The effect of *in vitro* digestion on selected biological activities of *Hypoxis sobolifera* corms

Anzél van Rooyen

Submitted in partial fulfillment of the requirements for the degree *Magister of Scientiae* in the Department of Biochemistry and Microbiology in the Faculty of Science at the Nelson Mandela Metropolitan University

January 2013

Supervisor: Prof. M. van de Venter

DEPARTMENT OF ACADEMIC ADMINISTRATION
EXAMINATION SECTION
PO Box 77000
Nelson Mandela Metropolitan University
Port Elizabeth
6013

Enquiries: Postgraduate Examination Officer

Nelson Mandela Metropolitan University
for tomorrow

DECLARATION BY CANDIDATE

NAME: Anzél van Rooyen

STUDENT NUMBER: 204015278

QUALIFICATION: Masters in Biochemistry

TITLE OF PROJECT: The effect of *in vitro* digestion on selected biological activities of *Hypoxis sobolifera* corms

DECLARATION:

In accordance with Rule G4.6.3, I hereby declare that the above-mentioned dissertation is my own work and that it has not previously been submitted for assessment to another University or for another qualification.

SIGNATURE: _____

DATE: _____

ACKNOWLEDGEMENTS

The author wants to thank the following people:

- To the Father, the Son (Jesus Christ) and the Holy Spirit, I give You all the glory, honour and praise.
- To my husband Hennie, thank you for all your love, patients and support during the hectic times, you made me believe I could do this.
- To my parents, Gerhard and Anita, your prayers have carried me through and your support is greatly appreciated. Thank you for all the tea and biscuits.
- To my parents-in-law, Amie and Magda, your prayers have carried me through and your support is greatly appreciated. Thank you for all the treats and dinners.
- To my brother, Du Toit, and brothers-in-law, Robert and Tertius thank you for your support, love and prayers.
- To my promoter, Prof Maryna van de Venter, thank you for all the help, advice and support, long distance correspondence and for believing in me. You are an amazing teacher!
- To Carel Smith for all your help with editing.
- To my friends and family, thank you for all your support.
- To Gerhardt Boukes for his patience, advice and help in my first year of masters.
- To Maranda Esterhuizen-Londt for printing and handing in on my behalf, all the help throughout the years, and the all the cups of tea.
- To the Biochemistry and Microbiology postgraduate and postdoctoral students for all their help and support.
- To Dr Carl Albrecht for his kind donation of hypoxoside and rooperol.
- To Mrs Yvette van Wijk from the Botanical Gardens in George for the donation of the *Hypoxis sobolifera* plants.
- To the Department of Biomedical Technology, NMMU North Campus, for bacterial strains from their culture collection.
- To the NMMU and NRF for financial support.

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ABSTRACT

In South Africa part of the cultural and religious beliefs of the African people is the use of traditional remedies to treat diseases. These remedies are obtained from medicinal plants (Steenkamp, 2003). One of the most frequently traded plants in the Eastern Cape is *Hypoxis*, commonly known as Afrika patat, or African potato. South African traditional healers instruct patients to brew the fresh *Hypoxis* corm as a tea and then ingest it (Steenkamp, 2006a). This prompted an investigation into the digestive stability of a traditionally prepared *Hypoxis* extract. The *H. sobolifera* extracts were digested using a simulated gastric/small intestinal digestion and their biological activity determined.

The hot water *H. sobolifera* extract before digestion only showed cytotoxic activity against cancer cell lines at very high concentrations which are not likely to be achieved under normal ingestion circumstances. In Chang liver cells on the other hand, chronic exposure to the hot water *H. sobolifera* extract increased glucose uptake in amounts similar to that of metformin. On the negative side, the glucose utilization stimulation was lost due to the simulated digestion process. The significant inhibition of AGEs by hot water *H. sobolifera* extract (IC_{50} of 6.3 µg/ml) is a very encouraging result as treatment in the management of diabetes. This activity was only slightly reduced by the *in vitro* digestion process. Also observed was enzyme inhibition activity by traditionally prepared *H. sobolifera*, with ∝-amylase being inhibited (IC_{50} of approximately 250 µg/ml) and therefore preventing or limiting starch breakdown.

From the DPPH results it was clear that *H. sobolifera,* even when digested, is a potent anti-oxidant (IC_{50} of 134.4 µg/ml when undigested compared to 162.9 when digested with β-glucosidase added to stomach digestive step). HPLC and TLC experiments revealed that rooperol which has previously been thought to be the compound responsible for the anti-oxidant activity in *Hypoxis* extracts, was absent from the traditional extract of *H. sobolifera* and therefore cannot be the sole compound exhibiting anti-oxidant activity; other compounds such as phenolics may be contributing. The phenolic and flavonoid content results revealed very high

concentrations of these compounds in the traditionally prepared *H. sobolifera* extract. These compounds may therefore play major roles in all of the biological activities observed from treatment with *Hypoxis* spp. The ROS results yielded interesting and promising results. Using standard or traditionally prepared *H. sobolifera* extracts, activation of differentiated U937 cells with PMA was greatly enhanced by co-treatment with the extracts, while extracts on their own did not cause significant activation. Future studies should investigate this property of the extracts as a promising immune booster.

The HPLC results showed that hypoxoside was undetectable in the hot water traditional extract and the TLC anti-oxidant experiment proved that rooperol is not present in the hot water traditional extract after treatment with β-glucosidase. This indicates that neither one of the *Hypoxis* compounds previously believed to be responsible for the biological activities observed are present in the extract when prepared the traditional way. Therefore, the biological activities observed in this study can be attributed to other phytochemical compounds.

ABBREVIATIONS

Abs	Absorbance
AGEs	Advanced glycation end products
AIDS	Acquired immunodeficiency disease syndrome
$AlCl_3$	Aluminum chloride
AP	Activation protein
ATCC	American type culture collection
BSA	Bovine serum albumin
CH_3CN	Acetonitrile
CNS	Central nervous system
COX	Cyclooxygenase
$cPLA_2$	phospholipase A_2
dH_2O	Distilled water
DCF-DA	2',7'-dichlorofluorescein diacetate
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EMEM	Eagle's minimal essential medium
FCS	Fetal calf serum
GIT	Gastrointestinal tract
H_2O	Water
H_2SO_4	Sulfuric acid
HBSS	Hanks buffered saline solution
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
i.d.	in diameter
ID	Intestinal digestion
IFN	Interferon
IL	Interleukin
iNOS	inducible nitric oxide synthase
LPS	Lipopolysaccharide

MeOH	Methanol
MIC	Minimum inhibitory concentrations
MNU	*N*-methyl-*N*-nitrosourea
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	Nutrient agar
NaCl	Sodium chloride
NaOH	Sodium hydroxide
Na_2CO_3	Sodium carbonate
$NaHCO_3$	Sodium bicarbonate
$NaNO_2$	Sodium nitrite
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NDGA	Nondihydroguairetic acid
NF	Nuclear factor
NMMU	Nelson Mandela Metropolitan University
NIDDM	Non-insulin-dependent diabetes mellitus
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAID's	Nonsteroidal anti-inflammatory drugs
PBS	Phosphate buffered saline
PBSA	Phosphate buffered saline (solution)
PMA	Phorbol myristate acetate
R^2	Regression correlation coefficient
R_t	Retention time
SD	Stomach digestion
SM	Sphingomyelin
STZ	Streptozotocin
TB	Tuberculosis
TCA	Trichloroacetic acid
TLC	Thin layer chromotography
TNF	Tumor necrosis factor
WHO	World Health Organization
γ, β and α	Gamma, Beta and Alpha, respectively

1 CHAPTER – LITERATURE REVIEW

1.1 AN OVERVIEW OF HYPOXIS

In South Africa, part of the cultural and religious beliefs of the African people is the use of traditional remedies to treat diseases. Most of these remedies are obtained from medicinal plants (Steenkamp, 2003). One of the most frequently traded plants in the Eastern Cape is *Hypoxis*, commonly known as Afrika patat or African potato. *Hypoxis* species have long, yellow starshaped flowers and are tuberous perennials (figure 1). The corms are brownish-black on the outside (figure 1) but yellow inside when freshly cut and are used as medicine against diseases such as the common cold and flu, hypertension, adult-onset diabetes, HIV/AIDS and cancer (Laporta *et al*., 2007a).

Figure 1: Photograph of the *Hypoxis sp.* (African potato) (Maduna, 2006).

1.1.1 Glycosides and Sterols

Several glycosides have been isolated from *Hypoxis*. The first glycoside isolated from *Hypoxis* was hypoxoside. It contains a pent-1-en-4-yne (pentynene) backbone. Rooperol, the aglycone of hypoxoside, is obtained by hydrolyzing hypoxoside via β-

glucosidase (figure 2) (Albrecht, 1995a). Potgieter *et al*. (1988) also chemically synthesized rooperol *in vitro*. However, the synthesis was limited because of the sensitivity of the pentynene backbone to basic and acidic conditions used for standard deprotonation, resulting in rearrangement of the pentynene backbone, therefore they used *tert*butyldimethylsilyl ether to produce significant amounts of stable rooperol (Potgieter *et al.*, 1988).

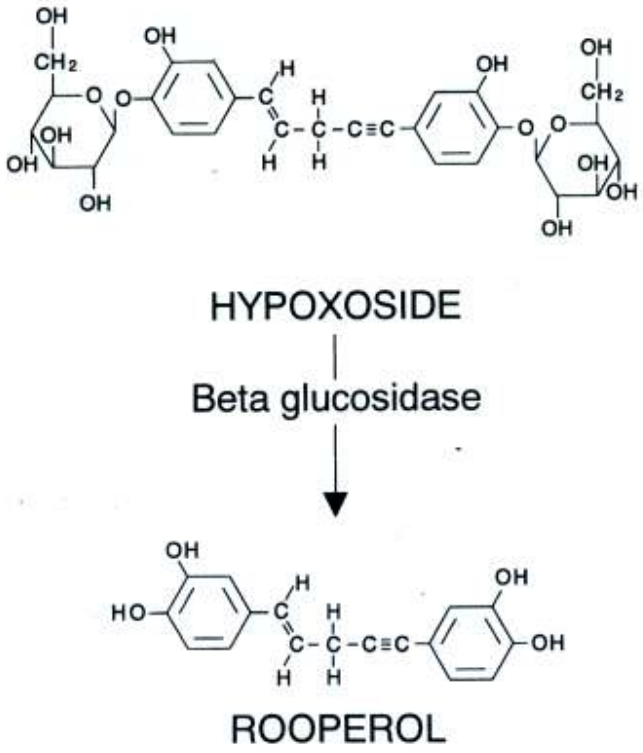

Figure 2: Hypoxoside, an inactive diglucoside, is deconjugated by β-glucosidase to form the cytotoxic and lipophilic aglucone, rooperol (Albrecht, 1995a).

Hypoxoside is a pale yellow water-soluble crystalline compound. β-glucosidase is found in the gastrointestinal tract and also released by rapidly dividing cancer cells. After hydrolysis by β-glucosidase, hypoxoside is converted to form a lipophilic aglycone, rooperol, which is a potent anti-oxidant.

In vitro tests against cancer cells proved that rooperol was cytotoxic, whereas hypoxoside was not (Albrecht *et al.,* 1995 and Smit *et al.,* 1995). This is important, as using a non-toxic prodrug which is selectively activated to become cytotoxic in the immediate vicinity of cancer cells could solve the problem of general cytotoxicity. This is different to chemotherapy and other chemical agents, since they cannot

eradicate cancer cells without harming normal cells (Mills *et al.*, 2005 & Albrecht *et al.*, 1995a). Rooperol is converted to phase II metabolites, glucuronides and sulphates. These metabolites are non-toxic to cells in culture, but treatment with glucuronidase activates the Rooperol metabolites. The degree of conversion of hypoxoside to rooperol, and rooperol to phase II metabolites is determined by the metabolizing activity of the gastrointestinal system and the liver (figure 3) (Albrecht *et al.*, 1995b).

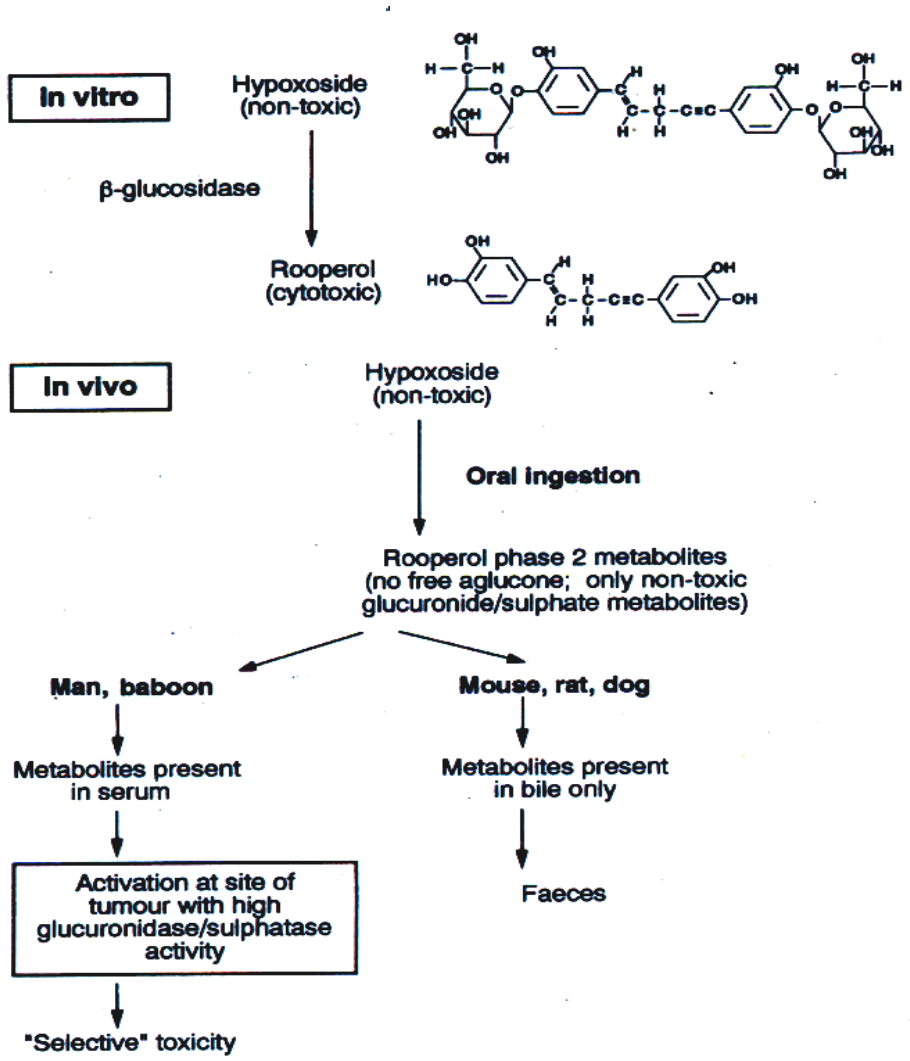

Figure 3: Hypoxoside as a non-toxic prodrug for cancer therapy (Smit *et al.*, 1995).

Along with the glycosides, other active compounds have also been identified in *Hypoxis*. Examples of these are sterols/sterolins. Sterols consist of a hydrophobic steroid nucleus with hydroxyl groups forming the hydrophilic heads. The hydrophobic tails are formed by sterane skeletons with side chains; therefore they are amphiphilic molecules (Boukes *et al.*, 2008). In plants, the sterols found are

known as phytosterols, while in humans the main sterol found is cholesterol (De Brabander *et al*., 2007). The main difference between human cholesterol and phytosterols is the ability of the phytosterols to be alkylated at C-24 with C_1 or C_2 substituents (Buchanan *et al*., 2000).

Plants have the ability to produce small amounts of cholesterol as well as other phytosterols such as β-sitosterol, campesterol and stigmasterol (figure 4).

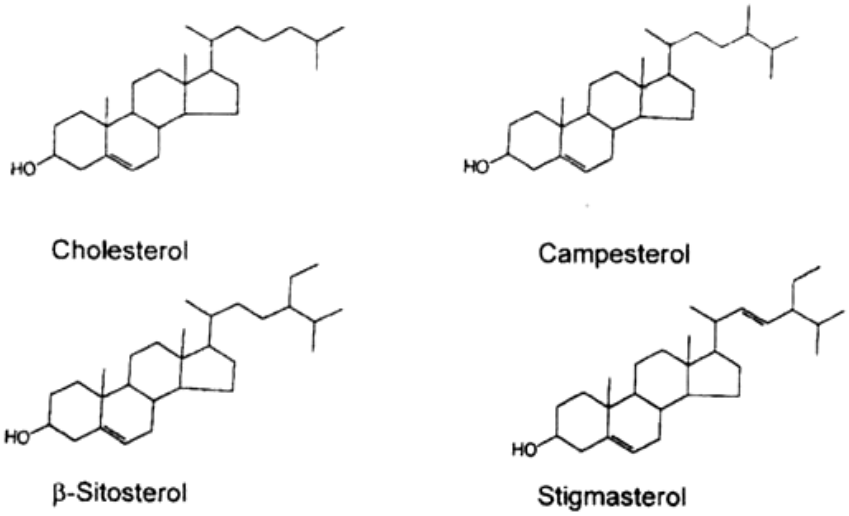

Figure 4: Structures of sterols (Awad and Fink, 2000).

As phytosterols are produced by plants, they enter the body through the diet. They are then absorbed in the intestine (Ling and Jones, 1995). *Hypoxis*, similarly, contain these phytosterols (Mills *et al*., 2005). Boukes *et al.* (2008) determined that *Hypoxis sp.* contain 8 sterols, β-sitosterol, campesterol, cholesterol, desmosterol, ergosterol, fucosterol, stigmasterol and stimastenol, with β-sitosterol being the main phytosterol found in chloroform extracts. As a result, it is possible for many of the medicinal properties of *Hypoxis* to be attributed to these phytosterols.

β-sitosterol has been proven to exhibit anti-inflammatory, anti-angiogenic, immune-modulating and anti-cancer properties in both *in vitro* and *in vivo* disease models. Raicht *et al*. (1980), for example, chemically induced colon cancer in rats, using *N*-methyl-*N*-nitrosourea (MNU). The carcinogen-induced rats were fed experimental diets, and one of the test groups was given added 0.2% β-sitosterol over 28 weeks,

after which the number of tumours in the intestine (from cecum to anus) was recorded. The rats treated with β-sitosterol showed a reduction in colon cancer (Raicht *et al*., 1980). Colon, prostate and breast cancer cell lines showed growth inhibition *in vitro* with β-sitosterol (Awad *et al*., 2003). Cell cycle arrest and stimulation of apoptotic cell death may result in these anti-proliferative effects on the cancer cells (Awad *et al.*, 2003 and Choi *et al*., 2003).

1.1.2 *Hypoxis* in treating diseases

There have been many claims of *Hypoxis* being effective against various diseases (Ojewole, 2006). *H. hemerocallidea* (African Potato) also known as a "wonder" and "miracle cure" herb, is used by indigenous populations in the belief that it alleviates immune related ailments such as HIV, AIDS, arthritis, common cold, flu, wounds, tumour, cancer (Grierson & Afolayan, 1999 and Singh, 1999), as well as hypertension, diabetes mellitus, psoriasis, gastric and duodenal ulcers, TB, urinary tract infections, asthma and some conditions affecting the central nervous system (CNS). Disorders such as epilepsy, childhood convulsions and heart weakness are also treated with *Hypoxia* (Ojewole, 2006).

1.2 BIOLOGICAL ACTIVITIES OF HYPOXIS – PUBLISHED REPORTS

1.2.1 ANTIMICROBIAL ACTIVITY

Prostatitis is a common inflammatory disorder of the prostate, and can appear in at least three forms: 1) chronic bacterial, 2) chronic non-bacterial, and 3) acute bacterial prostatitis. Acute bacterial prostatitis is usually caused by *Escherichia coli*, resulting in urinary tract infection, which spreads to the prostate. *E. coli* can also cause chronic bacterial prostatitis resulting from partial blockage of the male urinary tract, promoting the harbouring of bacteria (Steenkamp *et al*., 2006a). Considering this, Steenkamp (2006a) used *Hypoxis* as a possible antibacterial agent against *E.*

coli. It was found that for 100% *E. coli* inhibition, 62.5 μg/ml of *Hypoxis hemerocallidea* (extracted by ethanol or water) is required (Steenkamp *et al.*, 2006a). However, from the traditional preparation and ingestion of *Hypoxis* tea, only 18.5 μg/ml will be found in the serum (Steenkamp *et al.*, 2006a), therefore the dosage needs to be increased.

Katerere and Eloff (2008) performed anti-microbial studies on four selected cultures, *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli,* and *Pseudomonas aeruginosa*. The positive controls used were ampicillin (Gram-positive) and neomycin (Gram-negative). It was found that *Hypoxis* is active against all four cultures, although in varying concentrations and also that different parts of the plant are active on different cultures after specific extractions. It was found that the corms had more activity than the leaves on Gram-positive cultures while fresh leaves had greater activity on Gram-negative cultures (also see table 1). However, activity against all cultures was observed at high concentrations of *Hypoxis* extracts (Katerere and Eloff, 2008).

Table 1: Minimum inhibitory concentrations (MIC) of acetone (A) and ethanol (E) extracts of bulb and aerial parts of *H. hemerocillidea* (Katerere and Eloff, 2008).

	S. aureus	***E. faecalis***	***E. coli***	***P. aeruginosa***
Dried corm – A (mg/ml)	0.31	0.63	2.5	>5
Dried corm – E (mg/ml)	0.63	1.25	2.5	>5
Fresh leaf – A (mg/ml)	2.5	2.5	2.5	2.5
Fresh leaf – E (mg/ml)	0.63	0.31	0.31	0.63
Dried leaf – A (mg/ml)	1.25	2.5	0.31	0.63
Dried leaf – E (mg/ml)	2.5	0.63	2.5	>5
Ampicillin (μg/ml) (Gram +)	1.56	6.25	-	-
Neomycin (μg/ml) (Gram -)	-	-	0.20	0.78

Rooperol has also been suggested to be able to become a lead compound in the development of new anti-inflammatory, chemopreventive or antimicrobial drugs (Laporta *et al.,* 2007b).

1.2.2 ANTI-CANCER ACTIVITY

In 2007, it was estimated by the WHO that more than 1.2 million people would be diagnosed with breast cancer worldwide that year, with the majority being in European and North American countries (Awad *et al.*, 2007). Dietary factors have been considered as a reason for the increased incidence rate in some countries. The dietary factors considered were, specifically, the proportion of animal versus plant fats, since it seems that in women in less developed countries, where a more vegetarian diet is followed, the incidence rate of breast cancer is lower (Awad *et al.*, 2007 and Choi *et al.*, 2003). It appears that the phytosterols in the plants contribute to this protection against breast cancer, along with some other cancers such as colon and prostate cancers, common to Western societies (Awad *et al.*, 2007 and Awad & Fink 2000). Awad *et al*. (2007) suggested that the action of the phytosterols leading to protection against cancer may be by inducing apoptosis or programmed cell death in highly proliferative tumor cells.

Apoptosis (programmed cell death) is the process where unwanted or damaged cells are eliminated during cell development and other normal biological processes. One of the key role players in the apoptotic process is the family of enzymes known as caspases (cysteine-related proteases). They are found as inactive proenzymes in cells and are comprised of a small and a large subunit which, when cleaved, removes the prodomain and activates the caspases. Different caspases play a role in different biological pathways (Choi *et al*., 2003).

Apoptosis can occur through one of two main pathways. The first is the intrinsic pathway, which responds to an internal signal, activating a series of factors to ultimately activate caspase-3 the effector protease driving programmed cell death, thus inducing apoptosis (Awad *et al.*, 2007). A diverse array of non-receptor mediated stimuli is involved in the intrinsic signalling pathways that initiate apoptosis.

These stimuli produce intracellular signals that act directly on targets within the cell and are mitochondrial-initiated events, and may act in either a positive or negative fashion. Apoptosis may be triggered by negative signals since they involve the absence of certain growth factors, hormones and cytokines leading to failure of death program suppression; i.e. apoptosis is activated owing to the withdrawal of factors, and loss of apoptotic suppression. On the other hand, toxins, radiation, hyperthermia, hypoxia, viral infections and free radicals are examples of stimuli that act in a positive fashion (Elmore, 2007).

The second is the extrinsic pathway, which responds to an external signal (such as cholesterol), activating cell surface death receptors (Awad *et al.*, 2007). Transmembrane receptor-mediated interactions involving members of the tumor necrosis factor (TNF) receptor gene superfamily (death receptors) are involved in initiating apoptosis via the extrinsic signalling pathways. Within the TNF receptor family similar cysteine-rich extracellular domains are shared among members. The members also have an 80 amino acid cytoplasmic domain called the death domain, which plays a critical role in transmitting the death signal from the cell surface to the intracellular signalling pathways (Elmore, 2007).

β-sitosterol is similar to cholesterol in structure, and therefore Awad *et al.* (2007) wanted to assess the effect of cellular supplementation of β-sitosterol on the extrinsic pathway, as well as on cell growth, and membrane sterol content of MCF-7 human breast cancer cells. Growth inhibition (at concentrations of 8-16 μM) was found to be similar to previous studies in human prostate cancer LNCaP cells and human colon cancer HT-29 cells (Awad *et al.*, 2007). Awad *et al.* (2007) also found that the phytosterol concentrations at which cancer cell growth was inhibited are correlated to vegetarian diets. The membrane cholesterol content did not decrease, however, the membrane was enriched with the inclusion of β-sitosterol into the cell membrane, resulting in β-sitosterol representing more than half of the total membrane sterols. This may affect the activity of the extrinsic pathway components (Awad *et al.*, 2007).

Tellingly, caspase 3 (one of the executioner caspases in apoptosis, which leads to fragmentation of DNA) and caspase 9 (which activates caspase 3) activation increased when β-sitosterol was added to HCT116 colon cancer cells and human breast carcinoma cells (Choi *et al*., 2003).

To date, cancer treatment in the forms of chemotherapy and radiation has affected both cancerous and normal cells. Chemotherapy does not have tumour cell selective toxicity and therefore has been a disappointing trade-off between efficiency and toxicity (Albrecht *et al.,* 1995a). Selective eradication of cancer cells, without harming normal cells, by a chemical agent has yet to be discovered. Albrecht *et al*. (1995a), suggested that the use of a non-toxic pro-drug that can selectively be activated to be cytotoxic only in the immediate vicinity of the cancer cells, could be one way of overcoming this persistent and reoccurring problem. Albrecht *et al*. (1995a) used hypoxoside to investigate their hypothesis of using a non-toxic pro-drug which can be activated later to become cytotoxic at the desired location and time. Hypoxoside was chosen as it can be readily converted to the cytotoxic aglucone, rooperol, by β-glucosidase (Kruger *et al.,* 1994 and Albrecht *et al.,* 1995a).

Albrecht *et al*. (1995b) found that the FCS used in the culture media had endogenous β-glucosidase which caused rooperol to be progressively released from hypoxoside, causing false inhibition for hypoxoside cytotoxicity testing. However, after heat-inactivating the FCS for 1 hour at 56 °C, Albrecht *et al*. (1995b) found hypoxoside to be non-toxic, while pure rooperol had 50% inhibition at 10 µg/ml. Albrecht *et al*. (1995b) also did pharmacokinetic studies on mice with hypoxoside dosed intragastrically, and while β-glucosidase from bacteria in the colon deconjugated the hypoxoside to form rooperol, they found no hypoxoside or rooperol in the serum, only phase II byproducts (sulphates and glucoronides) were present in portal blood and bile. In contrast, however, Albrechts *et al*. (1995b) found the metabolites from orally ingested hypoxoside, rooperol, dehydroxy-rooperol and bis-dehydroxyrooperol, reached relatively high concentrations. Albrecht *et al*. (1995b) concluded that the complete first-phase metabolism of rooperol into non-toxic conjugates, which may be activated in tumours with high deconjugase activity, shows the promising properties of rooperol as an oral pro-drug for cancer therapy in humans. Smit *et al*. (1995) used the findings of Albrecht *et al*. (1995b) that the

conjugated metabolites, like the glucoside, are also non-toxic but can be activated by treatment with glucuronidase, to achieve selectivity in cancer chemotherapy. Smit *et al*. (1995) suggested this, since certain tumours contain relatively high levels of glucuronidase, and activation of rooperol metabolites at the site of the tumour is an attractive approach. Smit *et al*. (1995) therefore did a phase I trial of hypoxoside as an oral pro-drug for cancer therapy, and found no toxic effects in clinical examinations, biochemical or haematological measurements that could be ascribed to the ingestion of hypoxoside. Smit *et al*. (1995) concluded that short- and long term therapy (up to 5 years) with relatively high hypoxoside dosages did not result in any obvious toxic effects.

Other biological and chemical properties of compounds used in ethnopharmacological treatments, of disorders such as inflammation, infectious, parasitic and viral diseases, should be considered when selecting plants used to treat cancer as these could bear relevance to cancer or a cancer symptom (Cordell *et al.,* 1991 and Steenkamp and Gouws, 2006). For the discovery of new drugs in cancer treatment, it is also vital that the search for new biologically active compounds continues (Steenkamp and Gouws, 2006).

Steenkamp and Gouws (2006) investigated the cytotoxicity of various South African plants used by traditional healers to treat cancer. They used aqueous extracts, as all the remedies were prescribed by traditional healers as infusions. One extract was from *H. Hemerocallidea*, as it has been reported by Ojewole (2002) to display anti-inflammatory activity, which relates to cancer (Steenkamp and Gouws, 2006).

Steenkamp (2006b) determined the cytotoxicity of *Hypoxis hemerocallidea* against the breast cancer cell line MCF-7. Extracts were considered active if less than 50% survival after 72h exposure was recorded. Steenkamp (2006b) found that *H. hemerocillidea* extracts inhibited MCF-7 cell growth at an extract concentration of 50 μg/ml. Rooperol has 4-hydroxyl moieties in its structure which could pertain to its anti-carcinogenic and anti-proliferative capacity (Laporta *et al.,* 2007b).

1.2.3 ANTI-OXIDANT ACTIVITY

Anti-oxidants are used as a defense system in aerobic organisms, against the reactive oxygen species (ROS) produced from molecular oxygen from their aerobic respiration (Matés *et al.*, 1999). ROS in low levels is an essential component in many biochemical processes in our bodies, but in high concentrations ROS may cause oxidative stress and damage to the cell (Matés *et al.*, 1999; Mosoko and Eloff, 2007). Anti-oxidants react with these ROS to render them harmless through scavenging/quenching of these charged molecules, restoring the balance in the cells (Masoko and Eloff, 2007; Katalinic *et al.*, 2006; Matés *et al.*, 1999). Ou *et al.* (2003) compared this balance with the Chinese theory of yin-yang, where yin refers to anti-oxidant process and yang represents the oxidation process. If the balance shifts in favour of the oxidation process it can cause cells to lose their structure, function and eventually result in cell dysfunction and death (Masoko and Eloff, 2007). The onus rests on the anti-oxidants to restore this balance, in both an enzymatic and non-enzymatic manner (Matés *et al.*, 1999). Anti-oxidants have 3 mechanisms of action to restore this balance, namely preventive (proteins with the ability to bind metals such as iron and copper); repairing (repairing or eliminating damaged biomolecules); and scavenging (enzymes and non-enzymes capable to locate excesses of free radicals/ROS (Núñez-Sellés, 2005). Examples of enzymatic anti-oxidants are: superoxide dismutase (SOD), glutathione peroxidise (GP) and catalase (CAT) (Matés *et al.*, 1999; Röhrdanz and Kahl, 1997).

1.) Superoxide dismutase (SOD) – catalyses the dismutation of highly reactive superoxide anion (O_2^-) to molecular oxygen (O_2) and the less reactive species H_2O_2. In humans there are three forms of SOD, all of which can bind single charged anions (Matés *et al.*, 1999; Röhrdanz and Kahl, 1997).
2.) Catalase (CAT) – reacts with H_2O_2 to form water (H_2O) and molecular oxygen (O_2), thus destroying or detoxifying the peroxide, to protect the cells. CAT, therefore, plays an important role in the adaptive response of cells to acquire tolerance to oxidative stress, but is not essential for normal conditions in some cell types (Matés *et al.*, 1999; Röhrdanz and Kahl, 1997).

3.) Glutathione peroxidase (GP) – contains selenocysteine which is essential for enzyme activity, and catalyses reduction of hydroperoxides using glutathione, which is one of the most important anti-oxidant defence mechanisms. Hydrogen is a shared substrate between GP and CAT, however, only GP can react effectively with lipid and other organic hydroperoxides, making it an essential and major source of protection against low levels of oxidative stress (Matés *et al.*, 1999).

In order to form more reactive species, superoxides and peroxides have to react with metal catalysis. However, SOD, GP and CAT prevent this reaction by removing the superoxides and peroxides. Imbalance in anti-oxidant or free radical scavenging systems can result in conditions such as inflammation, hypersensitivity and autoimmune conditions. It could also cause other conditions such as increase DNA damage as a result of inflammatory cells activating pro-carcinogens to DNA-damaging species through ROS-dependent mechanisms and cancers when continuous damage occurs due to toxic oxygen also known as degenerative disease of old age (Matés *et al.*, 1999).

Examples of non-enzymatic anti-oxidants are: vitamin C (ascorbic acid), vitamin E ($\propto$-tocopherol), glutathione, β-carotene, vitamin A (Núñez-Sellés, 2005; Matés *et al.*, 1999) and phenolic compounds (Rashid *et al.*, 2010). One of the most commonly consumed beverages is tea. Both black and green tea are rich in polyphenolic compounds, collectively known as the tea flavonoids, which have high anti-oxidant activity (Katalinic *et al.*, 2006).

(E)-1,5-bis (4'-β-D-glucopyranosyloxy-3'-hydroxyphenyl) pent-4-en-1-yne, also known as hypoxoside, is a characteristic secondary metabolite of African potato. The hypoxoside is converted within the human body to rooperol by colonic bacterial β-glucosidase (Laporta *et al.*, 2007a and Drewes *et al.*, 1984).

Rooperol and a known strong anti-oxidant, nondihydroguairetic acid (NDGA), share a structural resemblance (Drewers *et al.*, 1984), the only difference being in the C-bridge connecting the two catechol molecules. NDGA has a 2,3-dimethyl-1,4-

butonediyl bridge and rooperol has a pent-4-en-1-yne-diyl bridge. Therefore rooperol is a potential candidate for anti-oxidant studies. Nair *et al*. (2007) investigated the African Potato for potential anti-oxidant activity as well as its major phytochemical constituents, hypoxoside and rooperol (the aglycone of hypoxoside), independently. African potato was also tested for free radical scavenging ability, using DPPH and high levels of activity were found for rooperol, but no significant levels were found for hypoxoside (Nair *et al*., 2007).

1.2.3.1 Reactive Oxygen Species (ROS) relationship with anti-oxidants

Reactive oxygen species (ROS) is a phrase used to describe the ubiquitous molecules that result from the partial reduction of molecular oxygen (one electron at a time) and free radicals (these same chemical species, when they have one unpaired electron) (Barbieri *et al.*, 2003; Hoidal 2001; Turrens, 2003; Thannickal and Fanburg, 2000; Gac *et al.*, 2010; Maneesh and Jayalekshmi, 2006, Held, 2010). ROS molecules are highly reactive and diffusible (Barbieri *et al*., 2003) oxidizing agents (Maneesh and Jayalekshmi, 2006) of which superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) are the most commonly known and studied (Thannickal and Fanburg, 2000; Barbieri *et al*., 2003; Hoidal, 2001; Turrens, 2003; Maneesh and Jayalekshmi, 2006; Held, 2010).

Once a pathogen or microbial invader has successfully been recognized by the host innate immunity, ROS is produced by an oxidative burst, which is one of the earliest cellular responses to the threat (Torres *et al.*, 2006; Spooner and Yilmaz, 2011). Free oxygen radicals are used by the immune system to prevent the colonization of tissues by microorganisms due to their high toxicity to pathogens (Spooner and Yilmaz, 2011). Normal tissue has low-levels of oxidants, and infected cells have high levels of oxidants due to the respiratory burst; this difference in oxidant levels is a significant aspect of cell physiology during infection. Intracellular redox profiles can be affected by ROS and influence a wide variety of signalling networks (Spooner and Yilmaz, 2011; Circu and Aw, 2010). During multiple viral infections the disruption of redox balance is common in infected cells. Anti-oxidants are naturally found in cells and they counter (inhibit or prevent) the damaging effects of

oxidation in animal tissues (Huang *et al.*, 2005). However, when the redox balance is disrupted during multiple viral infections, the anti-oxidant enzyme levels decrease and the cellular anti-oxidant stores with molecules such as glutathione are depleted. There is also a concomitant increase in ROS production, leading to oxidative stress and increased vulnerability to cell responses such as apoptosis, tumourogenesis and immune response, thereby causing the infected cells to die (Gac *et al.*, 2010; Spooner and Yilmaz, 2011; Circu and AW, 2010). This killing of the infected cells leads to increased viral spread to neighbouring cells and throughout the organism (Gac *et al.*, 2010; Spooner and Yilmaz, 2011; Circu and AW, 2010).

ROS molecules act on multiple facets within cells; they are directly harmful to microbes that invade tissues, while also acting as signal molecules for inflammation and immune responses as seen by the downstream results observed by Spooner and Yilmaz (2011), when they demonstrated the role for ROS as a pro-inflammatory gene transcription and cytokine-based signal response during infection. Membrane associated NADPH oxidase complex and the mitochondrial electron transport chain are two sources of ROS which have been best characterized during host cell-microbe interactions (Spooner and Yilmaz, 2011).

Grandvaux *et al*. (2007) stated that NADPH oxidase has several functions in different cells, for example during microbial infection of host cells, it is a primary ROS production site (Grandvaux *et al.*, 2007), similar to mitochondria (Spooner and Yilmaz, 2011). The mechanism of action is the use of rapid respiratory burst in phagocytic cells leading to ROS production as well as carrying out the electron transferring reaction, in the membranes of phagosomes, endosomes and the cell membrane, from NADPH to molecular oxygen (Grandvaux *et al.*, 2007; Spooner and Yilmaz, 2011). To ensure dedicated activation of NADPH oxidase and its downstream signalling intermediates, the powerful induction of ROS and inflammatory signalling cascades are under multiple levels of control.

Ochsendorf (1998) found that during infection of the male genital tract by microorganisms, leukocytes are activated and generate increased levels of ROS which disrupt or impair sperm function when seminal plasma is not present (during inflammation of epididymis or after semen preparation). However, when seminal

plasma is present, ROS does not appear to damage the spermatozoa, and the ROS produced by the leukocytes are critical for the techniques of semen preparation for assisted reproduction (Ochsendorf, 1998). From his findings, Ochsendorf (1998) suggested it was the powerful anti-oxidants in the seminal plasma that protected the spermatozoa against the detrimental effects of ROS.

Inflammation, for decades, was recognized as a simple allergic reaction, but is now considered to underline pathophysiology of a much broader spectrum of diseases than previously expected (Johar *et al.*, 2004). Frequently ROS, associated with inflammatory response, contribute to the tissue-damaging effects of inflammatory reactions. In the metabolism of aerobic organisms, the formation and degradation of ROS are key components. The activities of several kinases, transcription factors, cell death machinery and proteins such as COX-2 and iNOS are regulated by ROS (Johar *et al.*, 2004). ROS also has the ability to transform proteins to autoantigens and/or increase the susceptibility of proteins to degradation. In the development and manifestation of inflammation, neutrophils play a crucial role and they are the major source of free radicals at the site of inflammation (Johar *et al.*, 2004). Extracellular and intracellular signals are used for cell-cell communication by all multicellular organisms in diverse physiological processes (Thannickal and Fanburg, 2000). These cell-signalling pathways are used in processes such as normal tissue homeostasis maintenance, development/growth of organs, repair responses to tissue damage, and cell death (Barbieri *et al.*, 2003; Irani, 2000; Thannickal and Fanburg, 2000). ROS is a by-product of metabolism from mitochondria and other cellular sources and has been regarded as toxic with the potential to cause damage (Thannickal and Fanburg, 2000). ROS has been shown to be crucial as second messenger and an integral component of membrane receptor signalling (Rhee, 1999) and regulation (Thannickal and Fanburg, 2000).

Oxidative stress has been implicated in various disorders and diseases in humans (Rhee, 1999; Thannickal and Fanburg, 2000) and plants (Gupta, 2011). In humans one such disease is cancer (Cross *et al.*, 1987). ROS and oxidative stress also plays a role in apoptosis (Hampton and Orrenius, 1997; Lavrentiadou *et al.*, 2001; Turrens, 2003; Johar *et al.*, 2004). In his review Hoidal, 2001, suggested that to consider ROS (oxidative stress) only as pro-apoptotic is too narrow a view and that

the potential for inhibition of apoptosis by ROS has been overlooked. The enzymes involved in apoptosis are caspases, which are redox sensitive, when activated (Hampton and Orrenius, 1997). Prolonged oxidative stress has been shown to prevent activation of caspases and therefore prevent apoptosis (Hampton and Orrenius, 1997). Hoidal (2001) stated that apoptosis may be triggered by an initial burst of ROS, while excessive ROS at the early stage may block apoptosis in a variety of models.

The major defences against ROS are anti-oxidants, via preventive-, scavenger- and repairing mechanisms (Núñez-Sellés, 2005), and these anti-oxidants can be enzymatic (Maneesh and Jayaleksmi, 2006) or non-enzymatic (del Rio *et al.*, 2002). Examples of enzymatic anti-oxidants are as mentioned previously, superoxide dismutase (SOD), catalase (CAT) and gluthathione peroxidise (GP) (Röhrdanz and Kahl, 1997; Matés *et al.*, 1999; Núñez-Sellés, 2005). Examples of non-enzymatic anti-oxidants are vitamins (Núñez-Sellés, 2005), flavonoids and phenolics (Rashid *et al.*, 2010) and molecules such as ascorbate and glutathione (del Rio *et al.*, 2002).

1.2.3.2 Polyphenolic compounds relationship with anti-oxidants

The therapeutic potential of medicinal plants to act as anti-oxidants has recently caused an increase in interest as the anti-oxidants can play a role in reducing tissue injury/damage due to free radical scavenging. The search for novel anti-oxidants has resulted in many new plant species being investigated (Pourmorad *et al.*, 2006; Zhishen *et al.*, 1999 and Wojdylo *et al.*, 2007). In the search for new anti-oxidant-rich species another suggestion for the increased activity seen in some plants compared to others may be as a result of their phenolic compounds (Cook and Samman, 1996). Two categories of plants commonly contain polyphenolic compounds, namely edible and inedible plants (Wojdylo *et al.*, 2007). There is also a wide range of different polyphenolic compounds, all with different structures (Cook and Samman, 1996). Frankel (1995) identified one major group of polyphenolic compounds as flavonoids which have known properties, including free radical scavenging, anti-inflammatory action, as well as inhibition of hydrolytic and oxidative

enzymes. Another major group polyphenolic compounds is phenolic acids (Wojdylo *et al*., 2007).

Wojdylo *et al*. (2007) investigated the relationship between phenolic compounds (and their content) and anti-oxidant activity on 32 species of herbs. They found that the typical phenolics that possess anti-oxidant activity are mainly phenolic acids and flavonoids. Pourmorad *et al*. (2006) also found a relationship between total flavonoid and phenol contents with anti-oxidant activity. Cook and Samman (1996) suggested that the mechanism of action of flavonoids was through the process of chelating or scavenging of free radicals. Phenolics, however, are a class of anti-oxidant agents which act as free radical terminators (Pourmorad *et al*., 2006). Pourmorad *et al*. (2006) suggested that the high radical scavenging activity of *Mellilotus officinalis* can be explained by the high content of phytochemicals such as phenol and flavonoids.

Flavonoids exist commonly in leaves, flowering tissues and pollens and are therefore found widely in the plant kingdom (Zhishen *et al*., 1999). Flavonoids have been studied and found to have a number of activities in which they play a role. These include cancer, anti-oxidant- and reactive oxygen species formation (Gupta *et al*., 2010; Zhishen *et al*., 1999; and Rashid *et al*., 2010). In an overview done on flavonoids and their chemical nature, Gupta *et al*. (2010) stated that it is in fact their chemical structure that is responsible for their anti-oxidative activities. Gupta *et al*. (2010) also compiled a list of all the structural properties of the major flavonoids (see figure 5). From the list it can be seen that flavonoids comprise of two benzene rings (A and B) and they only vary in the (C) ring (Gupta *et al*., 2010 and Cook and Samman, 1995).

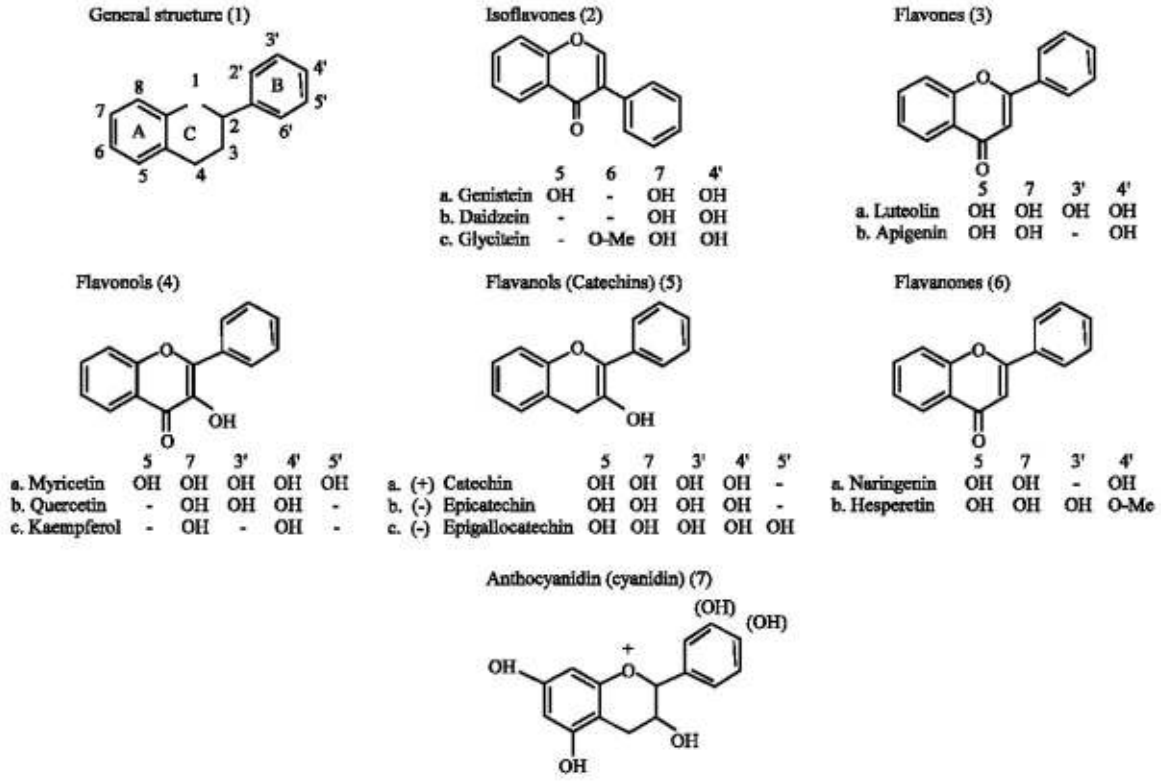

Figure 5: Chemical structures of the major classes of flavonoids (Gupta *et al*., 2010)

Flavonoids are present in a number of plants, in various parts of the plant, as well as in various concentrations (Gupta *et al*., 2010). Flavonoids show anti-oxidant activity (Zhishen *et al*., 1999) which is believed to be related to the -OH and O_2^- scavenging properties and variations in the (C) ring (figure 5) (Zhishen *et al*., 1999 and Gupta *et al*., 2010). Flavonoids can be absorbed in the body in two ways, one of which is the transformation into their glucouronides and sulfates (Gupta *et al*., 2010). This is similar to the way hypoxoside is metabolised (Mogatle *et al*., 2008). The major part in this absorption of flavonoids occurs in the intestines through degradation by die microflora in the gut (Gupta *et al*., 2010) in much the same way that hypoxoside is converted to rooperol by β-glucosidase in the colon (Mogatle *et al*., 2008). The bacterial enzymes involved in this absorption process of flavonoids catalyse several reactions which produce phenolic acids, and these phenolic acids possess a radical scavenging ability which can be compared to their intact precursors (Gupta *et al*., 2010).

Gupta *et al*., 2010 also reported that flavonoids have more target sites (compared to phenolic acids) for free radicals resulting in them acting as anti-oxidants. The O_2^- and -OH in flavonoids which can be scavenged directly are also suggested as the mechanism of anti-oxidant activity in flavonoids, resulting in growing interest among researchers (Zhishen *et al*., 1999).

1.2.4 ANTI-INFLAMMATORY ACTIVITY

Prostaglandins have been associated with many inflammatory diseases and are found in almost all animal tissues, glands and cells. Prostaglandin, a C20 fatty acid family member, is synthesized from the substrate arachidonic acid, present in phospholipids (Shale *et al*., 1999). This synthesis occurs through the combined action of cytosolic phospholipase A_2 ($cPLA_2$) and cyclo-oxygenase 1 and 2 (COX-1&-2) (Awad *et al.*, 2005). Prostaglandins are known to cause pain and fever associated with redness (erythema) and swelling (edema) (Shale *et al*., 1999). To reduce inflammation, pain and fever, COX-1 and COX-2 enzymes are targeted by nonsteroidal anti-inflammatory drugs (NSAID's). Examples of NSAIDs are COX-2 inhibitors, such as aspirin and ibuprofen (Smith *et al*., 1996).

Prostaglandin biosynthesis is catalyzed in the first step by the integral membrane proteins cyclo-oxygenase 1 and 2 (COX-1 and COX-2) (figure 6). COX-1 and COX-2 are also known as prostaglandin endoperoxide H synthase 1 and 2. COX-1 and COX-2 are structurally similar even though their respective genes are found on different chromosomes. The expression and biology of COX-1 and COX-2 are also different regardless of the fact that they are homodimeric, heme-containing, glycosylated proteins with 2 catalytic sites. Another difference between COX-1 and COX-2 is the fact that COX-1 is always present and in a near-constant concentration while COX-2 is inducible. In selected cells and tissues such as monocytes, platelets, endothelium, seminal vesicles and renal collecting tubes, COX-1 is constitutively produced at high levels, because it regulates housekeeping activities involved in renal and platelet function. COX-2 is involved in biological processes such as immunity, reproduction, neurotransmission, pancreatic secretion, renal physiology and bone resorption and therefore can be induced in response to bacterial

endotoxin, cytokines, growth factors tumour promoters and hormones. It is produced in endothelial cells, monocytes, fibroblasts and ovarian follicles (Smith *et al*., 1996).

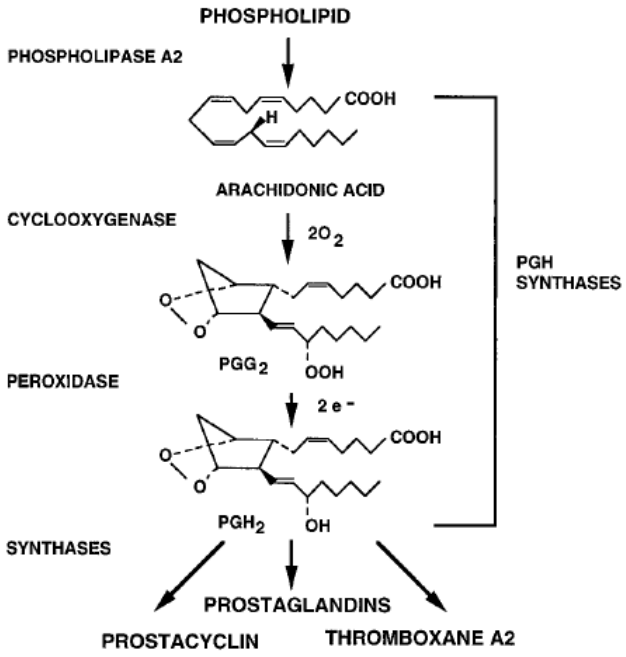

Figure 6: Prostaglandin biosynthesis pathway (Smith *et al*., 1996)

Aqueous extracts of African potato have been demonstrated to have anti-inflammatory activities. *H. hemerocallidea* ethanolic extracts showed high inhibitory effects on COX-1 catalyzed prostaglandin biosynthesis, whereas aqueous extracts showed mild inhibition of COX-2 as well (Laporta *et al*., 2007a). However, inhibition of the 5-lipoxygenase pathway is the most likely way that rooperol has as anti-inflammatory response (Guzdek *et al*., 1996).

Laporta (2007b) studied enzymatic COX-1 and COX-2 inhibition by hypoxoside and rooperol for the first time. Laporta (2007b) also hypothesized that rooperol is the main compound responsible for COX-1 and COX-2 inhibitory activities of whole extracts. Rooperol is expected to also have fewer side effects since it is more COX-1 selective. Since rooperol also has anti-oxidant activity, it may also modulate redox-sensitive cell signalling pathways (Laporta *et al.,* 2007b).

Respiratory burst (increased metabolic activity) forms superoxide during phagocytosis, the defense mechanism against bacteria in which phagocytes are

involved. Hydrogen peroxide, hydroxyl radical and hypochlorous acid are all reactive oxygen intermediates produced from superoxide that is formed during oxygen reduction via NADPH oxidase (located in phagocyte cell membrane). The abovementioned reactive oxygen intermediates act as microcidal agents, however when produced in excess these may damage the host. Another product of respiratory burst which has bactericidal activity is nitric oxide (NO), which may be produced in macrophages via inducible nitric oxide synthase (iNOS). NO respiratory burst results from opsonized particles, certain cytokines (TNF-α, IFN-γ), endotoxin, activators of kinase C (phorbol esters) and N-formylated chemoattractant. Reactive oxygen and nitrogen intermediates interact to form peroxynitrite, which increases cytotoxicity and inflammation. When peroxynitrite attacks a protein, the permanent product involves the nitration of tyrosine residues on the 3-carbon atom adjacent to the oxygen. Inhibition of NOS and NADPH oxidase simultaneously will suppress inflammatory arthritis (Guzdek *et al*., 1996, 1997 and 1998).

Rooperol is capable of inhibiting NO production and respiratory burst using an unknown mechanism and Guzdek *et al*. (1996, 1997 and 1998) suggested it might involve lipopolysaccharide (LPS) priming, interaction with the enzymatic pathway of oxygen radical formation. Rooperol may also inhibit inflammation by suppressing NF-κB, TNF-α, IL-1β and IL-6 synthesis by macrophages. Diseases such as asthma, pathogenesis of septic shock and rheumatoid arthritis are caused by overproduction of cytokines; therefore potential treatment includes suppressing production of cytokines (Guzdek *et al*., 1996). The production of TNF-α, IL-1β and IL-6 in human and rat alveolar macrophages can be inhibited by rooperol, as well as in human blood monocytes and human promonocytic cell line U937 (Guzdek *et al.*, 1998). This occurs due to rooperol decreasing the mRNAs encoding the inflammatory cytokines. mRNA concentration decreases may be as a result of changes in transcription rates or mRNA stability. The transcription factors (nuclear factor [NF]-κB and activation protein [AP]) required for cytokine gene transcription may also interact with rooperol, and as rooperol is also an anti-oxidant agent it may inhibit the oxidation step involved in activating NF-κB. This may involve reactive oxygen species and several anti-oxidants, thus inhibiting inflammatory cytokine synthesis (Guzdek *et al*., 1998).

1.2.5 ACTIVITY IN DIABETES MELLITUS

Diabetes mellitus affects humans from all walks of life, all cultures, all races, and with varying financial status. Approximately 25% of the world population is estimated to be affected by diabetes mellitus. Diabetes can result from target organs being insensitive to insulin or low blood insulin levels, both leading to abnormal carbohydrate metabolism (Maiti *et al*., 2004 and Erasto *et al*., 2005). Over the past two decades the number of people suffering from diabetes has been rising steadily, and is increasing more amongst black South Africans due to severe changes in their diet as they become accustomed to a Western diet and lifestyle. This has also lead to increased mortality from diabetes amongst black South Africans (Erasmus *et al*., 1999; Levitt *et al*., 1993 and Erasto *et al.*, 2005).

In the African cultures herbal medicines have always been used and due to the unwanted side-effects of synthetic drugs it is believed that natural/herbal medicines are safer because they are more harmonious with biological systems (Atal, 1983 and Erasto, 2003)

South Africa also has a severe problem with unemployment, leading to a large number of low or no income groups living in rural areas. South Africa, like other developing countries, is subject to shortages of funds, medical facilities and newly developed medicine, making them more dependent on their natural resources rather than modern anti-diabetic drugs (Louw *et al.*, 2002). The Eastern Cape, South Africa, is one of the provinces that is worst affected by poverty, with most of the people being rural dwellers, therefore the use of plants for the treatment of common diseases such as diabetes is widespread. Erasto *et al*. (2005) stated that in traditional medicine, the number of plants used in the treatment of diseases associated with physiological disorders such as diabetes is limited. These plant species are regarded as precious and highly valued. Erasto *et al*. (2005) documented the historic cultural knowledge of these plants as well as the experience of the traditional healers and herbalists. The local and scientific names of the plants used for the treatment of diabetes (14 plant species) in the Eastern

Cape as well as the parts of the plants used and the various methods of preparation and administration are summarized in table 2 (Erasto *et al*., 2005).

Table 2: Plants used for the treatment of diabetes in the Eastern Cape Province, South Africa (Erasto *et al.*, 2005).

Family & scientific name	Local name	Parts used	Preparation of medicine
Asteraceae *Helichrysum odoratissimum* L.	Imphepho	Whole plant	A fresh plant is crushed, boiled and the infusion taken orally.
Helichrysum nudifolium L.	Ichocholo	Leaves, roots	Fresh leaves or roots are boiled, and then taken orally.
Helichrysum petiolare H & B.L.	Imphepho	Whole plant	A fresh plant is crushed, boiled and the concentrated solution is taken orally.
Artemisia afra Jacq.	Umhlonyane	Leaves, roots	Leaves or roots are boiled, and then the infusion is mixed with sugar to mask the bitterness before being taken orally.
Vernonia oligocephala Sch. Bip.	Umhlunguhlungu	Leaves, twigs, roots	Fresh leaves, roots or twigs are pulverized, and the infusion taken orally.
Vernonia amygdalina Del.	Umhlunguhlungu	Leaves	Pulverized fresh leaves are soaked in water and the solution is taken orally.
Brachylaena discolor DC.	UmPhahla	Leaves	Leaves are boiled and the infusion is taken orally
Hypoxidaceae *Hypoxis hemerocallidea* Fisch. & C. A	**Inongwe**	**Corms**	**Fresh corms are crushed, boiled and taken orally.**
Hypoxis colchicifolia Bak. Asphodelaceae	Inongwe	Corms	Fresh corms are crushed, boiled and taken orally.
Bulbine natalensis (Syn. *B. latifolia*) Mill.	Ibhucu	Roots	Fresh roots are boiled, and the infusion is taken orally.
Bulbine frutescens L.	Ibhucu	Roots	The infusion is made from fresh boiled roots and is taken orally.
Apocynaceae *Catharanthus roseus*	Isisushlungu	Leaves	The infusion is made from boiled leaves and taken orally.
Apiaceae *Heteromorphica arborescens*. Hochst. Ex A. Rich.	Umbangandlala	Leaves, roots	The herb is made from boiled leaves or roots and taken orally.
Buddlejaceae *Chilianthus olearaceus.* Burch.	Umgeba	Leaves, twigs	The infusion is made from leaves or twigs and taken orally.

Erasto *et al*. (2005) found that four plants were frequently mentioned and highly recommended by both the traditional healers and rural dwellers. These are *Helichrysum odoratissimum*, *Helichrysum petiolare*, *Hypoxis hemerocallidea* and *Hypoxis colchicifolia*.

Day (1998) stated that in diabetic patients mineral deficiencies are common, aggravating insulin deficiency, and several minerals found in some medicinal plants may act as cofactors that signal intermediaries of insulin action and key enzymes of glucose metabolism. Therefore, even though the mode of action of the extracts from these anti-diabetic plants is uncertain, they may act, at least in part, through their fiber, vitamin or mineral contents and some secondary metabolites (Day, 1998 and Erasto *et al.*, 2005).

Ojewole (2003), illustrated that *H. hemerocallidea* corm possesses anti-inflammatory and hypoglycaemic properties. In South Africa, unreliable or anecdotal reports about *Hypoxis sp*. being used to manage and/or control painful, arthritic and inflammatory conditions, as well as for adult-onset, type-2 diabetes mellitus have been made. Ojewole (2006), investigated a corm of *H. hemerocallidea* aqueous extract for anti-inflammatory, analgesic (pain-relieving) and anti-diabetic effects in mice and also in experimental rat oedema and diabetes mellitus models.

In adult-onset, type-2 non-insulin-dependent diabetes mellitus (NIDDM) there are various classes of synthetic oral hypoglycaemic agents, including sulphonylureas, biguanides, thiazolidinediones, and alpha-glucosidase inhibitors (Ojewole, 2006). Ojewole (2006) used chlorpropamide as the reference hypoglycaemic agent in their study. Chlorpropamide is a member of the first-generation sulphonylureas. The release of endogenous insulin from pancreatic β-cells is stimulated by sulphonylureas. Insulin-producing pancreatic β-cells are known to be obliterated by streptozotocin (STZ) although this is dose dependent (Ojewole, 2006). Therefore Ojewole (2006) used the STZ-treated rat model to experimentally represent a NIDDM diabetic state, with residual or remnant insulin production by the pancreatic β-cells. In total pancreatectomy, daily administration of insulin is required for the survival of the animal, whereas in the diabetic state of STZ-treated diabetic rats this is not the case (Ojewole, 2006).

Ojewole (2006) found that when distilled water alone was used as acute treatment of the fasted normal and fasted STZ-treated diabetic rats, it did not produce any significant change in the blood glucose concentrations of the animals. The plant's

aqueous extract, however, did cause significant reductions in the blood glucose levels of the fasted normal and STZ-treated diabetic rats. The mechanism of the hypoglycaemic effect of the plant's extract is unknown at present (Ojewole, 2006).

It was suggested by Ojewole (2006) that the phytosterols and sterolins found in *H. hemerocallidea* corm are most likely not exclusively responsible for the anti-inflammatory and anti-diabetic activities of the corm's aqueous extract used in their study. The reason for this suggestion is that these compounds are usually more abundant in alcoholic extracts, rather than in aqueous extracts, of the corm (Ojewole, 2006).

1.3 IN VITRO DIGESTION

To study the digestive stability and absorption of several essential nutrients and other dietary bioactive compounds, the coupled simulated gastric/small intestinal digestion with Caco-2 human intestinal cell model has mostly been used. The reason for using this model is that the results obtained can be qualitatively compared to bioavailability data from human studies. The human Caco-2 intestinal cells act as surrogate for assessing the uptake and transepithelial transport of various nutrients, other dietary bioactive components and drugs (Bhagavan *et al.*, 2007).

Effective absorption from the gastrointestinal tract (GIT) into the circulation and delivery to the appropriate location in the body is needed to achieve biological effects of compounds in specific tissues and organs. Data on the absorption, metabolism, tissue, and organ distribution and excretion is required in studies to determine the bioavailability of any class of phytochemicals. However, this is very complex in human and animal subjects therefore *in vitro* digestion, mimicking the physiochemical and biochemical conditions found in the upper GIT, can be used. In intestinal mucosa cells β-glucosidase enzymes have been identified that are able to deglycosylate certain flavonol glycosides. This allows the glycosides to be converted to their aglycones, to be absorbed passively through the gut lumen. The *in vitro* digestion model is useful when studying a wide range of experimental

conditions and when screening large numbers of samples because it is simple, inexpensive and reproducible (Bhagavan *et al.*, 2007).

β-glucosidase is a glucosidase enzyme which acts upon β1→4 bonds linking two glucose or glucose-substituted molecules (i.e., the disaccharide cellobiose). It splits off terminal glucose units from oligosaccharides. It is an exocellulase with specificity for a variety of β-D-glycoside substrates. It catalyzes the hydrolysis of terminal non-reducing residues in β-D-glucosides (including gentinobiase and cellobiase) with concomitant release of glucose. Cellulose (from plants), considered the most abundant organic compound in the biosphere, is a linear polysaccharide composed of β-bond (β1→4) linked glucose molecules, and β-glucosidases are required by organisms (such as certain fungi, bacteria, and termites) that can consume it (McBain, 2001 and Lawrence, 2000).

1.4 AIM OF THIS STUDY

The aim of this study was to investigate the effect(s) of *in vitro* digestion of an aqueous extract of *H. sobolifera* on previously demonstrated *in vitro* anti-cancer, anti-inflammatory, anti-oxidant and anti-diabetic activities. *H. sobolifera* was used, as Boukes *et al*, 2008 found that it had greater activity for some biological processes, and because this *Hypoxis* species is growing and spreading across the Eastern Cape and Southern Cape of South Africa, while *H. hemerocallidea* is being depleted. This was attempted by using the traditional extraction method to obtain an aqueous *H. sobolifera* extract. This extract was then be subjected to a simulated digestive system, samples were taken at the "stomach" and "intestinal" steps of the simulated digestion. Thereafter the extracts were used as treatments in the following experiments:

1. Cytotoxicity activity against 3 cancer cell lines, MCF7, HT29 and HeLa as well as other cell lines including U937.
2. Antimicrobial activity against ATCC cultures as well as cultures from the NMMU Biomedical Technology culture collection.

3. Anti-diabetic activity: glucose uptake and glycogen storage in L6 and Chang liver cells; inhibition of glycation and enzyme inhibition.
4. Anti-inflammatory activity: phagocytosis in U937 cells.
5. Anti-oxidant activity with focus on relevant active compounds.

2 CHAPTER – METHODS AND MATERIALS

The study was conducted on *Hypoxis sobolifera* var. *sobolifera* (figure 7). The plants were originally from the Botanical Gardens in George and donated by Mrs Yvette van Wijk. They were transplanted into a garden in Nelson Mandela Bay where they grew for approximately three years before they were harvested. They were identified by Ashika Singh from the National Botanical Institute in Durban and a voucher specimen was deposited in the NMMU Herbarium, number PEU14840, in the Botany Department.

Figure 7: Photograph of one of the *Hypoxis sobolifera* var *sobolifera* plants used in this study.

2.1 Plant Extracts

The extract was prepared according to the method used by traditional healers in the Eastern Cape, South Africa. First, the *Hypoxis* corm was peeled and grated, then crushed with a blender. Boiling water was poured over the sheared *Hypoxis* and incubated overnight at room temperature, shaking regularly in order to prepare an extract. The crud was filtered out after 24 hours and the filtrate centrifuged so that the supernatant could be freeze-dried under vacuum and the dry weight could be obtained. Freeze-dried extract was stored in air tight containers in the dark at 4°C.

2.2 Digestion

Method was adopted from Adelakun *et al.* (2009). For the *in vitro* digestion, 5 g of the *Hypoxis* extract was added to 50 ml of simulated gastric juice (0.2 g NaCl dissolved in 66 ml dH_2O to which 13 mg pepsin and 0.3 ml cHCl (11.65 mol/dm^3) was added, pH was adjusted to 1.2-1.8 using HCl and NaOH, and final volume made up to 100 ml). The mixture was gently mixed, flushed with nitrogen and sealed. The tubes were incubated for 1 hour at 37°C in an orbital shaker. The pH of the mixture was adjusted to 6 with 1N $NaHCO_3$. Twenty five ml was then transferred to another 50 ml Falcon tube, flushed with nitrogen and kept on ice (labeled stomach digestion - SD). To the rest of the digestive sample (labeled intestinal digestion - ID), porcine pancreatin stock [12 mg/ml] and bile extract stock [72 mg/ml] were added. The pH was adjusted to 6.9 with 1N NaOH and the final volume increased to 50 ml. The tube was flushed with nitrogen, sealed and incubated at 37°C in an orbital shaker for 2 hours. While the intestinal digestion was incubating, the stomach digestion tube was centrifuged @ 15 000 x g for 35 minutes at a temperature of 4°C. The clear supernatant was transferred in 1 ml aliquots into microcentifuge tubes (Eppendorf®, hereinafter referred to as Eppendorf tubes), flushed with nitrogen and frozen at -80°C. After incubating for 2 hours the intestinal digestion tubes were also centrifuged @ 15 000 x g for 35 minutes at 4°C. Again the clear supernatant was transferred in 1 ml aliquots into Eppendorf tubes, flushed with nitrogen and frozen at -80°C.

The same protocol was followed for 50 g of freshly grated *H. sobolifera* as well as a control containing no *Hypoxis*. This was also repeated with 5 g of *H. sobolifera* extract but with β-glucosidase [100 μg/ml] added to the intestinal digestion process.

The activities of the digested extracts were compared to that of the undigested extract in a number of *in vitro* assays as described in the next few sections. All extracts were reconstituted in DMSO and diluted to the required concentrations in culture medium or incubation buffer as appropriate for the specific assay.

2.3 Routine Cell Culture and Subculturing

2.3.1 Cancer Cell lines

Colon, cervical and breast cancer cell lines represented by HT-29, HeLa and MCF-7 (Highveld Biological, Johannesburg), respectively, were cultured in 10 cm culture dishes in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS) in a humidified 5% CO_2 incubator at 37°C. Cell growth medium was replaced every 2^{nd} – 3^{rd} day and cells grown to a confluency between 70-80% before subculturing using trypsin for detachment.

The suspension culture of lymphoma cells represented by U937 (Highveld Biological, Johannesburg) was cultured in 25 ml culture flasks in RPMI 1640 medium supplemented with 10% FBS in a humidified 5% CO_2 incubator at 37°C. Cell growth medium was replaced every 3^{rd} to 4^{th} day.

2.3.2 Other Cell lines

Liver and muscle cell lines represented by Chang liver (Highveld Biological, Johannesburg) and L6 (Japanese Collection of Research Bioresources Cell Bank), were cultured in 10 cm culture dishes in EMEM and DMEM medium, respectively,

and supplemented with 10% FBS in a humidified 5% CO_2 incubator at 37°C. Cell growth medium was replaced every 2nd – 3rd day and cells grown to a confluency of 70-80% before subculturing using trypsin for detachment.

2.4 Cytotoxicity

2.4.1 MTT Assay

The number of viable cells was determined by assessing the reduction of yellow, water-soluble tetrazolium (3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide) to purple formazan, which is insoluble in aqueous solutions. Metabolically active cells reduce the yellow tetrazolium salt to an insoluble purple formazan. HeLa, HT-29 and MCF-7 cells were seeded in 96-well plates at 6000 cells per well and incubated for 24 hrs. The various *H. sobolifera* extracts were added to cells at various concentrations in 200 µl aliquots and incubated for 48 hrs. After removing the extracts, 100 µl MTT (0.5 mg/ml) was added and the plates were incubated for another three hours. MTT was then removed and DMSO added to dissolve the MTT formazan. The plates were agitated before the absorbance was read at 540 nm on a Labsystem Multiskan MS Plate Reader (Freshney, 2000).

2.4.2 CellTiter-Blue™ Cell Viability Assay

The number of viable cells was determined by the reduction of low intrinsic fluorescent, blue, resazurin to pink resorufin, which is highly fluorescent. Metabolically active cells reduce resazurin to resorufin (www.promega.com).

U937 cells were seeded in 96-well plates at 5000 cells per well and *H. sobolifera* extracts were added to cells at various concentrations in a total volume of 200 µl/well and incubated for 24 hrs. Following incubation, 20 µl of CellTiter-Blue® (Promega) containing the resazurin was added per each 100 µl in the well and the plates were incubated for another three to four hours. One hundred µl was then

removed from each well and placed in a black 96-well plate. The fluorescence was read at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. If the fluorimeter had set filter pairs, the pair that was closest to the prescribed wavelengths was used, for example 544 nm for excitation and 590 nm for emission were used.

2.5 Antimicrobial Screening

Nutrient agar (NA) was prepared to which *H. sobolifera* extract (dissolved in DMSO), ampicillin (control for Gram-positive bacteria), neomycin (control for Gram-negative bacteria), DMSO or nothing was added. A Mast Diagnostics® (UK) multipoint inoculator was used for this assay and therefore ±20 ml of growth medium containing the test samples was poured into each petri plate instead of the usual ±10 ml. The final concentrations in the NA were 1 mg/ml and 0.1 mg/ml for the *H. sobolifera* extract, 1% for DMSO, 100 μg/ml and 50 μg/ml for the antibiotics. Several different strains of bacteria were used for this assay, i.e. *Pseudomonas aeruginosa* (ATCC 37853), *Staphylococcus aureus* (ATCC 43300), *Staphylococcus aureus* 1802, *Enterococcus faecalis* (ATCC 29212), *Enterococcus faecalis* 3314, *Escherichia coli* 3080 and *Escherichia coli* (ATCC 35218). All cultures were obtained from the culture collection of the Department of Biomedical Technology, NMMU North Campus. The strains with ATCC numbers were purchased by them and the other strains (non-ATCC numbers) are clinical strains were isolated and supplied to the Department of Biomedical Technology by the National Health Laboratory Services in Port Elizabeth, and is part of their identification system but irrelevant in this study.

The bacterial cultures were inoculated into nutrient broth tubes and grown overnight at 37 °C to an optical density of between 0.2 – 0.5. One ml of the each of the overnight cultures was transferred to a Mast Diagnostics® (UK) multipoint inoculator which was used to inoculate the agar plates. The agar plates were incubated at 37 °C for 18-24 hours. After incubation, the plates containing the *Hypoxis* extracts were compared to the control plates.

2.6 Detection of hypoxoside using HPLC

All chromatographic systems consist of 2 phases, stationary and mobile phases. In the case of HPLC the stationary phase is a column and the mobile phase is a liquid. The mixture of analytes, i.e. the sample, is applied to the stationary phase. The mobile phase is then passed through the stationary phase in order to separated components of a sample mixture. Chromatographic separation is the result of the analytes continuously passing back and forth between the two phases, thus causing differences in their distribution coefficients resulting in separation. Distribution coefficient (K_d) describes the way in which a compound distributes between the two phases (Wilson & Walker, 2005).

$$\frac{concentration\ in\ phase\ A}{concentration\ in\ phase\ B} = K_d$$ (Wilson & Walker, 2005)

H. sobolifera samples extracted using different methods were resuspended in methanol at a concentration of 10 μg/ml, membrane filtered and compared to a hypoxoside standard and each other using a Beckman System Gold high performance liquid chromatograph, equipped with Solvent Module 128 and Diode Array Detector Module 169, to determine which extract contained the highest concentration of hypoxoside. A hypoxoside standard (100 μg/ml), a kind donation from Dr Carl Albrecht, was prepared in methanol and filtered through a 0.2 μm syringe filter was also analyzed in order to determine the retention time for hypoxoside and to have a standard with which to calculate concentrations. The column used to separate the compounds in the sample was a Nucleosil C18 column (Supelco, 5 μm, 150 x 4.6 mm i.d.). The mobile phase used was dichloromethane and water, CH_3CN:H_2O (20:80, v/v, degassed) in an isocratic mode at a flow rate of 0.5 ml/min (as described by Nair and Kanfer, 2006). Ten microliters of the standard and the extract were injected respectively and the chromatograms recorded.

2.7 Anti-diabetic Assays

2.7.1 Glucose Utilization

Glucose utilization was measured in Chang liver cells and L6 (rat skeletal muscle) cells as described by van de Venter *et al*. (2008). Glucose uptake is a 5 day process. First, the cells, when at a confluence of 90%, were trypsinised, counted and suspended at 30 000 cells/ml, following which 200 μl of the evenly resuspended cell suspension was seeded per well into 96-well plates (i.e. 6000 cells/well). The 96-well plate was incubated at 37°C for 5 days without changing the medium. On the third day, 10 μl of the plant extracts (final concentration 12.5 μg/ml) and positive controls [metformin or insulin (final concentration 1 μM)] were added to each of the relevant wells according to plate layout shown below. Incubation was continued until day five when the glucose uptake experiment was done.

On the day of the glucose uptake experiment (day 5), 20 μl of the spent medium was removed from all sample and control wells and transferred to a new 96-well plate. This was kept on ice to be used for determination of the % glucose uptake in response to chronic exposure to the treatments. The remaining medium was then aspirated from the cells and 50 μl of the incubation medium or control or extract was added per well according to the plate layout, and incubated at 37 °C for three hours. After the incubation period 20 μl was removed from each well and transferred to a clean 96-well plate, for determination of acute glucose utilization response. These aliquots were diluted for use in the glucose oxidase assay by adding 80 μl of deionised water to each well. Fifty μl of each of the diluted samples was transferred to a new 96 well plate and 200 μl of glucinet reagent (Bayer) was added per well. The 96-well plate was then incubated at 37 °C for 15 minutes after which the absorbance was read at 492 nm using a microtiter plate reader.

Plate layout for glucose utilization assay

	1	2*	3	4	5	6	7	8*	9	10	11	12
A	Control	Metformin	Extract 1 (50ug/ml)	Extract 1 (50ug/ml)	Extract 2 (50ug/ml)	Extract 2 (50ug/ml)	Control	Metformin	Extract 3 (50ug/ml)	Extract 3 (50ug/ml)	Extract 4 (50ug/ml)	Extract 4 (50ug/ml)
B												
C												
D												
E												
F												
G												
H												

*For the Chang liver cells, 1 µM metformin was used and for the L6 cells 1 µM insulin was used as the positive control.

2.7.2 Glycogen staining

This staining procedure was obtained and performed as per Cook (1982). In summary, for the sample preparation Chang liver and L6 cells were cultured in 24 well culture plates and allowed to grow for 24 hours in the presence of the various treatments. First, the cells, when at a confluence of 90%, were trypsinised, counted and suspended at 30 000 cells/ml, following which 1 ml of the evenly resuspended cell suspension was seeded per well into 24-well plates (i.e. 30 000 cells/well). The various treatments were added to each well at final concentration 12.5 µg/ml and the cells incubated at 37°C for 24 hours. Treatments used were positive controls, 1 µM metformin (chang liver cells), 1 µM insulin (L6 cells), hot water extract, DMSO and control.

Once confluent, the cells were washed with PBSA and fixed using Carnoy's Fixative, consisting of 60% absolute ethanol, 30% chloroform and 10% glacial acetic acid. The fixative was left on the cells for an hour at room temperature, after which it was removed and the cells were exposed to fresh Best's carmine solution (Sigma-Aldrich) for 5 minutes. After 5 minutes the Best's carmine solution was removed and the cells washed with Best's differentiator, which consists of 18% methanol, 37%

absolute ethanol and 45% dH_2O, and thereafter rinsed with fresh absolute ethanol. The washing and rinsing steps were repeated three times. Cook (1982), predicted that the results will be as follows upon microscopic viewing: Glycogen – will be stained red, some mucin/fibrin – will be stained weak red and nuclei – will be stained blue.

2.7.3 Anti-glycation

Glycation, also known as the Maillard reaction, is the non-enzymatic reaction of protein, such as hemoglobin, with glucose and measurement of glycated hemoglobin levels in patient blood has been used in the management of diabetes (Wauttier *et al*., 1994 and Yonei *et al*., 2010). The Maillard reaction can roughly be divided into early and late, which leads to the formation of advanced glycation end products (AGEs) in the late stage through a series of complex reactions (Wautier *et al*., 1994; Matsuura *et al*., 2002 and Yonei *et al*., 2010).

This procedure was modified from Matsuura *et al.* (2002). Four hundred μl of BSA solution (100 g BSA dissolved in 100 ml of 50 mM phosphate buffer, pH 7.4) was mixed with 90 μl of 1.11 M glucose (20 g glucose dissolved in 100 ml distilled water) in screw cap Eppendorfs, to which 10 μl of the various extracts or the positive control (10 mM aminoguanidine) was added and incubated in a heating block at 60 °C for 30 hours. Blanks were also prepared by using 10 μl of water instead of the extract/positive control, mixed with 400 μl of BSA and 90 μl of 1.11 M glucose. Half of the blanks were incubated along with the rest of the samples at 60 °C, the other half were incubated at 4 °C for 30 hours. Following incubation, the samples were cooled to room temperature after which 100 μl aliquots were transferred to new Eppendorfs. Ten μl cold 100% TCA was added to each tube to stop the reaction. Each tube was vortexed and centrifuged at 15 000 x g for 7 minutes at 4 °C. The supernatant was removed and discarded without disturbing the pellet. The pellet, containing advanced glycation endproducts (AGEs), was dissolved in 400 μl of PBS (pH 10). The amount of AGEs was determined by transferring 200 μl of each

sample to a black 96-well plate and measuring the fluorescence intensity using an excitation wavelength of 370 nm and an emission wavelength of 440 nm.

2.7.4 Enzyme Inhibition Assay

2.7.4.1 Alpha-glucosidase assay:

The assay is based on the conversion reaction of p-nitrophenyl ∝-D-glucoside to ∝-D-glucose and p-nitrophenol by the enzyme ∝-glucosidase, the colour reaction can be measured spectrophotometrically at 400nm.

Before starting the assay the enzyme was prepared and the activity tested, since a crude extract was used instead of the pure enzyme.

Preparation of α-Glucosidase Solution from Rat Intestinal Acetone Powder

One hundred mg intestinal acetone powder was suspended in 3 ml of 0.01 M phosphate buffer and sonicated for 30 seconds, 12 times in an ice bath. The sample was then centrifuged at ± 3000 x g at 4°C for 20 min and the supernatant (crude enzyme solution) removed. The pellet was discarded and the supernatant kept on ice. Since the amount of α-glucosidase released into the solution varies, it was necessary to first quantify the total activity in this crude preparation. Dilutions made were: 0, 2-, 4-, and 8-fold.

α-Glucosidase Assay:

The reaction mixture consisted of 50 μl of 0.1 M phosphate buffer (pH 7.0), 25 μl of 0.5 mM 4-nitrophenyl α-D-glucopyranoside (dissolved in 0.1 M phosphate buffer, pH 7.0), 10 μl of test sample (concentration: 500 μg/ml) and 25 μl of α-glucosidase stock solution. The stock solution of 1 mg/ml in 0.01 M phosphate buffer, pH 7.0 was diluted to 0.04 Units/ml with the same buffer, pH 7.0 just before assay. This reaction mixture was then incubated at 37°C for 30 min. Then, the reaction was terminated by the addition of 100 μl of 0.2 M sodium carbonate solution. The enzymatic hydrolysis of substrate was monitored by the amount of p-nitrophenol released in

the reaction mixture at 410 nm using a microplate reader. All experiments were carried out in triplicate.

2.7.4.2 Alpha-amylase assay:

The original Wohlgemuth principle has been modified several times to be more suitable for the determination of alpha-amylase with colorimetric methods yielding the most satisfactory results. Alpha-amylase activity of an extract is determined through the kinetic measurement of its reaction with a standard limit dextrin substrate made from starch (Perten, 1966).

Our microplate-based starch–iodine assay was carried out as follows. Assay reactions were initiated by adding 40 μl of starch (Sigma S-2630) solution (2.0 g/L) and 40 μl of enzyme in 0.1 M phosphate buffer at pH 7.0 to microplate wells. To minimize evaporative loss during incubation, a plastic mat was used to cover the microplate in combination with using a temperature block equipped with a hot lid. After 30 min of incubation at 50 °C, where the assayed enzymes were most active, 20 μl of 1M HCl was added to stop the enzymatic reaction, followed by the addition of 100 μl of iodine reagent (5 mM I_2 and 5 mM KI). Following colour development, 150 μl of the iodine-treated sample was transferred to a transparent flat-bottomed 96-well microplate and the absorbance at 580 nm (A_{580}) was measured using a microplate reader (Bio-TEK Power Wave XS spectrophotometer, Winooski, VT, USA).

2.8 Anti-inflammatory activity assays

2.8.1 Phagocytosis

A Vybrant, Phagocytosis Assay Kit was designed by InVitrogen to provide a model system for quantitating the effects of drugs or other environmental factors on phagocytic function. This is achieved by observing and quantitating the process of

phagocytosis in human polynuclear cells and mouse macrophages by following the internalization of a foreign particle - such as fluorescently labeled immune complexes and bacterial particles. Detecting the intracellular fluorescence emitted by the engulfed particles is the basis of this technique The InVitrogen Vybrant Phagocytosis Assay Kit contains fluorescein-labeled *Escherichia coli* (K-12 strain) BioParticles®.

The Vybrant® Phagocytosis Assay kit was modified for use on a flow cytometer to observe phagocytosis in cells. U937 cells were differentiated with 100 nM vitamin D3 ($1,25(OH)_2D_3$) for 48 hrs. Differentiated U937 cells were centrifuged at 250 x g for 5 min at room temperature. The supernatant was discarded and the cells washed with complete medium. The cells were then evenly resuspended in fresh complete medium at $1x10^6$ cells/ml. Two hundred µl of cells was aliquotted into Beckman Coulter flow cytometry tubes. Extracts were made up to 125 µg/ml in fresh RPMI160 medium with 10% FBS and 200 µl was added to the tubes; 0.25% DMSO was used as a negative control, while PMA was the positive control. The tubes were then incubated for three hours at 37 ºC. Fluorescein-labelled *E. coli* K-12 bioparticles were prepared by thawing a vial of fluorescent particles and a vial of concentrated Hanks buffered saline solution (HBSS). The HBSS was pipetted into the fluorescent vial and sonicated briefly. After the differentiated cells had been incubated with the extract for three hours, 50 µl of the fluorescent bioparticle suspension was added to each tube, gently mixed and incubated in the dark at 37 ºC for three hours. Tubes were centrifuged at 1000 x g for 5 minutes using an Eppendorf® 5804 centrifuge before aspirating the supernatant. The cells were then washed twice with PBS. Samples were resuspended in 500 µl of PBS and analysed on a Beckman Coulter® FC500 flow cytometer using the 488 nm laser for excitation and 525 nm for emission and recording green fluorescence on FL1.

2.9 Anti-oxidant Activity

2.9.1 DPPH Assay

The scavenging reaction between DPPH and an anti-oxidant (H-A) can be illustrated as:

$DPPH_{(purple)}$ (2,2-diphenyl-1-picrylhydrazyl) + H-A = $DPPH\text{-}H_{(yellow)}$ + A

Anti-oxidants react with DPPH, which is a stable free radical and is reduced to the DPPH-H and as consequence the absorbance decreases from DPPH radical to the DPPH-H form. The degree of discoloration indicates the scavenging potential of the anti-oxidant compounds or extracts in terms of hydrogen donating ability.

To a 50 μl sample, 450 μl of 50mM Tris buffer was added and 1,5 ml DPPH (0.1 mM DPPH solution made fresh on the day of the assay by dissolving 0.002 g DPPH in 50 ml ethanol) solution. This was mixed and incubated in the dark for 30 min. As a blank, 50 μl Tris buffer was used instead of the test sample. Fifty μl catechin (0.029 g catechin in 10 ml distilled water – [10 mM] prepared fresh on the day of assay) or ascorbic acid (1.7612 g ascorbic acid in 10 ml distilled water – [0.1-1 mMl] prepared fresh on the day of assay) were used as positive controls. In this experiment the positive control, ascorbic acid was used and the following dilutions were prepared:

[Positive control] mM	Volume Stock (μl)	Volume Tris Buffer (μl)
1	100	900
0.8	80	920
0.6	60	940
0.4	40	960
0.2	20	980

After 30 min at room temperature the absorbance was read at 518 nm on a Labsystem Multiskan MS Plate Reader and the percentage inhibition calculated according to the following equation:

$$\% \text{ inhibition} = [(Abs_{blank} - Abs_{sample})/ Abs_{blank}] \text{ X } 100$$

2.9.2 Reactive Oxygen Species

To detect/measure the level of intracellular ROS, the stain 2',7'-dichlorofluorescine diacetate (DCF-DA) was used as it is membrane permeable, which allows the intracellular ROS to be measured. DCF-DA can be oxidized by ROS to form fluorescent 2',7'-dichlorofluoresein (DCF) (Ibrahim *et al.*, 2012).

U937 cells were differentiated with 100 nM vitamin D3 ($1,25(OH)_2D_3$) for 48 hrs. The differentiated U937 cells were centrifuged and resuspended in PBS at $1x10^6$ cells/ml and stained with 2.5 µM DCF-DA for 30 minutes at 37 ºC in the dark. Cells were centrifuged at 1000 x g and washed three times with PBS to remove any remaining stain and the cells resuspended in RPMI1640 medium with 10% FCS. Flow cytometry tubes were labelled and aliquots of 200 µl of test sample or medium control were added to each tube, before adding an equal volume of the cell suspension and mixing gently. This was incubated at 37 ºC in the dark for 90 minutes. Extracts were tested at a final concentration of 125 µg/ml and 100 ng/ml PMA was used as a positive control. Extract combined with PMA was also tested. After the incubation period, EDTA was added to a final concentration of 50 mM to stop the reaction, before reading sample fluorescence on a Beckman Coulter® FC500 flow cytometer. The FL1 fluorescence was measured at excitation of 488 nm and emission of 525 nm and data collected in triplicates and the averages plotted linearly as a percentage comparison with the fluorescence intensity of the positive control cells (PMA).

2.9.3 TLC DPPH

To confirm that rooperol was indeed the anti-oxidant active compound a DPPH assay with TLC was performed. TLC is based on the principle of separation, similar to other chromatographic methods. The separation depends on the presence of a stationary and mobile phase and the relative affinity of compounds towards these

two phases. The stationary phase can be found as a thin coating on a glass or metal foil plate (Wilson and Walker, 2005).

The method was adapted from Bektas (2005). First, the aqueous extracts were dissolved in DMSO and methanol to a final concentration of 50µg/5µl. Spots ranging from 5 - 15 µl were made on the TLC plate. The TLC plate was developed using a solution of methanol and ethyl acetate in a 1:1 ratio. After developing, 0.2% DPPH (0.04 g in 20 ml made up in MeOH) was poured over the plate, and left to stain for 30 minutes at room temperature. If the purple colour of the DPPH turns to yellow the spot is positive for anti-oxidant activity. After colour development the plate was photographed. Rooperol was used as a standard (650 µg rooperol dissolved in 52 µl DMSO, followed by the addition of 13 µl MeOH). DMSO was also run as a standard.

2.9.4 Total content of compounds in extract

2.9.4.1 Total phenolic content assay:

Folin-Ciocalteu is a reagent used in the detection of phenolic groups, by oxidizing the phenolic compounds. In the first step the reagent detects compounds of a phenolic nature. The incorporation of cupric ions improves the sensitivity. This results in a copper complex. In the second step Fiolin-Ciocalteu (yellow), formed from a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$) is reduced by the copper complex (from step one) to tungsten (W_8O_{23}) (blue) and molybdenum (Mo_8O_{23}) (blue). The reduced Folin is blue and can be detected at an absorbance range of 500 – 750 nm with a spectrophotometer and is proportional to the total quantity of phenolic compounds originally present (Plummer, 1988).

This method was modified from Slinkard and Singleton (1979) and Rashid *et al.* (2010) and used to perform the assays in 96-well plates. One hundred µl Folin-Ciocalteu reagent was mixed with 20 µl of extract at various concentrations. Then

immediately 80 µl of 7.5% Na_2CO_3 was added and incubated at 30 ºC for 90 min. After incubation the plate was read at 765 nm on a Labsystem Multiskan MS Plate Reader. This was repeated on second plate with gallic acid at different concentrations in order to set up a standard curve. The results from the extracts were then read off from the gallic acid standard curve and expressed as gallic acid equivalents (mg gallic acid/g dried extract).

2.9.4.2 Total flavonoid content assay:

The aluminium chloride colorimetric method is based on the principle that aluminium chloride forms acid stable complexes with the C-4 keto group as well as either the C-3 or C-5 hydroxyl group of flavones and flavonols. In flavonoids it reacts with the orthodohydroxyl groups in the A- or B-ring to form acid labile complexes (Kiranmai *et al.*, 2011).

This method was modified from Zhishen *et al.* (1999) and Rashid *et al.* (2010) and used to perform the assay in 96-well plates. To 80 µl of dH_2O, 20 µl of 500 µg/ml extract was added, along with 6 µl of 15% $NaNO_2$. After 6 min, 6 µl of 10% $AlCl_3$ was added and the plate left for another 6 min. Thereafter 80 µl of 4% NaOH was added along with 8 µl dH_2O to make the final volume up to 200 µl. After mixing, the plate was left to stand for 15 min and then read at 510 nm on a Labsystem Multiskan MS Plate Reader. This was repeated with rutin at different concentrations in order to set up a standard curve. The results from the extracts were then read off from the rutin standard curve and expressed as rutin trihydrate equivalents (mg rutin trihydrate/g dried extract).

2.9.4.3 Total carbohydrate assay:

Carbohydrates are dehydrated by cH_2SO_4 (18 M) to form furfural and its derivatives. The furfural reacts with anthrone (10-keto-9, 10-dihydroanthracene) to give a blue-green complex (Plummer, 1978).

To determine the total amount of carbohydrates in the extracts an anthrone assay was used, as per Plummer (1971). A rapid and convenient method for the determination of hexoses, aldopentose and hexuronic acids in polysaccharides, either free or present, is the basis of the anthrone reaction. In the method, anthrone reagent reacts with carbohydrates and forms a coloured complex. One hundred and sixty µl anthrone (made from 2 g/l anthrone mixed with concentrated H_2SO_4) was mixed with 40 µl of 500 µg/ml extract. The samples were mixed well and then boiled for 10 min (covered). After boiling, the samples were cooled to about room temperature in a waterbath to avoid condensation on cuvette and then read at 540 nm on a Labsystem Multiskan MS Plate Reader. A glucose standard was set up and used as positive control.

3 CHAPTER – RESULTS AND DISCUSSION

3.1 Plant Extracts

In the Eastern Cape province of South Africa, poverty and poor living conditions are a reality that cannot be overlooked. In this community, the cultural traditions from decades of survival are still a large part of everyday living. As a result of years of distrust in Western societies and trust in the knowledge of their ancestors, traditional medicine is still used to treat diseases and ailments (Erasto *et al.,* 2005).

In the Eastern Cape, one of the most common plants used is *Hypoxis* (Erasto *et al..,* 2005). The corm is used for treatment of diseases such as cold, flu, diabetes, cancer and many others as discussed in section 1.1.2. The patient is given the fresh corm which has to be peeled and grated. Boiling water is then poured over the grated corm pieces and allowed to brew like a tea; or it can be boiled for 10 minutes. The resulting tea is then drunk and the process repeated several times a day as treatment.

Hypoxis extracts have been studied over several years to determine the credibility of the medicinal claims of this popular plant. The plant extracts obtained by using methanol, chloroform and ethanol extracts have proved that *Hypoxis* does have medicinal properties, see section 1.2 for published reports. In traditional medicine, however, aqueous infusions are ingested and the published results have not attempted to validate the use of these infusions before and/or after passage through the digestive system.

Based on the above argument, this study aimed to calculate the percentage yield of the aqueous extract as well as investigate the effect of digestion on the biological activity of the aqueous extract.

In order to determine the percentage yield, the wet weight of the freshly peeled and grated *H. sobolifera* was recorded after which it was homogenized and boiling water

was added (five times the wet weight). The crud was filtered out after 24 hours and the filtrate centrifuged so that the supernatant could be freeze-dried and the dry weight could be obtained. The yield is the dry weight of the extract expressed as a percentage of the wet weight of the starting material (table 3).

Table 3: Percentage yield of *Hypoxis* aqueous extracts from *H. sobolifera* using the traditional medicine method over 24 hrs.

	Initial wet weight (g)	Final dry weight (g)	% yield (w/w)
H. sobolifera	284	23.14	8.15

The yield could not be calculated after digestion due to the gastric and intestinal juices contributing to the total solutes. Based on the initial input of 100 mg/ml of *Hypoxis sobolifera* extract into the digestive system, the extract concentrations of each of the digested samples was taken as 100 mg/ml. This was used to compare the activities of digested extracts to the same concentrations of pure (undigested) extract.

3.2 Cytotoxicity on cancer cell lines

There have been reports of plant phytosterols providing protection against cancer. Positive results of growth inhibition by phytosterols on HT29 colon cancer cells were published by Awad & Fink (2000) and MCF-7 breast cancer cells by Awad *et al*. (2007) using β-sitosterol. Boukes *et al*. (2008) showed that *Hypoxis sp*. also contained the plant sterol β-sitosterol. As a result, *Hypoxis* spp. were tested as possible agent against cancer. In 2011 Boukes *et al.* confirmed that *Hypoxis sobolifera* has *in vitro* anti-cancer properties at concentrations of 125 – 500 µg/ml. However, the extracts used by Boukes *et al.* (2011) were chloroform extracts. It was still unclear if traditionally prepared extracts would be active and what effect digestion would have on the cytotoxicity of *Hypoxis sobolifera*.

In this study the *H. sobolifera* aqueous extract, prepared using the traditional method, was tested before and after digestion for cytotoxic effects on the same cell

lines previously used by Boukes (2011). These cell lines included the following: HT29, MCF-7 and HeLa cells representing colon, breast, and cervical cancer cells respectively. U937, human leukemic monocyte lymphoma cells were also used. Using U937 cells, Boukes (2010) showed anti-inflammatory effect, as well as cytotoxicity.

Cisplatin (cis-diamminedichloroplatinum (II) or cis-DDP) was used as positive control since it is one of the most effective chemotherapeutic agents, and is used to treat several types of human cancers (Wozniak *et al.,* 2004). Cisplatin's anti-cancer activity is believed to result from its interaction with DNA, and these cisplatin-DNA adducts inhibit processes like transcription, replication, translation and DNA repair (Wozniak *et al.,* 2004). Although clinically successful, the use of cisplatin to treat cancer can cause severe side effects and resistance, intrinsic and acquired, leading to limits in its application. The most important dose-limiting factor is the hepatotoxicity and nephrotoxicity caused in high doses, however impairment of kidney function being the main side effect (Wozniak *et al.,* 2004). Wozniak *et al.* (2004) stated that administration of anti-oxidants may reduce the side effects of cisplatin.

Initial investigations of all *H. sobolifera* extracts at 125 and 250 µg/ml showed no significant inhibition and in some cases even growth stimulation (results not shown). Only when concentrations were increased to 1.25, 2.5 and 5 mg/ml was significant inhibition observed with >50% inhibition only observed with an extract concentration of 5 mg/ml (figure 8).

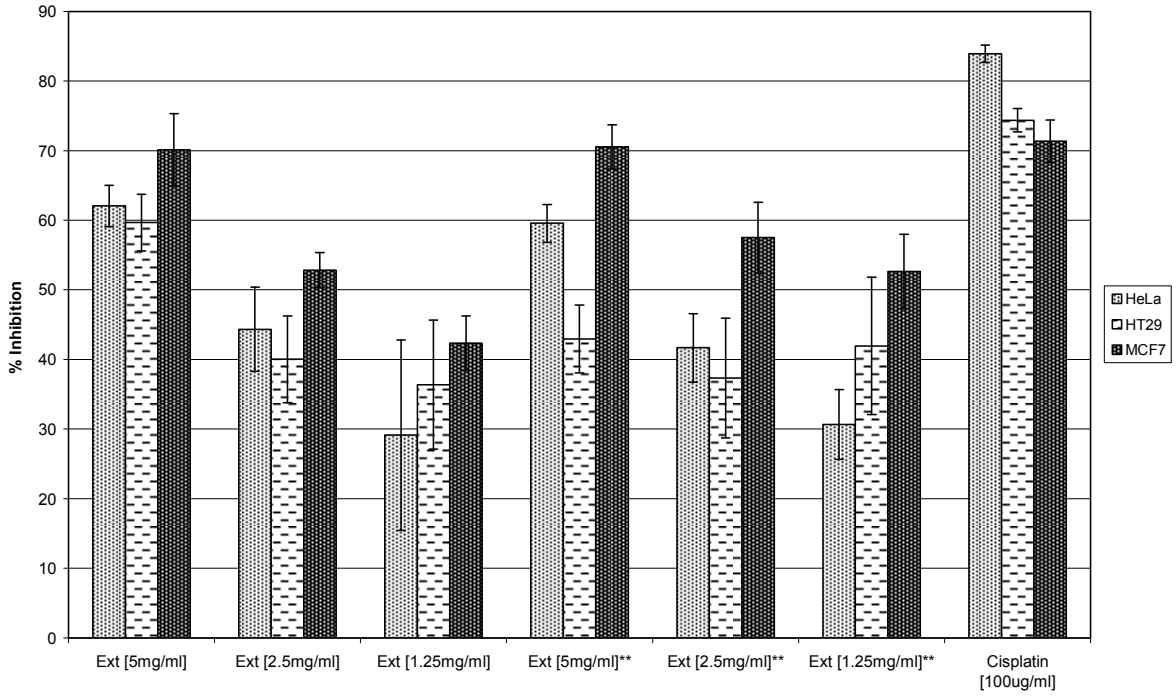

Figure 8: Percentage inhibition activity of undigested *H. sobolifera* hot water extracts at various concentrations against 3 cancer cell lines using the MTT assay. (Ext [x mg/ml]**): undigested hot water extract at the same concentrations but with 100 µg/ml of β-glucosidase enzyme added to convert any hypoxoside present in the extract to rooperol. Results are presented as mean ± SD of quadruplicate wells from a single experiment. The experiment was repeated twice, yielding similar results. All the data points shown were significantly higher (% inhibition) than the vehicle control at a level of $p<0.05$ or lower as determined by one way ANOVA test.

The IC_{50} value for the undigested hot water *H. sobolifera* was determined to be approximately 3.098 mg/ml. Unexpectedly, addition of β-glucosidase to convert any non-toxic hypoxoside to toxic rooperol did not decrease the IC_{50}. These high concentrations will be impossible to obtain *in vivo* under normal ingestion conditions and were therefore considered unrealistic to work with in further experiments. Concentrations of 500 µg/ml and 250 µg/ml were investigated again knowing that the cytotoxicity effects observed, if any, would be very low and calculation of IC_{50} values impossible. After correction of digestive extracts with digestive control values (digestive juices containing no extract), growth stimulation was observed (figure 9). Only results for 500 µg/ml are shown as no significant effects were seen at 250

µg/ml. No significant cytotoxicity was observed for DMSO vehicle control and β-glucosidase control (results not shown).

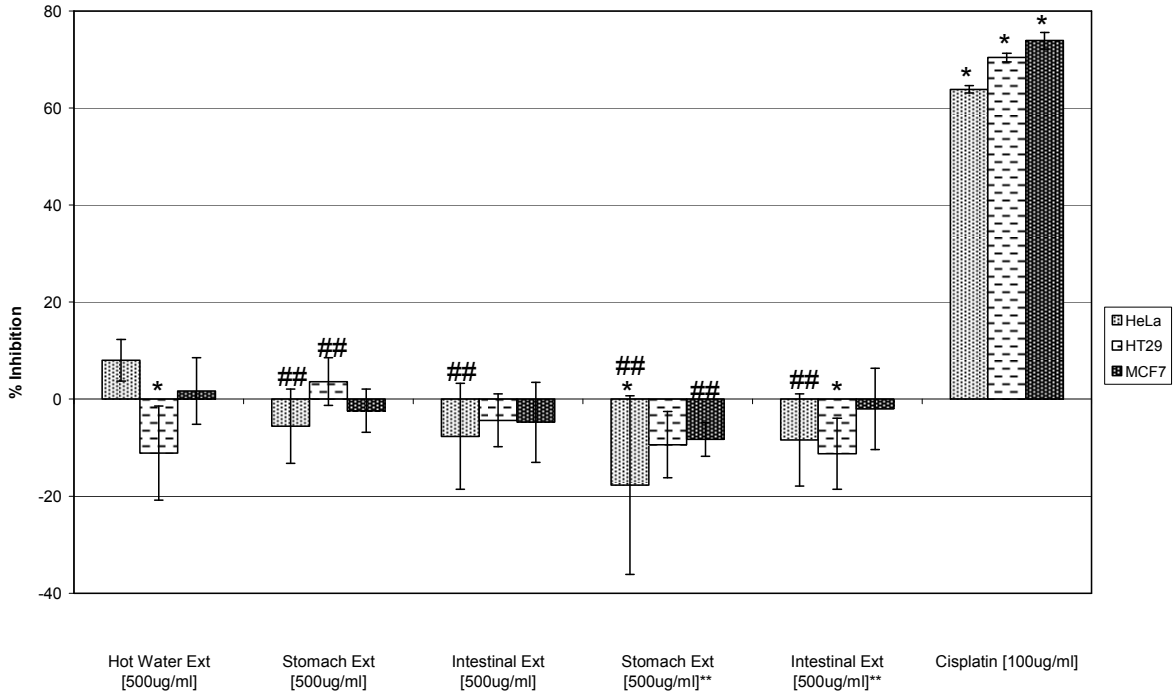

Figure 9: Percentage inhibition activity of undigested and digested *H. sobolifera* hot water extracts at lower concentrations against 3 cancer cell lines using the MTT assay. Stomach/Intestinal Ext [500 µg/ml]** represent digested hot water extract with 100 ug/ml of β-glucosidase enzyme added to convert any hypoxoside present in the extract to rooperol. Results are presented as mean ± SD of quadruplicate wells from a single experiment. The experiment was repeated twice, yielding similar results. Significant results represented as *$p<0.05$ compared to vehicle control, $^{\#\#}p<0.05$ compared to undigested hot water extract, all determined by one way ANOVA test.

From the results obtained for the cytotoxicity tests performed on the three cancer cell lines with the various extracts, it can be seen that when prepared in the traditional way, *H. sobolifera* extracts have no significant cytotoxicity at concentrations in the µg/ml region. It is also clear from figure 9 that after stomach and intestinal digestion respectively the activity that was observed from the hot water extract (before digestion) is reduced significantly ($^{\#\#}p<0.05$) and in some instances even growth stimulation of cancer cells could result, as seen with HT-29 cancer cells. This could be as a result of some of the digestive compounds used in

the simulated digestive experiment remaining in the extract after freeze drying, such as the bile used in the intestinal digestion step. De Jong *et al*. (2003) stated that bile acids may promote colon cancer cell growth. Sitosterol, on the other hand, has been shown to inhibit HT-29 colon cancer cell growth (Awad and Fink, 2000). *H. sobolifera* contains sitosterol in chloroform extracts (Boukes *et al.,* 2008) but the sitosterol content of the traditionally prepared extract used in the present study was not determined, therefore, more investigation into this is required.

No significant cytotoxic activity was observed from *H. sobolifera* extracts on U937 cells at concentrations ranging from 50 µg/ml to 250 µg/ml (results not shown).

As mentioned, phytosterols, especially β-sitosterol has been suggested to have anti-cancer properties (De Jong *et al.,* 2003) and Boukes *et al*. (2008) identified β-sitosterol as the major phytosterol present in three species of *Hypoxis*, namely, *H. hemerocallidea*, *H. stellipilis* and *H. sobolifera*, when using chloroform extracts. Boukes *et al*. (2011) also found that *Hypoxis* spp. were being sold indiscriminately in herbal shops under the common name African potato. Several researchers over the years investigated and reported on the anti-cancer activity of *Hypoxis* compounds such as hypoxoside, rooperol and their metabolites (Albrecht *et al.,* 1995; Smit *et al.,* 1995; Steenkamp and Gouws, 2006; Drewes *et al.,* 2008 and Kruger *et al.,* 1994). Therefore Boukes *et al*. (2011) investigated and compared the cytotoxicity of the three *Hypoxis* spp. previously shown to contain β-sitosterol by Boukes *et al*. (2008). Cell lines used by Boukes *et al*. (2011) were HT-29, HeLa and MCF-7, treated with chloroform extracts of the various *Hypoxis* spp. The authors illustrated cytotoxicity against MCF-7 cancer cells with extracts from all three *Hypoxis* spp. *H. hemerocallidea* and *H. sobolifera* also had cytotoxicity against HT-29 and HeLa cancer cells but at high concentrations, while *H. stellipilis* promoted the growth of HT-29 and HeLa cancer cells (Boukes *et al.,* 2011). In this study growth stimulation was observed in all three cell lines, HT-29, HeLa and MCF-7 when treated with digested extracts, while undigested only stimulated growth in HT-29, contradictory to what Boukes *et al*. (2008) found using *H. sobolifera*. The undigested hot water *H. sobolifera* extracts exerted a very small percentage of inhibition in HeLa and MCF-7 cells.

The different results obtained with the hot water aqueous extract in this study and the chloroform extract in published reports and the observation that β-glucosidase did not increase the cytotoxicity of the hot water extract, prompted an investigation into the hypoxoside content of the hot water *H. sobolifera* extract. Hypoxoside content is required in order to confirm whether or not hypoxoside was the active compound responsible for the cytotoxicity activity observed at very high concentrations (figure 8) since the addition of β-glucosidase at these high concentrations did not increase the cytotoxicity of the extracts. If not it would confirm that other compounds are responsible for the activity. The hypoxoside content was determined using HPLC (section 3.4). Boukes *et al.* (2008) showed that chloroform extracts of *H. sobolifera* contained no hypoxoside and therefore the cytotoxicity of *H. sobolifera* may be due to its β-sitosterol content.

3.3 Antimicrobial Screening

Due to the ever increasing bacterial infections in rural areas the need for traditional (non-Western) remedies for treatment is also increasing (Katerere and Eloff, 2008). *Hypoxis* is one example of the plants being used in these remedies. Published reports of antimicrobial activities show that even with pure chloroform and ethanol extracts the amount needed for positive antimicrobial activity is very high. It is unlikely that such high concentrations of hypoxoside or any other active compound(s) will be obtained from the traditional tea and as mentioned previously, the effect of the digestive system on the activity of the extract is still unknown. It was therefore crucial that the actual antimicrobial activity after digestion had to be determined. Several different bacterial cultures were used in order to determine if *H. sobolifera* is effective against clinical and/or cultured strains. According to the literature, for *Hypoxis* to be effective it has to be used in large quantities but that is not practical in traditional medicines. Therefore more feasible concentrations were tested and compared to various controls.

DMSO was used to prepare a stock solution of the undigested extract for two reasons. Firstly, the extract did not dissolve completely in water, and secondly, the concentrated DMSO sterilized the extract before it was further diluted with nutrient

agar. As seen in figure 10 (1), the final concentration of DMSO in which the extracts were dissolved (1%) did not inhibit the growth of any of the cultures. The cultures used were from both the Gram-positive and Gram-negative groups and therefore positive antibiotic controls for both groups were used, namely Ampicillin and Neomycin respectively. The bacterial cultures were grouped as seen in table 4.

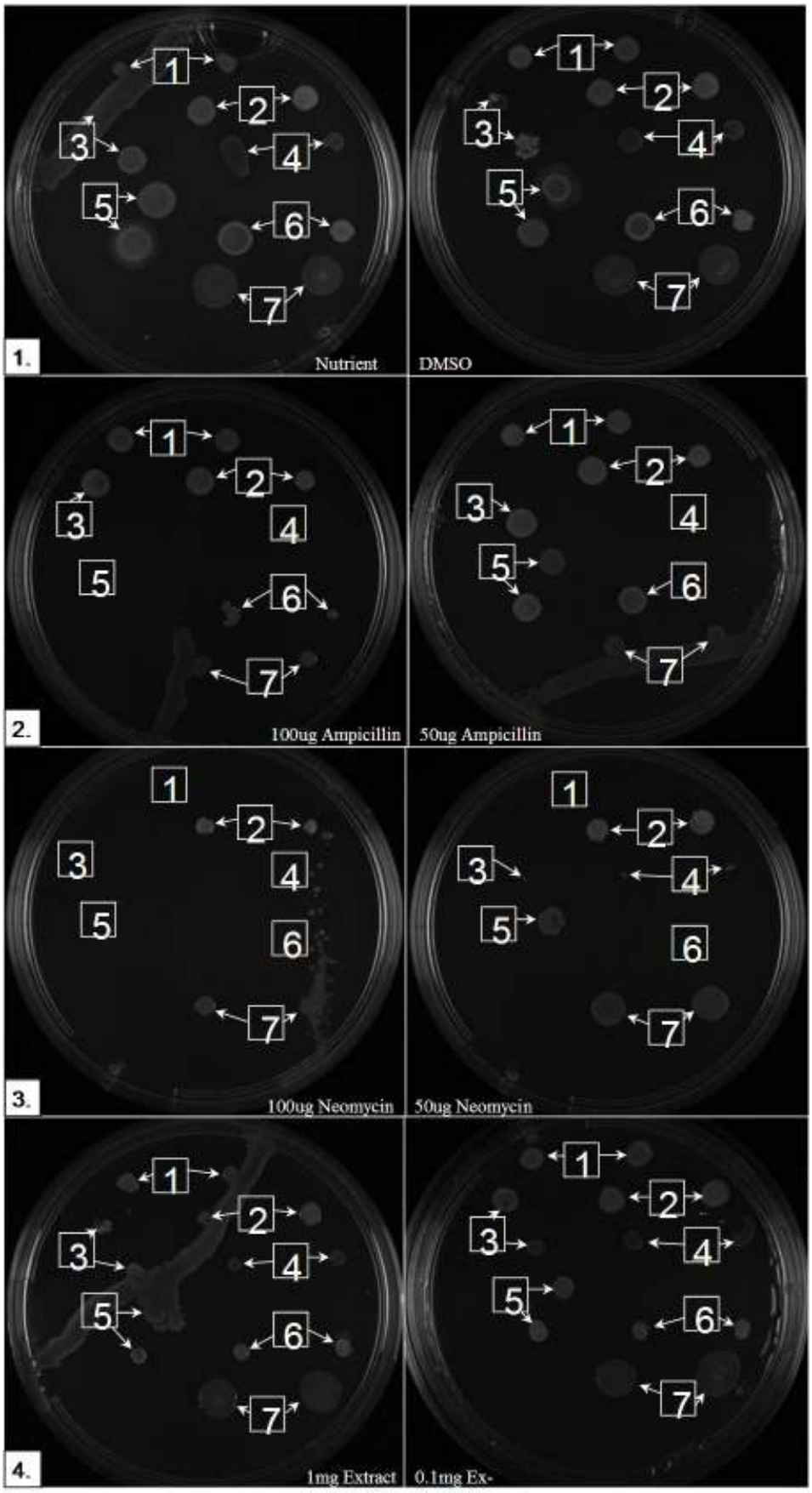

Figure 10: Photograph of nutrient agar plates inoculated with different bacterial strains using a Mast Diagnostics® (UK) multipoint inoculator. Photographs represent the following

plates containing nutrient agar mixed with: 1.) (left) Negative control, (right) 1% DMSO vehicle control; 2.) 100 µg/ml and 50 µg/ml of Ampicillin; 3.) 100 µg/ml and 50 µg/ml of Neomycin; 4.) 1 mg/ml and 0.1 mg/ml of hot water extracts of *H. sobolifera*. All plates were inoculated with different strains of bacteria. After inoculation plates were incubated at 37 ºC for 18-24 hours before the photographs were taken. The cultures used were numbered as follows 1→ *Escherichia coli* (ATCC 35218), 2→ *Escherichia coli* 3080, 3→ *Enterococcus faecalis* (ATCC 29212), 4→ *Enterococcus faecalis* 3314, 5→ *Staphylococcus aureus* (ATCC 43300), 6→ *Staphylococcus aureus* 1802, and 7→ *Pseudomonas aeruginosa* (ATCC 37853).

Table 4: Gram-positive or Gram-negative grouping of bacterial cultures used

Strain name	Gram-positive	Gram-negative
Escherichia coli (ATCC 35218)		√
Escherichia coli 3080		√
Enterococcus faecalis (ATCC 29212)	√	
Enterococcus faecalis 3314	√	
Staphylococcus aureus (ATCC 43300)	√	
Staphylococcus aureus 1802	√	
Pseudomonas aeruginosa (ATCC 37853)		√

From the results shown in figure 10 (2) it is clear that the Ampicillin at the above shown concentrations was not very effective against the Gram-positive strains but it at least decreased their amount of growth compared to the negative (DMSO) control, and this can therefore be used as a guideline to determine if the *H. sobolifera* extract showed any activity.

From the results shown in figure 10 (3) it is clear that the Neomycin at the above mentioned concentrations was very effective at complete antimicrobial activity against Gram-negative strain *Escherichia coli* (ATCC 35218) and in *Escherichia coli* 3080 and *Pseudomonas aeruginosa* (ATCC 37853) it had sufficiently decreased their amount of growth compared to the negative (DMSO) control, and can therefore be used as a guideline to determine if the *H. sobolifera* extract showed any activity.

From the results shown in figure 10 (4) it is clear that, while *H. sobolifera* at the specified concentrations was not very effective as antimicrobial agent against any of the bacterial strains, it did decrease the amount of growth in sample number 4 → *Enterococcus faecalis* 3314, when compared to the negative (DMSO) control. However, even the Neomycin was an effective antimicrobial agent against number 4 → *Enterococcus faecalis* 3314. It therefore seems that *H. sobolifera* is not a viable antimicrobial agent as it has no significant activity at conceivable concentrations. Katerere and Eloff (2008) have shown that MIC values for acetone and ethanol extracts of *Hypoxis hemerocallidea* were mostly in the mg/ml range against the same cultures used in this study. This concentration range (mg/ml) is not viable for traditional extraction and therefore the highest concentration tested in the present study was 1 mg/ml. It can be concluded that *H. sobolifera*, when prepared according to the traditional method, does not hold any significant antimicrobial activity and no further antimicrobial tests were done on the digested extracts.

3.4 Detection of Hypoxoside using HPLC

The activities reported in literature for *Hypoxis* extracts have mostly been linked to the presence of hypoxoside and/or phytosterols. Several glycosides and phytosterols have been identified in various *Hypoxis* spp. in recent years, e.g. hypoxoside, β-sitosterol and other sterols/sterolins (Nicoletti *et al*., 1992; Steenkamp and Gous, 2006; Awad and Fink, 2000; Lagarda *et al*., 2006; Pegal, 1976&1980). Boukes *et al*. (2008), investigated the quantities of these compounds (hypoxoside, β-sitosterol and other sterols/sterolins) in three species of *Hypoxis*. This came about after they had found that in South Africa various species of *Hypoxis* are indiscriminately sold and used, even though no evidence existed that all species contain equal quantities of these compounds. Hypoxoside presence has been confirmed by Nicoletti *et al*. (1992) in several South African *Hypoxis* spp. while Nair and Kanfer (2006) identified it in *H. hemerocallidea* using HPLC. Boukes *et al*. (2008), however, found no previous evidence of the presence of hypoxoside in either *H. stellipilis* or *H. sobolifera*. Boukes *et al*. (2008) therefore used *H. hemerocallidea, H. stellipilis* and *H. sobolifera* in their study to identify and quantify

the various hypoxoside and sterol/sterolin contents using HPLC as described by Nair and Kanfer, 2006.

Table 5: Presence of β-sitosterol and hypoxoside per 5mg of chloroform *Hypoxis* extract

	β-sitosterol	**Hypoxoside**
H. hemerocallidea	√	√
H. stellipilis	√	√
H. sobolifera	√ (highest %)	Undetected

Adapted from Boukes *et al.* (2008)

Boukes *et al*. (2008), detected no hypoxoside in their chloroform extract of *H. sobolifera* (see table 5) and suggested that a more polar solvent may be a better solvent for hypoxoside extraction. They detected hypoxoside when using ethanol, methanol and acetone as extraction solvents, however, when they used water hypoxoside was also not detected. Boukes *et al*. (2008), concluded that *H. sobolifera* does contain hypoxoside but in much smaller amounts than in *H. hemerocallidea* and *H. stellipilis*, and thus needs to be extracted using chemicals such as ethanol, methanol or acetone. This is significant since the traditional healers or herbalists in South Africa only use water and boiling to make *Hypoxis* extracts for medicinal treatmens.

The lack of cytotoxic and antimicrobial activity of the *H. sobolifera* extract in the present study, which contradicted some of the previously published literature showing cytotoxicity (Albrecht *et al.,* 1995; Smit *et al.,* 1995; Kruger *et al.,* 1994; Steenkamp and Gouws 2006) and antimicrobial (Steenkamp *et al.*, 2006a; Katerere and Eloff, 2008) activity, prompted an experiment to detect and quantify the glycoside hypoxoside in this extract. The hypoxoside content of the hot water extract used in this study was compared to that of a cold water extract and an ethanol extract of the same plant, *H. Sobolifera,* that were obtained from Dr Trevor Koekemoer (NMMU).

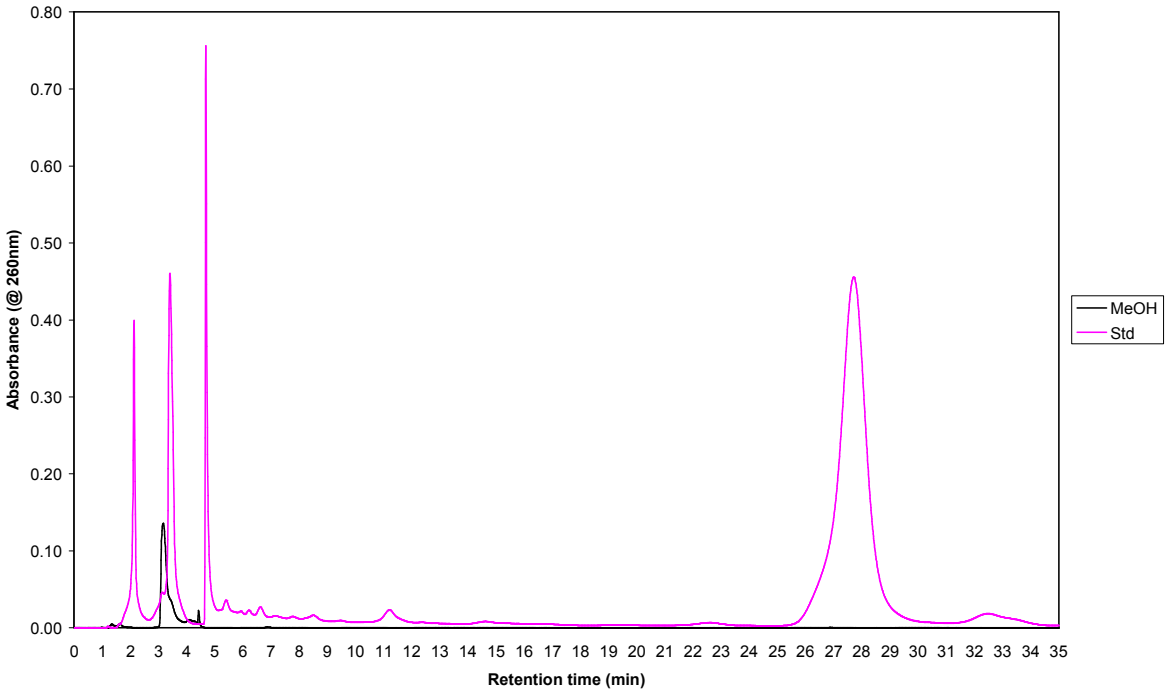

Figure 11: HPLC chromatogram of methanol (black) used as the vehicle control for the hypoxoside standard and test extracts during this experiment, as well as hypoxoside standard (purple) at concentration of 100 µg/ml. The Nucleosil C18 column, a mobile phase of CH_3CN:H_2O (20:80 v/v) and a flow rate of 0.5 ml/min were used to obtain hypoxoside separation. The hypoxoside standard eluted at a retention time (R_t) of 27.63 min.

Hypoxoside standard was detected after 27.63 min (figure 11). Of the three extracts of *H. sobolifera* tested none had any peak that eluted at R_t 27.63 min (figures 12). In fact no compounds were detected close to that time except for a compound detected at 28.9 min in the cold water extract (figure 12). This compound may be one of the two analogues of hypoxoside as identified by Kruger *et al*. (1994), namely dehydroxyhypoxoside (one -OH group) or bis-dehydroxyhypoxoside (no -OH groups). Kruger *et al*. (1994), determined that a decrease in OH groups is associated with an increase in retention time and as a result both analogues eluted after hypoxoside. However, further investigation is needed to confirm this.

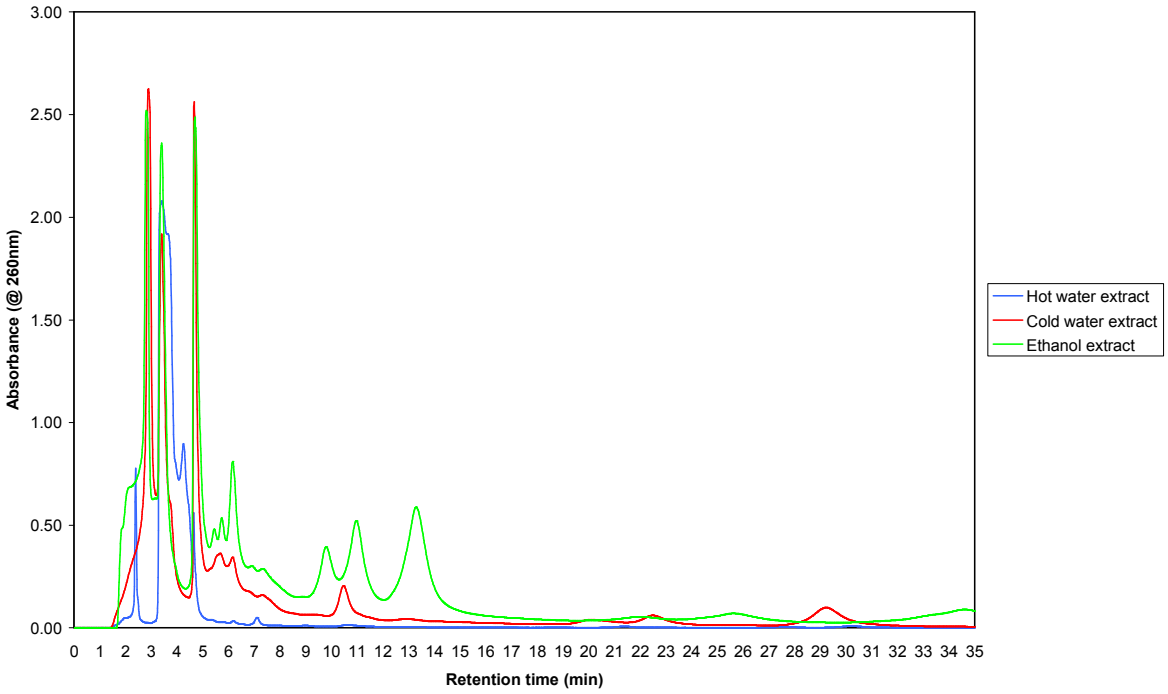

Figure 12: HPLC chromatogram of hot water (blue), cold water (red) and ethanol (green) *H. sobolifera* extracts at concentration 10 µg/ml. The Nucleosil C18 column, a mobile phase of $CH_3CN:H_2O$ (20:80 v/v) and a flow rate of 0.5 ml/min were used to obtain hypoxoside separation. The hypoxoside standard eluted at a retention time (R_t) of 27.63 min, with nothing being detected at 27.63 min in the extract.

From these results, it can be concluded that no detectable amount of hypoxoside was present in any of the three *H. sobolifera* extracts (hot- and cold- water or ethanol) tested. This method has previously been shown to be sensitive enough to detect hypoxoside in the low µg/ml range (Boukes *et al.* 2008). Any hypoxoside present but not detected in our extracts would be unlikely to contribute to any biological activities of the extracts. Due to the possible presence of an analogue of hypoxoside in the cold water extract, it was decided to include this extract in further assays as a comparison to the hot water extract. The cold water extract was, however, not subjected to *in vitro* digestion.

3.5 Antidiabetic Screening

3.5.1 Glucose Utilization & Glycogen staining

Diabetes is a growing concern in all countries across the world. However, adult-onset type 2 diabetes mellitus can be prevented and even controlled or managed through diet and some synthetic oral hypoglycaemic agents. The two most common oral medications are metformin and sulfonylurea (Lawrence *et al*., 2006).

In 80% of rural African communities this is, however, not the case as they still rely on the use of plant remedies as treatment (Zibula and Ojewole, 2000). As a result, one of the commonly used plants for type 2 diabetes treatment, called *H. hemero-callidea* or locally known as “African potato”, has been examined for hypoglycaemic activity (Erasto *et al*., 2005; Zimbula and Ojewole, 2000; Ojewole, 2006 and Mohamed and Ojewole, 2003).

Zibula and Ojewole (2000), investigated a methanol extract of African potato for possible hypoglycaemic activity in normal and diabetic animal models. The *Hypoxis* extract was found to be less potent than the positive control, glibenclamide (Zimbula and Ojewole, 2000). The authors suggested that it is not unlikely that the *Hypoxis* extract acts in the same way as glibenclamide, by stimulating insulin release which enhances cellular uptake and utilization of glucose in rats to induce hypoglycaemia. Mahomed and Ojewole (2003) and Ojewole (2006) also investigated aqueous extracts of *Hypoxis hemerocallidea* for hypoglycaemic activity, on normal and diabetic rats and found the aqueous extracts to also be positive for hypoglycaemic activity compared to positive controls insulin and glibenclamide.

Metformin is widely regarded and used as anti-hyperglycaemic agent for type 2 diabetes as it lowers blood glucose without causing overt hypoglycaemia by increasing the functional properties of insulin- and glucose-sensitive transporters, which is associated with increased glycogen synthase activity and glycogen storage (Wiernsperger and Bailey, 1999). Metformin enhances the effect of insulin and

therefore acts on the liver to suppress gluconeogenesis, and oppose the effects of glucagon by reducing hepatic extraction of substrates such as lactate. Metformin can also have effects independent of insulin, but can, however, not substitute the hormone insulin, and affects type 2 diabetes by collectively reducing insulin resistance and glucotoxicity (Wiernsperger and Bailey, 1999).

In the glucose utilization assay performed in the present study, human Chang liver cells and L6 (rat skeletal muscle) cells were used with metformin and insulin as the respective positive controls. In the assay the glucose in the culture medium is measured for each of the test samples before addition to the cells. After exposure of the cells to the treatments/test samples the glucose is measured again and the percentage glucose taken up by the cells determined. This experiment was performed as acute (exposure to 50 µg/ml extract for 3 h), chronic (48 h exposure to extracts at 12.5 µg/ml) and a combination of acute and chronic treatments (12.5 µg/ml for 48 h followed by 50 µg/ml for 3 h). Insulin stimulates glucose uptake into skeletal muscle (Wiernsperger and Bailey, 1999) and was therefore used as positive control in L6 cells, while metformin was used in Chang liver cells as positive control.

From the results in figure 13 it can be seen that the vehicle control DMSO (0.25%) did not have any effect on L6 glucose utilization. The positive control, insulin, significantly increased the glucose utilization in the two groups of cells treated for 48 h (chronic treatment and chronic + acute treatment). The lack of any significant effect in response to acute treatment may be attributed to the fact that the cells were not induced to differentiate and therefore would not have expressed high levels of the insulin responsive glucose transporter GLUT4. There was a small but insignificant increase in glucose utilization in L6 cells upon acute treatment with undigested hot water extract. Chronic exposure to this extract did not have any effect when compared to control cells. Interestingly, digestion of the hot water extract of *H. sobolifera* caused a significant decrease ($p<0.05$) in the acute response of the cells compared to that of the undigested hot water extract. The cold water extract caused a statistically significant decrease in acute glucose utilization compared to control. The response was also significantly lower than that of the hot water extract ($p<0.05$).

From figure 14 it could be seen that chronic treatment with metformin caused an increase in glucose utilization in Chang liver cells comparable with that of the hot water *H. sobolifera* extract. Also, both the hot water extract and metformin responses were significantly increased ($p<0.05$) compared to the control. Similar to what was observed in L6 cells, the cold water extract was ineffective in stimulating glucose utilization and digestion of the hot water extract significantly reduced the effect of the extract to levels comparable to that of the unstimulated control. There were no significant differences in the acute glucose utilization responses measured. DMSO (0.25%), the vehicle control, showed no glucose uptake stimulation in any of the test results compared to control cells.

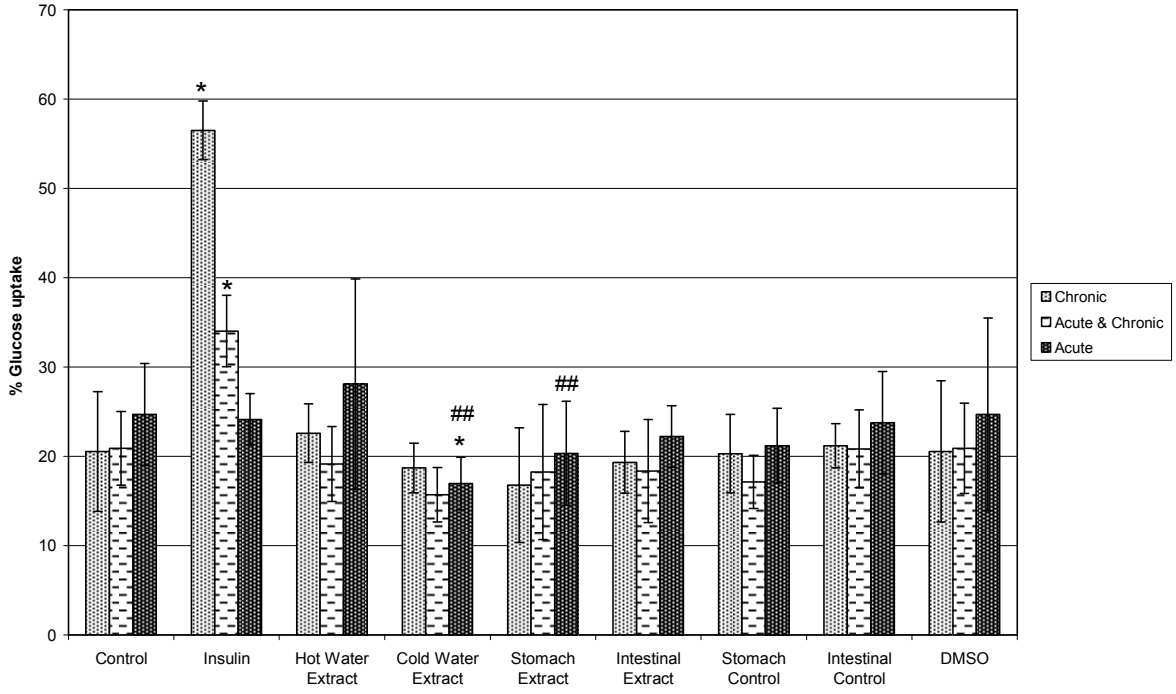

Figure 13: Percentage glucose uptake of L6 cells in the presence of insulin as positive control or *Hypoxis* test extracts. The experiment was performed for acute (50 µg/ml for 3 hours), chronic (12.5 µg/ml for 48 hours) and a combination of chronic & acute treatments (12.5 µg/ml for 48 h followed by 50 µg/ml for 3 h). Data points represent the mean ± SD of 8 replicates from a single experiment. The experiment was repeated three times yielding similar results. Significant results represented as *$p<0.05$ compared to negative control, $^{\#\#}p<0.05$ compared to undigested hot water extracts, all determined by ANOVA test.

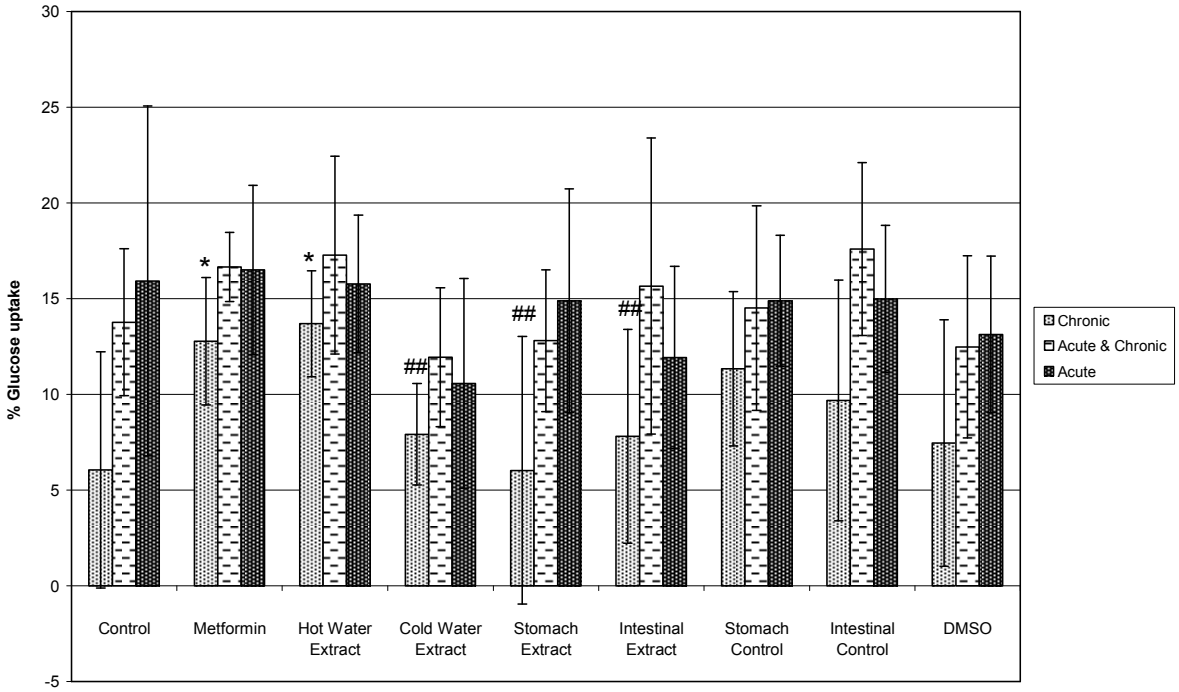

Figure 14: Percentage glucose uptake of Chang liver cells in the presence of metformin as positive control or *H. sobolifera* test extracts. The experiment was performed for acute (50 µg/ml for 3 hours), chronic (12.5 µg/ml for 48 hours) and a combination of acute & chronic (12.5 µg/ml for 48 hours followed by 50 µg/ml for 3 hours) treatments. Data points represent the mean ± SD of 8 replicates from a single experiment. The experiment was repeated three times yielding similar results. Significant results represented as *$p<0.05$ compared to negative control, $^{\#\#}p<0.05$ compared to undigested hot water extract all determined by ANOVA test.

For the glycogen staining, the same cell lines were used as in glucose utilization experiment. Treatments used were positive controls, 1 µM metformin (Chang liver cells), 1 µM insulin (L6 cells), 12.5 µg/ml hot water extract, 0.25% DMSO and control (figures 15 – 16).

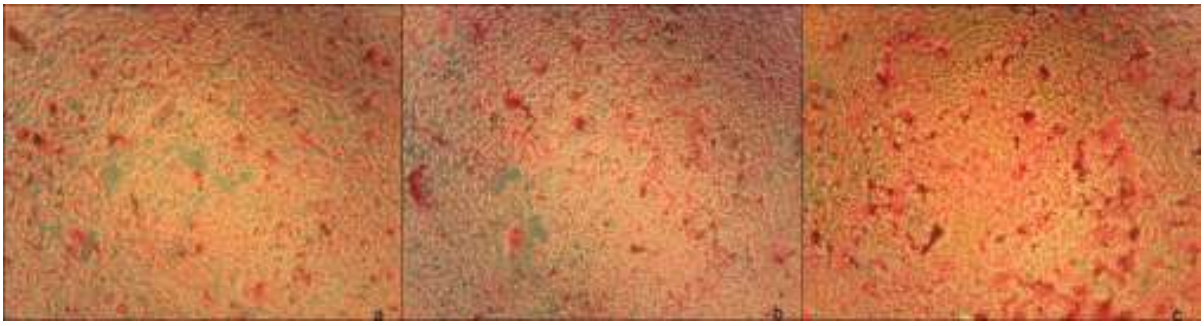

Figure 15: Photomicrographs of Chang liver cells treated for 48 h and stained with Best's carmine solution for glycogen content, (a) control cells; (b) 1 µM metformin treated cells; (c) 12.5 µg/ml hot water *H. sobolifera* extract treated cells.

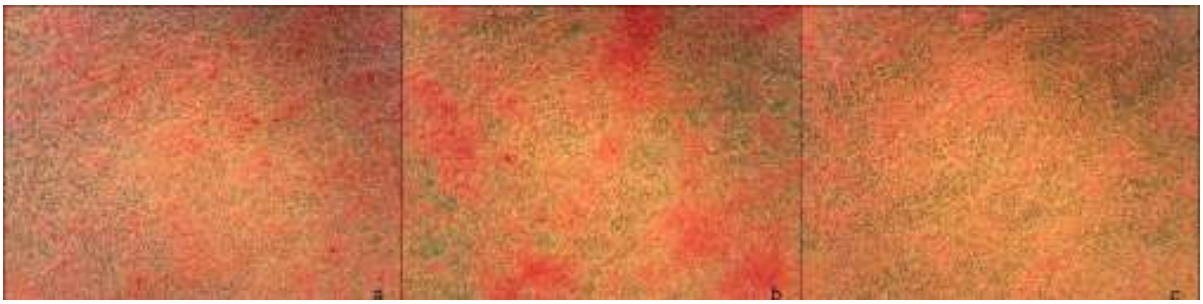

Figure 16: Photomicrographs of L6 cells treated for 48 h and stained with Best's carmine solution for glycogen content, (a) control cells; (b) 1 µM insulin treated cells; (c) 12.5 µg/ml hot water *H. sobolifera* extract treated cells.

From the photographs of the glycogen-stained cells, both Chang liver and L6, it is clear that merformin and insulin treated cells have more glycogen stored (red colour) than the control which is mostly mucin/fibrin (weak red colour). The hot water *H. sobolifera* extract had more glycogen and mucin/fibrin than the control and almost as much as the metformin (Chang liver cells) and insulin (L6) treated cells. This verifies the results obtained in the glucose uptake assay.

3.5.2 Antiglycation

The Maillard reaction is not only limited to proteins; glycation of phospholipids, resulting from the reaction between lipids and glucose, occurs under hyperglycaemic conditions in the cell membranes, which is also related to

exacerbation of chronic diabetic complications (Yonei *et al*., 2010). The glycation of proteins changes the structure and function of the affected protein. Examples of these changes are resistance to protease, changes in activity of glycated enzyme, or immobilization due to crosslinkage (Matsuura *et al*., 2002).

Inhibition of the formation of advanced glycation end products (AGEs) can prevent or improve the accumulation of AGEs and thus reduce the cause of diabetic complications. Examples of such inhibiting agents are aspirin, aminoguanidine (AG), and vitamin B6 (Matsuura *et al*., 2002). As with most inhibitory compounds, the effective dose of AG is very high and chronic administration is inevitable as a result of diabetic complications. This has resulted in the desire to find and develop more potent (Matsuura *et al*., 2002) and natural compounds.

It follows that a greater percentage inhibition of the formation of AGEs will result in better management for diabetics, with fewer complications as a result of glycation. Also, natural compounds such as plant extracts may have fewer side effects and may even increase health, for example through possible anti-oxidant activity of extracts.

From the results presented in figure 17 it can be seen that both the hot and cold water *H. sobolifera* extracts caused significant inhibition of AGEs formation at concentrations in the low µg/ml range. The cold water extract was more active than the hot water extract, as can be seen from their IC_{50} values of 6.3 µg/ml for hot water extract and approximately 0.35 µg/ml for cold water extract. An accurate value could not be calculated from the data obtained for the cold water extract because the percentage inhibition was still relatively high at the lowest concentration tested. The activity of the hot water extract was reduced after *in vitro* stomach digestion. At 5 µg/ml, less than 10% inhibition was observed compared to more than 40% inhibition at 6.25 µg/ml in the undigested extract. Addition of β-glucosidase did not improve the activity of the digested extract. This, together with evidence of the absence of hypoxoside in the hot water *H. sobolifera* extract as shown in a previous section, suggests that the active compound in this assay was not a glycoside that is activated through removal of its carbohydrate (or at least not one containing a bond cleaved by β-glucosidase). The reduction observed in the

antiglycation activity after digestion suggests that a concentration ≥50 µg/ml will need to be administered to ensure that a concentraton of around 50 µg/ml is available after the various digestive processes. The absence of cytotoxicity in the anti-cancer screening (sections 3.2 and 3.3) at 125 µg/ml suggests that this may be achievable *in vivo* without introducing toxic effects. This aspect, as well as other factors such as the amount that will finally be absorbed into the blood stream will need further investigation.

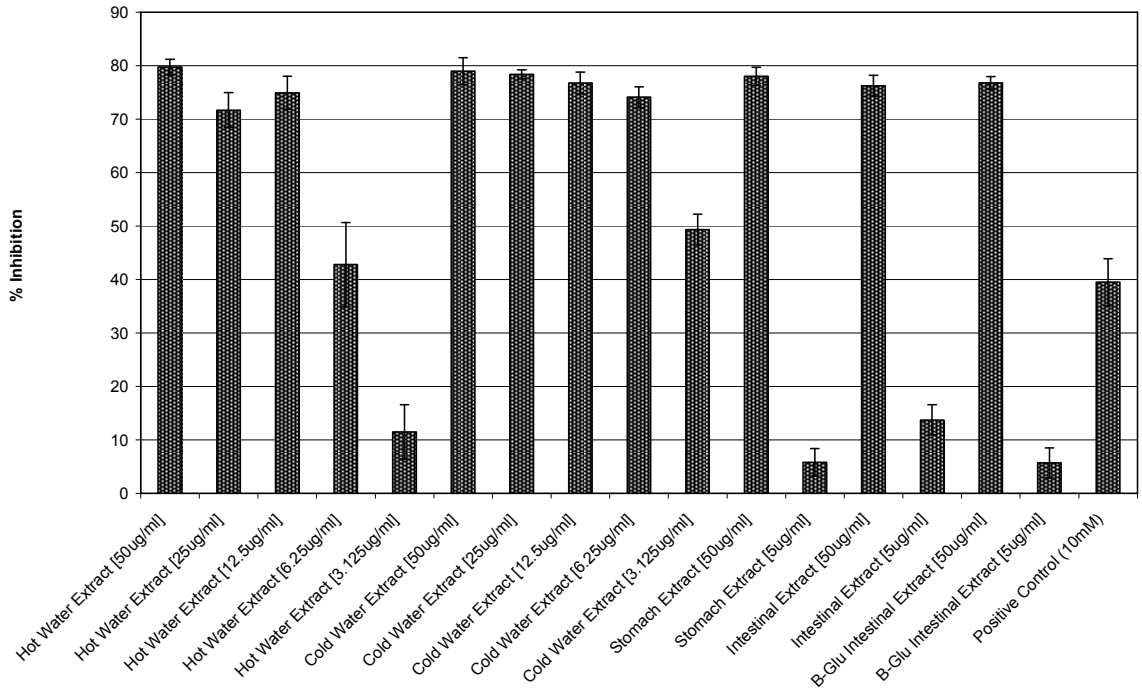

Figure 17: Percentage inhibition of advanced glycation end products by *H. sobolifera* extracts. The hot water extract was subjected to *in vitro* digestion (stomach extract and intestinal extract, the latter with and without the addition of β-glucosidase). Results shown represent the mean ± SD of 10 replicates from a single experiment. The experiment was performed three times yielding similar results. IC_{50} values for the hot water and cold water extracts were determined to be 6.3 µg/ml and approximately 0.35 µg/ml respectively. All the data points shown were significantly higher (% inhibition) than the vehicle control at a level of $p<0.05$ or lower as determined by ANOVA test.

3.5.3 Enzyme Inhibition Assays

There is a close relationship between nutrition and health and therefore the biggest challenge in type 2 diabetes is managing and maintaining blood glucose levels with few fluctuations, and to keep it as close to normal as possible (Kwon *et al*., 2008 and Elsenhans and Caspary, 1987). Two key enzymes have been identified as playing important roles in diabetes and the management thereof; these are α-amylase, responsible for starch breakdown to glucose and maltose, and α-glucosidase, responsible for starch and disaccharide breakdown to release glucose (Mogale *et al*., 2011 and Kwon *et al*., 2008). Inhibition of these two enzymes decreases the amount of blood glucose after a mixed carbohydrate diet by delaying the digestion and absorption of carbohydrates (Mogale *et al*., 2011 and Kwon *et al*., 2008). An example of such an inhibitor is acarbose, and it has been widely used as an antidiabetic agent (Gross *et al*., 2011; Mogale *et al*., 2011 and Kwon *et al*., 2008).

Acarbose, however, has been reported to have several side effects, mainly associated with gastrointestinal discomfort, including diarrhoea, flatulence, abdominal distension and meteorism (Mogale *et al*., 2011 and Kwon *et al*., 2008). Both Mogale *et al*. (2011) and Kwon *et al. (*2008) commented that the excessive inhibition of pancreatic α-amylase by acarbose might be the cause of the side effects. Kwon *et al*. (2008) suggested that α-amylase and α-glucosidase inhibition observed in extracts derived from plants will have a greater inhibition activity against α-glucosidase than against α-amylase with less or no side effects, compared to acarbose, by potentially reducing oxidation-linked hyperglycaemia. Kwon *et al*. (2008) hypothesised that the benefits for diabetes prevention lie in the anti-oxidant activity and α-glucosidase inhibitory potential linked to the high soluble-phenolic content.

3.5.3.1 Alpha-amylase assay:

From the results of the α-amylase assay it can be seen that at a concentration of 100 µg/ml of hot water extract the α-amylase enzyme is inhibited by more than 40% (figure 18), while acarbose [50µM], used as positive control, caused only 5%

inhibition. A higher concentration of acarbose is needed for a greater % inhibition. The hot- and cold water *H. sobolifera* extracts both inhibited the enzyme with IC_{50} values of approximately 250 µg/ml. Since α-amylase is a digestive enzyme and inhibition occurs in the intestinal lumen, it will be possible to achieve these concentrations *in vivo.* Therefore it can be suggested that traditionally prepared *H. sobolifera* extracts are viable agents for α-amylase, i.e. starch breakdown, inhibition and may prove beneficial in the management of type 2 diabetes.

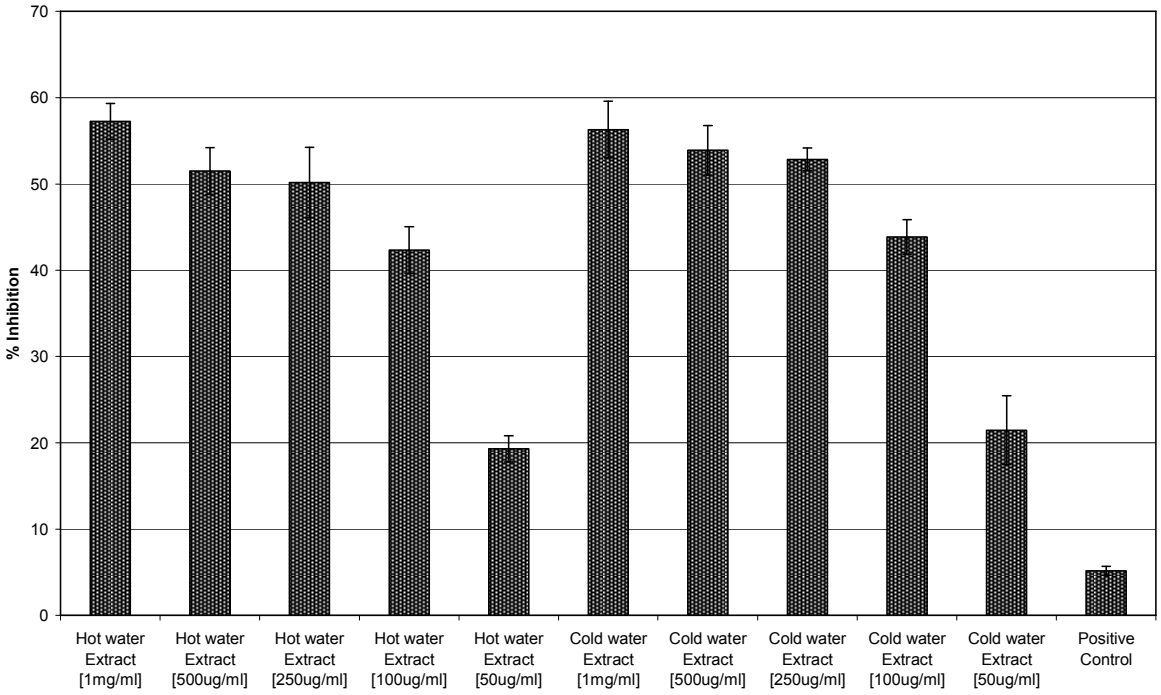

Figure 18: Percentage inhibition of α-amylase enzyme by *H. sobolifera* extracts. All results were corrected for vehicle control effects. Results shown represent the mean ± SD of 10 replicates from a single experiment. The experiment was performed twice yielding similar results. IC_{50} values for the hot water and cold water extracts were determined to be approximately 250 µg/ml for both. All the data points (% inhibition) were significantly higher than the vehicle control at $p<0.05$ or lower determined by ANOVA test.

Digested extracts could not be tested in this assay as the proteases present in the gastric and intestinal juices would have degraded the α-amylase, yielding meaningless results.

3.5.3.2 Alpha-glucosidase assay:

From the α-glucosidase assay no significant inhibition was observed since the colour of extract interfered with the assay and accurate results could not be obtained (results not shown).

Considering all the results obtained for antidiabetic screening it is clear that, even traditionally prepared, *Hypoxis sobolifera* may be beneficial in the management of type 2 diabetes. The effects observed on glucose utilization were small and appears to be lost after stomach and intestinal digestion and this effect will most likely not be seen *in vivo*. However, the antiglycation effect was only slightly reduced after digestion and the hot water extract caused significant inhibition of α-amylase. The hot water extract will therefore have the combined effect of preventing sudden increases in blood glucose levels by slowing down starch digestion and reduce diabetic complications through inhibition of AGE formation. The antihyperglycaemic effects of various *Hypoxis hemerocallidea* extracts published previously, may be partly explained by the α-amylase inhibition seen in this study (Lawrence *et al*., 2006; Erasto *et al*., 2005; Zimbula and Ojewole, 2000; Ojewole, 2006; Mohamed and Ojewole, 2003; Wiernsperger and Bailey, 1999 and Smit *et al*., 1995).

3.6 Anti-inflammatory activity

3.6.1 Phagocytosis

In Dale's (2008) historical review, the author referred to Majno's book the Healing Hand: Man and Wound in the Ancient World, in which Majno wrote that pus is a noble substance. Majno stated that pus is made of brave cells that all die in the line of duty, since they never sneak back into the blood vessels to escape their fate. Pus indicates an infection and also that the body is fighting it well (Dale, 2008).

Our immune system is divided into two types of immunity, namely acquired/adaptive and natural/innate (Dale, 2008). Phagocytosis plays a key role in our innate immune system. When pathogens and apoptotic cells are detected by receptors specific to them, phagocytic cells are activated in response. Phagocytes or phagocytic cells are involved in the synthesis and secretion of pro- and anti-inflammatory cytokines, chemokines (role in immune modulation) and inflammatory mediators during inflammation. The first line of antimicrobial host defence is the innate immune response, phagocytosis, which also impacts the adaptive immune response (Dale, 2008).

Boukes (PHD 2010), a previous student at NMMU, used chloroform extracts of three species of *Hypoxis*, one of which was *H. sobolifera*, and found small but statistically significant increases in phagocytic activity of differentiated U937 cells. The phagocytic activity detected by Boukes (2010) was the basis of this experiment. However, with the traditional aqueous extract, no significant phagocytic activity was observed (results not shown). This indicates that *H. sobolifera*, when prepared in the traditional way, will not have any effect on inflammation through phagocytosis. Rooperol, the aglycone of hypoxoside, has been shown to stimulate phagocytosis in differentiated and undifferentiated U937 cells (Boukes and Van de Venter, 2012). It is therefore likely that aqueous extracts from *Hypoxis* species that produce hypoxoside, will stimulate phagocytosis in the presence of β-glucosidase.

3.7 Anti-oxidant Activity

In living organisms oxidation plays an essential role during catabolism of fuel molecules for obtaining energy. Oxygen-centered free radicals and other reactive oxygen species (ROS) are constantly being produced in cells and could cause damage to tissues and cells or even result in cell death (Masoko and Eloff, 2007). To prevent these negative outcomes, the body has a well established system which maintains the balance in the cells between oxidants (ROS) and anti-oxidants (compounds that scavenge/quench the charged oxidant molecules rendering them harmless) (Masoko and Eloff, 2007). The resistance of cells to oxidant-induced damage to the genome and cell death can be severely affected by small deviations

in activity of anti-oxidant enzymes (Matés *et al.*, 1999). This balance in cellular oxygen is linked to three different classes of messenger molecules; these are growth factors, prostaglandins and nitric oxide (Matés *et al.*, 1999). During metabolism ROS are generated and these ROS can enter into reactions which, if not controlled, can affect certain cellular processes and lead to clinical manifestations (Matés *et al.*, 1999). An example of this clinical manifestation is when inflammatory mediators and ROS are generated by phagocytic cells; inflammation may influence anti-oxidant enzyme expression (Röhrdanz and Kahl, 1998).

Núñez-Sellés (2005) stated that the best anti-oxidant product is one that is able to prevent the excess of ROS, stimulate the endogenous repairing mechanism of anti-oxidants, and provide a large amount of chemical compounds to increase the endogenous scavenging mechanism of anti-oxidants. Considering the effect of excess ROS at cellular level may assist in the understanding of the relationship between oxidative stress and diseases (Núñez-Sellés, 2005). These cellular effects of excess ROS include modification and/or repression of gene expression, loss of cell integrity, modification of cell function, as well as activation or inactivation of key enzymes of cell function (Núñez-Sellés, 2005).

Previous studies have shown that dysfunction of glutathione metabolism due to oxidative stress may be a key factor in the development of Alzheimer's disease, and that oral vitamin E intake delayed the progression of Alzheimer's in patients with moderately severe impairment (Núñez-Sellés, 2005). Another report was that of anti-oxidants vitamin C [1000 mg/day] and vitamin E [400 IU/day] supplements reducing the incidence of pre-eclampsia in women at risk, by improving a range of biochemical markers of placental insufficiency and oxidative stress (Núñez-Sellés, 2005). Manzella *et al.* (2001) used chronic administration of the anti-oxidant vitamin E to show improvement in the ratio of cardiac sympathetic to parasympathetic tone in type 2 diabetic patients, and stated that such an effect may be mediated by a decrease in oxidative stress.

Katalinic *et al.* (2006), in their study of screening 70 medicinal plants for anti-oxidant capacity, showed that some medicinal plants are promising sources of natural anti-

oxidants. In their review, Heinrich and Gibbons (2001), stated that many of the valuable drugs of today only came into use after studies of folk remedies, and plant-derived drugs are a constant base of study as prototypes in an attempt to develop more effective less toxic medicines.

3.7.1 DPPH Assay

In this study the anti-oxidant activity of traditionally prepared *H. sobolifera* extracts (hot water; undigested and digested) and standard aqueous *H. sobolifera* extract (cold water; undigested) were compared to known anti-oxidant ascorbic acid, using the DPPH assay. The undigested cold water extract had an IC_{50} of approximately half that of ascorbic acid, while the hot water extract was less active with IC_{50} approximately double that of ascorbic acid (figure 19 and table 6). The IC_{50} values of all extracts tested can be seen in table 6.

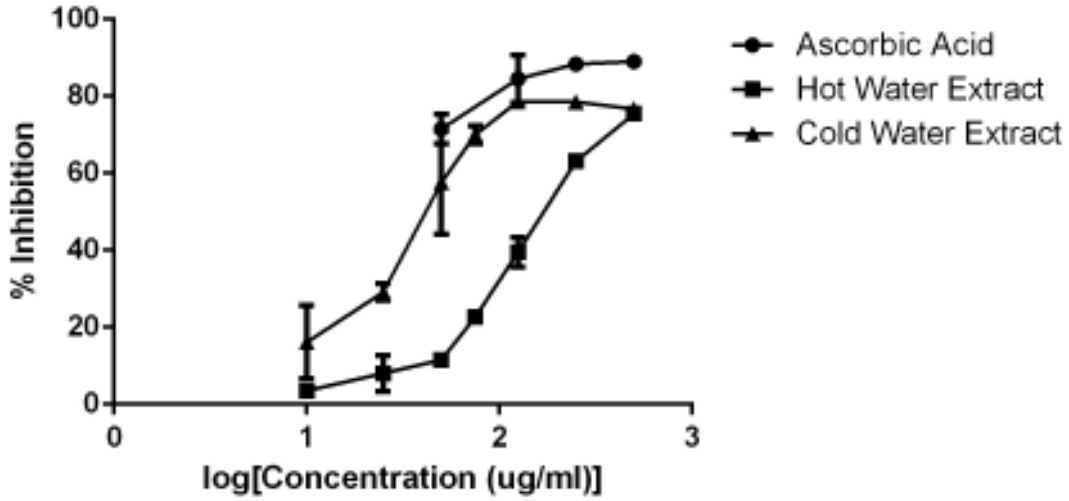

Figure 19: Dose response curve of DPPH representing anti-oxidant activity of hot and cold water *H. sobolifera* extracts tested against the positive control, ascorbic acid. Data points represent the mean ± SD of 8 replicates. The dose response curves were used to calculate IC50 values (see table 6).

Table 6: IC_{50} values obtained for the various *H. sobolifera* extracts using DPPH assay

	Ascorbic Acid	Hot Water Extract	Cold Water Extract	Stomach Extract	Intestinal Extract	β-Glu Stomach Extract	β-Glu Intestinal Extract
IC_{50} (µg/ml)	76.26	134.4*	39* ##	203.5*	>500*	162.9* ##	>500* ##

Stomach and intestinal extract refer to hot water extract subjected to *in vitro* stomach- and intestinal digestion, respectively. β-Glu refers to hot water extract with β-glucosidase added before being subjected to the said digestion processes.

* Significantly different compared to ascorbic acid $p<0.05$, while ## significantly different compared to undigested hot water *H. sobolifera* extract $p<0.05$.

From these results (table 6) it is clear that anti-oxidant activity of the hot water extract decreased significantly ($p<0.05$) after digestion IC_{50} increased from 134.4 µg/ml before digestion to 203.5 and >500 µg/ml after stomach and intestinal digestion, respectively. Addition of β-glucosidase to the digestive process only slightly increased the anti-oxidant activity compared to normal digested extracts. During the HPLC experiments on both the hot and cold water extracts, no hypoxoside was detected and so it is possible that very small quantities of hypoxoside (below the detection limit of the HPLC method used) may have been present in the extracts which could then be converted to rooperol and then have slightly more anti-oxidant activity. However, the original hot and cold water extracts had no β-glucosidase added and had significant anti-oxidant activity which had to be as a result of other phytochemicals present in the extracts. The possibility that the extract contained another glycoside that was activated through hydrolysis by β-glucosidase cannot be ruled out. Thus, although *H. sobolifera* is a viable anti-oxidant treatment, the traditional method of extraction and administration is not the best method as most of the activity is lost after digestion.

Hypoxoside obtained from African potato can be converted in the human body by colonic bacterial β-glucosidase into rooperol. Studies have shown that rooperol can be found in faeces while its phase II metabolites (glycosides, sulphates, mixed glucuronides and sulfuronides) can be found in the serum and urine (Kruger *et al.*, 1994). Rooperol structurally resembles a known strong anti-oxidant called nordihydroguairetic acid (NDGA) (Nair *et al.*, 2007). In this study strong anti-oxidant

activity was observed even though HPLC results (section 3.4) did not detect any hypoxoside.

NDGA interacts with the oxidative processes in human blood and so does rooperol (Nair *et al.*, 2007). In their study, Nair *et al.* (2007) investigated the free radical scavenging effects of aqueous *Hypoxis hemerocallidea* extracts and purified compounds compared to quercetin and found African potato extracts and rooperol compound had significant activity while hypoxoside showed no significant free radical scavenging activity. The anti-oxidant activity observed in this study therefore supports that of Nair *et al.* (2007) who concluded from their results that the anti-oxidant activity may be due to various other phytochemicals present in *Hypoxis* extracts since hypoxoside alone had no activity, indicating *Hypoxis* could have value as an anti-oxidant prodrug.

3.7.2 Reactive Oxygen Species

In aerobic organisms specific levels of ROS are required for normal cell function and therefore the formation and degradation of ROS are key components of metabolism due to the fact that oxidative stress results from excess ROS (Johar *et al.*, 2004). During infection, ROS production is rapidly increased to assist in pathogen eradication as well as signalling cascades related to inflammation, cell proliferation and immune responses (Spooner and Yilmaz, 2011). Most of the endogenous intracellular ROS is produced in the mitochondria through the mitochondrial respiratory chain, and an imbalance between anti-oxidant defences and ROS could occur due to inhibition of electron flow or exposure to xenobiotics.

In this study the effect of *H. sobolifera* extracts were investigated for their effect on ROS production. To measure the level of ROS, the stain 2',7'-dichlorofluorescein diacetate (DCF-DA) was used as it is membrane permeable, which allows the intracellular ROS to be measured. Non-fluorescent DCF-DA is oxidized by ROS to form fluorescent 2',7'-dichlorofluorescein (DCF) (Ibrahim *et al.*, 2012). Phorbol 12-myristate 13-acetate (PMA) is a compound known to induce ROS production and was therefore used as positive control. Extracts tested for effects on ROS

production were hot and cold water *H. sobolifera* extracts, as well as hot water *H. sobolifera* extracts after simulated stomach and intestinal digestion. DMSO was used and tested as vehicle control and unstained cells were used to establish if differentiation was achieved in the U937 cells. After treatment with the stain and various compounds, cells were analyzed using a flow cytometer with a 488 nm laser for excitation and emitted green fluorescence measured (FL1) channel.

When PMA, the positive control, was compared to the vehicle control, it showed PMA significantly increased ROS levels by more than 10%. The fluorescence intensity of positive control cells (PMA) was therefore taken as 100% and all other treatments compared to that, as shown in figure 20. It can further be seen that all the extracts caused only low, statistically insignificant percentage increases in ROS production when added to the cells on their own. However, when the cold- and hot water *H. sobolifera* extracts were combined with PMA, there was a significant (*$p<0.05$) increase in ROS production for both extracts. This increase in ROS production was much higher than that observed with PMA alone. The digested hot water extracts have lost this ability to enhance the effect of PMA on ROS production.

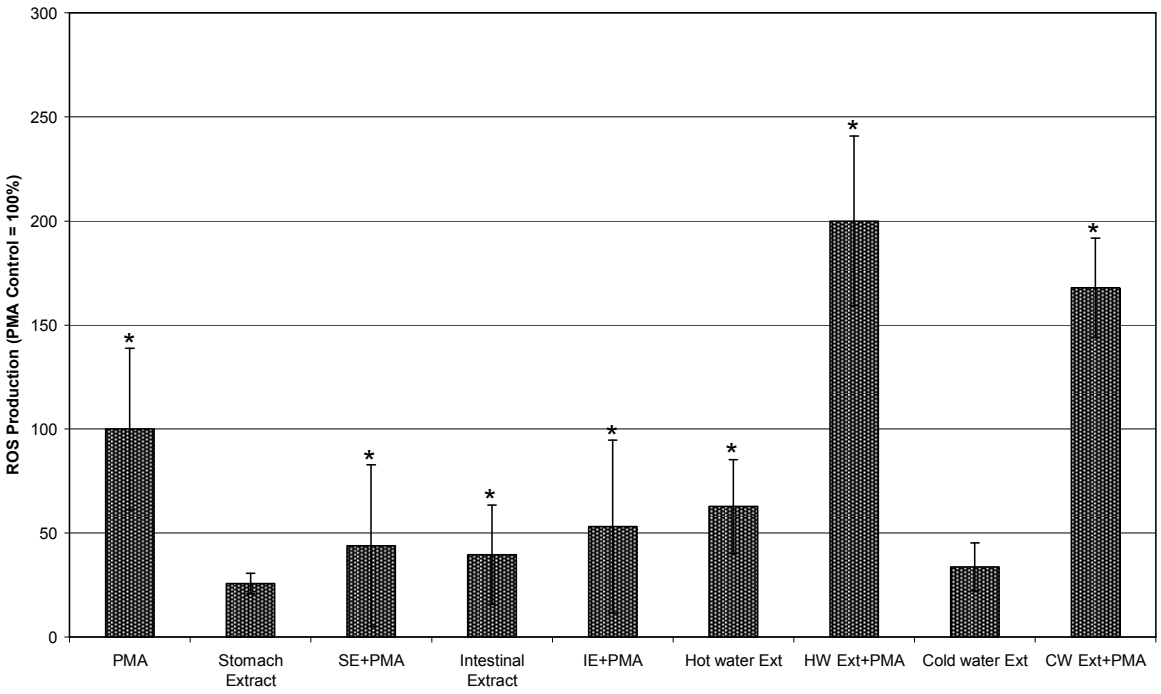

Figure 20: ROS production in U937 cells after treatment with *H. sobolifera* extracts alone and in combination with PMA. Percentages were calculated using the mean fluorescence

intensities obtained from flow cytometric analysis of DCF-DA stained cells. Values represent mean ± SD of four replicates from a single experiment. The experiment was performed three times, yielding similar results. Significant results represented as *$p<0.05$ determined by ANOVA test, compared to vehicle control.

The fact that ROS production in response to PMA was enhanced by the hot and cold water extracts suggests that *H. sobolifera* contains a potential immune booster which may strengthen the immune system's natural reaction to infection. This effect was, however, lost after stomach digestion and the traditional remedy taken orally will not have this property. Future studies should attempt the isolation of the active compound(s) and investigate the possibility of synthesizing more stable compounds for oral administration or other routes of administration.

3.7.3 TLC DPPH

TLC is based on the principle of separation, similar to other chromatographic methods. The separation depends on the presence of a stationary and mobile phase and the relative affinity of compounds towards these two phases. The stationary phase can be found as a thin coating on a glass or metal foil plate. The sample that needs to be tested/separated is applied as a spot near the end of the plate, usually about 1 cm from the bottom of the plate, called the spotting line (see figure 21). After the sample spots have dried the TLC plate is placed in a reservoir of mobile phase (solvent) that is allowed to pass over the plate by capillary action. There is little resistance to the mobile phase and so the solvent front moves rapidly across the layer. As it moves it transfers analytes in the test sample with a rate depending on their affinity to the stationary and mobile phase. The compounds with higher affinity for the stationary phase travel slowly while the others travel faster. This allows for separation of compounds. Once separation is complete, the individual (separated) compounds are visualized as spots at their respective levels of travel on the TLC plate, as shown in figure 22 (Wilson & Walker, 2005).

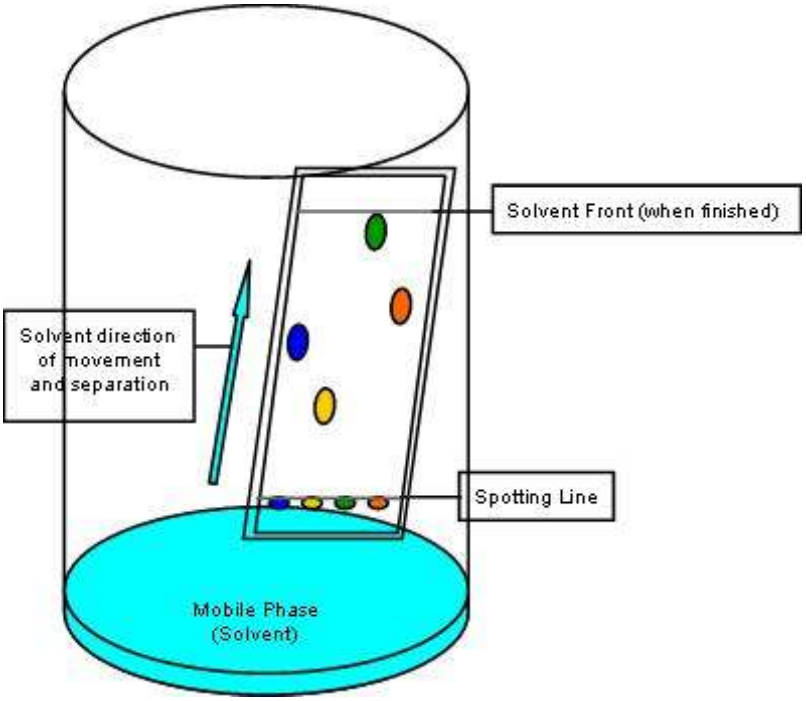

Figure 21: Diagram illustrating the principle of TLC plate separation of compounds (created by Anzél van Rooyen in Microsoft Publisher).

The active compounds of *Hypoxis* have always been believed to be hypoxoside and its aglycone, rooperol. As a result of all the evidence supporting and or showing the activity of these compounds, they have been purified and even chemically synthesized (Potgieter *et al*., 1988), making them available for use as standards.

Rooperol has previously been proven to have anti-oxidant activity while hypoxoside is inactive (Nair *et al*., 2007). In the present study, β-glucosidase was added to the extracts to convert any hypoxoside to rooperol. Even though HPLC showed no detectable hypoxoside (section 3.4) it is possible that a small amount of hypoxoside was present in the extract and could be converted to rooperol. Since rooperol has been suggested as the active compound, another approach was followed to confirm the absence or presence of this compound in the hot- and cold water *H. sobolifera* extracts. The method employed TLC and the extracts' DPPH scavenging activity with rooperol as standard for comparison.

All extracts were dissolved in DMSO and made up to the various concentrations with methanol to yield a final DMSO concentration of 0.5%. The test samples that were applied to the TLC plates were DMSO, the rooperol standard, and the following

extracts all pre-treated with β-glucosidase for conversion of any hypoxoside to rooperol: hot water extract (traditional extraction), cold water extract (conventional extraction) and the stomach and intestinal extracts obtained from *in vitro* digestion of the hot water extract. After the TLC plates were removed from the mobile phase solvent, the plates were allowed to dry, following which they were sprayed with a 0.2% DPPH solution and left at room temperature for 30 minutes. Yellow spots indicate positive anti-oxidant activity. Since rooperol is a known anti-oxidant and the standard used consists of the single compound, it can be used to indicate the location at which yellow/active spots of compound from the various extracts should be present if they contained any rooperol.

The TLC plates showed that DMSO, even at 100%, did not have any effect on the result. The rooperol lanes were yellow from near the application line up to a distinct yellow spot which is the distance travelled by rooperol. This indicates overloading but it was still possible to use the result for comparison to the extracts. For the undigested extracts, it was clear that only the cold water extract produced a yellow spot in the region where rooperol was found and the intensity of this spot increased with an increase in extract concentration. This could be an indication of some hypoxoside (detected as rooperol after β-glucosidase treatment) present in the cold water extract. More likely though is the possibility that this was the compound detected on HPLC (section 3.4) and suspected to be a hypoxoside analogue which was cleaved and thus activated by β-glucosidase. The results for the hot water extract confirmed the absence of hypoxoside as previously shown on HPLC (section 3.4). Simulated digestion of the hot water extract caused a slight reduction in intensity of the anti-oxidant profile on the TLC plate, as was expected from the previous DPPH study.

These results confirm that rooperol is not the only source of anti-oxidant activity in the African potato, as no rooperol was detected in the hot water *H. sobolifera* extract even at 150 µg/ml on the TLC plate, whereas the DPPH assay showed high anti-oxidant activity at 125 µg/ml and lower.

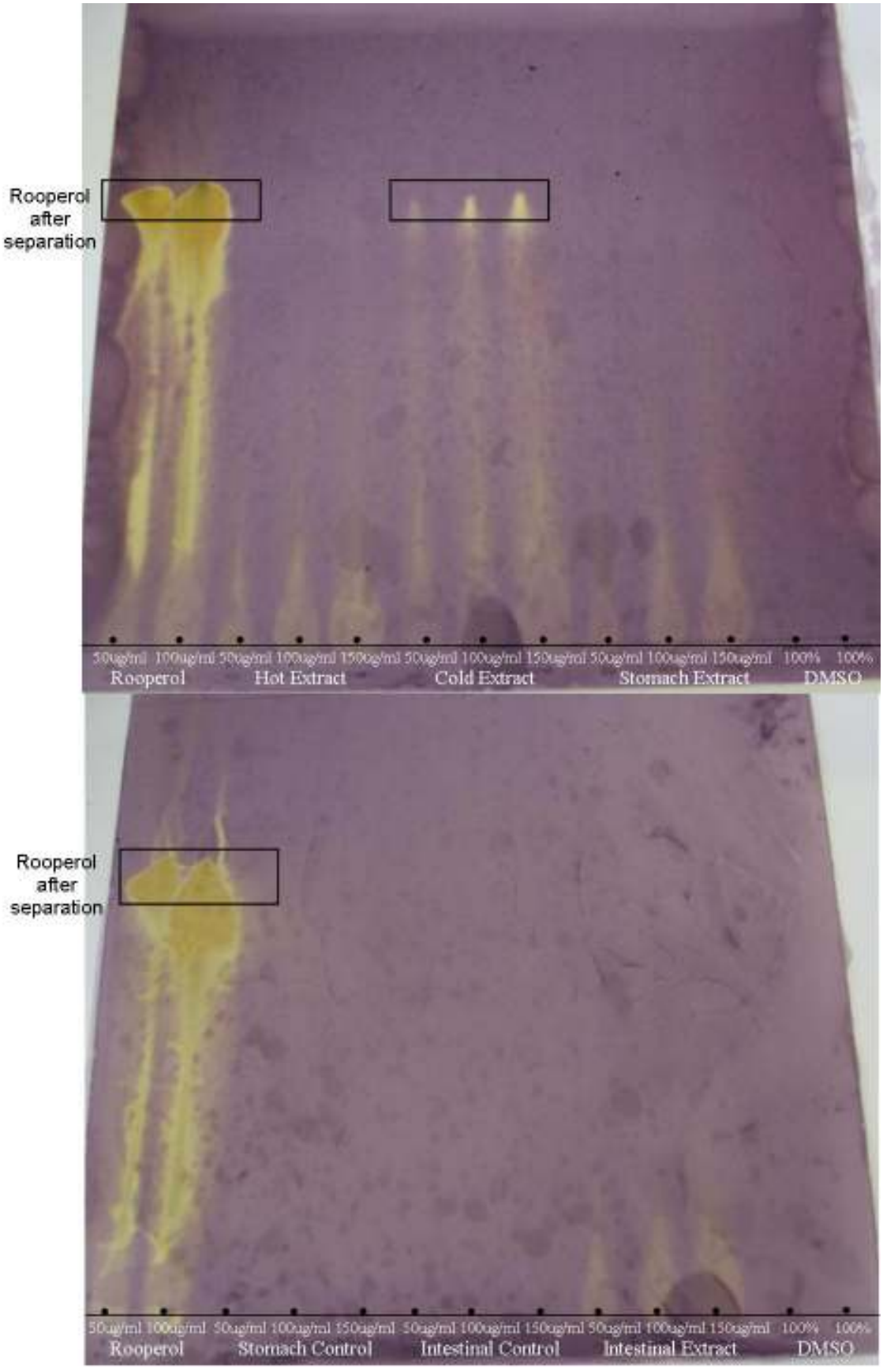

Figure 22: Photograph of TLC plates after separation and subsequent spraying with 0.2% DPPH. The spots on this plate represent the following. Top, from left to right: Positive

control Rooperol at 50 and 100 µg/ml; Hot water *H. sobolifera* extract at 50, 100 and 150 µg/ml; Cold water *H. sobolifera* extract at 50, 100 and 150 µg/ml; Stomach extract at 50 , 100 and 150 µg/ml (hot water extract subjected to simulated stomach digestion) and finally 100% DMSO as negative control. Bottom, from left to right: Positive control Rooperol at 50 µg/ml and 100 µg/ml; Stomach Control at 50 µg/ml, 100 µg/ml and 150 µg/ml (simulated stomach digestive fluid); Intestinal Control at 50 µg/ml, 100 µg/ml and 150 µg/ml (simulated intestinal juice); Intestinal Extract at 50 µg/ml, 100 µg/ml and 150 µg/ml (hot water extract subjected to simulated intestinal digestion) and finally 100% DMSO as negative control.

Ojewole (2005), showed scavenging activity by rooperol on HO^- in normal and diabetic rats caused dose-related hypoglycaemia, in this study the TLC plates confirmed that the hot water *H. sobolifera* extracts did not have any rooperol, suggesting that rooperol is not responsible for the anti-oxidant activity observed in the DPPH assay (section 3.7.1). Laporta *et al.* (2007) also found strong anti-oxidant activity in *Hypoxis* due to its strong capacity to inhibit free-radical-induced membrane lipoperoxidation.

3.7.4 Total content of compounds in extract

The high anti-oxidant activity of the *H. sobolifera* extracts in the absence of significant amounts of hypoxoside prompted phytochemical analysis of the major groups of compounds present in these extracts. Those analysed were total phenolics, flavonoids and carbohydrates.

3.7.4.1 Total phenolic content assay:

Zheng and Wang, 2001, confirmed a linear relationship between anti-oxidant capacities (oxygen radical absorbance capacity) and total phenolic content in herbs, both medicinal and culinary. The significance of this is that free radicals are generated as by-products of some biological reactions or as a result of exogenous factors. This involvement of free radicals is well documented in the pathogenesis of a number of diseases (Gyamfi *et al*., 1999 and Pourmorad *et al*., 2006). A

preventative intervention for the diseases can therefore be a possible use of a potent scavenger of free radicals (Pourmorad *et al*., 2006 and Gyamfi *et al*., 1999).

From the polyphenol content assay as performed by Rashid *et al*., 2010 (adapted from Slinkard and Singleton, 1979) the results for total phenolic content could be determined and expressed as gallic acid equivalents (mg gallic acid/g dried extract).

The gallic acid standard curve produced an R^2 value = 0.9491 and an equation of y=0.0798x (figure 23 in Appendix). From the extract samples absorbance values at 765 nm, the gallic acid concentration (x-value) for each sample could be determined. These values were then plotted on the standard curve to provide an indicator of the activity. Finally the results could be calculated as mg gallic acid per g of dried extract. These results are shown in table 7.

Table 7: Amount of gallic acid equivalents in various *H. sobolifera* extracts

***H. sobolifera* Extract**	**Gallic Acid Equivalents (mg Gallic acid/g dried extract)**
Hot water Extract	59.8
Cold water Extract	89.5
Stomach Extract	51.3
Intestinal Extract	46.6

The amount of phenolics detected in the cold water *H. sobolifera* extract was greater than that of the undigested hot water *H. sobolifera* extract, which could explain reason for the greater anti-oxidant activity observed with the cold water *H. sobolifera* extracts in the DPPH assay (section 3.7.1) as well as in the TLC plates (section 3.7.3). After digestion of the hot water *H. sobolifera* extracts, however, the amount of phenolics is substantially less; again this compliments the anti-oxidant results.

The Folin-Ciocalteu procedure used to measure the total phenolic content does not, however, give the full picture of the quantity and quality of the exact phenolic compounds in the extract and also tends to underestimate their activity levels

(Wojdylo *et al.*, 2007 and Katsube *et al.*, 2004) therefore further investigation is needed to review, for example, specifically flavonoid content.

3.7.4.2 Total flavonoid content assay:

Two methods of colorimetric screening exists for identifying flavonoids, the first is an aluminum chloride method developed by Zhishen *et al*., 1999. The second is a 2,4-dinitrophenylhydrazine utilizing reaction as developed by Nagy and Grancai (1996). Chang *et al*. (2002) investigated the two colorimetric methods to determine if different methods detect different flavonoids. After testing both methods on all major classes of flavonoids, Chang *et al*. (2002) found the aluminum chloride reaction was specific for flavones and flavonols, while the 2,4-dinitrophenylhydrazine reaction was specific for flavonones and flavanonols. Rutin is a widely used flavonoid in the industry as it has pronounced therapeutic activity (Gupta *et al*., 2010 and Rashid *et al*., 2010) and was therefore used as positive control in the aluminum chloride reaction.

In the flavonoid assay, the rutin standard curve for various concentrations (0 - 700 µg/ml) had a R^2 value of 0.9839 and an equation of y=0.0075x.

Table 8: Amount of rutin equivalents in various *H. sobolifera* extract

Extract	**Rutin Equivalents (mg rutin/g dried extract)**
Hot water Extract	183
Cold water Extract	268
Stomach Extract	154
Intestinal Extract	163

From table 8 it's clear that the cold water *H. sobolifera* extract has greater flavonoid content than the undigested hot water *H. sobolifera* extract, as well as the digested hot water extracts. The greater content of flavonoids in the cold water extract could

explain the reason for the greater anti-oxidant activity observed in the DPPH assay (section 3.9.1) as well as in the TLC plates (3.9.3). Also, it may be suggested that the flavonoids present were most probably flavones of flavonols as activity was recorded using the aluminum chloride colorimetric method. No significant colour development was observed above the background colour of the extracts using the 2,4-dinitrophenylhydrazine method (results not shown), and accurate results could not be obtained.

3.7.4.3 Total carbohydrate assay:

It is a well-known fact that bulbs and corms of plants act as storage organs for large amounts of carbohydrates (Ranwala and Miller, 2008; Cronk and Fennessy, 2001 and Zimmerman and Whigham, 1992). As, traditionally, the corm of the *Hypoxis* plant, or African potato, is used to treat patients it is necessary to determine the carbohydrate content of the traditionally prepared extracts, as this may affect some of the experiments (e.g. amylase, glucose uptake and glycogen staining assays) in this study as well as the patients themselves if, for example, they are diabetic.

To determine the carbohydrate content of the *Hypoxis* extracts, the anthrone method was used. The anthrone assay yielded carbohydrate content results >250 µg/g extract. This indicates that carbohydrates seem to be the main component of the extract. Depending on the type and identity of these carbohydrates, this may present a problem for diabetics and should be investigated further in order to accurately quantify the carbohydrates and determine if the specific carbohydrate could be responsible for any of the observed biological activities.

4 CHAPTER – SUMMARY AND CONCLUSION

In summary, the traditional preparation of *H. sobolifera* extracts yielded 8% dry extract and was successfully used in the simulated digestive system. The hot water *H. sobolifera* extract before digestion only showed cytotoxic activity against cancer cell lines at very high concentrations which are not likely to be achieved under normal ingestion circumstances. Hot water *H. sobolifera* extract showed no significant cytotoxic activity against U937 cancer cell lines when using Cell Titre Blue assay. In previous publications that reported on the *in vitro* cytotoxic properties of African potato extracts on cancer cells, the activity can be ascribed to the presence of either hypoxoside which is converted to active rooperol (Albrecht *et al.,* 1995; Smit *et al.,* 1995; Steenkamp and Gouws, 2006; Drewes *et al.,* 2008 and Kruger *et al.,* 1994) or phytosterols (De Jong *et al*., 2003 and Boukes *et al.,* 2008). The present study confirmed the absence of hypoxoside in the hot water *H. sobolifera* extract and a previous study done at NMMU has shown that the traditional method of preparation is not effective in extracting phytosterols from African potato (Du Plessis-Stoman *et al*., 2009).

As an antimicrobial agent, *Hypoxis* prepared traditionally is not a viable option, however, at high concentrations it may be. These extracts will therefore not be a good option for oral application against bacterial pathogens but if applied topically it may have some benefits. Due to the low/no activity observed for the undigested *H. sobolifera* extract in the cytotoxicity and antimicrobial assays, the digested extracts were not tested.

In diabetic treatment there are many areas to consider, however, in this study only a few where investigated. Hot water *H. sobolifera* extracts had activity in glucose uptake, and in L6 muscle cells there was a slight increase in glucose uptake but not as much as with insulin, the positive control. In Chang liver cells on the other hand, chronic exposure to the hot water *H. sobolifera* extract increased glucose uptake in amounts similar to that of metformin. With the glycogen staining experiment it could be observed that glycogen was stored in quantities comparable to, but not yet as high as, the respective positive controls. On the negative side, the glucose

utilization stimulation was lost due to the simulated digestion process. The significant inhibition of AGEs by hot water *H. sobolifera* extract is a very encouraging result as treatment in the management of diabetes. This activity was reduced but not completely abolished by *in vitro* digestion. The antiglycation activity of these extracts is probably related to their good anti-oxidant activities. Also observed was enzyme inhibition activity by traditionally prepared *H. sobolifera*, with ∝-amylase being inhibited and therefore preventing or limiting starch breakdown. Results for α-glucosidase inhibition were inconclusive. Considering previous reports on the *in vivo* hypoglycaemic activities of other *Hypoxis* species, and the differences in chemical composition between them and the hot water *H. sobolifera* extract already mentioned, it will be useful to test latter extract in similar animal models of diabetes.

As an anti-inflammatory agent *Hypoxis* still needs to be investigated further. For phagocytic activity the traditional preparation yielded no significant results. Inflammation is a complex process and anti-inflammatory activity can be exerted at many other molecular levels not included in our *in vitro* testing. One such example is the inhibition of cyclo-oxygenase (COX) enzymes.

From the DPPH results it was clear that *H. sobolifera,* even when digested, is a viable anti-oxidant treatment agent. The TLC experiment revealed that rooperol which has previously been thought to be the compound responsible for the anti-oxidant activity in *Hypoxis* extracts, was absent from the traditional extract of *H. sobolifera* and therefore cannot be the sole compound exhibiting anti-oxidant activity; other compounds such as phenolics may be contributing. The phenolic and flavonoid content results revealed very high concentrations of these compounds in the traditionally prepared *H. sobolifera* extract. These compounds may therefore play major roles in all of the biological activities observed from treatment with *Hypoxis* spp. The ROS results yielded interesting and promising results. Using standard or traditionally prepared *H. sobolifera* extracts, activation of differentiated U937 cells with PMA was greatly enhanced by co-treatment with the extracts, while extracts on their own did not cause significant activation. Future studies should investigate this property of the extracts as a promising immune booster.

In conclusion, *H. sobolifera* does not have the highest hypoxoside content of the various *Hypoxis* spp., even when using chloroform or ethanol for extraction (Boukes *et al.*, 2008). The HPLC results showed that hypoxoside was undetectable in the hot water traditional extract and the TLC anti-oxidant experiment proved that rooperol is not present in the hot water traditional extract after treatment with β-glucosidase. This indicates that neither one of the *Hypoxis* compounds previously believed to be responsible for the biological activities observed are present in the extract when prepared the traditional way. Therefore, the biological activities observed in this study can be attributed to other phytochemical compounds. These findings suggest that *Hypoxis sobolifera* may be a potential remedy in the prevention or treatment of various diseases. However, the traditional method of administration requires ingestion of the tea and in this study the results obtained suggested that very little, if any, of the activity is available after the various digestive steps. It is advisable that a more concentrated form of *Hypoxis* should be used rather than the traditional preparation.

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6 APPENDIX

Total phenolic content assay:

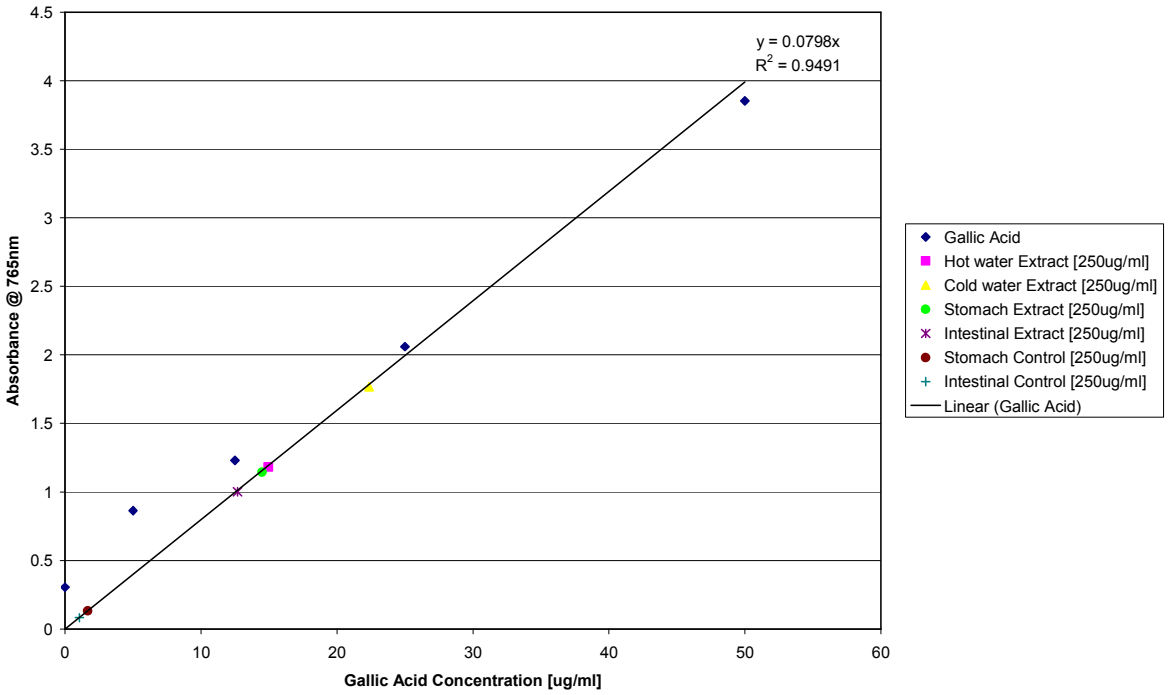

Figure 23: Gallic acid standard curve along with all the *Hypoxis* extracts tested.

Total flavonoid content assay:

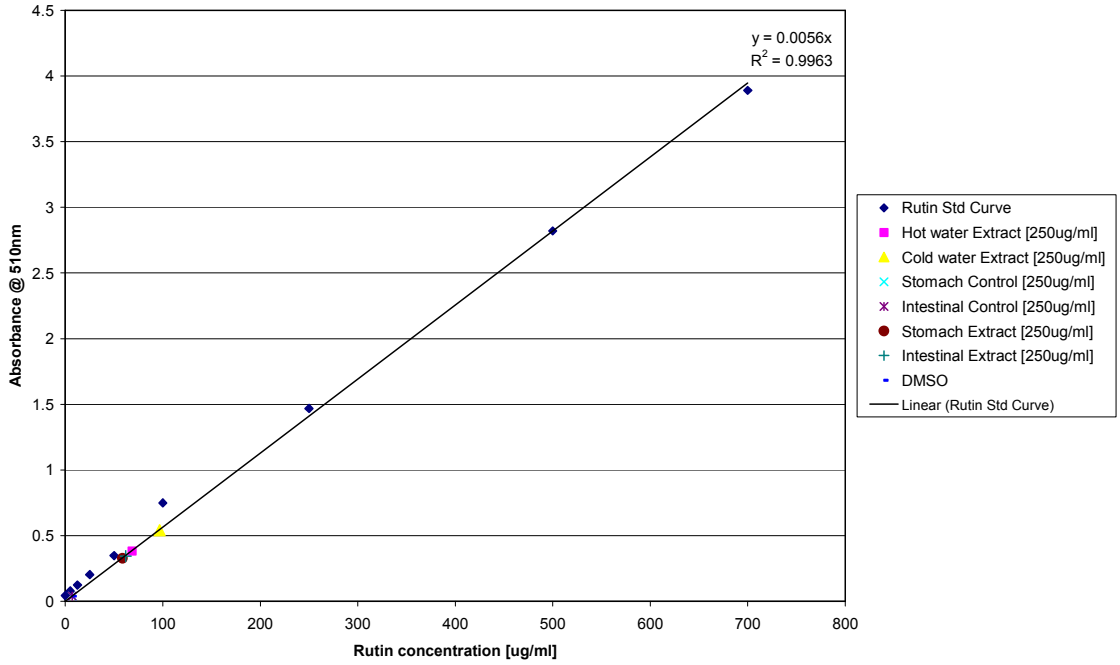

Figure 24: Rutin standard curve along with all the *Hypoxis* extracts tested using aluminum chloride reaction

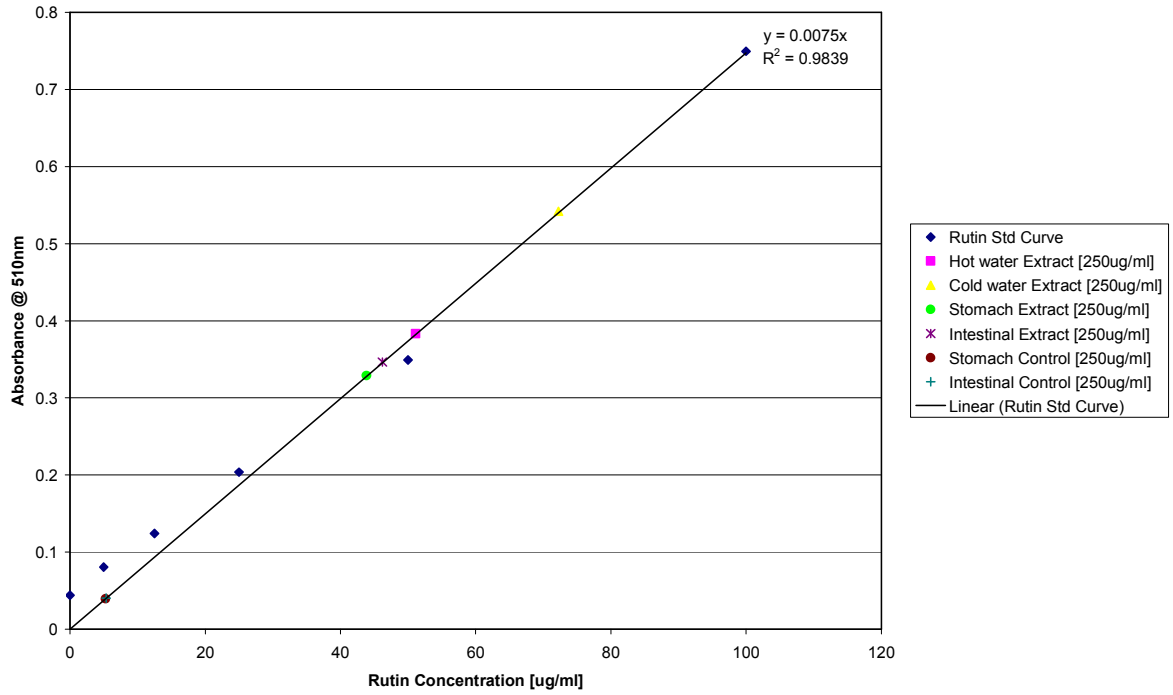

Figure 25: Rutin standard curve, using lower concentrations of rutin, along with all the *Hypoxis* extracts tested.

The extract concentration (x-value) was then plotted on the same graph (figures 24 & 25). After all extracts were plotted on the standard curve it was clear that the extracts all plotted to the bottom end of the standard curve. As a result, the standard curve was replotted to include only the lower concentrations of rutin (0 - 100 µg/ml). This standard curve had a R^2 value of 0.983 and an equation of y=0.0075x. The extract concentrations were then recalculated using the new equation and the points plotted on the new standard curve.

Total carbohydrate content assay:

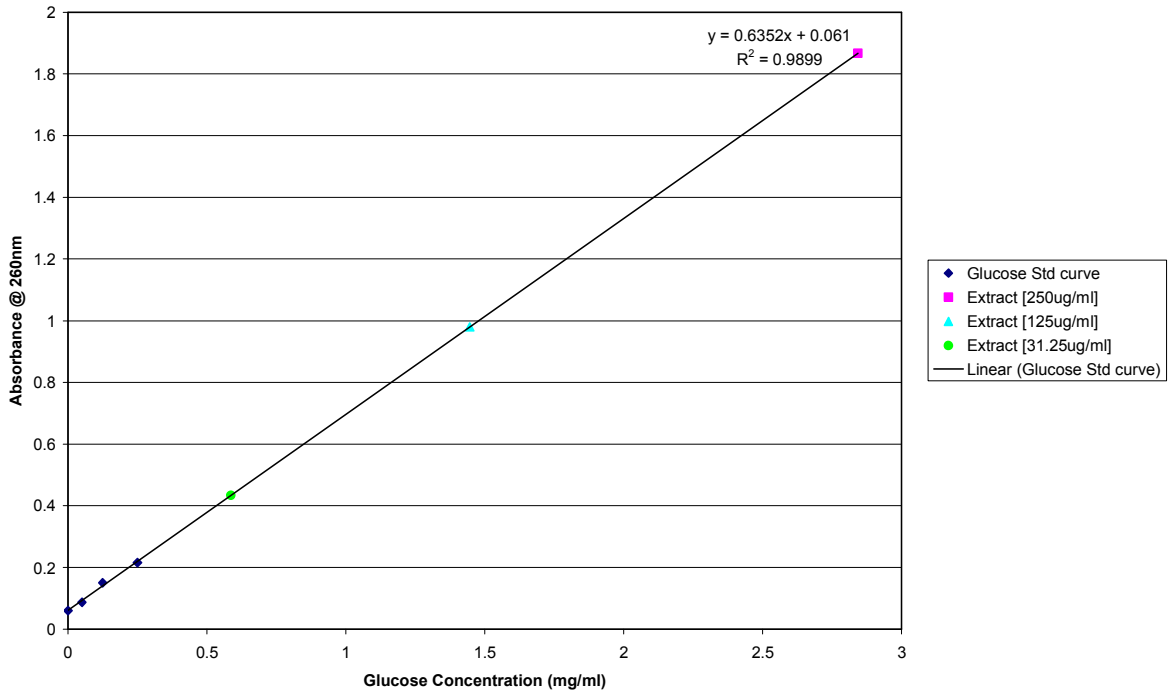

Figure 26: Graph showing the carbohydrate standard curve along with all the *Hypoxis* extracts tested.