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Comparison of secondary metabolite content and antimicrobial activity of four *Hypoxis* species used in traditional medicine

By

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Abstract

Hypoxis (commonly known as African potato, Ilabatheka, Inkomfe, sterretjie or yellow starflowers and also as monna wa maledu or thitidi) is a genus of the family Hypoxidaceae. The rootstocks of *Hypoxis* species, particularly *H. hemerocallidea*, are well-known to be used in traditional medicine for the treatment of different ailments, such as urinary tract infection, epilepsy, prostate cancer and onset diabetes. Several visits have been made to the Faraday Medicinal Market and Abey Bailey Nature Reserve in Johannesburg to determine the availability of *Hypoxis* plant materials. From these visits, it was discovered that different *Hypoxis* species are harvested and sold as the same plant commonly referred to as the African potato, and the treatment with these plants might be questionable as the secondary metabolites might differ. This was proven when a number of rootstocks bought from the medicinal market grew into plants showing distinct morphological differences when planted at the Agricultural Research Council (Roodeplaat, Pretoria). It is possible that the plants sold are used as the substitutes for the commonly main plant, which is *H. hemerocallidea* and a reality is that many of the problems related to the quality of medicinal plants are based on the substitution of the declared plants and when the substitution occurs the quality of the plants becomes compromised leading to the risks of public health. It is however, not known whether the substitution is done deliberately or unintentionally since the outward morphology of *Hypoxis* species

are not the same except their bright yellow flowers. The similarity within these species is on their underground rootstock. The dosage and toxicity of plant preparations is extremely important and, therefore adulteration is a concern where plant preparations are taken orally and the information about the plants used not being accurate. The aim of this study was to compare the secondary metabolite content of four *Hypoxis* species namely, *H. acuminata*, *H. hemerocallidea, H. iridifolia* and *H. rigidula*

Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) were used to analyze the secondary metabolite content of the plant extracts. Differences were also noted as one green compound was observed only in *H. acuminata* and *H. rigidula*. The HPLC results showed major differences in retention time in fresh material. The antibacterial activity of extracts of all four *Hypoxis* species showed similar results, although the activity differed amongst the microorganisms. The species showed high level of antioxidant activity that increased with increasing concentration in all four *Hypoxis* species. The species also showed no toxicity when tested *in vitro* on Vero cells however, they seemed to be toxic to cancer cells (Hela cells) but with a higher concentration. Hypoxoside was isolated and identified as the purple colour band on the TLC fingerprint and was confirmed in all the species. It might be possible to replace or substitute different *Hypoxis* species for *H. hemerocallidea* for medicinal use as the chromatograms of the other three species correlate well with the chromatograms of *H. hemerocallidea* after storage.

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Acknowledgement

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List of Abbreviations

Ac	absorbance of the control
AO	antioxidant
AP	African Potato
ARC	Agricultural Research Council
As	absorbance of the sample
AIDS	Acquired Immune Deficiency Syndrome
BPH	Benign Prostate Hypertrophy
BSS	Beta sitosterols
CCD	counter current distribution
DNA	deoxyribonucleic acid
DMSO	dimethylsulphoxide
DPPH	1,1-diphenyl-2picrylhydrazyl
OH^-	hydroxyl group
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HYP	hypoxoside
GC	Gas Chromatography
INT	p-iodonitrotetrazolium violet
I%	inhibition percentage
IC_{50}	inhibitory concentration 50
MEM	Minimal Essential Eagle's Medium

MIC	Minimum Inhibitory Concentration
MTT	methyltrazolium
NMR	Nucler Magnetic Resonance
PDA	photodiode array
ROP	rooperol
RI	refractive index
TLC	Thin Layer Chromatography
TM	traditional medicine
RPMI	Roswell Park Memorial Institute
SD	standard deviation
STA	Stigmastanols
UV	ultra violet
VOPI	Vegetable and Ornamental Plant Institute
WHO	World Health Organisation
XTT	sodium 3 – [1- (phenyl amino-carbonyl)-3.4-Tetrazolium]- nis –[4- methoxy-6-nitro

CHAPTER 1

1. Introduction

South Africa has a wealth of botanical diversity, and approximately 3 000 indigenous plants have been identified to have potential medicinal properties (van Wyk, 2008). Traditional herbal medicines are naturally occurring, plant derived substances with minimal or no industrial processing that have been used to treat illness local or regional practices. Medicinal plants are getting significant attention in global health debates (Risa *et al*., 2004). The uses of plants in the indigenous cultures of developing countries are numerous and diverse. One of the reasons could be low accessibility of western health care in developing countries, but could also be that traditional medicine provides people with a good alternative (de Boer *et al*., 2005). All cultures have long folk medicine histories that include the use of plants.

In rural areas throughout Africa plant resources fulfil a wide range of basic needs such as shelter, medicines and food (Cunningham, 1993). South Africa has been known as a country with a strong history of traditional healing hosting a variety of around 30 000 plant species. Medicinal plants have always played an important role in the therapy within the traditional health care system in South Africa (Grierson and Afolayan, 1999). In certain African countries, up to 90% of the

population still relies exclusively on plants as a source of medicines (Hostettmann *et al*., 2000).

Studies done in South Africa by Brandt *et al*., (1995) revealed that an estimated number of 60-80% of South Africans consult herbalists, traditional healers and sangomas before visiting any other medical facilities. Mostly the plant preparation is utilized in different ways such as infusions and decoctions etc. (de Boer *et al*., 2005). This shows that traditional medical practice is still the main vehicle of health care delivery today especially in the rural areas of the country where conventional medical facilities are not within the reach of most people (Oluwule *et al*., 2007).

Traditional medicine (TM) is the oldest and most diverse of all medicine systems. Plants have formed the basis for traditional medicine systems, which have been used for many years in different countries (Fakim, 2006). Traditional medicine occupies a central place among rural communities of developing countries for provision of health care in the absence of an efficient primary health care system (Tabuti *et al*., 2003). The vegetation and floral biodiversity of Africa provide African traditional health practitioners with an impressive pool of natural pharmacy from which plants are selected as remedies and/or as ingredients to prepare herbal medicines for an excess of human and veterinary disorders (McGaw and Eloff, 2008).

Recently in some laboratories, African medicinal plants have been selected for the investigation of their chemical constituents and pharmacological activities, in an attempt to establish a scientific basis for their folkloric, ethnomedical uses (Ojewole, 2006). Plant-derived traditional medicines are gaining popularity among a wider audience because of their affordability, accessibility and the global trend of seeking alternative methods of improving the quality of life. In the 20th century, herbal remedies spread to different nations where they have been processed and marketed as formulated pharmaceutical preparations. Studies done in the United States of America revealed that 29-85% of patients infected with the Human Immunodeficiency Virus (HIV) use dietary supplements and herbal medicines (Tilburt and Kaptchuk, 2008).

The traditional health practitioners of southern Africa have widely scrutinized the tuberous rootstock of *Hypoxis* (African Potato) for an array of ailments such as arthritis, hypertension, gastric and duodenal ulcers, yuppie flu, tuberculosis, asthma, prostate hypertrophy, type 2 diabetes mellitus, urinary tract infection and some central nervous system (CNS) diseases/illnesses especially epilepsy, and also as a natural antenatal remedy to prevent premature abortion or miscarriage (Zibula and Ojewole, 2000; Steenkamp, 2003; Musabayane *et al*., 2005; Nair *et al*., 2007; Nyinawumuntu *et al*., 2008; Ojewole *et al*., 2009). The widespread use of *Hypoxis* species in African traditional medicine has promoted several investigations and introduction into the market of lipophile extracts (Sibanda *et al*., 1990). Many websites, popular magazines and even the South African

Ministry of Health promote preparations of *Hypoxis* as an agent that can boost immunity in Human Immunodeficiency Virus Acquired Immune Deficiency Syndrome (HIV/AIDS) patients. Irrespective of the evidence, many Africans claim benefits from using the root of *H. hemerocallidea* (Mills *et al*., 2005). Recently considerable interest has been generated in the therapeutic properties of *Hypoxis* in treating prostatic hypertrophy and AIDS.

1.1. Adulteration and substitution of medicinal plants

Adulteration of medicinal plants is one of the common malpractices in medicinal plant material trade (Mitra and Kannan, 2007). Adulteration encloses a series of deliberate or accidental situations. It is described as intentional substitution with another plant species, however, unintentional adulterations also exist due to various reasons such as confusion in vernacular names between indigenous systems of medicine and local dialects. Most commonly deliberate adulterations or substitutions occur as follows:

- Substitution with a species or subspecies with lesser content of active constituents.
- Substitution with genera of related species.
- Careless collection
- Substitution with genera of completely different plants (Mitra and Kannan, 2007).

According to Mishra *et al*., (2009), the increase in demand of medicinal plants for commercial purposes also lead to the indiscriminate and unscientific collection as the quality of the material collected is not considered or given a higher priority. Adulteration and substitution of medicinal plant has become a major problem in drug discovery (Ahmed *et al*., 2010). Therefore, it is necessary to cultivate and authenticate medicinal plants remedy the adulteration.

1.2. Safety and quality control of medicinal plants

Quality is the basic requirements that food and beverages must comply with to guarantee the safe consumption of a product (Salgueiro *et al*., 2010). Quality control of medicinal plants materials intends to ensure the identity, purity and the content of the desired compounds, using a combination of both physical and chemical tests (Shinde *et al*., 2009; Salgueiro *et al*., 2010). The efficacy and safety of the medicinal plants rely on the quality and proper identification of the raw material or the original plant source and as such identification of plants with botanical verifications is essential as contamination due to misidentification of plant species is common (Igbal *et al*., 2010). In the past, the medicinal plants were largely collected from the wild without concerns about their taxonomic identity, purity, safety and efficacy (Ahmed *et al*., 2010). However, with further developments and improvements in communication there is a demand for high quality, safe, effective and clean plant products and their formulations with different substances have been growing significantly in the industrialized world.

Standardization of medicinal plant products is the prime need of the current time and is recognized by World Health Organization (WHO) (Shinde *et al*., 2009). Quality standards that raw material must fulfil are described and established in national, European or other international monographs. Important information to follow when determining quality control of a raw material of medicinal plants are definition of a species, the identification of the species and the chromatographic profiles by (Salgueiro *et al*., 2010).

Definition should include the correct botanical identity of the plant source with complete scientific name (genus, species and variety with author‘s name). The common name must also be included. Correct identity guarantee that the exact species, the proper plant part and accurate processing. Identification confirms that the raw material is not contaminated with other botanical species. Chromatographic profiling is an important test used for plant identification. There are several methodologies such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas liquid chromatography (GLC) that can be applied to determine the chromatographic profile of a plant or to develop the methods for the quantification of marker compounds. The more complex aspect of quality assurance relates to the standardization of medicinal plants is that the constituents of medicinal plants can vary as a result of different factors such as physiological, genetic and environmental factors (Salgueiro *et al*., 2010).

1.3. Motivation of the study

Different *Hypoxis* species are harvested and sold as the same plant commonly referred to as the African potato. Since substitution and adulteration of medicinal plants is a major challenge throughout the world, the safety and quality of the raw medicinal plants is an utmost need. The *Hypoxis* rootstocks bought from Faraday medicinal plant market grew into plants showing distinct morphological differences when planted at the Agricultural Research Council (Roodeplaat, Pretoria) which showed clearly that *Hypoxis* are undergoing a process of adulteration and substitution. It is possible that the plants sold are used as the substitutes for the commonly main plant, which is *H. hemerocallidea* and a reality is that many of the problems related to the quality of medicinal plants are based on the substitution of the declared plants and when the substitution occurs the quality of the plants becomes compromised leading to risks to public health. It is very important to be aware that the primary identity of the plant material can be decided by identification, biological or morphological, microscopic, chemical and biochemical methods.

1.4. Aim of the study

The aim of this investigation was to compare the secondary metabolite content and antimicrobial activity of four *Hypoxis* species using both fresh and stored plant material of *H. hemerocallidea, H. acuminata, H. iridifolia* and *H. rigidula*.

1.5. Objectives of the study

The specific objectives of this study were:

- To compare the secondary metabolite content of four *Hypoxis* species
- To compare their antibacterial activity
- To compare their antioxidant activity
- To evaluate and compare the cytotoxity against Vero and cancer cell lines (Hela cells).
- And, to isolate and identify the compounds associated with the similarities and differences of these species.

1.6. Structure of thesis

The thesis was written according to format and style used in the South African Journal of Botany.

Chapter 2 - gives a literature review, where the distribution, medicinal value, agricultural practices and morphology of *Hypoxis* are discussed. This chapter elaborates further on the uses and practices associated with each species.

Chapter 3 - reports on the comparison of the secondary metabolites associated with the four *Hypoxis* species. This chapter shows the similarities and differences of these species when analysed using Thin Layer Chromatography (TLC) and

High Performance Liquid Chromatography (HPLC) for both fresh and stored material.

Chapter 4 - describes the comparison of antibacterial and antioxidant activity of the *Hypoxis* species. Both the antibacterial and antioxidant activity were analysed using the serial dilution micro-plate assay.

Chapter 5 – evaluates and compares the cytotoxicity between the species on Vero and cancer cell lines.

Chapter 6 - comprises the isolation of compounds due to similarities and differences noted on the TLC fingerprint in Chapter three

Chapter 7 - gives a general discussion and conclusions and gives the future prospects with regards to this study.

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CHAPTER 2

Literature review

2.1. Morphology and classification of *Hypoxis*

Figure 2. 1 Yellow flowers of Hypoxis

Hypoxis (commonly known as African potato, Ilabatheka, Inkomfe, sterretjie or starflowers and also as monna wa maledu or thitidi) is a genus of the family Hypoxidaceae. The epithet *Hypoxis* was coined from the Greek word 'hypo' which means below and 'oxy' meaning sharp, which refers to the rootstock that is pointed at the base. *Hypoxis* was previously placed in Amaryllidaceae and Liliaceae families based on the similarity in appearance to members within these families. However, it was discovered that the aerial parts of *Hypoxis* are covered with soft hair and this feature differentiates them from Amaryllidaceae, and is

now currently placed in a small family named after it (Betto *et al*., 1992). According to Singh (2007) an ongoing taxonomic revision of *Hypoxis* in southern Africa has shown several taxa to be synonymous with the other already published species (Singh, 2007).

The plant family of the Hypoxidaceae belongs to the monocotyledons and includes other genera such as *Spiloxene, Rhodohypoxis, Pauridia, Saniella* and *Empodium* which occur world-wide except for the most parts of Europe and northern Asia (Pegel, 1979; Sibanda *et al*., 1990; Singh, 1999). *Rhodohypoxis* is closely related to *Hypoxis,* but may be distinguished from it by its white, pink or red flowers (Singh, 1999). The members of the Hypoxidaceae are perennial geophytic herbs with an underground rootstock and abundant adventitious roots, which enable them to survive unfavourable conditions (Singh 1999; Nair *et al*., 2006). Externally the rootstock is black brown and bright yellow to yellowish green internally when freshly sliced (Fig. 2.2). It darkens rapidly when exposed to air and exudes resinous yellow juice.

The yellowish colour within the rootstocks differs from species to species e.g. the rootstock of *H. rigidula* is darker than *H. iridifolia*. The rootstocks differ greatly in diameter ranging from 1 cm up to 12 cm (Appleton and van Staden, 1995). The plants are recognised by their bright yellow star-shaped flowers and are also called yellow stars (Fig. 2.1). The number of flowers per inflorescence varies from two to twelve. Leaves of *Hypoxis* arise directly from the apex of the

rootstock and the arrangements differ from species to species. Leaf bases in some species are enclosed in a sheath that creates a false stem. They range from linear to broadly lance-shaped and are usually hairy, which is the main distinguishing factor (Singh, 1999; Singh, 2007).

Figure 2. 2. Rootstocks of *Hypoxis* (unsliced and freshly sliced)

2.2. Distribution of *Hypoxis*

Hypoxis is indigenous to the southern part of Africa and has an estimation of 90 species world-wide and is almost cosmopolitan in distribution. The types of *Hypoxis* species include *H. hemerocallidea* which is more popular than the other species, *H. iridifolia*, *H. acuminata*, *H. rigidula*, *H. obtusa, H. latifolia, H. nitida*, *H. cochicifolia, H. angustifolia, H. interjecta, H. argentea* etc (Siegfried *et al*., 1984). *Hypoxis* occurs throughout most of the warm temperate and tropical zones of the world. In South Africa, the genus is distributed in five provinces namely, KwaZulu-Natal, Eastern Cape, Mpumalanga, Limpopo and Gauteng (Fig. 2.3), and also in Lesotho and Swaziland. The centre of diversity for *Hypoxis* appears to be KwaZulu-Natal and the Eastern Cape. *Hypoxis* is predominantly a

grassland genus preferring full sunlight even though few species are able to tolerate shaded conditions found in forest margins. However in Mozambique, *H. hemerocallidea* has a very wide geographical distribution ranging from savannahs to wet forests. *Hypoxis* rootstocks have a high tolerance to fire, of which it was claimed that fire promotes the growth of new leaves and flowering in the genus (Singh, 1999).

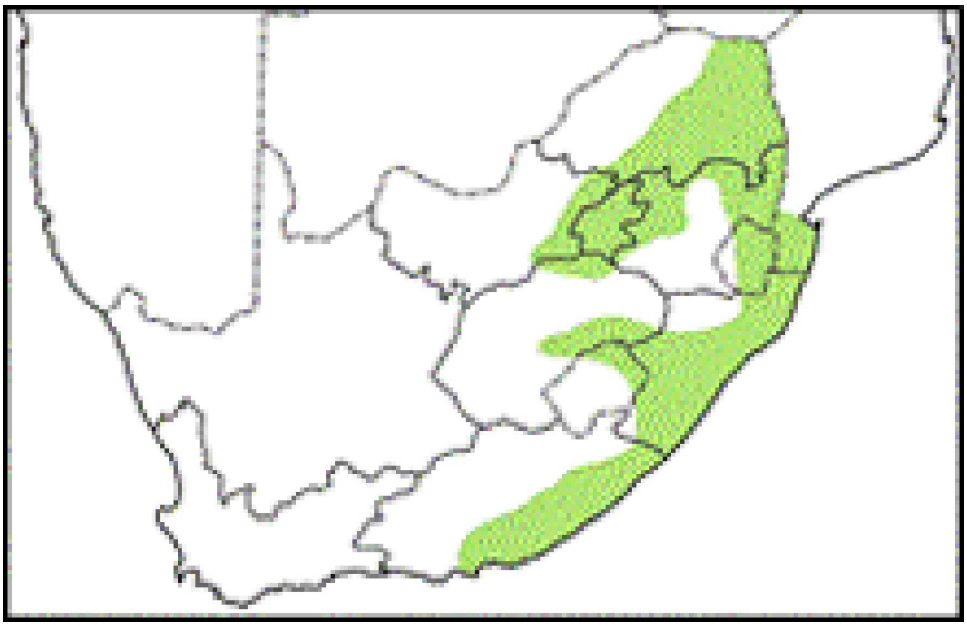

Figure 2. 3. Map illustrating the distribution of *Hypoxis* spp (Matsibisa 2007)

2.3. Economic and cultural value

It is estimated that the use of over counter medications totals over R2 billion annually in South Africa, however, over 70% of this total is spent on plant derived pharmaceuticals alone which exclude herbal remedies sold by informal street

vendors and those given by traditional healers. In the United States, the market for dietary supplements and herbal remedies doubled between 1996 and 1998 to a total of 12 billion dollars annually (Zeisel 1999).

The trade in traditional medicines forms part of a multi–million rand hidden economy in southern Africa stimulated by high population growth, rapid urbanisation, unemployment and the high cultural value of traditional medicines (Dold and Cocks, 2002). Among one of the most widely available medicinal plants sold in various markets in South Africa is the rootstock of plants belonging to the genus *Hypoxis* (Abegaz *et al*., 1999). Because of its attractive bright yellow flowers, it is also cultivated for its ornamental qualities (Singh, 1999; Singh, 2004). It was recorded that 71% of the traders in Maputo mentioned to sell *H. hemerocallidea* (Singh, 2004).

Hypoxis hemerocallidea was found to be one of the top ten most frequently sold plant species (selling approximately 11000 kg/year valued at R322 500) (Dold and Cocks, 2002). The fact that African potato treats various diseases and more recently Human Immunodeficiency Virus Acquired Immune Deficiency Syndrome (HIV/AIDS) related diseases has put *Hypoxis* under the spotlight (Drewes *et al*., 2008). According to the Endangered Wildlife Trust the species is impacted, because it is used both traditionally and by Western pharmaceutical companies, and the current rates of removal are not sustainable, therefore the conservation status of the plant needs to be reviewed.

The cultural uses of *Hypoxis* include as a charm against lightning, thunder and storms and also as an emetic for fearful dreams. The long keeled, hairy leaves that are tough and flexible are used for weaving, tying and binding. Several leaves can be twisted together into a rope, which is valuable for tying roofs when thatching and sewing grain baskets, therefore people generate an income (Roberts, 1990).

2.4. Medicinal uses of *Hypoxis*

The plant family Hypoxidaceae includes several genera of which the genus *Hypoxis* has been of medicinal interest (Kruger *et al*., 1994). Generally *Hypoxis* has been ingested by different tribes in southern Africa for a diversity of ailments. The genus *Hypoxis* is locally vulnerable, because of its medicinal value. Traditionally the rootstocks of *Hypoxis* are used for different ailments such as asthma, tuberculosis, urinary tract infection, headache, heart weakness, dizziness, duodenal and gastric ulcers, internal cancer tumours and some central nervous system ailments especially epilepsy (Kruger *et al*., 1994, Hutchings *et al*., 1996; van Wyk *et al*., 2002; Mills *et al*., 2005; Nair *et al*., 2006). In Mozambique the tea from *Hypoxis* was used to replace lost blood and it was used by soldiers during injuries. Traditional healers in southern Mozambique use the same tea in conjunction with other plants to combat “bad blood” in diabetes patients (Bouic *et al*., 1996).

The high concentration of phytosterols such as β-sitosterols has yielded an important breakthrough in the fight against benign prostate hypertrophy (BPH) (Mills *et al*., 2005). The strong decoctions of rootstocks are also used as purgatives (Mills *et al*., 2005). The majority of pharmalogical and clinical reports have led to the registration of patents and the commercialization of the extract of African potato (AP) in Germany using the trade name Hartzol (Nair *et al*., 2006).

Plant decoctions are given to weak children as tonics. A registered patent referring to drugs containing *Hypoxis* claims anti-tumour activity (Hutchings *et al*., 1996). Infusions of the rootstock are used as emetics (a medicine causing a person to vomit when suffering from food poisoning) and to treat dizziness. The extract of the rootstock was recorded to treat viral infection and reduce the rate of CD4 lymphocytes counts (Liebenberg *et al*., 1997). The decoction of other species of *Hypoxis* is recorded as being used as a steam bath against venereal diseases such as lice (Buwa and van Staden, 2006).

2.5. Agricultural practices of *Hypoxis*

Countries that have been cultivating medicinal plants for many years are well aware of the importance of good agricultural practices to ensure product quality and safety (Fennell *et al*., 2004). Commercial agriculture in South Africa has in the past mostly been focusing on existing traditional crops for their land use

activities. The biodiversity of the indigenous plants of South Africa offers a valuable source for investigation into the new crops (Reinten and Coetzee, 2002).

Research by the Agricultural Research Council (ARC) focusing on the commercialisation of indigenous plant material, has contributed to the cultivation practices of crops. Research has been conducted in a number of fields such as floricultural uses, medicinal plants such as *Hypoxis, Siphonochilus, Ruta* etc and indigenous vegetables such as *Amaranthus*. The focus areas of medicinal plant research are propagation and multiplication, cultivation, bioactivity etc. *Hypoxis* is the most prominent genus investigated, and recently they have developed a new method for the seed germination of *H. hemerocallidea* and *H*. *iridifolia.* However, *Hypoxis* was reported to be difficult when propagating from seeds, since the seeds remain dormant for about a year after flowering. It was discovered that complete mechanical removal of the hard seed coat lead to partial germination (Hammerton and van Staden, 1988).

Some of South African *Hypoxis* species with medicinal and horticultural value namely *H. hemerocallidea*, *H. obtusa*, *H. acuminata* and *H. rigidula* were micropropagated, and the method was successful. Over 90% of the regenerated *Hypoxis* species survived acclimitization. The best rooting was achieved using Murashige and Skoog medium without growth regulators (Appleton and van Staden, 1995), but as per Ndong *et al.,* (2006) shoot organogenesis appeared to

be the function of cytokinin activity since auxins alone did not initiate shoot formation. Rootstock division was reported to be a more rapid and successful method of propagating these plants (Hammerton and van Staden, 1988).

Due to the high rate of trade of medicinal plants, agricultural practice is extremely important to save some of the medicinal plants that are threatened by extinction. Despite the high rate of trading, agricultural practices will also help in regulating quality, efficacy and safety of the medicinal plants. It is therefore important to apply some of the developed propagation methods to rescue these plants from extinction.

2.6. Overview of selected *Hypoxis spp.* used for this study

2.6.1. *Hypoxis hemerocallidea* Fisch. C.A. Mey & Ave`-Lall

Figure 2. 4. *H. hemerocallidea*

Location and Botanical description

The species is distributed in the Savannahs regions of South Africa (van Wyk and Malan, 1997). This species was previously known as *Hypoxis rooperi*. It is the best-known species of the family Hypoxidaceae. The species has long, strap-shaped leaves and yellow, star-shaped flowers. The broad and slightly hairy leaves are arranged in three ranks, thus one above the other to form three distinct groups of leaves spreading outward from the centre of the plant (Fig. 2.4). The size of the leaves is up to 30 cm long and bright yellow flowers bearing soft hairs on the margin. The flowering time is from October to January. It is widely distributed in the grassland and woodlands of South Africa (van Wyk and Malan, 1997).

Traditional uses

Medicinally it is recorded as being 'zifozonke' meaning that the plant can be used to treat many diseases (Oluwule *et al*., 2007). The crushed rootstock is recorded to treat pimples and wounds (Matsiliza and Barker, 2001). *Hypoxis hemerocallidea* has been claimed to be an amazing and wonder plant medicine in the fight against various modern human disorders, including HIV/AIDS-related diseases, arthritis and hypertension. The rootstock may be useful in the management of adult onset, noninsulin-dependent, type 2, diabetes mellitus (Musabayane *et al*., 2006).

Pharmacological and Phytomedicine research

Evidence based laboratory investigations confirmed that aqueous extracts of *H. hemerocalidia* had pharmacological properties such as antinociceptive (reducing sensitivity to painful stimuli) (in mice), antioxidant, anti-inflammatory and antidiarrhoeal properties (Ojewole, 2006; Ojewole *et al*., 2009; Nair *et al*., 2007. According to Ojewole (2006) the findings of an experimental animal study indicate that *H. hemerocallidea* rootstock aqueous extracts possesses antidiabetic properties in the mammalian laboratory animal models used. Both hypoxoside and rooperol isolated from *H. hemerocallidea* showed antioxidant activities which increased with an increasing of the compound (Lindsey *et al*., 2002; Laporta *et al*., 2007). The methanol extracts of this species showed also to

inhibit the synthesis of prostaglandins (Laporta *et al*., 2007). The aqueous extract of the rootstock of H. hemerocallidea showed to possess the antibacterial activity when tested in rodents (Ojewole *et al*., 2009). The rootstock of *H. hemerocallidea* has yielded three cytokinins, identified as zeatin, zeatin riboside and zeatin glucoside (Hutchings *et al*., 1996).

Hot aqueous extracts of the fresh rootstock was recorded to treat benign prostatic hyperplasia (BPH). Its extract is being increasingly utilized in pharmacological preparations focused on prostate health (Laporta *et al*., 2007). It was also recorded that the methanolic extract produced a greater anti-inflammatory effect than the aqueous extract (Steenkamp, 2003). β-sitosterols were found to have a vitamin-like action on the functioning of the immune system Researchers reported that sitosterols decrease the testosterone levels through inhibition of 5α reductase, or by decreasing the binding of dihydrotestosterone within the prostate (Bouic *et al*., 1996).

2.6.2. *Hypoxis rigidula* Baker

Figure 2. 5 *H. rigidula*

Location and Botanical description

The species grows in marsh margins in Lesotho (Roberts, 1990). This species is usually 30-60 cm high and the leaves are soft, thin, and strongly ribbed, covered with white hairs forming a false stem at the base (Fig. 2.5). The flowers are 30-40 mm in diameter (Roberts, 1990). The flowering time is September and October. Internally the rootstock is much brighter than *H. hemerocallidea* and *H*. *iridifolia*. The species is occasionally hybridised with *H. obtusa,* and leaves are used to make floor polish by local tribes in the Escort area of Kwa-Zulu Natal (Singh, 2004).

Traditional uses

Traditionally the leaves are used to make ropes and hat trimmings. *Hypoxis rigidula* was among the other *Hypoxis* species that were selected by Drakensberg baboons as their source of food (Whiten *et al*., 1991). The species are claimed to contain protein, lipids, phenolics and fibre (Whiten *et al*., 1991). No pharmacological and phytomedicine research have been reported on this species.

2.6.3. *Hypoxis acuminata* Baker

Figure 2. 6. *H. acuminata* Baker

Location and Botanical description

Hypoxis acuminata is found in damp grasslands in the Eastern Cape, Lesotho and Swaziland (Singh, 2006). The leaves are thin and very long like that of *H. rigidula* but it is differentiated from other species by its multi-stems (Fig. 2.6) and

it has small rootstocks, which are also bright yellow inside as compared to *H. hemerocallidea* and *H. iridifolia*. The flowers are also similar to the flowers of *H. rigidula*.

Pharmacological and Phytomedicine research

The aglycone geraniol was reported to be linked to glucose, which is connected to apiofuranose residue. However, glucose and other two unknown compounds were yielded when acuminoside was hydrolysed with cellulose (Bredenkamp *et al*., 1989). Two compounds namely, hypoxoside and a new geraniol acuminoside were isolated from *H. acuminata* using preparative High Performance Liquid Chromatography (Prep Pak 500/ C18) (Bredenkamp *et al*., 1989).

2.6.4. *Hypoxis iridifolia* Baker

Figure 2. 7. *H. iridifolia*

Location and Botanical description

This species occurs in grasslands, often in sandy soil of the Free State, KwaZulu-Natal, and Northern Cape and also in Swaziland. *Hypoxis iridifolia* is a herbaceous geophyte with a rootstock densely covered with bristle hairs (Fig. 2.7). The flowers are star-shaped and lemon yellow on the inner segments. The flowering time is in August. The leaves are spirally twisted when matured (Van Wyk and Malan, 1997).

Traditional uses

Medicinally, the species is used as a purgative in Lesotho.

2.7. Secondary metabolites associated with *Hypoxis*

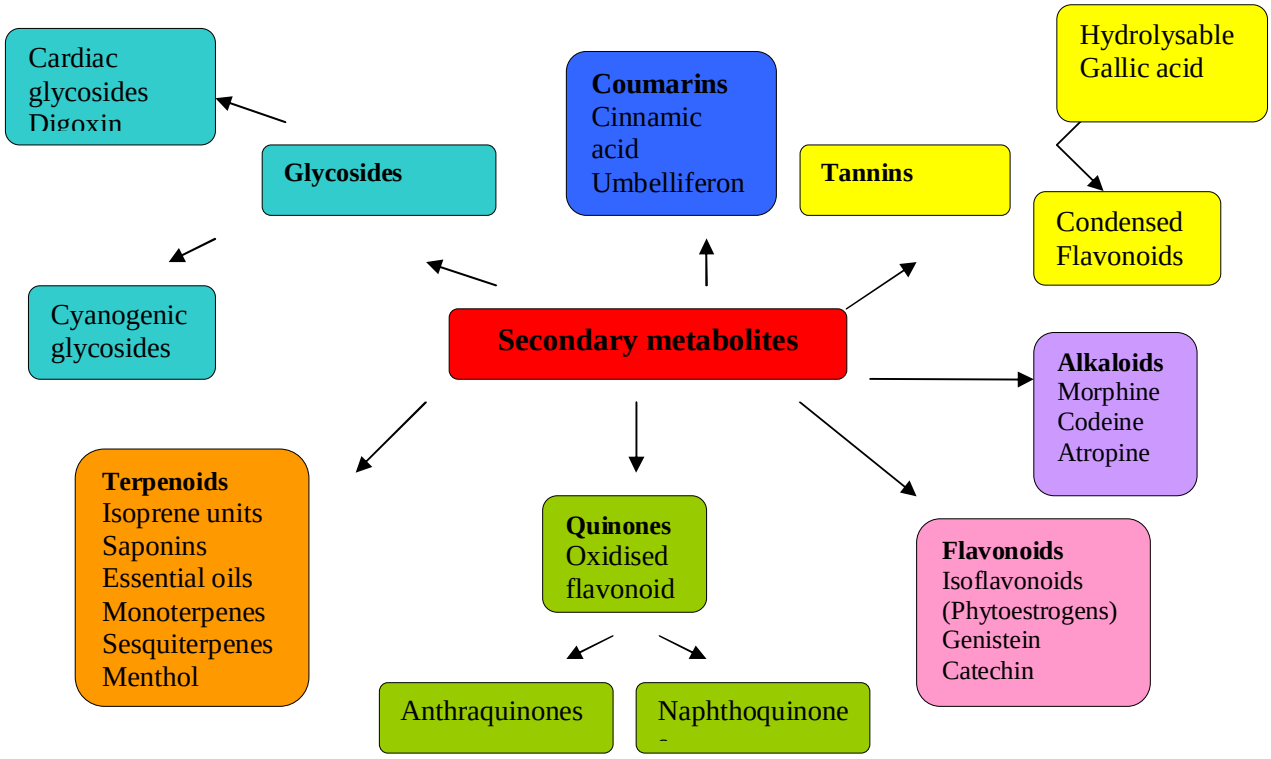

Figure 2. 8. Schematic diagram showing different types of secondary metabolites (Adapted from Pengelly, 2004)

Nature's ability to synthesize highly diverse chemicals, some of which posses exquisitely selective biological activities is well known to today's scientists involved in drug discovery. Secondary metabolites have historically served as templates for the development of many important classes of drugs. They are organic compounds that are not involved in the normal growth, development or reproduction of plants (Fig. 2.7). Some act as chemical defence against animals. They provide invaluable resources that have been used to find new drug molecules (Fakim, 2006).

Secondary metabolites differ from primary metabolites in having a restricted distribution in the plant kingdom. Particular secondary metabolites are often found in only one plant species or a taxonomically related group of species, whereas primary metabolites are found throughout the plant kingdom (Taiz and Zeiger, 1998). According to Fakim (2006) analysis of natural products as a source of new drugs over a period 1981-2002 showed that 67% of the 877 small molecules, new chemical entities are formally synthetic but 16 (4%) correspond to the synthetic molecules containing a pharmacophore derived directly from natural products. However, in the area of the anti-infectives (anti-bacterial, antifungal, parasitic and viral), close to 70% are naturally derived. It is clear that the nature will continue to play a vital role in the drug discovery process.

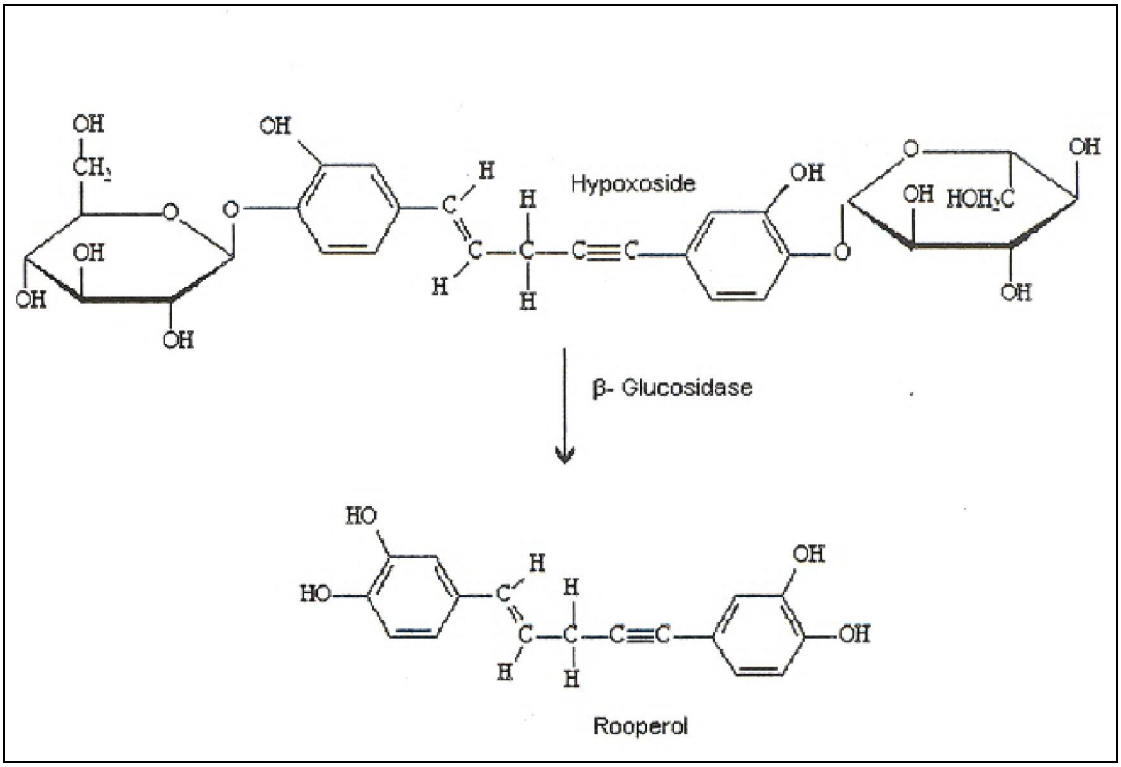

Figure 2. 9. **Structures of hypoxoside and rooperol (Nair *et al*., 2007).**

2.7.1. Hypoxoside

The value of *Hypoxis* species is claimed to be in its content of sterols such as hypoxoside. Hypoxoside (HYP) is the trivial name for (E)-1,5 bis(4'-B-D-glucopyranossyloxy-3'-hydroxyphenyl)pent-1-en-4-yne, which is a norligan diglucoside isolated from the rootstock of the family Hypoxidaceae. This compound has characteristics of glycosides (Fig. 2.8). Glycosides are molecules in which a sugar group is bonded through its anomeric carbon to another group via glycosidic bond (Fakim, 2006). Hypoxoside has reported to have an uncommon aglycone structure consisting of a C6 (aromatic) –C3-C2-C6 (aromatic) skeleton (Nair *et al*., 2007). Hypoxoside was first isolated from the

rootstock of *Hypoxis obtusa* by Marini- Bettolo *et al*., (1982) also from *H. hemerocallidea* and later in *H. rigidula*, *H. latifolia*, *H. acuminata*, *H. nitida* and *H. angustifolia* (Marini- Bettolo *et al*., 1982). Hypoxoside is reported to be a unique feature in this genus (Nair *et al*., 2006). According to Singh, (1999) not all *Hypoxis* spp. contains hypoxoside. Species that have a much deeper yellow colour internally contained hypoxoside in high levels. There is a seasonal variation in the concentration of the diglucoside and the maximum concentration remains to be established (Drewes *et al*., 1984). Studies done by Boukes *et al*., (2008) also revealed that there are some variations of both the sterol and hypoxoside contents between three different *Hypoxis* species (*H. stephillis*, *H. sobolifera* and *H. hemerocallidea*).

The hypoxoside compound is useful as a pro-drug for anti-inflammatory use and may also serve as a convenient starting material for preparing rooperol and rooperol derivatives (Nair *et al*., 2006). Thus once hypoxoside is ingested, it is readily converted to rooperol (Fig. 2.8). Extracts of plants of the *Hypoxis* genus have been found to be active in tests against mouse p388 lymphotic leukaemia cell cultures (Drewes and Liebenberg., 1987).

2.7.2. Rooperol

Rooperol (ROP) may be obtained by treating hypoxoside with a β-glucosidase to remove the attached glucose groups (Fig. 2.8). β-glucosidase is an enzyme found predominantly in the gastrointestinal tract (Mills *et al*., 2005). Rooperol is recorded as being the active compound that has immune-modulating properties and is claimed to be used by patients suffering from cancer and HIV. Low concentrations of rooperol could be selectively cytotoxic for the tumour cells and stimulate collagen synthesis, thus inhibiting tumour growth, invasion and metastasis (Dietzsch *et al*., 1999).

Pharmacokinetic studies have indicated that rooperol can be found in faeces, and metabolites are found in the serum and urine as its glycosides, sulfates, mixed glucuronides and sulforonides. These metabolites when deconjugated back to rooperol, were found to be cytotoxic to cancerous cells (Nair *et al*., 2007). It is also recorded as being a lipoxygenase inhibitor and effective against mutagenesis in the Ames test (Hostettman *et al*., 2000).

2.7.3. Other secondary metabolites

Beside rooperol and hypoxoside, nyasoside was also one of the compounds isolated from *H. nyasica*. The compound was reported to be biologically related to hypoxoside as they are di-glucosides (Marini-Bettolo *et al*., 1985). β-sitosterols is an ever present plant sterol with a cholesterol–like structure, which was

isolated from *H. hemerocallidea* in the late 1950's and identified as the active compound against BPH. In addition to hypoxoside and rooperol, the rootstocks are reported to contain β-sitosterols, sterol, monoterpene glycosides, stanols and stigmastanols (STA) (Laporta *et al*., 2006; Nair and Kanfer, 2008). However, Nair and Kanfer 2008 reported the lower content of sterols and sterolins in the aqueous *Hypoxis* extract that they were analysing.

The result of the chemical analysis of *H. hemerocallidea* showed, that the species have different classes of secondary metabolites namely, glycosides, polyphenols, saponins, steroids and tannins (Oluwule *et al*., 2007). It has been shown that the members of this family contain various sugars and sugar derivatives such as mucilage, hemicellulose, polysaccharides and specific sugars such as xylose, glucose, mannose, fructose, and sucrose and glucoronic acid. Phenolic compounds have also been detected such as caffeic acid, ferulic acid as well as quercetin (Pegel, 1979).

2.8. The effect of storage on the chemical composition of medicinal plants

Based on the long history of medicinal plant use, traditional medicines are believed to be safe, however, absence of regulation of the medicinal plant trade in aspects such as collection, processing and storage provides no guarantee (Street *et al*., 2008). Besides that in many African countries, traditionally used medicines are sold in markets or prescribed by traditional healers, the time collected and period stored is not noted. The physical condition and infrastructure

in the medicinal markets are underdeveloped and plant materials are clearly exposed to serious contamination such as microbial, insect attack and temperature (Stafford *et al*., 2005).

According to Mander (1997), 84% of the rural clinic patients he interviewed would prefer hygienic packed indigenous medicines and modernised hygienic trading sites (Mander, 1997). Because of this major problem of storage and dependence on plants as medicines, it is important to study their safety and efficacy. This is because environmental pollution, misidentification and adulteration provide further grounds for concern (Masoko *et al*., 2005).

Plant storage is the most important part of the procedure, because failure to store plant material correctly means that all the time and effort of collecting and drying has been wasted. Any fungal contamination on any part of the plants leads to large differences in the chemical composition and biological activity of that extracts. The conditions contributing to decomposition of plant material are governed by the composition of the plant material and by various environmental factors (Fennell *et al*., 2004).

The effect of storage of plants on pharmacological activity seemed to be varied between the species. For instance, according to McGaw (2001) extracts of *Schotia brachypetala* leaves did not show changes in chemical composition and antibacterial activity after being stored for 18 months where as the TLC

fingerprints of *Alepidia amatymbica* and *Drimia robusta* showed to document chemical changes when analysed by Fennell *et al*, (2004), although antibacterial activity was retained, some chemical breakdown was reported during storage.

According to Stafford *et al*., (2004) the *in vitro* biological activity of nine frequently used South African medicinal plants stored for various lengths of time at 20˚C showed similar trends of chemical break down but retained biological activity. There is lack of literature on the effect of storage on *Hypoxis* species. In general, changes in the TLC fingerprint of a material as a result of storage confirmed that storage has an effect on plant material although this is species specific (Fennell *et al*., 2004).

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CHAPTER 3

Analysis and comparison of secondary metabolite content of four *Hypoxis* species used in traditional medicine in South Africa

3.1. Introduction

Throughout the history of mankind many infectious diseases were treated with plant extracts (Shale *et al*., 1999, Buwa and van Staden, 2006). The majority of plants are harvested from wild populations within South Africa and its neighbours for the medicinal plant trade (Dold and Cocks, 2002). The most important factors when it comes to the medicinal plant industry are the chemical constituents, since they are responsible for the plant's efficacy in treating illness and diseases (Louw *et al*., 2002). Plants produce a diverse array of secondary metabolites with various functions such as defence against herbivores, diseases and parasites (McGaw and Eloff, 2008). These secondary metabolites are claimed to possess chemical structures that are not available in synthetic compound libraries. There are approximately 250 000 plant species in the world, and 5-15% of these species are reported for being tested for potentially useful biological active compounds (Pieters and Vlientick, 2005).

The medicinal value of these plants lies in some chemical substance that produces a definite physiological action on the human body (Dzingirai *et al*., 2007). However, there is a need to link scientific proof and clinical validation with chemical standardization, biological assays, animal models and clinical trials. Extraction and characterization of several active phytocompounds from these medicinal plants gave birth to some high activity profile drugs (Palombo *et al*., 2001). It has been reported that renewed interest in African traditional medicine has attracted the attention of not only government and private research laboratories and institutes, but also pharmaceutical industries so as to rationalize the scientific and therapeutic values of traditional medicine (Fakim, 2006).

Hypoxis, (particularly *H. hemerocallidea*) commonly known as African potato, is a plant species that has been long used as traditional medicine in Southern Africa (Peter *et al*., 2008). Laboratory studies reported that the most important chemical constituent of African potato extracts is hypoxoside, which is a biologically inactive pro-drug with an uncommon aglycone structure, consisting of diphenyl-1-en-pentane skeleton (Marini-Bettollo *et al*., 1982).

The most recent information is that a *Hypoxis* extract is used as a major constituent with tea tree oil (*Melaleuca alternifolia*), aloe (*Aloe barbadensis*), comfrey (*Symphytum officinale*) and others in the production of skin products (creams), and these creams are specifically used for clearing skin blemishes (Drewes *et al*., 2008). More research has been conducted on one species

namely, *H. hemerocallidea* and numerous compounds associated with the medicinal properties have been identified in relation to this species. Little research has been carried out on other *Hypoxis* species (Boukes *et al*., 2008).

The main aim of this chapter was to analyse and compare the secondary metabolite content of four *Hypoxis* species utilized in traditional medicine. This is due to the fact that different species of *Hypoxis* are sold and utilized without any evidence that they contain the same secondary metabolites. Both fresh and stored plant material were used in this study. Chromatography is a separation technique whereby the components of a mixture may be separated by allowing the sample to be transported through packed bed of material by fluid mobile phase (Igbal *et al*., 2010). The techniques used for the chemical analyses were Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC). Thin layer chromatography is rapid and easy to use but is not quantitative, whereas HPLC is the preferred technique since it gives a good separation of the different chemical components and is quantitative (Diederichs, 2006).

3.2. Materials and methods

3.2.1. Plant collection

Four *Hypoxis* species namely; *H. hemerocallidea, H. iridifolia*, *H. rigidula* and *H. acuminata* were used in this study. *Hypoxis hemerocallidea* and *H. iridifolia* were collected at the Agricultural Research Council-Roodeplaat Vegetable Ornamental

Plant Institute (ARC-VOPI) Pretoria (South Africa), 25˚35 24.2 S , 28˚ 21 46.9 E, whereas *H. rigidula* and *H. acuminata* were collected at Waterkloof Airforce Base, Pretoria (South Africa), 25˚ 49 03.5 S, 28˚ 14 05.3 E. Ten samples of each species were collected in April. Five were used as fresh samples and the other five were used as stored samples to evaluate if there may be any change of chemical composition as compared to the fresh plant material.

Voucher specimens of the four species were assigned (NR 101, NR 102, NR 103 and NR 104) and deposited in the H.G.W.J Schweickerdt Herbarium (PRU) at the University of Pretoria. The samples were stored as unprocessed material (just like in the medicinal plants market) for two months (60 days) in a maize bag at room temperature.

3.2.2. Extraction of plant material

Extracts were prepared from both the fresh and the stored rootstocks, respectively. Five rootstocks were harvested and extracted with four different solvents, namely; ethyl acetate, ethanol water (8:2), methanol and petroleum ether. Grated twenty grams of the plant material was first extracted with 150 ml of petroleum ether with magnetic stirring for 15 min followed by filtration and repeated twice. The same plant material was sequentially extracted with ethyl acetate and methanol. Another 20 g was used for the ethanolic water (8:2) extract where the extraction was carried out under reflux at 70˚C for 30 min. All the extracts were evaporated to dryness using rotary evaporation (Buchi)

(Heidolph), and a yellowish brown powder was obtained from both the methanol and ethanol water extracts. The colour intensity of the methanol extracts differed amongst species. The petroleum ether and ethyl acetate extracts were not used through the entire study due to insufficient amount of crude extract. Methanol and ethanol water crude extracts were kept in bottles and refrigerated until use.

3.2.3. Chemical analysis of plant extracts

Thin Layer Chromatography (TLC)

Aluminium TLC plates (20 x 20 cm) covered with silica gel (0.2 mm thickness) were used. A 10 mg/ml solution was prepared from both methanol and ethanol water (8:2) extracts. Five hundred micrograms was applied on silica TLC plates and developed in a TLC tank and three solvent systems were used. These were: 100% chloroform, chloroform: ethyl acetate (4:6) and ethyl acetate: acetic acid: formic acid: H_2O (100:10:10:15). The plates were viewed under UV light with both the short and long wavelengths (254 and 366 nm) and fluorescent bands were marked with a soft pencil. The plates were sprayed with freshly prepared vanillin solution as a colouring reagent (7.5 g vanillin, 250 ml methanol and 5 ml of H_2SO_4). The plates were heated after they were treated with vanillin. Finally the different colours of the spots were also marked with a soft pencil. The TLC fingerprints of the different species were used to identify the differences and similarities between the extracts.

High Performance Liquid Chromatography (HPLC)

This chemical analysis was carried out on a HPLC system equipped with a quaternary pump (2LC-10 AD), photodiode (PDA) UV detector SPD-10A (V), Refractive index (RI) detector and auto sampler (SIL-10AD) (LC Solution, Shimadzu, Japan). The chromatographic separation was achieved on a Symmetry C18 reverse phase silica gel HPLC column (5µm, 250 mm x 4.6 mm i.d.) (Waters). Ten milligram per millilitre of the dissolved extract (methanol and ethanol water (8: 2)) was transferred into vials and inserted in the auto sampler. The chromatographic separation was performed at 25 min at a flow rate of 1 ml/min with a mobile phase composed of water and acetonitrile. The multigradient solvent system used was acetonitrile: water (4:6), which gradually changed to acetonitrile: water (8:2) using a wavelength of 260 nm.

3.3. Results

Thin Layer Chromatography (TLC)

Both methanol and ethanol water (8:2) extracts were evaluated in this study. Their TLC fingerprints were similar since they are both highly polar solvents therefore only TLC fingerprints for both fresh and stored methanol extracts are shown. The TLC fingerprint (Fig. 3.1) of the fresh rootstock extracts showed similarity between all four different species. Numerous bands appeared on the

chemical fingerprint of the fresh prepared material as compared to the chemical fingerprint of the stored (Fig. 3.2). Compounds 4 (purple), 1 (blue), 2 & 5 (pinkish) were common in all the species (Fig. 3.1). Compounds marked with pencil are those that were visible under UV light (366 nm) whereas the other compounds were only visible after the TLC plate was sprayed with a vanillin (Fig. 3.2).

The difference noted was seen with compound 6 (green) that was visible in the *H. rigidula* (C) and *H. acuminata* (D) extracts, while absent in the *H. hemerocallidea* (A) and *H. iridifolia (*B) extracts and also compound 3 (reddish pink), which was only present in species (A) *H. hemerocallidea* and (B) *H. iridifolia*. The green compound is totally absent on the fingerprint analysed from the stored material. All pink bands that are clearly visible on the fresh prepared TLC seemed to fade after storage. The solvent system of ethyl acetate: acetic acid: formic acid: H_2O (100:10:10:15) showed better separation when compared to other solvent systems used. The retention factor (Rf) was calculated as the distance travelled by the compound divided by the distance travelled by the solvent (Dhont, 1980) (Fig. 3.1 and Fig 3.2).

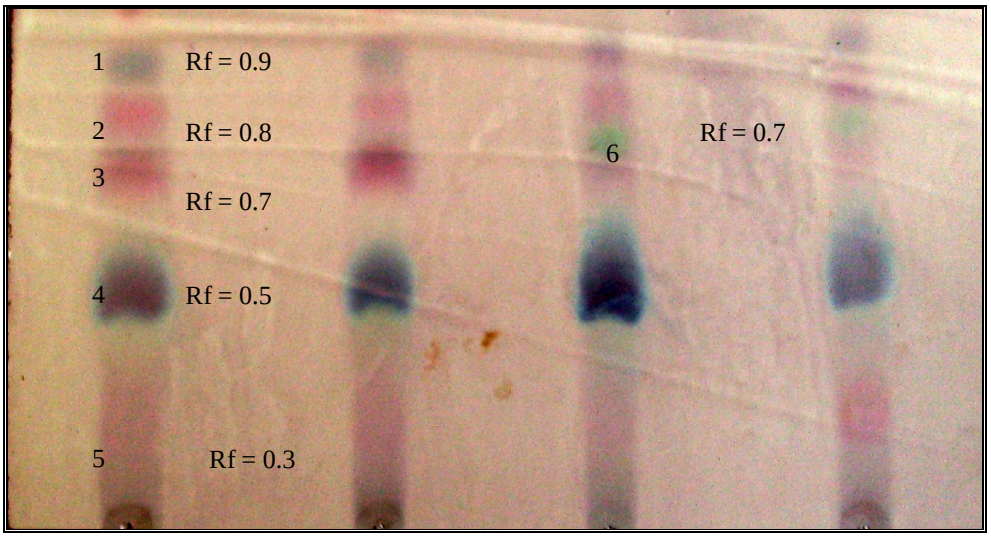


Figure 3. 1 . Fingerprint of four methanol extracts of fresh *Hypoxis* species. (A= *H. hemerocallidea*, B= *H. iridifolia*, C= *H. rigidula* and D= *H. acuminata*). Solvent system: ethyl acetate: acetic acid: formic acid: H_2O (100: 10: 10: 15)

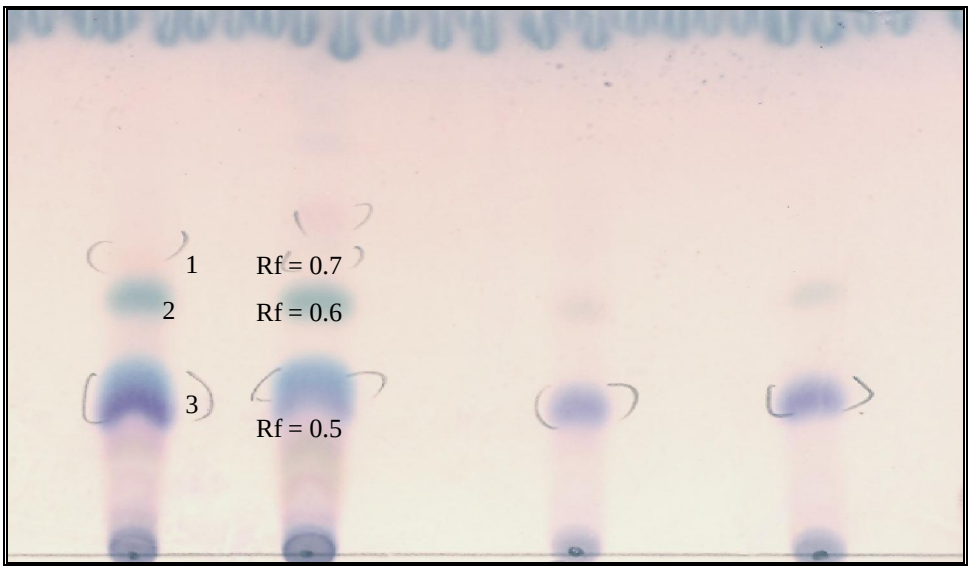
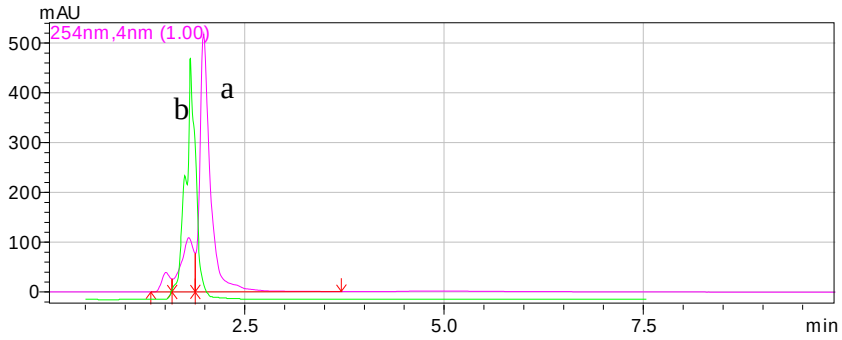


Figure 3. 2. Fingerprint of four methanol extracts of stored *Hypoxis* species. (A= *H. hemerocallidea*, B= *H. iridifolia*, C= *H. rigidula* and D= *H. acuminata*). Solvent system: ethyl acetate: acetic acid: formic acid: H_2O (100: 10: 10: 15)

High Performance Liquid Chromatography (HPLC)

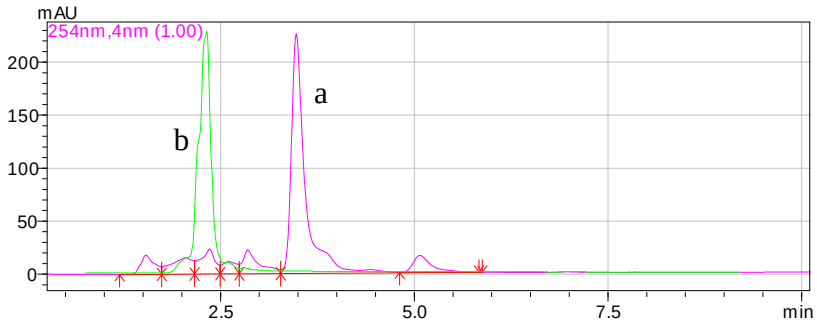
In general the methanol extracts chromatograms for fresh material showed some variation. The chromatograms varied greatly with regard to retention time. Two species namely, *H. acuminata* and *H. hemerocallidea* did not show any variation in both fresh and stored material (Fig. 3.3 A and D). Numerous minor peaks were also observed in all the species and they seemed to be in lesser content with regard to both methanol and ethanol water extracts. The major difference in fresh material was noted in *H. rigidula* (Fig. 3.3 C a), and in *H. iridifolia* (Fig. 3.3 Ba). These two species varied to the other species in terms of their retention times. Nevertheless after storage the major peaks observed in *H. rigidula* and *H. iridifolia* were showed to be similar to *H. acuminata* and *H. hemerocallidea* which was the interesting part of this study.

The visibility of minor peaks disappeared after storage. The degradation of these compounds after storage indicated that a period of two months had an effect on compounds contained in *Hypoxis* species (Fig. 3.3 Ab, Bb, Cb and Db and Fig. 3.3 Aa, Ba, Ca and Da). With the ethanol water (8: 2) extracts (Fig. 3.4) the profile of the fresh and stored materials were similar to the methanol extracts, however *H. iridifolia* showed to be different to the other species.



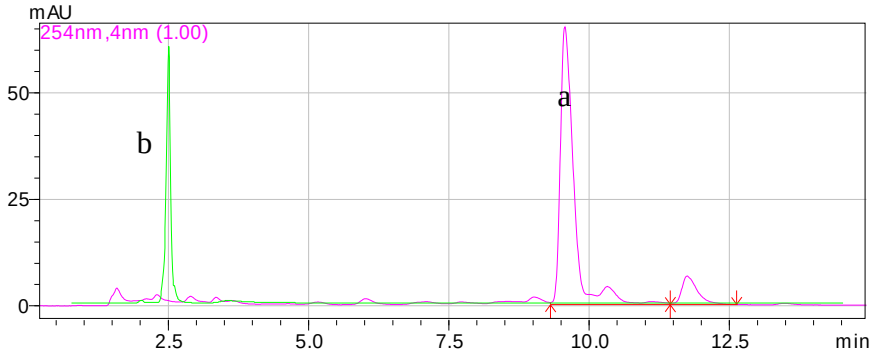
A. Fresh (a) and stored (b) material

Hypoxis hemerocallidea



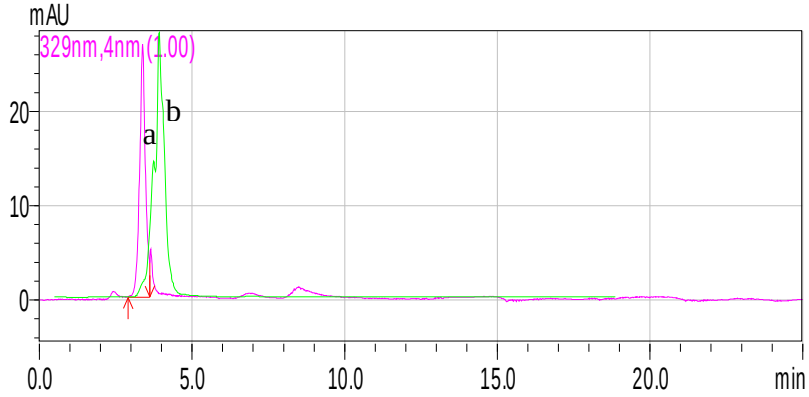
B. Fresh (a) and stored (b) material

Hypoxis iridifolia



C. Fresh (a) and stored (b) material

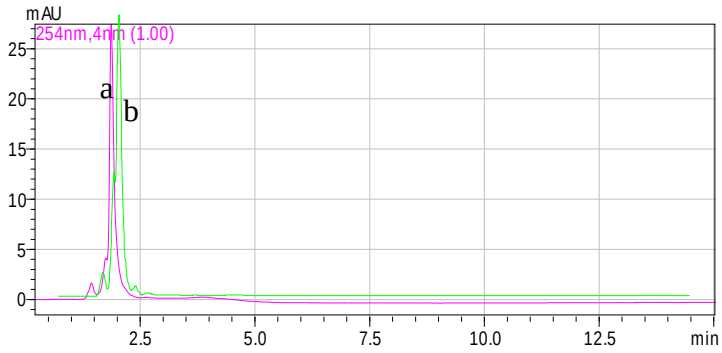
Hypoxis rigidula



D. Fresh (a) and stored (b) material

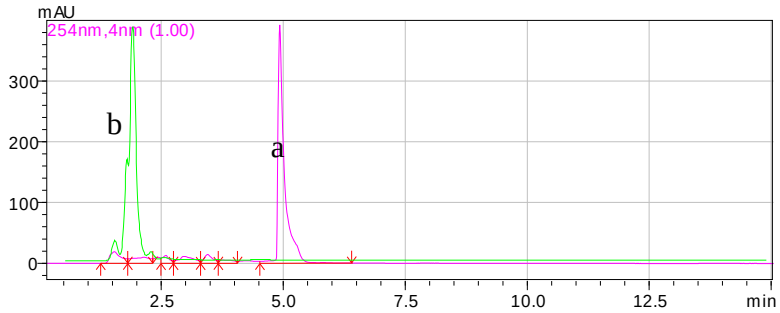
Hypoxis acuminata

Figure 3. 3. Chromatograms of (fresh and stored) methanol extracts from rootstocks of four *Hypoxis* spp.



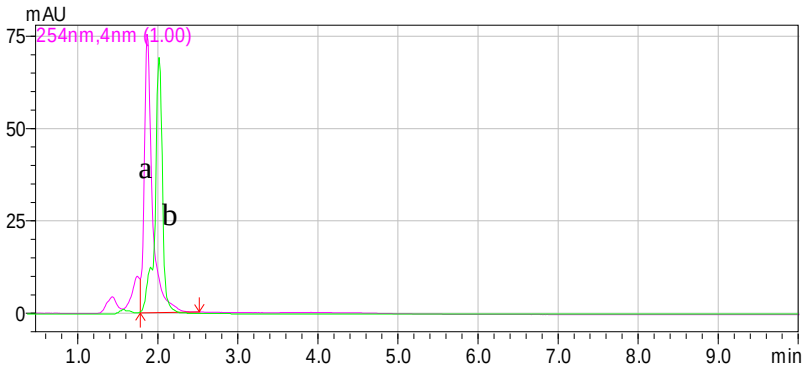
A. Fresh (a) and stored (b) material

Hypoxis hemerocallidea



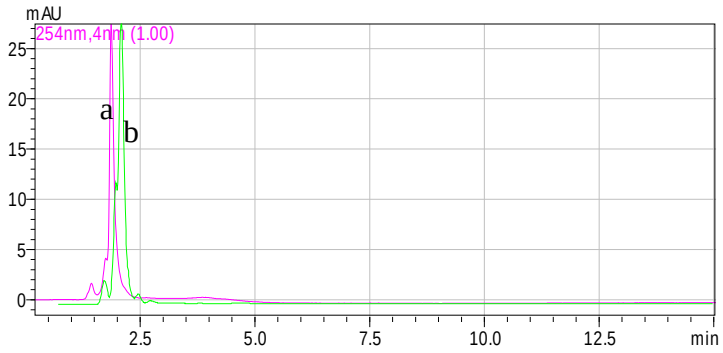
B. Fresh (a) and stored (b) material

Hypoxis iridifolia



C. Fresh (a) and stored (b) material

Hypoxis rigidula



D. Fresh (a) and stored (b) material

Hypoxis acuminata

Figure 3.4. Chromatograms of ethanol water (8:2) extracts from rootstocks of four *Hypoxis* spp.

3.4. Discussion

The presence of secondary metabolites explains the various uses of plants for traditional medicine. There are several standard methods used for the phytochemical screening of medicinal plants. Thin Layer Chromatography and High Performance Liquid Chromatography were used to compare the secondary metabolite content of four *Hypoxis* species. The TLC profile showed similarities between all four *Hypoxis* species, which is indicated by the blue (1), pink (2 & 5) and purple (4) bands observed on the TLC fingerprint (Fig. 3.1). A pink compound was also found by Katerere and Eloff (2008) when analysing the chemical composition of both the rootstocks and leaves of *H. hemerocallidea.* They described the pink compound as phytosterols. According to Boukes *et al*., (2008), the spots of the sterols standards (stigmasterols and β-sitosterols) migrated the same distance on the TLC plates. The spots that they observed in the TLC plates ranged from pink to blue colour when they were analysing the quantitative and qualitative content of sterols/sterolins and hypoxoside of three *Hypoxis* species. The green (6) and pink (3) compounds that were observed only in some species were also a concern because it differentiates these species.

The TLC fingerprint of the rootstocks extracts of *H. hemerocallidea* and *H. colchicifolia* evaluated by Adeyemi, 2009 showed less spots as compared to numerous bands observed in this study on the TLC fingerprint of the rootstocks extracts of *H. hemerocallidea*, *H. iridifolia*, *H. rigidula* and *H. acuminata*,

nevertheless, the variation might be of the different solvent used for extraction and also the solvent system used for TLC analysis. Even though there are a less number of bands on TLC fingerprint, the chromatographic profile seemed to be similar between the two species (Adeyemi, 2009). However, Muwanga, (2006) observed six coloured bands on their TLC study using a different solvent system to this study. The six coloured bands are similar to the ones observed in this study however the compounds were not isolated or identified.

The HPLC results showed that there were distinct differences of chemical constituents in the different species used for extraction when the fresh material was compared to the stored material. The disappearance of the minor peaks indicated a possible degradation of compounds in the stored material when compared to the fresh material. Only *H. hemerocallidea* and *H. acuminata* did not show any changes on the chromatograms for both the fresh and the stored material. It is very difficult to correlate the findings of this study with other researchers, since their studies focused mainly on the compounds hypoxoside and rooperol rather than analysing the whole plant extract and none of the compounds were isolated or identified.

Different methods and solvents have been used by different researchers to isolate hypoxoside, which resulted in different retention times (Rt) of hypoxoside. For instance the studies done by Kruger *et al.*, (1994) have shown hypoxoside as the peak eluted at Rt 8 and rooperol as the peak eluted at Rt 12, whereas, the

findings by Laporta *et al*., (2007) differed with the findings by Kruger. According to their studies rooperol eluted at Rt 15, however rooperol was not expected in this study since it is a product of hypoxoside after a process of hydrolysis with Beta-glucosidase. As per Nair and Kanfer, (2006) hypoxoside was shown as the peak eluted at Rt 10.23.

The profile of the chromatograms after storage indicates that *Hypoxis* species showed similarity in the major secondary metabolites. It might be possible to substitute different *Hypoxis* species for *H. hemerocallidea* as the chromatograms of the other species in stored extracts correlate well with the chromatograms of *H. hemerocallidea* although further study on the isolation of different compounds observed on the TLC and HPLC fingerprint is required. This might explain the interchangeable use of different *Hypoxis* species used in traditional healing. With regard to the results obtained in this chapter, it was concluded that storage has an effect on the secondary metabolite content of *Hypoxis* species, as was confirmed by the two techniques (HPLC and TLC) used in this study.

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Chapter 4

Comparison of antibacterial and antioxidant activity of four *Hypoxis* species

4.1. Introduction

Plant-derived compounds are the desired scientific interest as a source of safe and effective substitutes for synthetic antimicrobials (Fakim, 2006). An impressive number of modern drugs have been isolated from natural sources based on their use in traditional medicine. Plants produce a diverse range of bioactive molecules making them a rich source of medicine (Graham *et al*., 2000, Fakim, 2006). However, the process of drug discovery has become longer and expensive, while the world-wide need for more effective drugs continues to grow. Medicinal plants are considered as an important source of potentially useful structures for the development of new chemotherapeutic agents. They produce a variety of compounds that are known to have therapeutic properties (Fakim, 2006). Natural products are becoming more important in modern society (Louw *et al*., 2002). As such most of the antibiotics available today are coming from these natural products (Sarker *et al*., 2007).

In recent years, antimicrobial properties of medicinal plants have been increasingly reported from different parts of the world (Masoko *et al*., 2005). The

initial point towards drug discovery is the biological and phytochemical screening of plants extracts. The winning approaches for investigation of these drug preparations involve the selection of test crude extracts based on the combination of ethnopharmacology and traditional healer's knowledge (Tona et *al.,* 1998). Various strategies have been devised by various research groups to select plants for scientific examination. Some strategies involved in drug discovery include bioassays, isolation and identification of substances that have positive activity. Bioassays are important stages in assessing the pharmacological actions of plant extracts. Among the commonly used assays are the brine shrimp, antimicrobial (bacteria and fungi) and antioxidant assays (Fakim, 2006).

Various microorganisms have been tested against the crude extracts and aqueous solution of *H. hemerocallidea* where high antibacterial activity was reported (Katerere and Eloff, 2008). Initially the biological activity of *Hypoxis* was reported to be associated with the steroid glycosides namely betasitosterols and sitosterols, however, recent studies relate it to the glycosides of unsaturated aglycones or rooperol (Laporta *et al*., 2007a). For screening medicinal plants using crude extracts, chromatographic fractions or purified compounds for antibacterial activities, it is essential to employ an *in vitro* antibacterial assay (Sarker *et al*., 2007). In this chapter both the fresh and stored plant materials of the ethanol water (8:2) and methanol crude extracts were used to evaluate the antibacterial activity of four *Hypoxis* species, namely, *H. acuminata, H*

hemerocallidea, H. iridifolia and *H. rigidula*. The method is simple, rapid, reliable, safe and more cost effective than the disc diffusion method which is time consuming.

Antioxidants (AO) are substances or nutrients in our food which can prevent or slow the oxidative damage to our body. Antioxidants reduce the damage by neutralizing the free radicals before they can attack the cells and prevent damage to lipids, proteins, enzymes, carbohydrates and DNA (Umamaheswari and Chatterjee 2008). Naturally occurring antioxidants are found in plants and can be also found in fruits, vegetables, nuts and bark (Lindsey *et al*., 2002). Recently there is a demand of the investigation of antioxidant activity of secondary metabolites from medicinal plants for compounds with high potency and lower toxicities (Thabrew *et al*., 1998). It has been reported that natural antioxidative compounds from plants have brought much attention due to the concerns about the safety of synthetic antioxidants (Kai Jin and Ning 2007).

Several phenolic compounds are reported to trap the free radical directly or scavenging them through a series of reactions with antioxidants enzymes (Motlhanka *et al*., 2008). Nevertheless some of the secondary metabolites ascribed to be the best antioxidants are flavonoids such as quercetin from appleskin, myricetin from red wine and luteolin from red peppers (Motlhanka *et al*., 2008). Therefore some medicinal plants have now been recognised as a source of natural antioxidant compounds (Wagensteen *et al*., 2004).

People are using different *Hypoxis* species under the same name i.e. African potato and they are substituting other *Hypoxis s*pecies with the commonly known *H. hemerocallidea*, of which extensive research on the medicinal properties of this species has been conducted including the antioxidant activity. Since the antioxidant activity of *H. hemerocallidea* has previously been reported and not previously performed on the other species, it was therefore necessary to determine and compare the antioxidant activity between the four different *Hypoxis* species.

4.2. Materials and methods

The collection and preparation of the plant extracts was the same as described in Chapter 3. Both the methanol and ethanol crude extracts were used for evaluation in the experiments carried out in this study however, only methanol crude extracts results were given since both crude extracts (methanol and ethanol water) showed similar results.

4.2.1. Antibacterial activity

Antibacterial activity was assessed by serial dilution micro-plate assay developed by Eloff (1998). Two Gram positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*) and one Gram negative bacteria *(Escherichia coli)*, were

cultured and maintained on nutrient agar medium in Petri dishes. An innoculum of each microorganism was grown in nutrient broth for 24 hours. The sterilized 96-well plates were prepared by transferring 100 µl of broth into each well. One hundred microlitres of the plant extracts (50 mg/ml stock solution) was added in the first row followed by serial dilution.

Hundred microlitres of the 24 hour old bacteria in nutrient broth was added in all the wells. Ciprofloxacin was used as the positive control while 10% dimethylsulphoxide (DMSO) was used as the negative control. The plate was covered and incubated overnight at 37 ˚C. As an indicator of bacterial growth, 40 µl p-iodonitrotetrazolium violet (INT) was added to the wells. Minimum inhibitory concentration (MIC) values were recorded as the lowest concentration of the extract that inhibited the growth of the bacteria. The inhibition of the bacteria was indicated by the well in which there is no colour change after the addition of INT as INT turns pink when in contact with living bacteria. The test was done on both the fresh and stored plant material and was repeated three times.

4.2.2. Antioxidant activity

Antioxidant activity was determined by using the micro-dilution method (du Toit *et al*., 2001). A stock solution of 2 mg/ml of methanol extract was prepared by dissolving 2 mg of the crude extract into a 1 ml of ethanol. Vitamin C (Ascorbic acid) was used as a standard solution (positive control). The 96-well plates were

prepared by adding 200 µl of distilled water into all wells, followed by the addition of 20 µl of plant extract in the first row and then serially diluted. Ninety microlitres of 1.1-diphenyl-2-picryl hydrazine (DPPH) was added into the wells as an indicator of the radical scavenging activity. The plate was then covered with aluminium foil and left at room temperature for 1 hr. The clear colour in the well indicated the antioxidant activity of the extracts. The absorbance was measured at 516 nm using a spectrophotometer. All the samples were prepared in triplicate. Percentage inhibition (I %) was calculated using the formula,

I% = (Ac-As)/ Ac x 100. Where Ac is the absorbance of the control and As is the absorbance of the sample. The IC_{50} was calculated using the graph prism pad package.

4.3. Results

4.3.1. Antibacterial activity

All four methanol crude extracts (whether prepared from fresh or stored rootstocks) showed no activity against *Staphylococcus aureus* and was not highly active against *Escherichia coli*. The extracts showed activity against *Enterococcus faecalis* however, the MIC value was above 1mg/ml. The MIC values were the same as per microorganism (Table 4.1).

Table 4. 1. MIC values of methanolic extracts of four different *Hypoxis* species

Plant	MIC (mg/ml)		
	E.c	E.f	S.a
H. acuminata fresh	6.25	3.125	>12.5
H. hemerocallidea fresh	6.25	3.125	>12.5
H. iridifolia fresh	6.25	3.125	>12.5
H. rigidula fresh	6.25	3.125	>12.5
H. acuminata stored	6.25	3.125	>12.5
H. hemerocallidea stored	6.25	3.125	>12.5
H. iridifolia stored	6.25	3.125	>12.5
H. rigidula stored	6.25	3.125	>12.5
Ciproflaxin	$2.5*10^{-7}$	$2.5*10^{-8}$	$2.5*10^{-8}$

Bacteria: E.c., *Escherichia coli*; E.f., *Enterococcus faecalis*; S.a *Staphylococcus aureus*. MIC: minimum inhibitory concentration obtained in the serial dilution assay.

4.3.2. Antioxidant activity

The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) is a stable, nitrogen–centered free radical which produces a violet colour in ethanol solution (Umamaheswari and Chatterjee 2008). When DPPH is in contact with antioxidant compounds, it

becomes yellow (Middleton *et al*., 2005). The DPPH assay was used to determine free radical scavenging activity. The free radical scavenging effects of the four methanol extracts of different *Hypoxis* species were compared with that of Vitamin C (Ascorbic acid). The free radical scavenging activity of these extracts showed high activity at the higher concentrations (Fig 4.1).

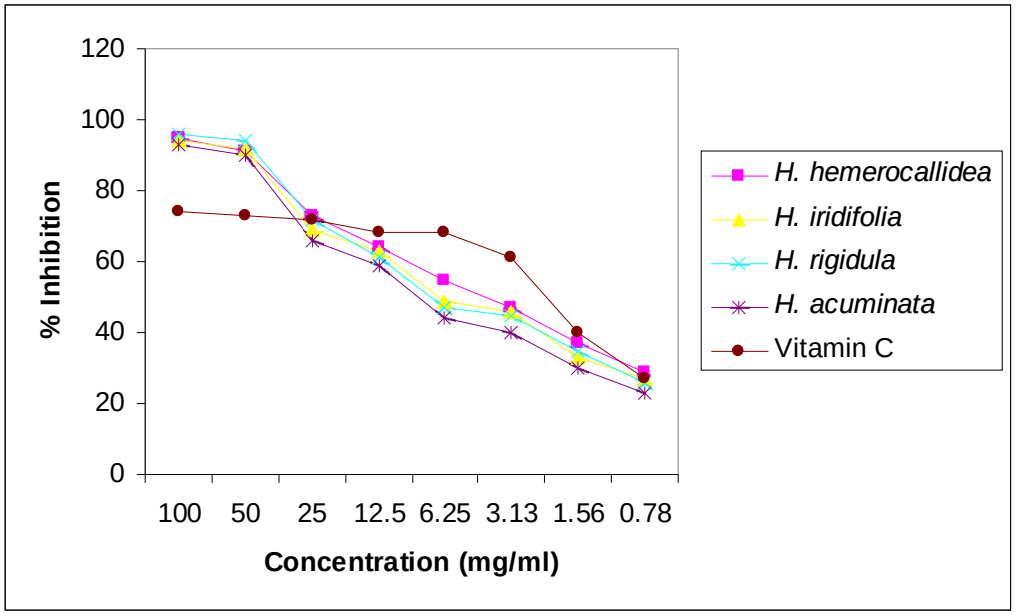

Figure 4. 1. Antioxidant activities of four *Hypoxis* methanol extracts using the DPPH assay

4.4. Discussion

Numerous publications on the bioactivity of *Hypoxis,* in particular *H. hemerocallidea* have been reported, however, not much attention has been reported with regards to other species (Brown *et al*., 2008; Ojewole, 2008; Ojewole *et al*., 2009). The juice secreted from the rootstocks of *Hypoxis* species was reported to be medicinally used in the treatments of minor wounds and pimples (Matsiliza and Barker, 2001), but antibacterial activity of the methanol extracts of the rootstocks of four *Hypoxis* species showed to be inactive against *S. aureus* as compared to the acetone leaves and rootstocks extracts that showed to be most effective by Katerere and Eloff (2008). The authors reported that the acetone crude extracts of rootstocks of *H. hemerocallidea* showed activity at the concentration of 0.31 mg/ml. However, the extracts were less potent than the control compound, ciprofloxacin.

The better activity was also confirmed by Laporta *et al*., (2007a) when *H. rooperi* methanol extract inhibited the growth of *S. aureus, E. coli* and *Klebsiella pneumoniae* with the MIC_{50} values below 1.56 mg/ml. The findings of this study concurs with findings by Steenkamp *et al*., (2006) where laboratory studies have shown that both ethanol and aqueous extracts of *H. hemerocallidea* rootstock inhibited the growth of *E. coli* at 6.25 mg/ml. Adeyemi (2009) reported the antibacterial activity of *Hypoxi hemerocallidea* and *H. colchicifolia* ethanol rootstocks extracts against *E. coli*, *S. aureus*, *K. pneumoniae* and *Bacillus*

subtilis. The antibacterial effect against *E.coli* for both species was similar to the findings of this study. In both species the MIC values of the ethanol rootstocks extracts was similar to the petroleum ether and dichloromethane rootstocks extracts. Petroleum ether leaf extract of *H. colchicifolia* showed antifungal activity with an MIC of 0.78 mg/ml as compared to 6.25 mg/ml of *H. hemerocallidea* (Adeyemi, 2009). *H. hemerocallidea* reportedly inhibited the growth of *Mycobacterium smegmatis* tested using the bioautography method (Muwanga, 2006).

The DPPH assay was used to compare the antioxidant activity of four *Hypoxis* species. The species showed high levels of activity that increased with increasing concentration which is similar to studies done by Nair *et al*., (2007), where free radical scavenging activity of the aqueous *Hypoxis* preparations using a similar assay showed high activity with higher concentrations. However Nair *et al*., (2007) also mentioned that hypoxoside, did not show any significant free radical scavenging activity when tested using Ferric Reducing Activity Plasma (FRAP). Therefore this could mean that there are some other secondary metabolites contained in *Hypoxis* extracts that are responsible for the antioxidant activity. The findings are supported by the studies done by Laporta *et al*., (2007b) where they mentioned that extracts derived from the *H*. *rooperi* rootstocks might have a potential use as a strong antioxidant in nutraceuticals or dietary supplements. According to Lindsey *et al*., (2002), the aqueous and boiled extracts of *H. hemerocallidea* exhibited very good antioxidant activity, and this statement was

also confirmed by Steenkamp *et al*., (2006) that methanol and aqueous extracts of *H. hemerocallidea* possessed free radical scavenging activity.

The antioxidant activity of extracts is associated with the compounds with the most hydroxyi groups (Salah et al., 1995, Steenkamp et al ., 2006). The similarity of the genus *Hypoxis* was not seen only on antibacterial or antioxidant properties, but also on anticonvulsant properties. Both ethanol and water extracts of *H. hemerocallidea*, *H. angustifolia* and *H. colchicifolia* showed dose-dependent anti-convulsant activity (Risa *et al*., 2004). Although the storage had an effect at the secondary metabolite content, it did not affect the biological activity of these species. Based on the results obtained from the antioxidant assay, it may be concluded that both the methanol and ethanol water extracts of the rootstocks of four *Hypoxis* species showed the potential of *in vitro antioxidant* activity and the study suggest further *in vivo* analysis.

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Chapter 5

In vitro cytotoxic evaluation of four *Hypoxis* species on Vero and Hela (cancer) cells

5.1. Introduction

Medicinal plants are valued as great importance to the health of communities. Currently the majority of the commonly used medicinal plants are investigated because of their chemical constituents and pharmacological activities in a purpose of developing a scientific basis for their ethnomedical use (Ojewole, 2003; Musabayane, 2005a). However, there are general and herb-specific concerns regarding medicinal plants and their ability to produce toxicity and adverse effects (Firenzuoli and Gori, 2007). Furthermore, accidental herbal toxicity occurs not only as a result of lack of pharmaceutical quality control in harvesting and preparation, but occurs because herbal remedies are believed to be harmless (Saad *et al*., 2006).

Generally cultural widespread use in a traditional medical system may indicate safety, but not efficacy of treatments, especially in medicinal plants where tradition is almost relying on magical-energetic principles and also lack of

complete information about the composition of plant extracts (Firenzuoli and Gori, 2007). Safety and efficacy data are available for an even smaller number of plants, their extracts and active ingredients and preparation (Springfield *et al*., 2005). In addition toxicity associated with medicinal plants may result from misidentification or mislabelling of the plant species, use of incorrect parts of plants, contamination or adulteration with pharmaceutical agents (van Breemen *et al*., 2008). The toxicity or adverse effect of a plant may also be associated to the presence of toxic compounds in particular plant organs, for instance toxic compounds may be found in the leaves but not on the fruits or may be on the seeds but not on the roots and etc (Salgueiro *et al*., 2010). Therefore the evaluation of clinical efficacy and safety of herbal medicines with the goal to know if they are efficient to treat diseases and if their use is free of damage to the health of consumers is surely the most important aspect either for medical community or public opinion (Firenzuoli and Gori, 2007).

Hypoxis hemerocallidea is a declared plant that people are substituting with other species, and this species has been researched extensively. The cytotoxicity is another dimension that has been investigated and *H. hemerocallidea* extracts has reported to contain the secondary metabolites that have promising anticancer properties (Theron *et al*., 1994; Albrecht *et al*., 1995; Boukes *et al*., 2008). However, it was found that numerous publications have focused on pure compounds (hypoxoside and rooperol) and their cytotoxic effects rather than the whole extracts. Since the cytotoxic effects of *H. hemerocallidea are* known and

as substitution thereof is part of the problem of this study, it was necessary to evaluate and compare the *in vitro* cytotoxic activities of crude extracts of four different *Hypoxis* species against normal (Vero) and cancer cell (Hela) lines.

5.2. Materials and Methods

Collection and preparation of the plant extracts was followed according to the procedure as described in chapter three. The same methanol crude extracts used in the previous chapters were used for evaluation in the experiments done in this study. There are common standardized test methods that are used in the laboratories for toxicological studies. These methods are based on the crude extracts or on the active compounds isolated. The extracts are applied to the cells in different dilutions and cytotoxicity is assessed by XTT (sodium 3´-[1-(phenyl amino-carbonyl)-3.4-tetrazolium]-nis-[4-methoxy-6-nitro) benzene sulfonic acid hydrate) and MTT (methyltrazolium) tests. Usually MTT is used to assess the viability or the metabolic state of the cells (Saad *et al*., 2006).

5.2.1. Preparation of Vero cell cultures

Vero cells (African green monkey kidney cell) were purchased from Highveld Biological (South Africa). The cells were maintained in complete Minimal Essential Eagle's Medium (MEM) containing 10% foetal bovine serum and antibiotics. Cells were sub-cultured in a ratio of 1:3 on every third or fourth day.

The study was carried out in the Cell Culture laboratory at the Department of Plant Science, University of Pretoria.

5.2.2. Determination of toxicity in Vero cells

The cytotoxicity assay was performed as described by Zheng *et al.,* (2001). Vero cells in complete MEM medium were dispensed into the wells of a 96-well plate (10^4 cells per well). Toxicity of the four different *Hypoxis* extracts on the Vero cells was assayed using XTT. Hundred microlitres (µl) of Vero cells (1x 10^5 cells/ml) were seeded into a microtiter plate and incubated for 24 h to allow the cells to attach to the bottom of the plate. This was followed by serial dilution of the extracts with the concentration ranging from 3.125-400µg/ml and the microtiter plate was incubated for 48 h. The positive drug control (Actinomycin D) was included in the assay. The XTT reagents were added to a final concentration of 0.3 mg/ml and the cells were incubated for 1-2 h.

Zearalenone was used as a positive control at a concentration ranging from 10-0.6 µg/ml. Absorbance of the colour was read using an ELISA plate reader with the optical density of 490 nm and a reference wavelength of 690 nm. The assay was carried out in triplicate. The results were statistically analysed with the Graph Pad Prism 4 statistical programme and the IC_{50} value was calculated by the programme. IC_{50} was defined as the concentration of the compounds at which absorbance was reduced by 50% (Zheng *et al*., 2001) The method used to

determine the toxicity of the cancer cell lines (Hela) was similar to the method described above.

5.3. Results

The results in this study done on the Vero cells showed that extracts of all four *Hypoxis* species are non-toxic (Fig. 5.1). The IC_{50} value of the samples was not calculated because the IC_{50} value was greater than the highest concentration tested which is 400 µg/ml. However, the results on Hela cells (cancer cells) (Fig. 5.2) showed that the extract prepared from rootstocks of *H. iridifolia H. hemerocallidea*, *H. rigidula* and *H. acuminata* were unable to inhibit the growth of cancer cells at the lowest concentration but seemed to be moderate cytotoxic at a very high concentration 400 µg/ml. The IC_{50} for all four *Hypoxis* species extracts tested against the cancer cells are shown in Table 5.1.

The results obtained in this study proved that the extract prepared from the rootstocks of *Hypoxis* species is not toxic even though the extracts showed to contain numerous secondary metabolites. According to Steenkamp *et al*., *(*2006), the American National Cancer Institute guidelines set the limit of activity for crude extracts at a 50% inhibition (IC_{50}). Thus the crude extracts are evaluated at a concentration of 50 µg/ml for antiproliferative activity against one cell line and extracts that significantly inhibit the growth of the cells (≥ 50%) at relatively low concentrations (50 µg/ml) are recognised as anticancer agents. However, in this

study the IC_{50} was found to be greater than 50% and the extracts are active at a relatively high concentration.

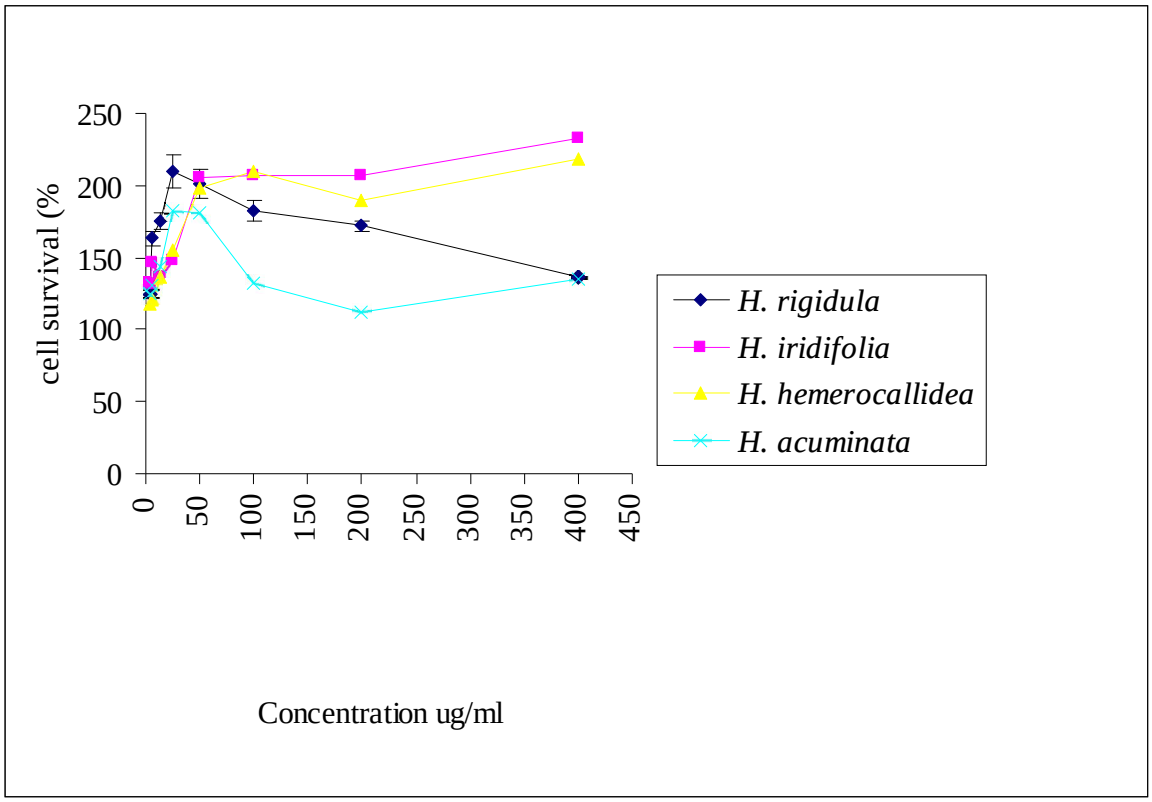

Figure 5. 1. Cytotoxic activities of four *Hypoxis* species on Vero cell lines

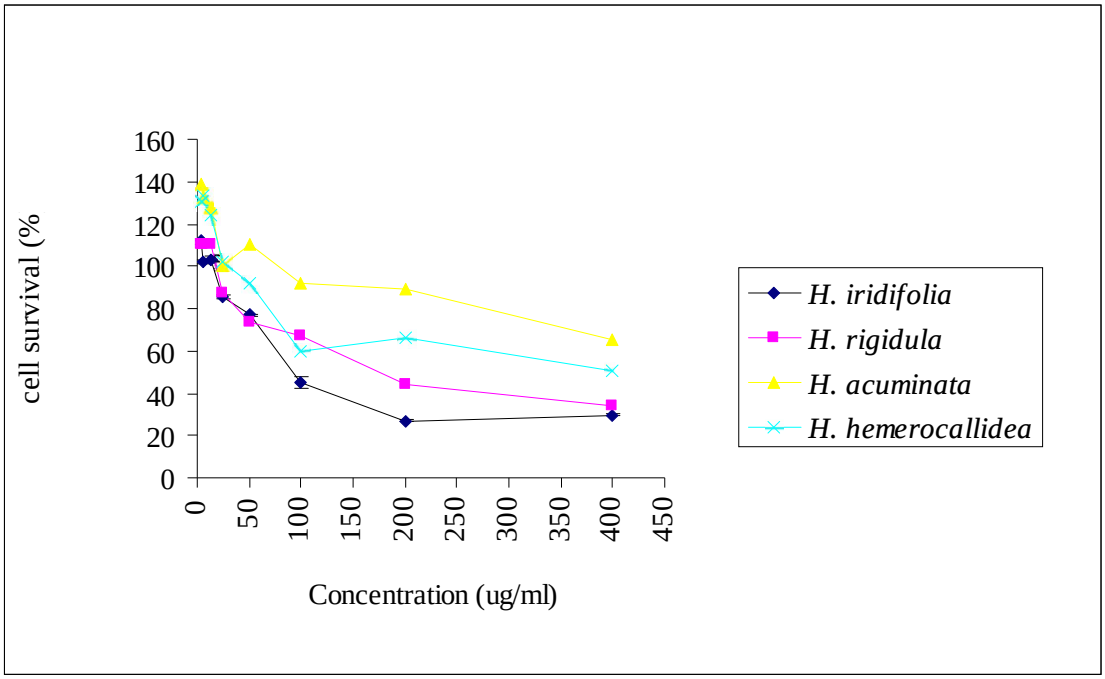

Figure 5. 2. Cytotoxic activities of four *Hypoxis* species on (Hela) cancer cells

Table 5. 1. IC_{50} values of methanol extracts of four *Hypoxis* for cytotoxicity tests against (Hela) cancer cells (µg/ml)

Plant extracts	Mean IC_{50} value ± S.D
H. iridifolia	85.46±0.152
H. rigidula	139.6±0.198
H. acuminata	>400
H. hemerocallidea	>400
Actinomycin D	0.07±0.358

5.4. Discussion

Many toxic responses attributed to medicinal plants may result from misidentification of the plant species, contamination or adulteration with pharmaceutical agents. Alternatively, toxicity may result from systematic or organ-specific damage by plant constituents, which would require *in vitro* or *in vivo* toxicity studies similar to pharmaceutical development studies (Firenzuoli and Gori, 2007).

Hypoxoside is reported to have low toxicity (Drewes *et al*., 2008). When analysing the methanol extracts of four *Hypoxis* species against the normal (Vero) cells, the results indicated 100% viability of the cells. Although in this experiment the whole extract of the species was analysed. The findings of this study correlate well with the findings by Theron *et al*., (1994) in which hypoxoside had no effect on the viability of B16-F10-BL-6 mouse melanoma cells. However, after treating hypoxoside with β-glucosidase, rooperol showed cytotoxic effect with an IC_{50} of 20 µM (Theron *et al*., 1994).

According to Mills *et al.,*(2005) a phase I trial in cancer patients failed to establish any clinical, haematological, or biological toxicity that could be ascribed to the ingestion of hypoxoside. It was recorded that a further clinical study has shown that after three months of treating benign prostate hypertrophy (BPH) with

hypoxoside, 39% of patients were symptom free and in a further 53% symptoms of BPH improved and no side effects have been reported (Franklin, 2001, Steenkamp, 2003). When B16-F10-BL-6 mouse melanoma cells were cultured in a medium containing heat-inactivated foetal calf serum, the hypoxoside was shown to be non-toxic to the cell. A phase I clinical trial on the efficacy of hypoxoside as a putative oral pro-drug in the treatment of lung cancer found that the majority of patients survived longer than their estimated prognosis (Dietzsch *et al*., 1999).

No toxic phenomenon or organ changes were detected when the safety of methanol extracts of *H. rooperi* was pharmacologically investigated for both the acute and chronic toxicity (Pegel, 1979). In the study of oral acute toxicity of *H. rooperi* (former name of *H. hemerocallidea*) extract (Laporta *et al*., 2007) revealed that no deaths occurred in either the control or *H. rooperi* groups during the treatment where a single dose of 2000 mg/kg b.w.t (body weight) was administered to mice over a period of two weeks. These results were confirmed by examining (post mortem) the mice after the experiment and the post mortem analysis showed no abnormalities on vital organs such as brain, heart, lungs, kidneys or intestines. However, according to Musabayane *et al*., (2005a) chronic infusion of *Hypoxis* extracts may cause a decrease in glomerular filtration rate, and can also elevate plasma creatinine concentrations in rats, which suggest an impaired kidney function. *Hypoxis* extracts have been reported to interact with HIV drug metabolizing enzymes which lead to drug resistance, toxicity or

treatment failure (Mills *et al*., 2005). Nevertheless, the co-administration of *H. hemerocallidea* with efavirenz (EFV) (HIV-1 reverse transcriptase inhibitor) did not show to affect the pharmakinetics of efavirenz (Mogatle *et al*., 2008).

The *in vitro* cytotoxicity tests done by Albrecht *et al*., (1995) showed hypoxoside to be nontoxic to cancer cells at a concentration up to 100`µg/ml, but in this study *H. iridifolia* showed to be toxic at the same concentration whereas the other three species showed to be toxic at concentration higher than 100`µg/ml when tested against Hela cancer cells. However, the authors (Albrecht *et al*., 1995) reported that after hypoxoside had been hydrolysed to its aglucone rooperol, cytotoxicity was obtained at a concentration ranging from 2-10 µg/ml.

In conclusion, the methanolic extracts of all four *Hypoxis* species showed to be toxic to cancer cells at a higher concentration. Despite the fact that the whole extract was analysed instead of the compound itself, *Hypoxis* has indicated the possibility of having an effect against cancer cells (Smit *et al*., 1995). In general terms all four *Hypoxis* species compared in this chapter proved to be 100% non-toxic to the normal cells although differ in cytotoxic activity against cancer cells.

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Chapter 6

Isolation of compounds from four *Hypoxis* species

6.1. Introduction

Traditional herbal medicines are naturally occurring, plant-derived substances that have been used to treat illness within local or regional healing practices. It is estimated that 80% of African populations use some form of traditional herbal medicine and the world-wide annual market for these products approaches US$ 60 billion (Tilburt and Kaptchuk, 2008). In Africa the use of medicinal plants is currently attracting attention by scientists in order to validate the ethnomedicinal applications as remedies against some diseases (Falodun *et al*., 2009)

The separation, identification and structure determination of biologically active compounds has been facilitated by continual development of chromatographic and spectroscopic methods of analysis (Phillipson, 2007). Chromatographic procedures are the most diverse and widely used techniques in the fractionation of extracts. Within a decade there were a number of dramatic advances in analytical techniques including Thin Layer Chromatography (TLC), Gas Chromatography (GC) and Nuclear Magnetic Resonance (NMR) (Misra, 2009).

Column chromatography is one of the oldest forms of chromatographic techniques. A tube is packed with a solid stationery phase, the sample mixtures applied to the top of the column and the mobile phase is allowed to move down through the column under gravity. Numerous publications have described different methods for the extraction, isolation and determination of hypoxoside from plants belonging to the genus *Hypoxis* (Drewes *et al*., 1984; Nair and Kanfer, 2006; Laporta *et al*., 2007).

Hypoxoside which is claimed to be the major compound found in the family of Hypoxidaceae was first isolated by Marini-Betollo *et al*., (1982) from the methanolic extract of *H. obtusa.* The compound was isolated by counter-current distribution (CCD) between H_2O AcOEt: N-BuOH. This compound was also isolated from the ethanolic extract of *H. rooperi* which was the previous name for *H. hemerocallidea*. The compound was isolated by Drewes *et al*., in 1984.

Drewes *et al*., (1984) reported the seasonal variation in concentration of this diglucoside compound. Vinesi *et al*., (1990) reported a HPLC method for separation of the glycoside fraction from *H. obtusa*. However, validatation data to support the use of this quantitative analysis of hypoxoside was not provided. The later report on isolation of hypoxoside was given by Kruger *et al*., (1994), Nair and Kanfer, (2006) and Laporta *et al*., (2007). Nair and Kanfer, (2006) employed reverse phase HPLC for the quantitative determination of hypoxoside using *H. hemerocallidea*, while Kruger *et al*., (1994) isolated hypoxoside from a

methanolic extract of dehydrated *Hypoxis* rootstocks using a preparative C_8 HPLC column and an isocratic mobile phase consisting of acetonitrile-water (15:85) at a flow rate of 100 ml/min. The aim of this chapter was motivated by the results observed on the TLC fingerprint (Fig. 3.1) where the fingerprint clearly illustrated the similarities and differences of the secondary metabolite content of four *Hypoxis* species. The aim of this investigation was to isolate and identify the compounds observed in the TLC fingerprint.

6.2. Materials and Methods

6.2.1. Extraction

The extraction procedure for all four *Hypoxis* spp. rootstocks was carried out similarly as described in Chapter three, but only the methanol extract was used as it gave more yield. Based on the TLC fingerprint (Chapter 3) only one species namely *H. rigidula* was used for isolation, as one of the objectives was to isolate and identify the compounds associated with the similarities and differences of these species and this species has both features.

Removal of non-polar impurities was carried out according to the extraction and isolation method by Nair and Kanfer (2006). Thirty-five grams of methanol crude extract of *H. rigidula* was dissolved in 250 ml of distilled water and was subjected to shaking for 10 min. The water-soluble extract was then filtered through Whatman no 1 filter paper and transferred to a 500 ml separating funnel.

The extract was first partitioned with 250 ml of water-saturated ethyl acetate repeated three times to remove non-polar impurities. The aqueous portion was further partitioned with 250 ml of water-saturated *n*-butanol and also repeated three times (Fig 6.1), and the non-aqueous layer was removed and thoroughly dried under vacuum to obtain a butanolic extract. This procedure facilitated the removal of non-polar and other mucilaginous impurities.

Figure 6. 1. Water-saturated n-butanol partitions

6.2.2. Isolation

6.2.2.1. Column Chromatography

A glass column was packed with silica gel (70-230 mesh) (Fig. 6.2). A glass column was chosen because separation on silica gel represents one of the

affordable methods for isolation of compounds. Six grams of the dried butanolic extract of *H. rigidula* mixed with silica gel (3 g of extract and 3 g of silica gel) was then loaded onto the column, and was first eluted with toluene: butanol: water: methanol (6: 4: 2: 1). The first 200 ml fraction was concentrated with rotary evaporation (Buchi) (Heidolph) and analysed using TLC. The column was further eluted with a different ratio of toluene: butanol: water: methanol (4: 4: 2: 1).

The column was again eluted with a different ratio of toluene: butanol: methanol: water (4: 4: 4: 1) and the TLC fingerprint showed only one purple band. The second fraction that showed several numbers of compounds was further evaluated using preparatory TLC plates. The solvent system used for TLC was ethyl acetate: acetic acid: formic acid: H_2O (100:10:10:15).

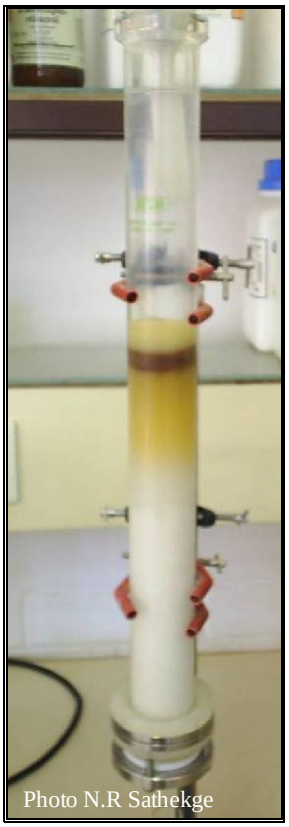


Figure 6. 2. Glass column packed with silica gel

6.2.2.2. Thin Layer Chromatography plates (preparative layer with UV 254)

Silica gel thin layer chromatography with preparative layer UV 254 20 x 20 cm, 2000 microns (Anatech) were used. These plates were used to isolate compound 5 that was observed in Chapter three (Fig 3.1). Fraction A (Fig 6.3) was concentrated on a rotary evaporator, and fifty milligrams was applied on the TLC plate and developed in the TLC tank. The same solvent system as explained in 6.2.2.1 was used. The plate was viewed under the UV light and the fluorescent bands were marked with a soft pencil. The bands with the green colour (compound 5) were isolated from the rest of the compounds by scratching with a

scalpel, followed by dissolving in methanol. The solution was filtered using a Whatman no 1 filter paper and concentrated on a rotary evaporator and analysed on aluminium TLC as previously explained.

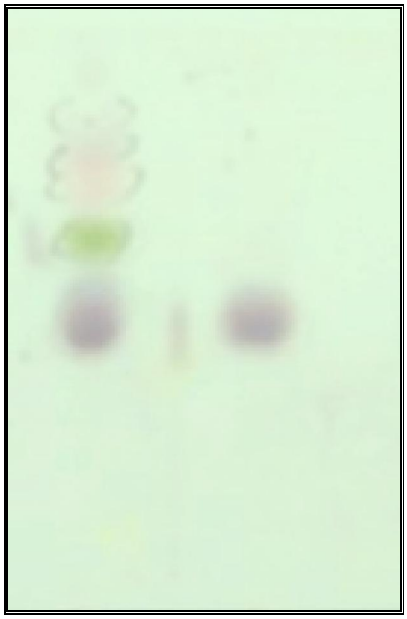
6.2.2.3. Nuclear Magnetic Resonance

Ten milligrams of the compound (Fig 6.3 B) was dissolved in 1 ml of methanol and analysed using ^{1}H and^{13}C NMR. Both the ^{1}H and ^{13} C NMR spectral data observed from this study was compared with the values reported for hypoxoside published by Laporta *et al*., (2007).

6.3. Results

6.3.1. Column chromatography

The first fraction that was eluted with toluene: butanol: water: methanol (6: 4: 2: 1) did not show any compounds when analysed using TLC. The second fraction showed several compounds when eluted with toluene: butanol: water: methanol (4: 4: 2: 1) (Fig 6.3 A). However, after changing the mobile system of the column to toluene: butanol: methanol: water (4: 4: 4: 1), only one compound was eluted (Fig 6.3 B).



A B

Figure 6. 3. Fraction, A eluted from toluene: butanol: water: methanol (6: 4: 2: 1) and B was eluted from toluene: butanol: methanol: water (4: 4: 2: 1)

6.3.2. Thin Layer Chromatography plates (preparative layer with UV254)

The method of isolation using preparative TLC is time consuming and needs accuracy when cutting the bands. The green compound 6 (Fig 3.1) was noted only on *H. rigidula* and *H. acuminata*. The green compound isolated by TLC is shown in Figure 6.4 below. The structure of this compound was not analysed due to little yield obtained, therefore further work will be conducted and therefore the identification of this compound is not included in the dissertation.

Figure 6. 4. Compound isolated using preparatory TLC (Methanol extract) Mobile system: (ethyl acetate: acetic acid: formic acid: H_2O (100: 10: 10: 15)

6.3.3. Nuclear Magnetic Resonance

The purple compound (Fig. 6.3 B) was isolated from the polar fraction of the plant extract and identified as hypoxoside based on spectroscopic data of ^{1}H and ^{13} C NMR. This purple compound was confirmed in all four species by the TLC fingerprint (Chapter 3). The ^{1}H NMR spectra showed signals as presented in Table 6.1. The data obtained in this study was tabulated below as the isolated sample. The ^{1}H NMR spectra showed signals at 7.08 (d, J= H2, H-2'), 7.06 (d, J= H2, H-2''), 6.87 (d, J= H2, H-5''), 6.85 (d, J= H2, H-2), 6.82 (d, J= H2, H- 6') 6.87 (d, J= H2, H-2), 6.77 (6.77 (d, J= H2, H6''), 3.23 (2H, m, H-3) in addition to signals of glucoside unit which showed a nomeric proton at 4.73 with other signals between 3-4 ppm. The ^{13} C NMR showed 17 carbon signals of the

compound backbone (Table 6.1) in addition to 6 carbon of the sugar unit. Therefore the above data is similar to the data of hypoxoside published by Laporta *et al*., (2007), although, the data of hypoxoside published by Laporta *et al*., (2007) was analysed using *H. hemerocallidea* and the data from this study was of *H. rigidula*. The structure of hypoxoside (Fig. 6.5) was also confirmed by Nair *et al*., 2007.

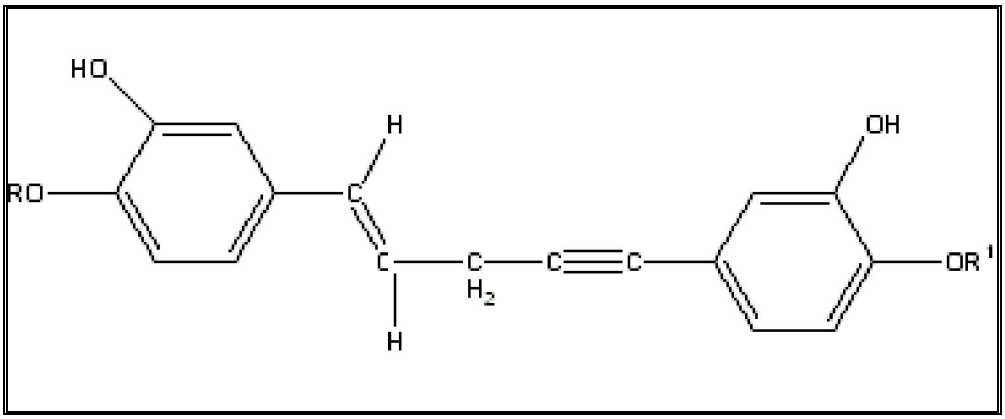


Figure 6. 5. The structure of hypoxoside (R and R^1 = ß-D –glucose)

Table 6. 1. ^{1}H and ^{13}C Nuclear Magnetic Resonance chemical shifts of hypoxoside

No. of C	Laporta (2007) Rf	sample ^{13}C NMR	No of H	sample ^{1}H NMR
C1	131.6	131.7	H1	6.53
C2	121.6	124.4	H2	6.08
C3	23.2	23.2	H3	3.23
C4	86.2	86.3		
C5	83.2	83.2		
C1’	114.4	114.4		
C2’	118.2	118.2	H2’	7.00
C3’	148.3; 148.0	148.2; 147.9		
C4’	146.8; 146.2	146.7; 146.1		
C5’	120.0	120.0	H5’	6.85
C6’	124.5	124.4	H6’	6.82
C1”	134.3	134.4		
C2”	118.5	118.6	H2”	7.00
C3’’	[d]	-		
C4’’	[d]	-		
C5’’	114.3	114.4	H5’’	6.87
C6’’	119.3	119.4	H6’’	6.77+
C1-Gluc	104.1; 103.7	103.7; 104.1	H1-Gluc	4.73
C2-Gluc	78.2	78.1	H2-Gluc	3.00-4.00
C3-Gluc	74.8	74.7	H3-Gluc	same as above
C4-Gluc	71.2	71.1	H4-Gluc	same as above
C5-Gluc	77.6	77.4	H5-Gluc	same as above
C6-Gluc	62.3	62.3	H6-Gluc	same as above

[d] signal may be interchanged

\+ hidden under the solvent peak

Rf reference

6.4. Discussion

Earlier history regarding the isolation of hypoxoside reported that, hypoxoside was firstly isolated in 1982 by Marini-Bettolo *et al* from the methanolic extract of *H. obtusa* and later by Drewes *et al*., (1984) from *H*. *rooperi*.

In this study, two compounds were isolated, and one compound (Fig 6.3 B) was analysed by nuclear magnetic resonance (NMR) and was identified as hypoxoside. However, the second compound in Figure 6.4 was not identified due to the yield that was not sufficient. According to Drewes *et al*., (2008), the isolation of hypoxoside from *H*. *hemerocallidea* by conventional column chromatography was difficult and a time consuming procedure. However in this study, isolation of hypoxoside was successfully isolated according to the method developed by Nair and Kanfer (2006), but the solvent system was slightly modified.

Despite the fact that hypoxoside was identified and published years ago, numerous papers reported the isolation of hypoxoside by HPLC other than glass column chromatography and they gave less detailed information on TLC. Therefore it was inappropriate to compare the results of this study with the other studies since separation of compounds were clearly determined by TLC method. Hypoxoside was reportedly found in other *Hypoxis* species such as *H. nyasica* and *H. angustifolia* as well as the related species *Spiloxena schlechteri* (Vinesi *et al*., 1990; Nicoletti *et al*., 1992), In agreement with the results of Vinesi *et al*., (1990)

hypoxoside was showed to be present in all four *Hypoxis* species analysed in this study.

A water extract of *H. sobolifera* var *sobolifera* showed, however, no hypoxoside content when compared with *H. hemerocallidea* and *H. stellipillis*. The same species showed undetectable levels of hypoxoside when extracted with chloroform. Both the sterol and hypoxoside contents were shown to be varied in all the three species (Boukes *et al*., 2008). du Plessis-Stoman *et al*., (2009) confirmed the absence of sterols in a water extract of *H. hemerocallidea* when investigating the presence of sterols and sterolins in the herbal medicines.

This study provides no evidence of active compounds as the study dealt mainly with the whole extract rather than the isolated compounds. However, it is well reported that hypoxoside is a major constituent of *Hypoxis* species and is converted to ROP (rooperol) upon hydrolysis by the enzyme ßeta-glucosidase (Dietzsch *et al*., 1999; Theron, 1994). The determination of hypoxoside in this study was achieved by thin layer chromatography (Chapter 3), isolation using column chromatography and identification using nuclear magnetic resonance (Chapter 6). As the outcomes of this study, hypoxoside was confirmed to be contained by all four *Hypoxis* species.

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Chapter 7

7.1. General discussion

The need for this study was realized after several visits were made by the Agricultural Research Council (ARC) to the Faraday Medicinal Plants Market in Johannesburg and to the Abey Bailey Nature Reserve. The purpose of these visits was to determine the costs and availability of *Hypoxis* species plant material at the markets. However, from these visits it was discovered that different *Hypoxis* species were harvested and sold as the same plant, commonly referred to as the African potato. This was proven when a number of rootstocks bought from the medicinal market grew into plants showing distinct morphological differences when planted at the ARC. Treatment of particular ailments with these plants might be questionable as the secondary metabolites might differ leading to different medicinal properties. It was assumed that the plants sold were used as substitutes for the main plant, which is *Hypoxis hemerocallidea*. The dosage and toxicity of plant preparations is extremely important and, therefore adulteration was a concern where plant preparations are taken orally and the information about the plants used not being accurate.

Hypoxis hemerocallidea was reported as ‘zifozonke’ meaning that the plant can be used to treat many diseases (Oluwule *et al*., 2007) and as such many scientists developed an interest in investigating it at different research areas. This species

was investigated more than any other species belonging to the genus *Hypoxis* and a list of published papers proved this species to be active to numerous medicinal properties such as antibacterial, anti-inflammatory, anticonvulsant, antiduretic, antidiarhoeal, antioxidant, antinociceptive, and antidiabetic (Ojewole 2008; Zibula and Ojewole, 2000). Numerous methods have been published on how to analyse the chemical composition of *Hypoxis* species but none of the published papers give detailed information about the differences between the *Hypoxis* species. The main aim of this study was to compare the secondary metabolite content and antimicrobial activity of four *Hypoxis* species. Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) were used to compare the secondary metabolites while different bioassays were used to compare their antibacterial and antioxidant activity and also to evaluate their cytotoxicity to normal and cancer cell lines.

Thin Layer Chromatography seemed to be an effective method that showed the best comparison between these species. The results shown in this study demonstrates that the extracts derived from four different *Hypoxis* species namely *H. acuminata*, *H. hemerocallidea, H. iridifolia* and *H. rigidula* contain several secondary metabolites, which were indicated by the different coloured spots. From these coloured spots, both the similarities and differences were noted and at least six compounds were noted in all four *Hypoxis* species. The similarity was clearly indicated by compounds 1, 2 and 4 which were present in all four species. The difference was noted in the concentration of compound 3 which seemed to be

present in *H. hemerocallidea* and *H. iridifolia* and absent in *H. rigidula* and *H. acuminata*. The same difference was noted again in compound 5 where it was more visible in *H. acuminata* and less in the other three species. The main difference was noted in compound 6, which was noted only in *H. rigidula* and *H. acuminata* but totally absent in *H. hemerocallidea* and *H. iridifolia*.

With regard to HPLC the results confirmed the distinct differences in *H. rigidula* and *H. iridifolia* extracts prepared from the fresh rootstocks. However, when analysing the extracts prepared from stored rootstocks the results showed that all species had the same chromatograms which indicate clearly that some of the compounds observed in fresh rootstocks were degraded. Therefore for this reason it might be possible to substitute *H. hemerocallidea* with the other species since the *Hypoxis* sold at the medicinal markets are kept for a long period.

The antibacterial and antioxidant activity of these species also confirmed the similarities between the species. The radical scavenging activity was used as a criterion for the antioxidant properties of these species. The extracts of all species, both fresh and stored inhibited the growth of *E. faecalis* with the same concentration of 3 mg/ml and *E. coli* with 6.25 mg/ml. All species showed to be inactive against *S. aureus*, with a MIC of 12.5 mg/ml. Since there was no previous information about the effect of storage on the biological activity of *Hypoxis* species, this study discovered that storage does not affect the biological activity of these species over a period of two months. The effect of storage was mainly on the degradation of other compounds which were not linked to the biological activity of

these plants. In addition to the bioassays, *in vitro* cytotoxicity tests against Vero cells (Monkey kidney cells) confirmed that all four species showed 100% viability of the cells. The study of cytotoxicity on normal cells (Vero) confirmed that the extracts are not toxic, which means that the decoction of the rootstocks is possibly safe for human ingestion. Further studies on the cytotoxicity tests against the cancer cells confirmed that the species demonstrated a cytotoxic effect to Hela cancer cells, although the extracts seemed to be toxic only at a higher concentration. The cytotoxicity observed in the experiments performed in this study was detected from the whole extract. Hypoxoside was isolated and identified as the compound with the purple colour band on the TLC fingerprint (Fig 3.1). The NMR values of this compound correlated well with the values from the previously published papers (Laporta *et al*., 2007). The compound was isolated using glass column chromatography.

Adulteration and substitution of medicinal plant seemed to be the serious problem that is encountered around the world today, and is mainly due to failure of correct identification of raw materials. The argument behind this study was based on the fact that different species are used as the substitutes of the commonly known species being *H. hemerocallidea*, and the *in vitro* results obtained from this study confirmed that all four *Hypoxis* species exhibited the same biological properties with regard to antibacterial and antioxidant activities. The TLC fingerprint described the detailed secondary metabolite content of the four species. In terms of the cytotoxicity, all the species confirmed to be non-toxic to the normal cells even

when the cells were exposed to extremely high concentrations. Therefore adulteration and substitution of *Hypoxis* species seems to be not a major problem and as such there is a possibility of substituting *Hypoxis* species namely, *H. acuminata*, *H. iridifolia* and *H. rigidula* investigated in this study with the commonly known one (*H. hemerocallidea*) for medicinal use however, further investigation of other *Hypoxis* species is still required.

The outcomes of this study recommend further identification of compounds observed on the TLC fingerprint. The green compound which is compound 6 (Fig. 3.1) differentiates *H. iridifolia* and *H. rigidula* from *H. hemerocallidea* and *H. acuminata* and also the reddish pink noted as compound 3 in Chapter three needs to be identified. The outcomes of this study also recommend more research in relation to the chemotypes and DNA finger printing of different *Hypoxis* species because genetic factors are also responsible for chemical variability in the plant kingdom. There is a need for further investigation of other *Hypoxis* species that are not yet analysed, since other scientists proved variation or absence of hypoxoside in some of the *Hypoxis* species. Some of the scientists mentioned to buy the rootstocks of this species at the medicinal market, which was still a challenge to this study since the sellers themselves cannot differentiate the rootstocks of the *Hypoxis* species they are selling. Quality control of the medicinal plants still needs to be addressed in different continents of the world. The lack of regulation of medicinal plants constitutes a real problem and can results in several health risks. In conclusion to this study the following suggestions have been formulated:

- Cultivation of medicinal plants is needed as it would maintain a regular supply of good quality and genuine raw materials to the users and that will prevent exploitation of natural habitat and WHO guidelines on good agricultural practices for medicinal plants may be followed (WHO, 2003)
- A focal national organization should be established to deal with the management of medicinal plants and drug discovery, this will help in the monitoring of medicinal from collection till consumption.
- Recent data about cost, availability and annual consumption should be collected from medicinal plant market of South Africa.
- Combination of physical, chemical and biological techniques and tools need to be appropriately applied.
- Collectors must have sufficient knowledge of the plant they have to collect such as identification, characteristics and the habitat requirements (Zschocke *et al*., 2000).
- They should have sufficient knowledge about the best time to harvest and the primary processing to guarantee the best quality (Zschocke *et al*., 2000).
- A clear regulatory framework would be of great importance to everyone involved in the field of phytomedicine being a farmer, collectors, producers, industry, regulatory bodies and the consumers however, the government need to give the platform or implement the policies with regard to regulation of medicinal plants.

- Research and development programmes are required for developing quality products and to educate all people engaged with medicinal plants and its cultivation.
- Medicinal plants gardens should also be established on regional basis in order to create awareness to the local people about proper identification of plants.

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