM extracts towards COX-1, suggest that lipophilic compounds are involved in the enzyme inhibition process. Lipophilic compounds have a good resorption through the cell membrane even at lower concentrations compared to polar compounds (**ZSCHOCKE and VAN STADEN, 2000**). Lipophilic compounds are seldom found, and if they are, they are often in very minute quantities in water extracts (**TUNÓN *et al*., 1995**). This therefore, explains the high activity of these extracts in spite of the low extract yields obtained when using lipophilic extraction solvents.

Table 4.1. COX-1 enzyme inhibitory activity (%) of bulb/corm and leaf extracts of *T. violacea*, *H. hemerocallidea, D. robusta* and *M. plumbea* in spring, summer, autumn and winter seasons.

Plant species	Plant part	Extract	Inhibition (%)			
			Spring	Summer	Autumn	Winter
Tulbaghia violacea	Bulb	PE	88.5 ± 2.15^{a}	96.5 ± 1.43^{b}	91.8 ± 2.61^{a}	99.8 ± 1.18^{b}
		DCM	90.4 ± 2.54^{a}	95.1 ± 4.60^{b}	88.7 ± 1.58^{a}	94.3 ± 3.80^{b}
		EtOH	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
		Water	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
	Leaf	PE	94.7 ± 2.02^{c}	88.2 ± 3.73^{b}	79.5 ± 3.20^{a}	81.8 ± 3.74^{a}
		DCM	89.1 ± 1.66^{b}	50.3 ± 3.55^{a}	88.1 ± 3.92^{b}	91.4 ± 4.31^{b}
		EtOH	25.6 ± 2.22^{a}	27.3 ± 2.13^{a}	33.1 ± 1.11^{b}	29.3 ± 3.50^{ab}
		Water	0.00^{a}	0.00^{a}	0.00^{a}	7.71 ± 2.81^{b}
Hypoxis hemerocallidea	Corm	PE	90.5 ± 2.89^{b}	89.3 ± 1.37^{b}	49.5 ± 2.54^{a}	87.2 ± 1.58^{b}
		DCM	77.4 ± 2.96^{c}	52.3 ± 4.57^{a}	78.8 ± 0.71^{c}	71.6 ± 3.90^{b}
		EtOH	66.0 ± 4.32^{a}	67.7 ± 5.14^{a}	74.5 ± 4.12^{a}	86.9 ± 5.97^{b}
		Water	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
	Leaf	PE	77.5 ± 4.12^{b}	80.2 ± 0.07^{c}	70.8 ± 1.24^{a}	76.0 ± 2.91^{b}
		DCM	87.9 ± 2.18^{c}	60.0 ± 2.61^{a}	70.6 ± 2.37^{b}	57.2 ± 3.02^{a}
		EtOH	12.8 ± 3.60^{a}	10.8 ± 3.50^{a}	13.6 ± 4.22^{a}	8.80 ± 3.11^{a}
		Water	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
Drimia robusta	Bulb	PE	84.6 ± 1.73^{b}	88.1 ± 1.99^{c}	73.7 ± 3.12^{a}	94.1 ± 2.42^{d}
		DCM	68.2 ± 2.41^{b}	59.2 ± 1.93^{a}	58.0 ± 1.65^{a}	83.1 ± 1.78^{c}
		EtOH	7.10 ± 0.09^{c}	9.80 ± 3.23^{c}	3.42 ± 1.03^{b}	1.67 ± 0.18^{a}
		Water	12.2 ± 0.36^{c}	9.10 ± 2.11^{b}	11.1 ±1.21^{bc}	5.12 ± 0.56^{a}
	Leaf	PE	90.9 ± 4.57^{b}	90.9 ± 0.30^{b}	75.4 ± 3.29^{a}	-
		DCM	73.8 ± 4.36^{ab}	70.3 ± 0.88^{a}	75.5 ± 0.53^{b}	-
		EtOH	1.22 ± 0.14^{b}	0.00^{a}	0.00^{a}	-
		Water	0.00^{a}	0.00^{a}	0.00^{a}	-
Merwilla plumbea	Bulb	PE	85.0 ± 3.67^{c}	82.0 ± 2.16^{c}	68.9 ± 1.40^{a}	78.2 ± 0.87^{b}
		DCM	82.4 ± 4.42^{c}	83.9 ± 1.35^{c}	68.3 ± 1.31^{b}	43.6 ± 2.51^{a}
		EtOH	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
		Water	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}

Plant species	Plant part	Extract	Inhibition (%)			
			Spring	Summer	Autumn	Winter
Merwilla plumbea	Leaf	PE	90.0 ± 2.20[b]	91.1 ± 2.19[b]	93.0 ± 1.31[b]	83.9 ± 2.16[a]
		DCM	83.3 ± 3.48[c]	55.8 ± 2.57[a]	85.2 ± 2.31[c]	62.1 ± 0.85[b]
		EtOH	0.00[a]	3.70 ± 3.95[b]	4.10 ± 0.93[b]	0.00[a]
		Water	0.00[a]	0.00[a]	4.11 ± 0.07[c]	1.30 ± 0.11[b]

Values in a row with different letters are significantly different at $P \leq 0.05$ ($n = 4$)

PE = Petroleum ether, DCM = Dichloromethane, EtOH = 80% ethanol

COX-1 inhibition (%) by indomethacin at a final concentration of 5 µM was 61.3 ± 2.18

Inflammation is a complex pathophysiological process that involves an interaction of a variety of signalling molecules and mediators in a series of enzyme catalysed reactions (**WHITE, 1999**). Lack of and/or insignificant inhibitory activity of the water extracts, from the COX-1 inhibitory assay, does not therefore, disqualify these extracts as anti-inflammatory agents. Plant extracts exert their enzyme inhibitory effects through a spectrum of different modes of action and target sites (**CAPONE *et al*., 2007**). It is possible, therefore, that the active compounds in the water extracts could work at alternative sites in the complex inflammation process. A number of other studies with different plant species have reported good inhibitory activity from water extracts (**TAYLOR and VAN STADEN, 2001; JÄGER and VAN STADEN, 2005; FAWOLE *et al*., 2009**). This again points to the possibility that the activity of the different extracts is caused by different COX-1 inhibitors, either active individually or in synergism with other compounds in the extract.

In all plant species screened in this study, the inhibitory activity of both bulb and leaf extracts were comparable, with both plant parts exhibiting good activity (moderate to high levels) in PE and DCM extracts. The results from this study further confirm the traditional uses of the four plant species in the treatment of pain related ailments such as, gastro-intestinal ailments, stomach ache, wounds, urinary tract infections and cancer. A conservation strategy that would take into consideration the sustainable harvesting of medicinal plants and simultaneously provide similar medicinal benefits would be the substitution of bulbs with leaves of these plants in the treatment of pain related ailments (**ZSCHOCKE *et al*., 2000; LEWU *et al.*, 2006**). Harvesting of leaves is considered less destructive, although intensive pruning can affect reproductive performance (**GAOUE and TICKTIN, 2007**).

COX-1 enzyme is expressed constitutively in most tissues and catalyses the production of prostaglandins involved in the prostanoid-mediated physiological functions such as gastric cytoprotection, maintenance of renal homeostasis, and normal platelet functions (**MORITA, 2002**). However, COX-1 has also been suggested to play a role in the inflammatory process (**SMITH *et al*., 1998**). Findings suggest COX-1 to have an important role in pain processing and sensitisation in the rat spinal cord after surgery (**ZHU *et al*., 2003**). Complete COX-1 inhibition is associated with some detrimental side effects. Because of these side effects such as

the damage to the gastrointestinal tract, anti-inflammatory agents with high COX-1 inhibitory activity are less desirable **(ANDERSON *et al*., 1996)**. Although plants screened in this study showed good COX-1 inhibition, considering these side effects, extracts with moderate activity may be preferable to use than those with high activity. Prolonged use of plant extracts with high inhibition activity may result in the manifestation of the damaging side effects.

4.3.2. COX-2 inhibitory activity

Table 4.2 shows the COX-2 inhibitory activity of plant extracts. Petroleum ether and DCM extracts of both bulb and leaf extracts of all the screened plant extracts showed good inhibitory activity (> 40%) in all seasons. However, the PE and DCM bulb extracts of *T. violacea* and *D. robusta* displayed inhibitory levels in all the seasons. Although the percentage inhibition levels varied significantly from season to season, extracts from bulbs of *T. violacea* (PE, DCM, EtOH) and *D. robusta* (PE, DCM) showed consistently high (73.7% to 100%) inhibition levels in all seasons.

The highest inhibition (%) activity was recorded in the PE extracts of *T. violacea* bulbs and leaves with both having 100% inhibition in autumn and spring respectively. The results for the COX-2 inhibitory activity, however, followed a somewhat similar trend as those of COX-1. Among the ethanol extracts, good COX-2 inhibitory activity was recorded from the *T. violacea* bulb and leaf extracts, with both exhibiting consistently high and moderate activity respectively, across all seasons. All water and the other ethanol extracts showed insignificant activity. In most of the plant species, extracts of the plant material gathered in winter appeared to have lower activity compared to the activity of those gathered in the other seasons. This could have been due to the senescing leaf tissues and the onset of dormancy in the bulbs/corms. The biochemical activity rate, and consequently the production of compounds in plants decrease drastically during dormancy and tissue senescence (**BIDWELL, 1974**).

Table 4.2. COX-2 enzyme inhibitory activity (%) of bulb/corm and leaf extracts of *T. violacea*, *H. hemerocallidea, D. robusta* and *M. plumbea* in spring, summer, autumn and winter seasons.

Plant species	Plant part	Extract	Inhibition (%)			
			Spring	Summer	Autumn	Winter
Tulbaghia violacea	Bulb	PE	94.3 ± 0.90^{a}	99.7 ± 1.79^{b}	100 ± 00^{b}	99.4 ± 0.01^{b}
		DCM	97.9 ± 3.48^{b}	90.7 ± 3.43^{a}	98.0 ± 0.17^{b}	89.1 ± 0.38^{a}
		EtOH	83.5 ± 3.18^{b}	81.4 ± 2.21^{a}	83.9 ± 0.71^{b}	79.7 ± 0.04^{a}
		Water	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
	Leaf	PE	100 ± 00^{c}	83.2 ± 2.01^{b}	82.3 ± 3.51^{b}	58.2 ± 4.38^{a}
		DCM	91.5 ± 2.38^{c}	40.1 ± 1.29^{a}	93.2 ± 1.49^{c}	62.0 ± 4.73^{b}
		EtOH	63.6 ± 2.06^{c}	59.8 ± 0.66^{b}	61.4 ± 2.03^{b}	51.2 ± 1.11^{a}
		Water	0.00^{a}	1.23 ± 0.89^{b}	0.00^{a}	0.00^{a}
Hypoxis hemerocallidea	Corm	PE	85.7 ± 1.61^{d}	80.1 ± 1.79^{c}	68.1 ± 1.54^{b}	63.2 ± 4.42^{a}
		DCM	86.5 ± 0.33^{b}	88.1 ± 2.11^{b}	88.6 ± 2.02^{b}	82.6 ± 1.25^{a}
		EtOH	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
		Water	12.4 ± 0.56^{c}	14.0 ± 1.86^{d}	10.2 ± 1.00^{b}	6.20 ± 0.80^{a}
	Leaf	PE	80.3 ± 3.71^{c}	79.4 ± 0.61^{c}	70.6 ± 1.26^{b}	51.0 ± 2.50^{a}
		DCM	73.1 ± 2.21^{c}	69.2 ± 2.74^{c}	63.8 ± 5.21^{b}	49.3 ± 2.25^{a}
		EtOH	8.08 ± 1.74^{b}	11.5 ± 1.51^{c}	7.81 ± 2.11^{b}	4.56 ± 0.84^{a}
		Water	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
Drimia robusta	Bulb	PE	73.7 ± 2.58^{a}	83.2 ± 2.31^{b}	86.3 ± 1.00^{c}	88.9 ± 0.08^{c}
		DCM	90.8 ± 1.51^{b}	89.7 ± 1.93^{b}	91.2 ± 2.33^{b}	79.4 ± 2.20^{a}
		EtOH	5.41 ± 1.25^{a}	8.11 ± 0.89^{b}	11.0 ± 2.88^{c}	6.11 ± 1.79^{a}
		Water	2.31 ± 1.22^{a}	5.69 ± 0.61^{b}	6.12 ± 0.92^{b}	2.11 ± 0.85^{a}
	Leaf	PE	85.2 ± 3.69^{b}	81.3 ± 1.19^{b}	65.8 ± 2.40^{a}	-
		DCM	80.3 ± 1.20^{c}	52.8 ± 0.98^{a}	62.3 ± 0.40^{b}	-
		EtOH	0.00^{a}	0.00^{a}	0.00^{a}	-
		Water	0.00^{a}	0.00^{a}	0.00^{a}	-
Merwilla plumbea	Bulb	PE	80.1 ± 6.40^{c}	71.5 ± 2.29^{b}	55.0 ± 4.74^{a}	51.2 ± 0.82^{a}
		DCM	76.1 ± 1.59^{c}	65.1 ± 3.06^{b}	56.4 ± 3.05^{a}	58.9 ± 3.72^{a}
		EtOH	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
		Water	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}

Plant species	Plant part	Extract	Inhibition (%)			
			Spring	Summer	Autumn	Winter
Merwilla plumbea	Leaf	PE	85.1 ± 1.00[b]	91.7 ± 0.34[c]	100 ± 00[d]	61.8 ± 3.54[a]
		DCM	78.1 ± 0.27[c]	82.7 ± 3.94[d]	67.1 ± 0.24[b]	58.4 ± 0.76[a]
		EtOH	0.00[a]	0.00[a]	0.00[a]	0.00[a]
		Water	0.00[a]	0.00[a]	0.00[a]	0.00[a]

Values in a row with different letters are significantly different at $P \leq 0.05$ ($n = 4$)

PE = Petroleum ether, DCM = Dichloromethane, EtOH = 80% ethanol

COX-2 inhibition (%) by indomethacin at a final concentration of 200 µM was 65.2 ± 2.74

The inducible COX-2 enzyme is thought to be responsible for the accumulation of prostaglandins in most acute inflammations (**VANE *et al.*, 1998**). However, there is increasing evidence that in some tissues such as the brain, reproductive organs (ovaries, uterus), kidney, and placenta, COX-2 is also synthesised at a constant rate, and is responsible for the synthesis of prostanoids responsible for the regulatory and homeostatic functions in these tissues (**HINZ and BRUNE, 2002**; **MITCHELL and WARNER, 2006**). Anti-inflammatory agents with selective COX-2 inhibition are most desirable as they attenuate the damaging side effects associated with inhibition of COX-1. Interestingly, ethanol extracts of both bulbs and leaves of *T. violacea* showed good inhibitory activity (51.2% to 83.9%) towards COX-2 compared to very low to insignificant levels (0% to 33.1%) for COX-1. The ethanol bulb extracts of *T. violacea* maintained high inhibitory levels (79.7% to 83.9%) in all seasons while leaf extracts had moderate (51.2% to 63.6%) activity. The two extracts could contribute to the development of remedies with specific COX-2 activity as this remains a considerable challenge.

The important pharmacological and biological differences between COX-1 and COX-2 enzymes are attributed to the small differences in their structure. The active site of COX-2 is larger than that of COX-1 (**GIERSE *et al*., 1996**). The development of COX-2 specific inhibitors therefore, in part, utilises this characteristic (**HABEEB *et al*., 2001**). Good COX-2 inhibitory activity in the ethanol extracts of *T. violacea* bulbs and leaves compared to COX-1, may be an indication that the active compound(s) in these extracts are specific targets of the COX-2 active site. COX-2 selective inhibitors are believed to induce selectivity through interaction with the secondary pocket of COX-2 which is absent in COX-1 (**HABEEB *et al*., 2001**). The secondary pocket present in COX-2 has been attributed to the presence of isoleucine (Ile^{523}) in COX-1 relative to the smaller valine (Val^{523}) in COX-2 (**LUONG *et al*., 1996**). It is therefore, an interaction between the active site chemistry and that of the molecular inhibitor that determines selectivity. Ethanol extracts of *T. violacea* are promising anti-inflammatory agents in the treatment of pain and inflammation with a reduced risk of serious gastrointestinal side effects than those showing similar activity in both enzymes. The active compounds in these extracts may be inhibiting COX-2 enzyme through this unique secondary pocket site.

4.4. Conclusions

Generally, bulb and leaf extracts of all the plant extracts showed comparable activity in both COX-1 and COX-2 assays. Petroleum ether and DCM extracts of all plant extracts showed good inhibitory activities towards both enzymes in all seasons. Despite the damaging side effects, NSAIDs that exert their therapeutic effects by blocking both COX enzymes are still used in the treatment of pain and inflammation in modern medicine (**KONG, *et al*., 2009**). Based on the results obtained in this study, supported with the current use of non-selective NSAIDs in modern medicine, both bulbs and leaves of the screened plant extracts can be used in traditional medicine in the treatment of pain and inflammation related ailments. However, prolonged use may lead to the manifestation of side effect symptoms. Considering the aspect of conservation of these medicinal plants, and in light of the comparable anti-inflammatory activity of their bulb/corm and leaf extracts, leaves may substitute for bulbs in the treatment of inflammation ailments. This could offer a practical strategy in the conservation of these medicinal plants.

Ethanol extracts of *T. violacea* offer prospects for the development of COX-2 selective inhibitors. Research aimed at identification and possible isolation of the active compound(s) in these extracts, and testing them *in vivo* will be necessary. The COX bioassay is an example of a mechanism-based assay that utilises subcellular structures (enzymes) to detect inhibitors of inflammation (**NOREEN, *et al*., 1998**). The actual inflammation process in living tissues is, however, very complex and involves a series of mediators and various other enzymes. In this regard, the *in vitro* effects of an extract should be appreciated as supporting evidence only, since the *in vivo* effects may be complicated by a plethora of chemical, physical and physiological factors. *In vivo* tests are therefore necessary to validate the effects of these extracts in living organisms.

CHAPTER 5
Phytochemical Properties

5.1. Introduction

Plants constantly interact with the rapidly changing and potentially damaging external environmental factors. Being organisms devoid of mobility, plants have evolved elaborate alternative defence strategies, which involve an enormous variety of chemical metabolites as tools to overcome stress conditions. Secondary metabolites play a major role in the adaptation of plants to the changing environment. Plants have an almost limitless ability to synthesise these metabolites.

As a result of biotic and abiotic stresses, such as temperature, light intensity, herbivory and microbial attack, plants generate these defence mechanisms, triggering many complex biochemical processes (**HOLOPAINEN and GERSHENZON, 2010**). Changes have been reported at a genetic or protein level that are brought about by stress conditions and are reflected in a profound alteration of the metabolite pool of the affected plants (**DANGLE and JONES, 2001; SZATHMÁRY *et al*., 2001; LORETO and SCHNITZLER, 2010**). The synthesis of secondary metabolites is, however, often tightly regulated, and is commonly either restricted to specific plant tissues or developmental stages, or induced in response to stimulation factors (**OSBOURN *et al*., 2003; WINK, 2003**). As a strategy for survival and for the generation of diversity at the organism level, the ability to synthesise particular classes of secondary metabolites is also restricted to selected plant groups.

The ability of plants to carry out *in vivo* combinatorial chemistry by mixing, matching and evolving the gene products required for secondary metabolite biosynthetic pathways, creates an unlimited pool of chemical compounds, which humans have exploited to their benefit. Plant secondary metabolites therefore, offer a diverse range of benefits to humans, that include among them, medicinal benefits. Plant phenolics are a group of compounds that are ubiquitous to plants and have been reported to offer a range of pharmacological benefits. Saponins are also among a group of plant compounds that offer medicinal benefits to humans.

A qualitative and quantitative insight into the potential amount and seasonal dynamics of these phytochemical compounds in medicinal plant use, is basic to the understanding of when to collect these plants. Coinciding medicinal plant harvesting with maximum biological activity of a particular plant species would serve as a logical way for the effective utilisation of plant-based medicine. In view of the medicinal value of phytochemical compounds, the four medicinal plants were evaluated for their phenolic and saponin constituents and the seasonal variation in their quantities between bulbs/corms and leaves.

5.2. Materials and methods

5.2.1. Preparation of plant extracts

Phenolic compounds were extracted from plant material as described by **MAKKAR** (**1999**). Ground plant samples (2 g) were extracted with 10 ml of 50% aqueous methanol by sonication on ice for 20 min. The extracts were then filtered under vacuum through Whatman No. 1 filter paper and used in the assays outlined below.

5.2.2. Determination of total phenolics

The amounts of total phenolic compounds in plant samples were determined using the Folin Ciocalteu (Folin C) assay for total phenolics as described by **MAKKAR** (**1999**) with slight modification (**NDHLALA *et al*., 2007**). In triplicate, 50 µl of each extract were transferred into test tubes and the volume made up to 1 ml with distilled water (950 µl). Folin C phenol reagent (500 µl, 1 N) and 2% sodium carbonate (2.5 ml) were added to each dilute sample. A blank that contained 50% aqueous methanol instead of sample extract was also prepared. The test mixtures were incubated for 40 min at room temperature and the absorbance was read at 725 nm using a UV-visible spectrophotometer (Varian Cary 50, Australia). A gallic acid standard curve was prepared from a freshly made 0.1 mg/ml gallic acid (Sigma-Aldrich) stock solution in distilled water. Total phenolic concentrations were expressed as gallic acid equivalents (GAE) derived from the standard curve.

5.2.3. Determination of condensed tannins (proanthocyanidins)

The butanol-HCl assay for the determination of condensed tannin was followed as described by **PORTER *et al*.** (**1985**). Three millilitres of butanol-HCl reagent (95:5, v/v) were added to 500 µl of each extract in test tubes, followed by 100 µl ferric reagent (2% ferric ammonium sulphate in 2 N HCl). The test combinations were mixed using a vortex and transferred into a water bath set at 100 °C for 60 min. A blank test was prepared for each sample extract by mixing the extract (500 µl) with butanol-HCl reagent (3 ml) and ferric reagent (100 µl), but without heating. Absorbance was then read at 550 nm using a UV-visible spectrophotometer against a suitable blank. Each extract had three replicates. Condensed tannin (% in dry matter) was calculated as leucocyanidin equivalents (LE) using the formula:

$$\text{Condensed tannin (\%)} = (A_{550\ nm} \times 78.26 \times \text{Dilution factor}) / (\%\ \text{dry matter})$$

where $A_{550\ nm}$ is the absorbance of the extracts at 550 nm, 78.26 is the molecular weight of leucocyanidin and the dilution factor was 0.5 ml/ (volume of extract taken). The formula assumes that the effective $E^{1\%,\ 1\ cm,\ 550\ nm}$ of leucocyanidin is 460.

5.2.4. Determination of gallotannin

The determination of hydrolysable tannin as gallotannins was done according to **MAKKAR** (**1999**) with modifications (**NDHLALA *et al*., 2007**). In triplicate, sample extracts (50 µl) were made up to 1 ml with distilled water. Sulphuric acid (100 µl, 0.4 N) and 600 µl of rhodanine were added to the diluted extracts. After 5 min of incubation at room temperature, 200 µl of potassium hydroxide (0.5 N) was added followed by 4 ml distilled water after a further 2.5 min. The mixtures were incubated for an additional 15 min at room temperature, after which the absorbance at 520 nm was read using a UV-visible spectrophotometer against a blank that contained 50% aqueous methanol instead of plant extract. A freshly prepared stock of gallic acid solution (0.1 mg/ml in 0.2 N sulphuric acid) was used for the standard curve. Gallotannin concentrations were expressed as gallic acid equivalents (GAE), derived from a standard curve.

5.2.5. Determination of flavonoids

The flavonoid content was evaluated as described by **HANGERMAN** (**2002**) with modifications. In triplicate, 50 µl of each extract were made up to 1 ml with methanol in test tubes before adding 2.5 ml of 5% methanol in HCl (v/v) and 2.5 ml vanillin reagent (1 g/100 ml glacial acetic acid). A blank that contained methanol instead of plant extract was prepared. After 20 min of incubation at room temperature, absorbance was read at 500 nm using a UV-visible spectrophotometer. A catechin standard curve was prepared from a freshly made 1 mg/ml catechin (Sigma-Aldrich) stock solution in methanol. The amount of flavonoids in the plant extracts were expressed as catechin equivalents (CTE), derived from a standard curve.

5.2.6. Qualitative determination of saponins

In duplicate, 10 ml distilled water were added to 0.1 g of ground samples in test tubes. The test tubes were corked and vigorously shaken for 2 min. The appearance of stable and persistent foam on the liquid surface for 15 min indicated the presence of saponins (**TADHANI and SUBHASH, 2006**). Presence of saponins was further confirmed by the formation of an emulsion upon addition of ten drops of olive oil into 2 ml of aqueous extracts.

5.2.7. Saponin extraction

Saponins were extracted from the dried plant material as described by **MAKKAR *et al*.** (**2007**).The dried and ground plant samples were defatted with hexane in a Soxhlet apparatus (Isopad KSEU, UK) for 3 h. After air-drying, saponins were extracted twice from the defatted samples (10 g) in 100 ml of 50% aqueous methanol by incubating at room temperature overnight with continuous stirring. The extracts were then centrifuged at 3 000 *g* for 10 min and the supernatant collected. The procedure was repeated with the original residue to obtain a second supernatant. The first and second supernatants were combined and filtered under vacuum through Whatman No. 1 filter paper. Methanol from the filtrate was evaporated from the solution under vacuum at 40 °C using a rotary evaporator

(Rotavapor-R, Büchi, Switzerland) to yield the saponin sample in aqueous phase. The aqueous phase was then centrifuged at 3 000 *g* for 10 min to remove water insoluble materials. The aqueous solutions were transferred to a separating funnel and extracted three times with an equal volume of chloroform to remove pigments. The concentrated saponins in the aqueous solution were then extracted twice with an equal volume of *n*-butanol. The *n*-butanol was evaporated under vacuum at 45 °C. The dried fractions containing saponins were dissolved in 10 ml of distilled water and freeze dried. The dried saponin extracts were kept in airtight glass sample vials at 10 °C in the dark until required for the assay.

5.2.8. Quantitative determination of total saponins

Total saponin content was determined using a spectrophotometric method as described by **HIAI *et al*.** (**1976**) with slight modifications. The crude saponin extracts were redissolved in 50% aqueous methanol to a concentration of 10 mg/ml. From this, aliquots of 250 µl (in triplicate) of each extract were transferred into test tubes and an equal volume of vanillin reagent (8 g/100 ml ethanol) was added followed by 2.5 ml of 72% (v/v) sulphuric acid. The test mixtures were mixed using a vortex and transferred into a water bath adjusted at 60 °C for 10 min. The tubes were cooled on an ice-cold water bath for 3 min and absorbance was measured at 544 nm using a UV-visible spectrophotometer against a blank that contained 50% aqueous methanol instead of plant extract. A diosgenin standard curve was prepared from a freshly made 0.5 mg/ml diosgenin (Sigma-Aldrich) stock solution in 50% aqueous methanol. The saponin concentrations in the plant extracts were expressed as diosgenin equivalents (DE) calculated from a standard curve.

5.2.9. Determination of total steroidal saponins

The amounts of total steroidal saponins in plant samples were determined according to the method described by **BACCOU *et al*.** (**1977**) with slight modifications. Crude saponin extracts were redissolved in 50% aqueous methanol (0.1 mg/ml) from which 300 µl aliquots (corresponding to a sapogenin content of between 1 and 40 µg) were transferred into test tubes and placed in a water bath set at 100 °C to remove the

methanol by evaporation. After cooling, 2 ml of ethyl acetate were added followed by 1 ml anisaldehyde-ethyl acetate reagent (0.5:95.5, v/v) and 1 ml sulphuric acid-ethyl acetate reagent (50:50, v/v). The test combinations were mixed using a vortex and incubated in a water bath at 60 °C for 20 min. After cooling for 10 min in a water bath at room temperature, absorbance was measured at 430 nm using a UV-visible spectrophotometer against a blank that contained ethyl acetate instead of plant extract. A freshly prepared stock of diosgenin (0.1 mg/ml in ethyl acetate) was used for the standard curve. Each plant extract was evaluated in triplicate and steroidal saponin concentrations were expressed as diosgenin equivalents (DE) calculated from a standard curve.

5.2.10. Statistical analysis

All determinations were done in triplicate. Data was subjected to one-way analysis of variance (ANOVA) using GenStat 12th edition (VSN International, UK). Significantly different means were separated using Least Significant Difference (LSD) technique ($P \leq 0.05$) and results presented as graphs (mean ± standard error). Graphs were done using GraphPad Prism version 4.0 for Windows (GraphPad Software Inc., USA) and SigmaPlot 2002 for Windows version 8.0 (SPSS Inc., USA).

5.3. Results and discussion

5.3.1. Total phenolic compounds

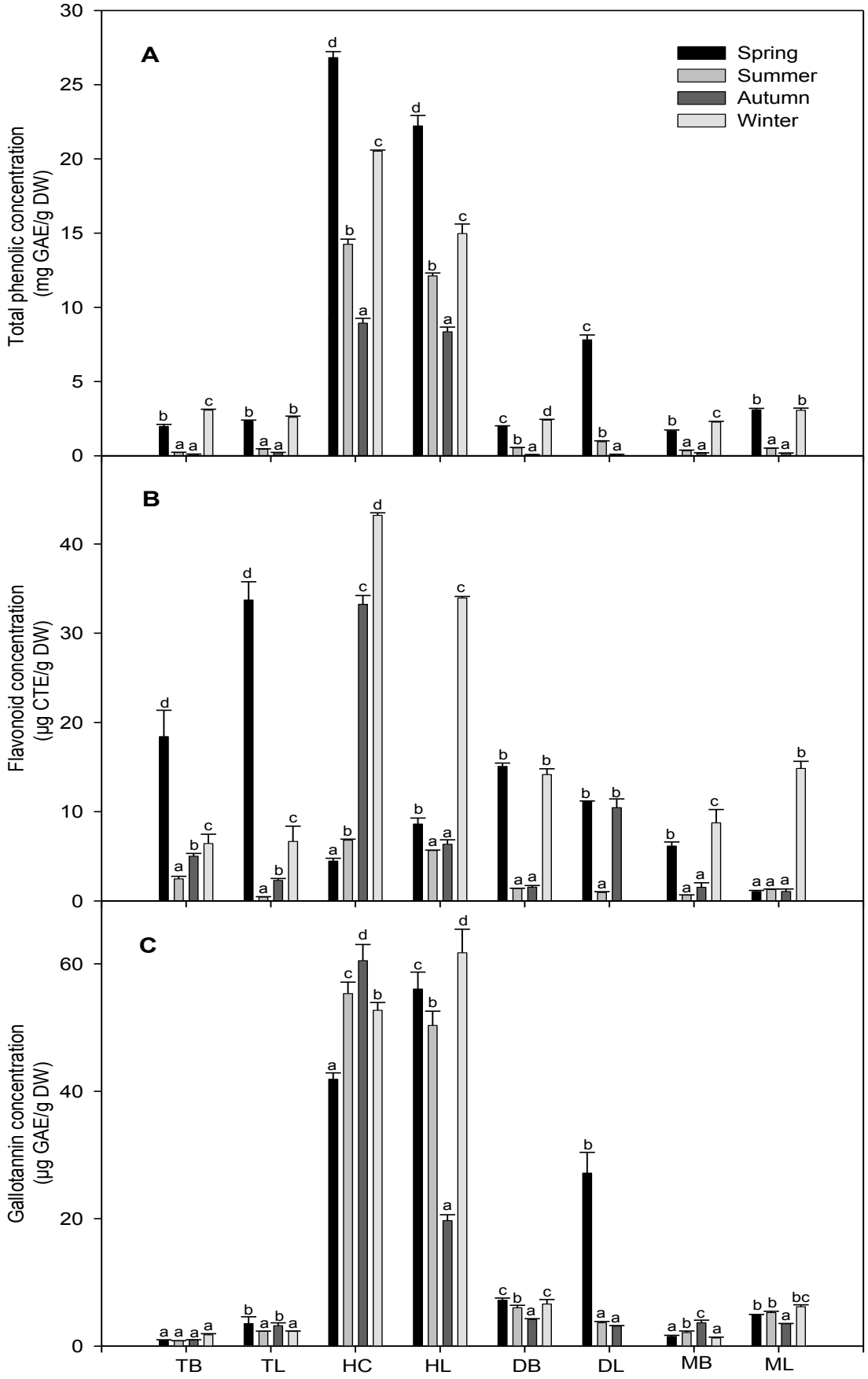
WATERMAN and MOLE (**1994**) described phenolics as compounds that can play a role in virtually any interaction a plant can have with its environment, biotic or abiotic. It is for this reason that plant phenolic compounds have received extensive utilisation in both modern and traditional medicine. Plant phenolic compounds are of important pharmacological value and are reported to have antioxidative, anticarcinogenic, antimicrobial and anti-inflammatory effects (**SHARMA *et al*., 1994; BRUNETON, 1995; KUDA *et al*., 2005**). Plants with high phenolic compounds are usually used in traditional medicine for the treatment of various ailments including infectious diseases. The total phenolic concentrations of the screened plant extracts in this

study are presented in **Figure. 5.1** (**A**). Higher concentration levels of total phenolic compounds in all the screened plant species were recorded in either spring or winter in both leaves and bulbs/corms than in other seasons. In *H. hemerocallidea* corm and leaf extracts, total phenolic concentrations were higher in the plant material gathered in spring yielded by those from the winter season. *Drimia robusta* leaf extracts also recorded high total phenolic content in spring. All the other plant extracts had higher phenolic content in winter followed by spring. Plant material gathered in autumn had the least amount of total phenolics. The highest amounts of phenolic compounds were recorded from *H. hemerocallidea* (corm) samples (26.82 ± 0.41 mg/g) in spring, while *D. robusta* (bulb) samples had the lowest (0.08 ± 0.01 mg/g) in autumn. Due to senescence, there were no records for *D. robusta* leaf samples in winter.

Phenolic compounds are produced in plants to serve a diverse range of purposes, such as defence against pathogens and different forms of environmental stress, among them heat stress, moisture stress and UV radiation (**DEY and HARBORNE, 1989; TREUTTER, 2001**). For this reason, the observed differences in phenolic concentrations between different seasons could be explained on the basis of the differences in the climatic, biotic and environmental conditions experienced in different seasons. In relation to the results of the present study, high phenolic concentrations in spring could be due particularly to the high and fluctuating temperatures experienced during this season. Considering that light and temperature plays an essential role in phenol biosynthesis (**JORGENSEN, 1994**), **AMARAL *et al*.** (**2004**) attributed the increase in the production of phenolic compounds to high values of solar radiation. Phenolic compounds, especially anthocyanins, which accumulate in the epidermis, can act as a darkening filter and protect the mesophyll from excessive solar radiation (**LARCHER, 1995**).

Another explanation for the increased phenolic content production is the known stimulation by light of most of the enzymes involved in phenolic biosynthesis. In particular, the activity of phenylalanine ammonia-lyase (PAL), a key enzyme in the synthetic pathway of phenolic compounds, increases under the influence of light (**DIALLINAS and KANELLIS, 1994; LIU *et al*., 2006**). This phenomenon could possibly be put forward to explain the high levels of phenolic compounds observed in

both spring and winter since the two seasons receive high light exposure due to less cloud cover. Numerous studies have long established this positive relationship between the intensity of solar radiation and the quantity of phenolics (**BRYANT *et al*., 1987; MOLE *et al*., 1988**). Anthocyanins and flavones are reported to increase in response to high visible light levels, and it is thought that these compounds help attenuate the amount of light reaching the photosynthetic cells (**BEGGS *et al*., 1985; TREUTTER, 2001**). Reduced water availability and high temperatures also influence high phenolic production in plants (**GLYNN *et al*., 2004; ALONSO-AMELOT *et al*., 2007**). Plants close their stomata and curtail photosynthesis during periods of water shortage and thus one might expect a negative relationship between water shortage and the synthesis of phenolics. A more definite role of phenolics in relation to plant water-relations have been proposed for lipophilic resins accumulated in *Diplacus* and *Larrea* species (**RHOADES, 1977**), where an integrated antidesiccant and UV screen defence role has been assumed. Furthermore, **HORNER** (**1990**) suggested a link between xylem pressure and tannin synthesis, and that the relationship can either be positive or negative, depending on the degree of water stress suffered by the plant. Water stress, however, typically characterises the winter season in this study area while high temperatures are characteristics of spring. It is possible therefore, that the two factors may have contributed to the higher phenolic production by plants in these two seasons.


A
B
C
Spring
Summer
Autumn
Winter
Total phenolic concentration (mg GAE/g DW)
Flavonoid concentration (µg CTE/g DW)
Gallotannin concentration (µg GAE/g DW)
TB
TL
HC
HL
DB
DL
MB
ML

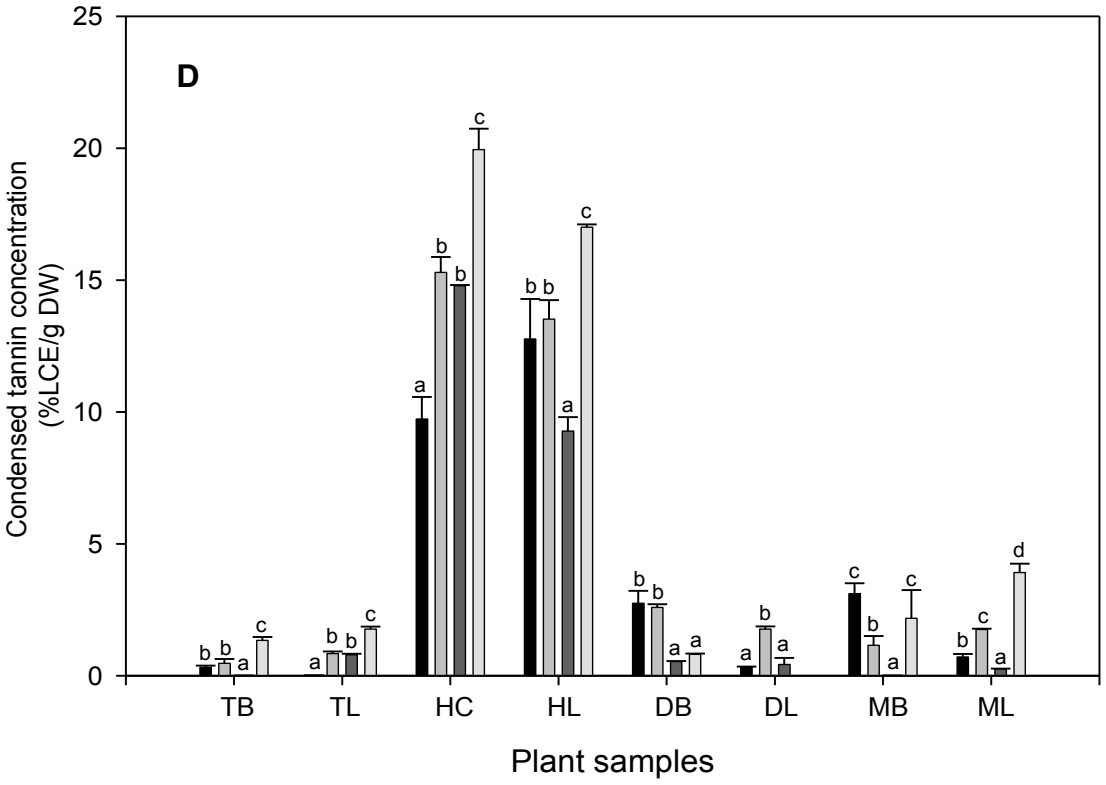


Figure 5.1. Total phenolic compounds as gallic acid equivalents (GAE) (A), flavonoid concentrations as catechin equivalents (CTE) (B), gallotannin concentrations as gallic acid equivalents (GAE) (C), percentage condensed tannin concentrations as leucocyanidin equivalents (LCE) (D) per dry weight (DW) in *T. violacea* bulbs (TB), *T. violacea* leaves (TL), *H. hemerocallidea* corms (HC), *H. hemerocallidea* leaves (HL), *D. robusta* bulbs (DB), *D. robusta* leaves (DL), *M. plumbea* bulbs (MB) and *M. plumbea* leaves (ML) in summer, autumn, winter and spring seasons. Results indicate the means ± standard error (n = 3). Significant differences ($P \leq 0.05$) within seasons in each plant sample are indicated by different letters.

Cold stress has been shown to stimulate an increase in phenolic production and their subsequent incorporation into the cell wall (**CHRISTIE *et al*., 1994**). The levels of PAL were shown to increase following exposure of maize seedlings and oilseed rape plants to low temperatures, resulting in a corresponding increase in their phenolic content (**CHRISTIE *et al.* 1994; SOLECKA and KACPERSKA, 1995**). In particular, levels of anthocyanins increases following cold stress and are thought to protect plants against this effect (**PENNYCOOKE *et al*., 2005**). Winter is characterised by low temperatures. The high levels of total phenolic compounds obtained in the current study in winter, are consistent with this fact and supports similar findings from previous studies (**PRASAD, 1996; PENNYCOOKE *et al*., 2005**).

Further to climatic factors, plant nutrient balance in the soil is thought to influence the production of phenolic compounds at the level of metabolic regulation in plants (**HERMS and MATTSON, 1992**). The carbon/nitrogen balance (CNB) hypothesis, as proposed by **BRYANT *et al*.** (**1983**), postulate that fertilisation with growth-limiting nutrients will lead to decreased concentrations of carbon-based secondary metabolites. CNB explains the concentrations of secondary metabolites in plant tissues as a function of the relative abundance of plant resources, particularly nitrogen. The theory rests on the assumptions that growth (primary metabolism) is a priority for plants over secondary metabolism, and that carbon and nitrogen are allocated to secondary metabolite production only after the requirements for growth are met. It also assumes that the rate of secondary metabolite production is determined by the concentrations of precursor molecules (**REICHARDT *et al*., 1991**). When growth is limited by nitrogen deficiency in plants, CNB predicts that carbohydrates will accumulate in plant tissues, leading to an increased synthesis of carbon-based secondary compounds like phenolics. The interpretation placed on such results is that the accumulation of phenolics when the carbon-nitrogen ratio is high, is due to a relative excess of fixed carbon being available to the plant. Thus the plant is only able to use the photosynthates for C-, H-, and O-containing metabolites, such as phenolics (**WATERMAN and MOLE, 1994**). Although many studies have yielded results that are consistent with this prediction, CNB has also failed repeatedly in several other similar studies (**KORICHEVA *et al*., 1998; RIIPI *et al*., 2002**). However, owing to the reported successes of this theory, its role in

determining the phenolic concentration trends observed in the present study cannot be overlooked.

In line with the opinion of the CNB theory, **JONES and HARTLEY** (**1999**) proposed a protein competition model (PCM) of phenolic allocation in plants. In contrast to the predictions of CNB, the PCM attempts to explain phenolic production on the basis of the metabolic origins of pathway constituents, alternative fates of pathway precursors, and biochemical regulatory mechanisms. Protein synthesis and the PAL-catalysed committed step of phenolic biosynthesis, both utilises the amino acid phenylalanine as a precursor (**DIALLINAS and KANELLIS, 1994**). This, therefore, presents a protein-phenolic competition for the limiting phenylalanine, leading to a process-level trade off between rates of protein versus phenolic synthesis. When rates of protein synthesis are high, rates of phenolic synthesis should be low and vice versa (**JONES and HARTLEY, 1999**). Because of these demands, the phenylalanine pool appears to be limiting in plants (**WEAVER and HERMANN, 1997**). Allocation to phenolic biosynthesis, therefore, may be determined by the competitive dynamics between protein and phenolic demands and these are controlled by the inherent growth demands and environmental cues. To this effect, the two theories further affirm the widely held view that the rate of secondary metabolite production in plants is a result of the interaction between the extrinsic (environmental) and the plant's intrinsic factors. The trend in phenolic concentrations observed in the current study in different seasons could therefore be a reflection of such interactions.

5.3.2. Condensed tannin, flavonoids and gallotannin content

An almost similar trend to that of total phenolic compounds was observed for flavonoids, gallotannins and condensed tannins (**Figure. 5.1 B, C and D**), with highest concentrations alternating between winter and spring seasons in most of the plant species. Exceptions were observed in *H. hemerocallidea* corms and *M. plumbea* bulbs, where there were higher concentrations of gallotannins in autumn than in other seasons.

Although the different classes of phenolic compounds in the present study were higher either in winter or spring season in most plant species, a look at the single compound classes shows that the level of seasonal fluctuations vary for each plant species. In *T. violacea*, for example, very high flavonoid levels were recorded in spring in both bulbs and leaves (18.4 ± 2.95 µg/g and 33.7 ± 2.04 µg/g respectively) compared to 2.5 ± 0.28 µg/g and 0.44 ± 0.03 µg/g respectively in the summer season. This sharp contrast in flavonoid levels between the two seasons, suggest that high flavonoid concentrations in spring were in response to some stimuli. Environmental conditions, especially UV-light radiation has been shown to affect the concentration of UV-absorbing compounds in plants (**RAO *et al*., 1996**). Several studies in this connection have identified flavonoids as the main group of UV-absorbing phenolics (**LI *et al*., 1992; KOLB *et al*., 2003**). It is possible that the observed differences in the present study are a result of light stimulation in spring. However, in comparing flavonoid fluctuations of *T. violacea* to that of *H. hemerocallidea* and *M. plumbea*, the trend was totally different, with *H. hemerocallidea* and *M. plumbea* yielding low levels in spring and higher levels in winter. This is despite the fact that the three plant species were growing under the same environmental conditions. This variation suggests a link between the different classes of compounds with their different functions in different plants and the stimuli triggering their biosynthesis. The stimuli that triggered high flavonoid production in *T. violacea* in spring might not have had the same effect on *H. hemerocallidea* and *M. plumbea.* The genotypic differences between the different plant species may be one of the contributory factors to the observed concentration trends of these different phenolic compounds. Different plants respond differently to different sources and magnitudes of environmental stimuli. The variations reported in this study may therefore, be a reflection of this phenomenon.

In addition to the variations between plant species and different seasons, there were, in some species, noticeable variations in the amount of total phenolic compounds, flavonoids, gallotannins and condensed tannins between bulbs/corms and leaves, although the trend in seasonal fluctuations in most cases were similar. The most remarkable concentration differences were noted in the total phenolics and gallotannins from *D. robusta* (spring) and flavonoids from *T. violacea* (spring), with both having higher levels in the leaves than in bulbs. On the other hand, *D. robusta*

and *M. plumbea* had higher tannin levels in the bulbs than in leaves during the spring season. In *H. hemerocallidea*, flavonoids, gallotannins and condensed tannin levels were all higher in the corms than in leaves in autumn.

While the biosynthesis and accumulation of phenolic compounds depend on exogenous factors, it is the endogenous factors, developmental stage and tissue differentiation (**TREUTTER, 2001; MIRDEHGHAN and RAHEMI, 2007**) that determine the site of synthesis in plants. Differences between leaves and bulbs observed in this study could therefore, be due to the inherent morphological differences and tissue differentiation between the two organs. Plant metabolic reaction to localised stress stimulation may be detected by the localised concentration of response-specific compounds (**FEUCHT, 1994**). Differences in phenolic levels between the leaves and bulbs reported in this study show a similar mechanism at work in these plants. The high levels of flavonoids, gallotannins and condensed tannins in winter are probably in response to cold and water stress experienced during this period. It has been frequently reported that, as the season progresses and/or leaves age, levels of phenolic compounds increase or at least rises to a plateau level (**PARKER, 1977; LINDROTH *et al*., 1987**). Considering the changing carbon-nutrient balance of growing leaves and assuming that carbon resources are more profitably used for growth in younger leaves than older ones, an allocation of carbon resources to phenolics during leaf senescence appears logical. There has been contrasting reports, however, of young leaves having higher phenolic compounds, especially tannins, than mature ones and vice versa (**SCHULTZ *et al*., 1982; WILSON, 1984; HELDT, 2005**). During leaf senescence in winter, metabolic cost is also minimised by concentrating and retrieving metabolites from senescing tissues and transporting them to other organs (**McKEY, 1979; WATERMAN and MOLE, 1994**). The high levels of flavonoids, gallotannins, and condensed tannins observed in the present study within bulbs and leaves in either spring or winter, may be indicative of such mechanisms at play. In spring, vegetative material starts to develop in plants, while in winter, leaves and other tissues are at a senescence stage.

Plants are not only faced with abiotic stress factors but also interact with biotic factors such as pathogens and insects. Their response to the biotic stress factors will

therefore vary depending on the type and magnitude of stimulation each plant species receives and on which part of the plant. In addition to the environmental stimulation, biotic factors may have contributed to the observed seasonal trends in each plant species. From the results obtained in this study, it is clear that the adaptive functions of phenolics in plants cannot be safely inferred from individual stimulations and that quantitative variation may not be simply in response to a single adaptive utility.

Flavonoids, gallotannins and condensed tannins have been reported to posses some antimicrobial and anti-inflammatory properties (**TAPIERO *et al*., 2002; ERDÈLYI *et al.,* 2005; TAPAS *et al*., 2008**). Several flavonoids and tannins have also been reported to possess other pharmacological benefits such as antioxidant, anticancer, anthelmintic, antivirus, anticarcinogenic and anti-parasitic activity (**TAPIERO *et al*., 2002; HIRATA *et al.*, 2005**). However, some phenolic compounds have been reported to impact negative physiological effects such as neurological problems, reproductive failure, goitre, and in more severe cases may lead to death, when consumed at higher concentrations (**MAKKAR *et al*., 2007**). Gallotannins have been implicated in this poisoning effect in animals (**MAKKAR *et al*., 2007**). The toxicity is due to higher levels of hydrolysable tannins and other phenolic compounds, beyond those that the liver can detoxify in the blood stream. Due to their characteristic protein binding and precipitation nature, consumption of higher levels of tannins may reduce the absorption of glucose and amino acids in the digestive system (**MAKKAR, 2003**). This is one of the challenges that medicinal plant users are faced with. While the high concentration of phenolic compounds in *H. hemerocallidea*, particularly tannins may be of good pharmacological benefit, extreme caution must be exercised to avoid the “poisoning” effect.

5.3.3. Saponin content

The qualitative froth test for the presence of saponins was positive for all the plant samples. Like phenolic compounds, saponin concentrations in this study were found to vary with season and plant organ and were consistently higher in winter than in other seasons (**Figure 5.2 A**). An exception was *D. robusta* leaves which had no leaf

samples in winter and total saponin levels were not significantly different in the other seasons. Generally, saponins are found in tissues that are most vulnerable to fungal or bacterial attack or insect predation (**WINA *et al*., 2005**). Therefore, it is believed that one of their roles in plants is to act as a natural chemical defence against potential pathogens, which would account for their antimicrobial activity (**OSBOURN, 1996; 2003**). The concentrations of saponins in different parts of the plant would therefore be expected to correlate with levels and sites of pathogenic stimulation. This also serves to explain the variations between bulbs and leaves as well as between seasons.

Summer season appeared to have favoured production and accumulation of steroidal saponins in *T. violacea* leaves, *D. robusta* bulbs and *M. plumbea* bulbs compared to other seasons (**Figure 5.2 B**). In *T. violacea* bulbs and *H. hemerocallidea* leaves, steroidal sapogenin levels were higher in autumn and spring respectively. Although seasonal fluctuations existed in different plant samples, the magnitude of the relative variation was less in the total saponin concentrations compared to those of phenolic compounds. An overall assessment of the published literature reveals a surprisingly poor understanding of the role of environmental factors (light, temperature, moisture, and nutrition) on saponin levels. Nonetheless, the marked seasonal variations in the steroidal saponins of some plant species suggest that one or more of these factors might have contributed to the observed differences. The consistently high saponin levels in all plant samples collected in winter, in particular, suggest the involvement of environmental factor(s) in their stimulation. This may be in response to cold temperatures that the plants were exposed to during the winter season.

Seasonal variations in steroidal sapogenin levels between leaves and bulbs/corms in most plant species did not follow a similar trend. For example, in *T. violacea*, higher levels were noted in autumn (4.68 ± 0.51 mg/g) followed by spring (2.69 ± 0.28 mg/g) in bulb samples while leaf samples had higher levels in summer (3.77 ± 0.43 mg/g) followed by spring (2.46 ± 0.42 mg/g). In *H. hemerocallidea,* on the other hand, corm samples maintained constant levels across seasons while leaf samples had markedly high levels in spring compared to the other seasons. These differences in steroidal sapogenin concentrations between the two plant parts,

suggests that these compounds may have been produced in response to specific and localised stimuli. The trends observed in the present study may be consistent with the hypothesis that their role in plants is for chemical defence against potential pathogens.

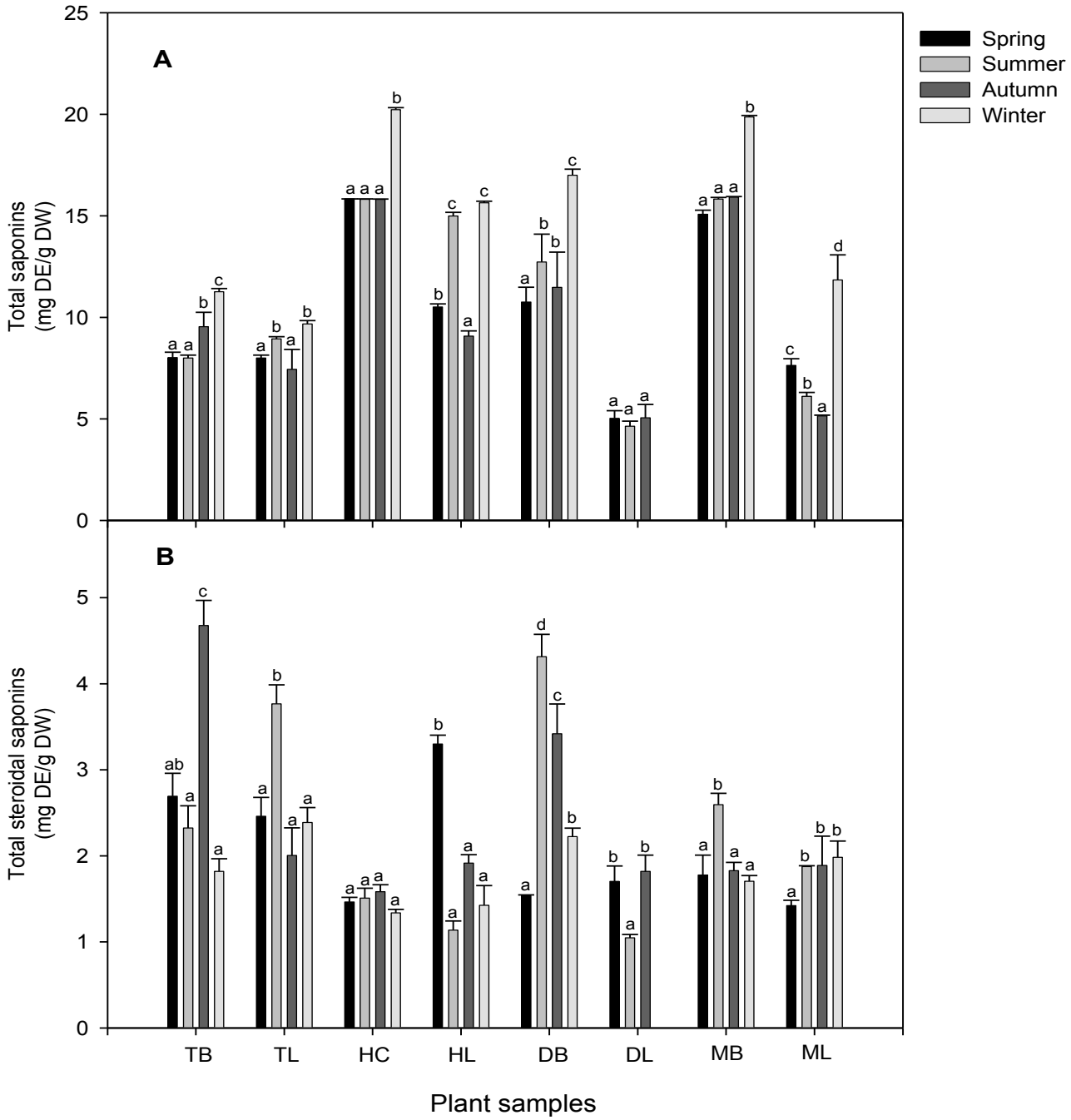


Figure 5.2. Total saponin concentrations (A) and total steroidal saponin concentrations (B) as diosgenin (DE) equivalents per dry weight (DW) in *T. violacea* bulbs (TB), *T. violacea* leaves (TL), *H. hemerocallidea* corms (HC), *H. hemerocallidea* leaves (HL), *D. robusta* bulbs (DB), *D. robusta* leaves (DL), *M. plumbea* bulbs (MB) and *M. plumbea* leaves (ML) in summer, autumn, winter and spring seasons. Results indicate the means ± standard error (n = 3). Significant differences ($P \leq 0.05$) within seasons in each plant sample are indicated by different letters.

Due to their toxicity to various organisms, saponins can be utilised for their insecticidal, antibiotic, fungicidal, and pharmacological properties. Saponins are reported to have antimicrobial (**BADER *et al*., 2000**), anti-inflammatory (**LI *et al*., 2002**) and haemolytic (**ODA *et al*., 2000**) properties. A number of the biological effects of saponins have been ascribed to their action on membranes. The affinity of the aglycone moiety for membrane sterols, particularly cholesterol, with which they form insoluble complexes (**GLAUERT *et al*., 1962; ARMAH *et al*., 1999**), is thought to be the major contributory factor for its haemolytic properties. Membrane fluidity is an essential characteristic that controls the enzyme activity of biological membranes and plays an important role in ion transport (**ENOMOTO *et al*., 1986**). The ability of saponins to affect this parameter may explain their biological activity in enzymes and microorganisms. Owing to their increased membrane permeability effect, saponins are considered to have some deleterious effects on all phases of the digestive metabolism in animals (**FRANCIS *et al*., 2002**). They are thought to interfere with the digestive bacterial flora in ruminants and in the process act as anti-nutritional factors. For these reasons, prolonged use and high concentrations of saponins in medicinal remedies may be harmful.

5.3.4. Phytochemical compounds and the biological activity

Collectively, plants produce a multitude of phytochemical compounds, constitutively and in response to stimulation. Biological activity in medicinal plants is a result of this pool of compounds. A critical view of the antimicrobial activity and the trend observed in seasonal fluctuations of phytochemical compounds in the present study, suggests a complex relationship between the two. In *T. violacea* (leaves and bulbs), total phenolic, gallotannin and tannin levels were generally low in all seasons but flavonoids levels were remarkably higher in spring than in the other seasons (18.41 µg/g in bulbs and 33.72 µg/g in leaves) (**Figure 5.1**). A closer look at the antimicrobial activity of these extracts reveals that some good antibacterial activity from the bulb (PE and DCM) and leaf (DCM) extracts were recorded in spring (**Table 3.1**). This may therefore, suggest that flavonoids might have contributed to the observed good antibacterial activity during this season. The winter bulb extracts of *T. violacea* demonstrated the best of the antibacterial activity recorded in the results of

this study, with MIC values as low as 0.195 mg/ml (**Table 3.1**). However, the flavonoids as well as gallotannin, and tannin levels during this season were surprisingly low in these extracts. The activity cannot, in such an instance, be attributed to phenolic compounds. The total saponin levels (**Figure 5.2 A**), on the other hand, were significantly higher in *T. violacea* bulbs in winter than in the other seasons. The total activity was correspondingly higher for *T. violacea* bulb extracts (36.4 ml/g) in the same season, an indication of the high concentration of the active compounds. It is highly likely therefore, that saponins might have contributed to this observed antibacterial activity. In summer and autumn, *T. violacea* DCM leaf extracts demonstrated good fungicidal activity (**Table 3.2**), with MFC values of 0.78 mg/ml. Steroidal saponin levels (**Figure 5.2 B**) were correspondingly higher in these extracts in summer but were significantly lower in autumn. Although this fungicidal activity may, in part, be attributed to the levels of steroidal saponins obtained in summer, the same may not be true for the fungicidal activity in autumn.

The high condensed tannin and gallotannin concentrations, particularly in summer, autumn and winter, in *H. hemerocallidea* corms, could have possibly contributed to the correspondingly good antibacterial and antifungal activity recorded for these seasons. This might have been complemented with the action of saponins that were consistently high across all seasons. The correspondingly high total activity for these extracts during the same seasons is again an indication of the concentrated levels of active compounds, results of which support condensed tannin and saponins as having a contributory effect on the observed antibacterial activity. Poor antibacterial and antifungal activity was shown by the *H. hemerocallidea* corm extracts in spring despite the higher total phenolic (26.82 mg/g) levels (**Figure 5.1 A**) recorded in this season than in the other seasons. In contrast with the corm extracts, leaf extracts of *H. hemerocallidea* exhibited good antibacterial activity in spring, which might have been a reflection of the high total phenolic (22.22 mg/g), gallotannin (56.10 µg/g) and saponin (3.30 mg/g) levels in this season. The lack of correlation between the antibacterial activity in *H. hemerocallidea* corm extracts and the peak in phenolic compounds suggest that individual classes of compounds, their different combinations and concentrations interact in determining the bioactivity of an extract. In autumn, on the other hand, *H. hemerocallidea* leaf extracts demonstrated good antibacterial (**Table 3.1**) and antifungal (**Table 3.2**) activity. However, total phenolic

content, flavonoids, gallotannin, condensed tannin and total saponin levels were surprisingly lower in *H. hemerocallidea* leaves during this season. The observed bioactivity might have been a result of other phytochemical compounds or synergistic effects of these at their respective concentrations and/or in combination with other compounds. In *D. robusta* leaf extracts, good fungicidal activity was recorded in spring and autumn, and an analysis of phytochemical composition during the two seasons, reveals higher levels of total phenolics (7.81 mg/g in spring) and gallotannins (27.15 µg/g in autumn) than in the other seasons. Flavonoid levels were significantly higher (11.16 µg/g in spring and 10.54 µg/g in autumn) in both seasons. A similar trend to that of flavonoids was observed for total steroidal sapogenin levels (1.70 mg/g in spring and 1.82 mg/g in spring), while total saponins were similar across all three seasons (no leaf samples for winter). The observed antifungal activity may have been a result of a combination of these chemical compounds. The higher levels of total saponins recorded in *M. plumbea* bulbs in winter, corresponds with good antibacterial activity observed in the same season, although the same plant extracts had poor antifungal activity in all seasons. These findings partly supports the observation by **DERITA *et al*.** (**2009**) who reported variations in the percentages of polygodial in *Polygonum acuminatum* extracts collected in the four seasons of the year and their corresponding variation in antifungal activity.

The relevant physiological effects of tannins upon microbes and enzymes, is their ability to complex with proteinaceous material (**HASLAM, 1996**). Considering the high tannin levels recorded for *H. hemerocallidea,* and in light of this fact, there is a highly likely possibility that the observed good anti-inflammatory activity in the extracts of this plant could have been due largely to the effects of tannins and saponins. COX-1 and COX-2 inhibitory activity displayed by the extracts in this study may have been due to phenolic compounds and saponins or a combination of these and various other compounds.

Due to the multiplicity of chemical compounds responsible for the biological activity in plants, the activity observed in this study cannot in any way be solidly attributed to the individual compounds observed in the present study. The interaction of a suite of chemical compounds in plants and the resulting activity is too complex. The interaction mechanism of the different compounds may result in some synergistic

effects where the presence of one compound potentiate or amplify the biological activity of another. In certain instances, however, the interaction may be antagonistic, where a chemical compound(s) reduces or inhibit the bioactivity of another compound. There is no doubt however, that the two mechanisms of action may have been at play among the compounds of the plant material screened in this study.

5.4. Conclusions

Generally, spring and winter seasons appeared to have favoured the production of high levels of both phenolic compounds and saponins in most plant species analysed in this study. Understanding the seasonal dynamics of the phytochemical concentrations in each medicinal plant species, may help medicinal plant users know when each of the plant species has high concentrations of both beneficial and harmful compounds. In addition, understanding the levels of these compounds in different plant parts may act as a guide for selection of the plant part to use in a given season and the application of plant part substitution as a conservation strategy. Although phenolic compounds and saponins were detected at varying levels in different seasons, issuing a definitive and conclusive recommendation on which plant part to collect in which season based on the results from this study may be premature and misleading. However, the results provide some preliminary insight into the seasonal trends in the physiological response of medicinal plants regarding phytochemical production. In seasonally changing environments, seasonal and ontogenetically constrained changes are hard to separate, and present complexity in the plant's metabolic response. Given that plants often employ a suite of defence mechanisms, it may, therefore, be more relevant to examine the phytochemical production trend under multiple stress conditions including pathogenic attack.

Due to the toxicity of some of these compounds at certain concentration levels, toxicological tests to ascertain the suitability of the different levels in different seasons are required. Research aimed at identifying and isolation of specific compounds responsible for the observed bioactivity is necessary. Moreover, tests on the interaction effects of different compounds will add to the better understanding of

their mechanisms of action in medicinal plants. In light of multiplicity of the phytochemical compounds produced by plants, the few phytochemical compounds screened in the present study may not have been suffice to provide a clear relationship between different levels of phytochemical compounds and their resulted biological activity. It would be therefore, desirable to carry out several similar studies encompassing a number of phytochemical compounds tested in a range of bioassays both *in vitro* and *in vivo.* Toxicological studies on these plants are also necessary considering that some compounds are toxic to humans when consumed at certain levels.

CHAPTER 6
General Conclusions

The rapid increase in population growth, cultural beliefs and inadequate provision of basic health care systems are considered to be the major driving forces in the extensive exploitation of medicinal plants in most developing countries. To this point, medicinal plants have become, not only a source of medicine but also provide income to many of the resource poor group of populations through trading. Plants undoubtedly provide these benefits to humans largely because of their inherent ability to provide a limitless pool of chemical compounds with diverse ranges of medicinal properties. Extensive scientific research efforts have been and continue to be directed in identifying and validating these compounds in the treatment of various ailments in the traditional circles. Significant successes have been made to this effect. This human to plant interaction has led to the progressive depletion of plant resources leading to the extinction of some plant species with the resulting loss of biodiversity and plant genotypes. The significance of medicinal plant conservation stems from this fact and the major cultural, livelihood or economic roles that they play in many people's lives. Medicinal plant conservation is, in some way, a microcosm of plant conservation as a whole.

In South Africa, like in all developing countries, medicinal plants are widely utilised in traditional medicine. The country is endowed with a very rich diversified flora and geophytes form part of this biodiversity. These slow growing groups of plants are utilised extensively in traditional medicine and most of the species are at the risk of over exploitation and extinction. Their conservation is therefore a priority.

This study sought to investigate the possible similarities in the pharmacological and phytochemical properties between bulbs and leaves across seasons in frequently used medicinal bulbs; with a view of promoting the use of the renewable plant parts (leaves) as alternative sources of medicinal remedies, thereby providing options in their conservation. Extracts of four medicinal bulbs frequently used in traditional medicine in South Africa, were evaluated for their seasonal pharmacological and phytochemical properties between their bulbs and leaves. The extracts were tested for antibacterial, antifungal properties and their ability to inhibit the two COX

isozymes (COX-1 and COX-2). Further to the pharmacological tests, samples from the four plant species were also subjected to phytochemical analysis to ascertain any possible seasonal variation in saponins (total saponins and total steroidal saponins) and phenolic compounds (total phenolics, condensed tannins, hydrolysable tannins and flavonoids).

Three of the four plant species (*T. violacea*, *H. hemerocallidea* and *M. plumbea*) exhibited a fairly comparable antibacterial activity between bulbs/corms and leaves, although in some cases the activity varied with the type of bacteria inhibited in each season. The leaves of these plant species can therefore be successfully used to substitute or complement bulbs in the treatment of diseases caused by bacterial strains in which they showed good activity. Bulb extracts of *D. robusta* did not show any good activity against any of the tested bacterial strains while leaf extracts showed good activity against *B. subtilis* in spring, summer and autumn and *S. aureus* in autumn. There were, however, no leaf samples for *D. robusta* in winter. The leaves of *D. robusta* therefore, demonstrated better antibacterial results compared to bulbs and can thus be used instead of bulbs during these seasons in the treatment of diseases caused by *B. subtilis* and *S. aureus*. Generally, most of the extracts expressed inhibitory activity against Gram-positive bacteria (*B. subtilis* and *S. aureus*) than the Gram-negative ones (*E. coli* and *K. pneumoniae*).

Based on the total antibacterial activity results from this study, a comparison between *T. violacea* bulb and leaf extracts reveal that, of the same bacterial strains that were inhibited by both extracts in each season, leaf extracts appeared to have more concentrated active compounds than bulbs in all the other seasons except winter. Going by this trend, harvesting of leaves for medicinal use in spring, summer and autumn, and bulbs in winter, will not only provide sustainable use for this plant species, but also offer better medicinal benefits. The shift in the concentration of active compounds from the leaves to the underground bulbs in *T. violacea* is probably a result of the translocation of such compounds from the senescing leaf tissues to the bulbs as a way of minimising metabolic costs. In *H. hemerocallidea*, corm extracts did not show good antibacterial activity in spring, while PE, DCM and EtOH leaf extracts showed good activity against the two Gram-positive bacterial strains. In summer, water and EtOH corm extracts of *H. hemerocallidea* were active

against *B. subtilis, E. coli* and *S. aureus* while no activity was shown by the leaf extracts in the same season. In autumn and winter, however, the corm (PE and water) and leaf (EtOH and water) extracts had a fairly comparable total activity, with both of their water extracts exhibiting some good antibacterial activity. In particular, water leaf extracts showed good antibacterial activity and high total activity against most of the bacterial strains including both of the Gram-negative bacteria. Based on these results, harvesting of leaves would be ideal in spring, autumn and winter, while bulbs could be harvested in summer.

The leaf extracts of all the screened plant species in this study demonstrated good fungicidal activity in autumn. Total activity indicates that water corm extracts of *H. hemerocallidea* had the highest concentration of fungicidal compound(s) with an activity of 433.3 ml/g in both autumn and winter. Corm and leaf extracts of *H. hemerocallidea*, were the only water extracts that showed good fungicidal activity. Water is the only readily available solvent for most of the rural medicinal plant users. The good antibacterial and antifungal activity shown by *H. hemerocallidea* water extracts indicate that medicinal plant users can derive better medicinal benefits from this plant by using water as a solvent. For the treatment of *Candida albicans*-related infections, the use of leaf material gathered from these plant species in autumn would likely yield better results. Both corm and leaf water extracts of *H. hemerocallidea* can be used for the treatment of candidal infections in autumn, while corm extracts can be used in winter. In spring, DCM leaf extracts of *D. robusta* and *M. plumbea* may be used for the treatment of candidal infections while in summer, using DCM leaf extracts of *T. violacea* would be ideal.

In the COX assays, generally all the PE and DCM extracts of all the screened plant species in this study showed moderate to high inhibitory levels (> 40%) in all the seasons. The EtOH corm extracts of *H. hemerocallidea* also demonstrated moderate to high inhibitory activity against COX-1 enzyme across all the seasons. In the COX-2 assay, EtOH bulb extracts of *T. violacea* exhibited high COX-2 inhibitory activity in all the seasons compared to insignificant levels in COX-1. On the other hand, ethanol leaf extracts of *T. violacea* exhibited moderate inhibitory activity against COX-2 across all seasons compared to insignificant levels in COX-1. This selective COX-2 inhibitory activity by these two extracts implies that the two extracts may be

used in the treatment of pain and inflammation with little detrimental effects. The comparable COX inhibitory activity of both leaves and bulbs of all the screened plant extracts in this study suggest that the two plant parts may be used interchangeably in the treatment of pain and inflammation in any season of the year.

The phytochemical composition (total phenolics, total saponins, total steroidal saponins, condensed tannins, gallotannins and flavonoids) were evaluated in each plant species using spectrophotometric methods. The results revealed that plant samples collected in winter had higher saponin levels for all the plant species evaluated compared with those collected in the other seasons. The level of variation from season to season was small in total saponins, with most plant species showing insignificant variations from one season to the other. However, total steroidal saponins were higher either in spring, summer or autumn and varied with plant species and plant part, with no distinct observable trend. Total phenolics in plant species and in bulbs/corms and leaves were either higher in spring or winter. Plant samples collected in autumn had the least levels of total phenolics in all the tested samples. Plant samples from *H. hemerocallidea*, recorded the highest levels of phenolic compounds. An almost similar trend to that of total phenolics was observed for flavonoids, gallotannins and condensed tannins in most plant samples, with higher levels either in spring or winter.

In trying to establish a link between the observed phytochemical production trends, there were in some cases existing relationships between the levels of some phytochemical compounds and the observed level of biological activities in different assays. For example, the good antibacterial activity exhibited by the bulb and leaf extracts of *T. violacea* in spring, coincides with the higher flavonoids levels obtained from the same plant samples in the same season.

The results obtained from this study demonstrate that the leaves of the screened plant species may substitute or complement bulbs in the treatment of certain ailments in traditional medicine. Thus, plant part substitution can be sustainably utilised in the conservation of these plant species while retaining the same medicinal benefits. The effective implementation of this conservation strategy may however, meet some resistance from traditional healers and other medicinal plant users.

Traditional healers are respected members in their communities and enshrined in the minds of the people because of their unique knowledge of healing plants. Scientific research works on traditional medicine should, therefore, establish close collaborative links with traditional healers so that the results can be more acceptable and applicable within communities.

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