An assessment of *Hypoxis hemerocallidea* extracts, and actives as natural antibiotic, and immune modulation phytotherapies.

By

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Declaration

I hereby declare that

" An assessment of *Hypoxis hemerocallidea* extracts

and actives as natural antibiotic, and immune modulation phytotherapies"

is my own work, only submitted to

The University of the Western Cape, with all research resources

used in this project duly acknowledged

by means of complete references.

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Catherine Muwanga

November 2006

Dedication

To my beloved parents, and extended family

who always believed in,

and expected success.

To Elizabeth, Lena and Pearl, my inquisitive children

who encouraged,

and supported me throughout this work.

♦

Chapter one

Overview

An assessment of *Hypoxis hemerocallidea* extracts, and actives as natural antibiotic, and immune modulation phytotherapies

Chapter 1

Overview

Folk healing, since ancient times, has always included medicines of plant origin. Traditional healing practices and herbal remedies have stood the test of time because of their strong cultural ties, and easy accessibility (1). Brandt *et al* (1994), working in rural South Africa estimated that 60-80 % of South Africans consult herbalists, traditional healers, and *sangomas,* before presenting their ailments in medical facilities. Most use crude herbal remedies in the form of water-based extracts, tinctures, and concoctions (2). In African settings, the tendency to self-medicate is highest in individuals afflicted with chronic diseases like *Human immunodeficiency virus* (HIV), cancer, diabetes and arthritis. This trend is aggravated by their poor social-economic situation, ignorance, and cultural beliefs which are often prohibitive to easy access to western medicine.

In the last half of the 20th century herbal remedies, as a form of healing, spread to first world nations where they have been progressively processed, and marketed as formulated pharmaceutical preparations. Studies done in the USA in 1999 revealed that 29-85 % of HIV patients, often well educated, regularly use dietary supplements, and herbal medicines (3). This unprecedented demand for herbal medicines, which appears to be universal, is set to rise even further with the parallel *Human Immunodeficiency Virus* (HIV), and tuberculosis (TB) epidemics. However, because of deleterious side effects, adulterations and interactions with conventional drugs, herbal remedies present an important challenge for clinicians.

Although considered to be 'safe', crude plant-derived medicines contain biologically active molecules with the potential to be harmless, therapeutic or toxic. In comparison, a scientifically formulated drug is a substance, or chemical entity other than food which is capable of preventing, treating or curing disease by causing change in cell, and tissue function to correct the effects of trauma or tissue pathophysiology (4, 5). Such a substance may be of plant, chemical, animal or microbial origin. It therefore follows, that plant- derived preparations, crude or otherwise are also essentially drugs. This underscores the fact that the public needs to be protected from their unregulated use.

During the nineteenth century, drugs and remedies discovered by individuals or small companies were introduced into public use without prior clinical trials. This went on until the Thalidomide disaster changed the entire course of drug discovery in the 1950s (6). Because of the disaster, carefully screened clinical trials, preceded by *in vitro*, and animal experiments, were introduced. As a result, the process of drug discovery has become even longer and more expensive, while the worldwide need for more effective drugs continues to grow (7, 8). The need for new drugs in cash-strapped third world countries has risen dramatically with the incidence of HIV infection, and statistics indicate that the largest market for anti-TB drugs is in third world countries where the financial incentives for drug discovery processes are lacking (7, 9).

According to the World Health Organisation (WHO) statistics, pneumonia still kills 3.9 million people annually, followed by diarrheal diseases at 1.9 million, tuberculosis (TB) 1.2 million, and malaria 0.6 million (10). The mortality figures of these preventable diseases are predicted to rise with the proliferation of multidrug resistant microorganisms in communities with high HIV prevalence, especially without new antibiotics to meet the escalating demand. In addition the number of new diseases has continued to rise in the last three decades. This includes new diseases like Ebola, Lyme disease, Avian flu and above all HIV infection (11).

The dire need for new drugs against TB in sub-Saharan Africa is demonstrated by the Maputo declaration made on the 26th of August 2005 (12). At a conference for the World Health Regional Committee for Africa, held in Maputo Mozambique, TB was given special priority, and declared an emergency in Africa (12). This unprecedented step was taken, following statistical reports indicating that the TB epidemic in Africa had more than quadrupled in the last 15 years to reach an annual fatality rate of 500,000, after outpacing all counter measures taken against it (12). Unless new and more effective drugs are made available soon, the future management of TB in Africa is very bleak (13, 14).

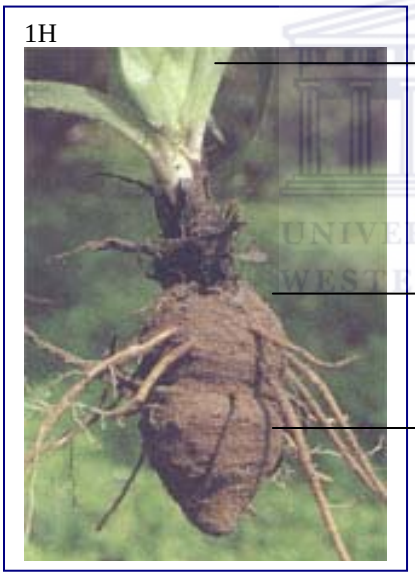
In recent years, in the search for new drugs and escalating public demand, researchers have turned to plant sources for lead molecules (15). *Hypoxis hemerocallidea* is a South African plant with a long history of medicinal use in a wide range of diseases (16, 17, 20-25). The regular use of crude-water based extracts from its corm by traditional healers to treat HIV related ailments remains controversial, and has been condemned by some clinicians (17).

Medicinal history and research into *Hypoxis*

Hypoxis hemerocallidea belongs to the genus *Hypoxis,* from the large lily family *Hypoxidaceae* (18, 19), with the synonyms *Hypoxis rooperii*, yellow star, *Inkomfe* in Zulu, and the street name of magic 'muti'. The name *African potato*, coined by the media, is a misnomer since it has no resemblance to potatoes.

Hypoxis hemerocallidea' leaves grow back in spring, and summer. Its leaves are strap-like, and grow out of a false sheath like stem which extends downwards into the underground corm.

Six-petal star shaped flower hence the name 'yellow star'. The ovary, inferior to the flower, is not visible.



The false stem encloses the leaves in a tubular, membranous sheath made from the remnants of old leaves.

Numerous, adventitious roots grow in concentric circles around the upper part of the rhizome.

The mature vertical rhizome grows to 7-10 cm diameters. It is a swollen, underground stem, whose cut surface is yellow-orange in colour which turns brown with oxidation. When the leaves die away in winter, the rhizome is able to survive the harsh freezing conditions. This tuber-like structure, must have developed as a revolutionary storage organ to survive high stress conditions.

Figure 1: The medicinal plant *Hypoxis hemerocallidea*. The name *hypoxis* comes from the Greek words *hypo* meaning below, and *oxy* meaning base of ovary or fruit. *Hemerocallidea* means resembling *hemerocallis*, the day lily. *Hypoxis hemerocallidea*, formerly known as *Hypoxis rooperii*, is known as *Inkomfe* in Zulu or kaffertulp in Afrikaans (18-20). Its underground rhizome, which is a storage structure, takes 2-3 years to mature to 7-10 cm in diameter. Extracts from the mature fresh rhizome yield between 3.5-4.5 % Hypoxoside, the main active compound (21). Hypoxoside, which is highly cytotoxic, is most likely a deterrent that must have developed over time to discourage animals from eating the plant, especially its rhizome. The variations in concentration of active constituents observed with *Hypoxis* depend on the season and age of the rhizome.

There are over 90 *Hypoxis* species spread throughout sub-Saharan Africa, North and Southern America, South East Asia, and Australia. Southern Africa holds a diversity of 45 species found in all regions except the Northern Cape in South Africa, and Botswana (18-22). *Hypoxis hemerocallidae* is one of the larger species of *Hypoxis* that grows to a height of 10-50 cm in summer, usually at altitudes of 50-1800 m above sea level (18, 22). The larger species, like *hemerocallidea, colchicifolia* and *obtusa*, have been so extensively harvested by local communities for medicinal purposes that ethno-botanists are concerned about the survival of the wild species (21, 23). The preservation of its wild species is of vital importance since *Hypoxis* are difficult to cultivate, they do not easily propagate vegetatively, and seed dormancy is not easy to break. Further, *In vitro* tissue culture propagations that have been attempted, and the cultured plants cultivated have had smaller rhizomes, and less output of Hypoxoside (23).

Hypoxis hemerocallidea, the African potato*,* is the most sought after species for traditional medicinal preparations, and the crude water-based extract from the crushed rhizomes is increasingly regarded as an effective tonic for not only healthy individuals, but for mostly HIV and AIDs sufferers (17, 21-25). Extracts from crushed *hypoxis* rhizome have been used for generations to treat a wide variety of ailments including the common cold, hypertension, psoriasis, urinary tract infections, prostate diseases, gastrointestinal complaints, and even mental disorders (16, 17, 2-24). Reports of its traditional use, especially for prostate diseases seem to be universal, having come from various parts of the world (24-25).

Keen scientific interest in *Hypoxis hemerocallidea*'s effects on the prostate, dates back to the early 1950s (24, 25). Since then on going research, although slow and isolated, has revealed the various pharmacological activities of at least two of its compounds. The first recorded scientific report of the medicinal activity of *Hypoxis* was in 1926 concerning the species *H. brasiliensis,* an edible tuber from South America (23). Scientific reports about *H. hemerocallidae* (*H. rooperii*) first appeared in 1930 from Natal in South Africa (23), but focused scientific research didn't follow until Liebenberg, a South African, brought scientific attention to *Hypoxis hemerocallidae* in the 1950s. Liebenberg who had witnessed the therapeutic benefits of the plant in rural farming communities, where it was widely used for prostate disease, was determined to seek scientific proof of its efficacy (24, 25).

Active compounds of *Hypoxis hemerocallidae*

Medicinal plants contain a variety of compounds ranging from alkaloids, terpenes, glycosides, tannins, and carbohydrates. Only two of the active compounds from *Hypoxis hemerocallidea* have been described so far, although it holds a number of other unidentified compounds which could be of further benefit (21-23).

Hypoxoside

The research that followed Liebenberg's introduction of *Hypoxis* to medical scientists in Dusseldolf Germany revealed that the active compound was a plant lipid from the plant's rhizomes. Betasitosterol (BSS), a plant sterol was later isolated and went on to become an effective treatment for Benign Prostatic Hypertrophy (BPH) (24, 25). BSS is still widely used as Hartzol in Germany and Western Europe whose South African equivalent is the Moducare preparation (24, 25).

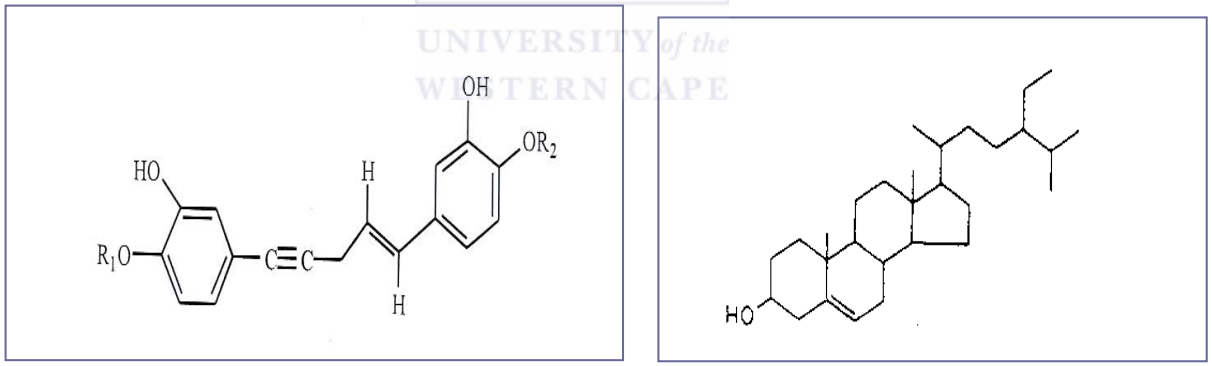
Thirty years after BSS was described, scientists in Southern Africa described an unusual molecule, also from the hypoxis rhizome (21). The dicatechol molecule Hypoxoside, first isolated from the corms of *Hypoxis obtusa* by Marini-Bettolo in 1982, and from *Hypoxis hemerocallidae* (*H. rooperii*) at the University of Natal in 1984 (21, 25), has intrigued scientists since then. The unusual molecule, a precursor for Rooperol, has been found in *H. hemerocallidae*, *H. rigidura, H. latifolia, H. acuminata, H. nitida,* and *H. acuminata* (21). However, not all *Hypoxis* species carry Hypoxoside (21, 22).

The unique Hypoxoside molecule consists of two benzene rings, with a pent-4-en-1-yne link, and a central methyl group (21, 26, 27, Figure 2A). It is believed that this unusual structure has a bearing on its biological activities, and toxicity, as well as its baffling pharmacokinetics observed in different animal studies (26, 36, 37).

Rooperol: Intricate pharmacokinetics

In 1995 Albrecht *et al* (26) conducted laboratory studies focusing exclusively on the pharmacokinetics of both Hypoxoside and Rooperol. Hypoxoside, when taken orally, is hydrolyzed by bacterial beta-glucosidase in the intestinal tract, to its bioactive aglycone Rooperol (21, 26). After the enzymatic cleavage of the two glucose molecules from Hypoxoside, it isn't clear how Rooperol, which is more lipophilic, is absorbed.

However, serum levels analyzed during the study, indicated that Rooperol was conjugated by the cytochrome P-450 system in the liver to a variety of secondary metabolites, including diglucuronides, and monoglucronides (26, 27). The conjugation appears to be a species-specific biochemical process, determining how much of each metabolite is excreted either via the biliary system back into the gastro-intestinal tract, or via the systemic circulation and kidneys (26). The study further revealed that neither Hypoxoside, nor Rooperol or their metabolites reach the systemic circulation in rodents, and small mammals. In such mammals Rooperol appeared to be completely metabolized in the liver, and all its metabolites excreted via the biliary system (26, 27).



A **B**

Figure 2 (A). The unique Hypoxoside molecule. Hypoxoside bears two glucose molecules at R1 and R2, which are cleaved off by β-glucosidase hydrolysis to form Rooperol. The glucose molecules make the Hypoxoside molecule more hydrophilic, while Rooperol is more lipophilic, and more absorbable than its precursor. Rooperol carries a hydroxyl molecule at R1 and R2. The two benzene rings, with a pent-4-en-1-yne link, and a methyl group may be responsible for the unique biological activities of the two molecules (21).
(B). The Betasitosterol molecule. Betasitosterol, a plant-derived lipid molecule, differs from cholesterol by only one ethyl group at carbon 24 in the side chain (28). The steroid nucleus, common to all human sex hormones, makes Betasitosterol an ideal mimic for human sex hormones, but doesn't explain its immunostimulatory activity (28).

Residual traces of Rooperol's metabolites were easily detected by High Performance Liquid Chromatography performed on bile samples, and portal vein blood. These findings indicate that rodents are unsuitable research models for the systemic effects of Rooperol, and Hypoxoside (26). In human subjects Rooperol's metabolites were detected in high concentrations in the systemic circulation, and urine (26, 27). But, Rooperol proved to be elusive, and could only be detected in human urine after incubation of urine samples with exogenous beta-glucuronidase enzymes which converted the inactive metabolites back into Rooperol (27).

It was therefore proposed that cellular diglucronide enzymes, in the immediate vicinity of targeted cells such as cancer cells (27), maybe able to convert the metabolites back into Rooperol, which is cytotoxic. If proven right, this hypothesis will make Rooperol a very highly selective therapeutic agent sparing normal tissue from injury during cancer chemotherapy.

In vitro research

Review of the literature indicates that most research on Hypoxoside has focused on its dramatic cytotoxic effects (27). Purified samples of Hypoxoside tested by the National Cancer Institute (NCI), in Bethesda USA in 1989, proved to be highly effective against a panel of 60 cancer cell lines. The *in vitro* disease orientated primary antitumor screen, consisting of 60 human cancer cell lines (experiment ID: 8909NS63), revealed that at an average concentration of only 8 μg/ml, Hypoxoside can inhibit 50 % of all cells tested (27). The cells tested included specimens from the most resistant human solid tumors. Samples included small-cell lung carcinoma, ovarian carcinoma, breast carcinoma, gastric carcinoma and colon carcinoma.

These findings were further confirmed in a British laboratory at The Huntingdon Research centre on five human cancer cell lines including colon, breast, uterus, melanoma and non-small cell lung cancer (NSCLC), with results very similar to the Bethesda study (27). Again, the cell lines selected represented some of the most resistant human tumors, where surgical intervention is the only option. In the Huntingdon study Hypoxoside inhibited 50 % of all 5 cancer cell lines at 4.1-8.2 μg/ml, and the NSCLC was found to be the most sensitive at only 1.1 μg/ml of Hypoxoside (27).

At the University of Cape Town, to observe the cytotoxic effects of Hypoxoside *in vitro*, human melanoma cell cultures were incubated with 50 μg/ml of Hypoxoside, and photographed at 2 hourly intervals, over a 24-hour period under an inverted-phase-contrast microscope. Cellular changes starting with cytoplasmic vacuoles were observed after 12 hours, followed by loss of individual chromosome integrity, and morphology. Progressive cellular disintegration was observed after 24 hours, proceeded by punctured holes in cell membranes, empty mitochondria, and fragmented nuclei (27).

A 1997 *in vitro* study by Guzdek *et al* in Cracow Poland, illustrated the unique activity of Rooperol on cells of the immune system. Phagocytic cells, including alveolar macrophages, were assessed for intracellular pathogen killing, and cytokine output before, and after exposure to Rooperol's tetra acetate esters (28). The secretion of inflammatory cytokines, potentially harmful to normal tissue, was inhibited in the presence of Rooperol esters, with the promotion of anti-inflammatory cytokine profiles. It was further observed that Rooperol's esters inhibited the synthesis of nitrous oxide, a toxic but vital compound in the macrophages' killing of intracellular pathogens (28). Rooperol's immunomodulatory activity, in particular its anti-inflammatory activity, and its not-so-well understood effects on cell-mediated immunity (CMI), warrant further in-depth research.

Betasitosterol

BSS is a steroid plant fat, which is ubiquitous throughout the higher plant kingdom. With a structure similar to cholesterol, and differing only by the presence of an ethyl group at the 24th carbon in the side chain, BSS competes with cholesterol for cholesterol esterase in the intestinal epithelial cells for absorption (29). Unlike cholesterol, phytosterols cannot be synthesized endogenously in man therefore, a daily intake of at least 100-300 mg consisting of 50 % BSS, and 30 % of its close cousin camphesterol, and 2-5 % Stigmasterol is a physiological requirement (29). It has been observed by researchers, that only 5 % of that intake is absorbed, which amounts to a total 800-1000 times less than cholesterol absorption (29).

Even after absorption, phytosterols are actively, and effectively expelled by the efflux activity of P-glycoprotein in the intestinal wall, and the ABC transporters in hepatic cells, so that excess amounts of phytosterols never reach the systemic circulation (28, 30).

Only in congenital mutations, or deletions of the gene coding for the ABC transporters do excess sterols accumulate in the body as subcutaneous xanthomas in a rare disease called sitosterolaemia (28). Excess BSS, like cholesterol, is atherogenic, and accumulates in tissues including blood vessels like the coronary, aorta, common and internal carotid arteries (28). However, the physiologically controlled minimal bio-available amounts are just sufficient to provide cardio-protective effects by lowering serum cholesterol, low density lipoproteins (LDL) and very low density lipoproteins and maintenance of immune homeostasis (VLDL) (29, 30, 31, Table 1). Phytosterols are effective estrogen mimics capable of competing with estradiol for beta-estrogen receptors (ERβ), and alpha-estrogen receptors (ERα) on estrogen sensitive tissues (32, 33). This makes phytosterols, like BSS, potential therapeutic agents in the management of oestrogen sensitive cancers which include breast and endometrial carcinoma (33, 33).

It is believed that BSS's success in the treatment of BPH is mainly due to its competitive blockade of testosterone in the prostate gland (24, 25). Details of a 1993 randomised, placebo-controlled, double-blind trial provided proof of BSS's efficacy in the treatment of BPH (25). The trial was carried out on a group of patients with BPH, in Germany. 200 healthy male patients, with BPH as the only problem, were followed up for 3-6 months on BSS, with a control group on placebo. A definite improvement in urinary flow, and a significant fall in residual urine were observed in a matter of weeks in those on BSS, while the placebo group had no improvement. In contrast Finasteride, a standard pharmacological treatment, takes over 6 months to show similar results remains unpopular due to side effects of reduced libido, erectile dysfunction, and gynaecomastia (25). The plant-derived BSS had no side effects over the same period.

The physiologically controlled bioavailability of phytosterols in humans provides minute serum concentrations which appear to be merely adequate for normal immune system function (29-31). A study published from Cape Town by Bouic *et al* confirmed the immunostimulatory effects of BSS in *in vitro* experiments (30, 31), and that concentrations of BSS in the range of 0.3-1.02 mg/100 ml are adequate for optimal immune system function (31). In further experiments it was demonstrated that BSS's immunomodulatory activity leads to CD4+ T-lymphocyte proliferation both *in vitro* and *in vivo*, and therefore CMI (30, 31).

The mixture of BSS: (betasitosterol-glucoside) BSSG was found to induce the secretion of IFN-gamma and IL-2 from activated T-cells, thus promoting a Th1 cytokine profile while inhibiting the Th2 cytokine profile (31). This is a feature that can be exploited to modulate protective CMI in chronic diseases like TB, and those like asthma, and autoimmune diseases where the suppression of Th2 cytokines may be therapeutic (31, 34, 35).

To confirm these promising results the research team conducted small pilot trials involving patients with a range of infections including TB, HIV infection, *Human papilloma virus*, and non-infectious diseases like rheumatoid arthritis, and allergy. They used the BSS: BSSG combination, in its optimal ratio of 100:1, administered orally over a period of six months to half of the 47 patients with drug-sensitive TB in the trial. Hematological findings, in a few weeks, included higher lymphocyte, eosinophil and monocyte counts in the BSS group (30, 31). While erythrocyte sedimentation rates (ESRs) remained high in the placebo group, the sterol group had significantly lower ESRs, gained weight, and had faster radiological improvement (24, 30, 31, Table 1).

To demonstrate the beneficial effects of BSS in HIV disease, 15 domestic cats with feline retrovirus infection were treated with BSS while 15 others were left untreated for a period of 3 years. While the control group deteriorated with falling $CD4^+$ T-lymphocyte counts, and steady disease progression, the BSS group maintained stable $CD4^+$ T lymphocyte counts (31).

An important observation made during the same experiment was that the synergistic combination of BSS: BSSG enhanced natural killer cells' (NK) activity against cancer cell lines *in vitro* (31). NK cells, with the help of $CD4^+$ T-helper lymphocytes, and $CD8^+$ cytotoxic T-lymphocytes, attack and kill cancer cells *in vivo* (36). The finding that BSS up regulates NK cells' activity against cancer cells confirmed previous findings on colon cancer cells by Raicht *et al* in 1980 (37) When BSS is added to the diets of mice, and rats that are treated with colon carcinogens it significantly reduces cell proliferative changes and tumor yields (37) In similar experiments, phytosterols have been reported to inhibit the growth of breast cancer cell lines *in vitro* and *in vivo* experiments on rodents (38).

Therapeutic manipulation of the host immune response, as an adjunctive therapeutic intervention, is a major goal in the management of cancer. Awad *et al* demonstrated the effects of dietary phytosterols on breast cancer cells in a group of mice (38). A breast cancer cell line, MDA-MB-231, was inoculated into the right inguinal mammary fat pad of mice fed on phytosterols. A control group of similar mice was fed only cholesterol. In 8 weeks the phytosterol group had 40 % less serum cholesterol and their tumor mass was 33 % smaller with 20 % less metastases, which led to the conclusion that phytosterols can slow the rate of breast tumor growth, and spread (32, 38). Although the exact mechanism of BSS activity against cancer cells is unknown, it is significant in view of potential synergism, or additive effects with the highly cytotoxic activity seen with Hypoxoside against cancer cell lines.

Table 1. Biological effects of Rooperol and Betasitosterol

Pharmacological activity or Clinical outcome	Rooperol and BSS	References
Cardiovascular system:		
1.Transient inotrophic effects without increased HR.....	Rooperols catechol mioety.	44
2. Cardioprotective effects. Lowering cholesterol levels.....	BSS competitive absorption.	29
Immune system:		
1. Sustains and maintains predominant Th1 cytokine profile....	BSS	30, 31
2. Stimulates CD4+ lymphocyte proliferation	BSS	24, 30, 31
3. Suppresses acute phase cytokines.....	Rooperol	28
4. Might suppress respiratory burst, and nitrous oxide activity in macrophages *in vivo*.....	Rooperol	28
5. Nutritional immunostimulant.....	BSS	31
Anti-inflammatory activity:		
1. Blocks acute phase inflammatory cytokines.....	Rooperol	28
2. Anti-inflammatory.....	Rooperol, BSS	16, 28, 30
3. Anti-oxidant activity.....	Rooperol, Hypoxoside	16
Endocrine , reproductive system:		
1. Diminishes effects of testosterone on prostate.....	BSS	25
2. Used in the treatment of male alopecia, balding.....	BSS	67
3. Potential contraceptive? male/female.....	BSS	41
4. Affects DHEA and cortisol ratio.....	BSS	24
Cancer:		
1. Interferes with chromosomal chromatin during miosis. Perforates cell walls and increases free oxygen radicals.	Rooperol	27
2. Antineoplastic properties.....	Rooperol, BSS	27, 32, 37, 38
Central nervous system:		
1. Appetite stimulus.....	BSS	24, 31
2. Mood enhancement.....	BSS	31
3. Anticonvulsant.....	Rooperol	68

*A literature review revealed significant overlapping between the pharmacological activities of Rooperol, and Betasitosterol. Despite their entirely different pharmacokinetics, the two compounds share numerous clinical outcomes. This overlapping activity highlights the importance of synergism in the study of *Hypoxis*, and may explain the efficacy of apparently minute concentrations of active compounds in crude extracts.

* HR-Heart rate, BSS- Betasitosterol, DHEA-dehydroepiandrosterone, TB- tuberculosis, HIV- Human immunodeficiency virus

Toxicology studies

The continuing use by the public of *H. hemerocallidea* in crude extracts, which are reportedly toxic, has raised serious scientific concern. Review of the current literature does not reveal full detailed toxicological research into *Hypoxis* species, but there has been some research done on hepatorenal toxicity, and bone marrow suppression by Rooperol, and Hypoxoside (16, 39, 40).

Due to their ubiquitous presence in higher plant species, which leads to their presence in most crude medicinal preparations, phytosterols have been widely researched as bioactive compounds as well as dietary supplements. BSS has been included in OTC (Over-the - counter) supplements for over 20 years (24, 25) and has been found to be free of serious side effects apart from endocrine disruption (41). Studies on the reproductive systems of rodents revealed some disturbing results, where BSS administered over a period of weeks caused sterility in female rats, and altered sperm morphology in the males (41). Similar effects, although not yet documented, are highly likely in larger mammals, including humans, in the long-term use of BSS. Studies done on rats by Malini *et al* in Madras, India confirmed the cardio-protective, hypo-cholesterolemic effect of BSS in rodents (42) and effects on rodent uteri (43). Since hormones execute their functions in such minute quantities, and with their effects being so insidious, and subtle, clinicians can easily miss the clinical effects of endocrine disruption.

In 1995, researchers at Karl Bremer Hospital, in Cape Town studied the pharmacokinetics, and toxicity of a hypoxoside preparation, in a small-randomised clinical study, in 24 lung cancer patients. A standardized *Hypoxis* extract, in 200 mg capsules was administered orally, in three divided doses of 1200, 2400, and 3200 mg per day to three different patient groups. The preparation contained 50 % Hypoxoside, and a synergistic combination of 10 % BSS, and 0.1% BSSG (43). Patients on the preparation were closely monitored over an 11-day period, and over that period, in spite of the large doses of Hypoxoside involved, no toxicity was reported (43). This small study confirmed three very important facts about Hypoxoside.

Neither Hypoxoside nor Rooperol was detected in circulation over those 11 days. Secondly, the lack of short-term toxicity, and the fact that toxicity if it occurs, is not immediate but is likely to be a cumulative, insidious process that may involve the liver, kidney, or bone marrow. Finally, the presence of the two sterols may have contributed to the lack of toxicity, and it was during the course of this tentative trial that lymphocyte proliferation was noted in response to phytosterol stimulation (42, 31) which paved the way for further detailed research by immunologists at Stellenbosch University in 1996.

Detailed toxicological studies on Rooperol are lacking. The chemical structure of Rooperol, with two catechol moieties, indicates potential sympathomimetic activity, as was demonstrated on the cardiovascular system by Coetzee *et al* who confirmed this fact in a very short study at the department of anesthesiology at Stellenbosch University Cape Town (44). Rapid intravenous infusions of Rooperol administered to anaesthetized baboons l caused a moderate, transient increase in stroke volume, and cardiac output, without inducing any tachycardia while hypoxoside had no effect (44). However, these cardiovascular effects were considered to be too transient to be of clinical importance.

In 2005, researchers at the University of Natal South Africa demonstrated renal toxic effects of the aqueous *H. hemerocallidea* extract on rat kidney. Their study focused on the short, and long-term effects of the aqueous extract administered in intravenous, and oral doses to separate groups of rats. The urine output is reported to have fallen significantly both in the acute, and chronic phases (40). A similar fall was noted in electrolytes secreted in urine, followed by elevated serum creatinine levels in affected rats (40). These findings suggested possible renal tubular damage.

However, the same investigators, using the method of Lorke, later tested graded oral *H. hemerocallidea* aqueous extracts on mice at doses ranging from 50 to 1600 mg/kg. The mice were observed for 48 hours in comparison to a control group fed on water alone. They found the extracts to be safe below 1600 mg/kg, and only very high doses of 1948 ($\pm$ 57) mg/kg were found to be toxic, or lethal. A final conclusion was then reached that aqueous extracts, taken orally in moderate doses, were after all free of toxicity (39, 40). Their latest results were attributed to better technique, and facilities.

Literature published in early 2005, implicated *Hypoxis* extracts in pharmacological interference with the function of intestinal p-glycoprotein, and inhibition of cytochrome P-450 in hepatocytes (45). Interference at these sites has the potential for significant disruption of drug substrates *in vivo*. This implies that the use of whole *Hypoxis* extracts may disrupt Rifampicin's bioavailability in TB treatment, and the protease inhibitors in Highly Active Antiretroviral Therapy (HAART) (45). There is therefore, a genuine risk of treatment failure either with HAART or TB treatment, with the likely development of resistant species. HIV clinicians have expressed great concern regarding the indiscriminate use of *Hypoxis* extracts by their patients (17). The continued support for herbal medicines from the Southern African Development Community (SADC), consisting of 14 member states, has added to the controversy involving herbal remedies in HIV medicine (17).

It is interesting to note that most reports of toxicity involve isolated active compounds from *Hypoxis*, rather than whole extract. Studies on whole extracts have not proved to be as toxic, or prone to side effects as the isolated Hypoxoside, Rooperol, and Betasitosterol. Similar reports, comparing toxicity between isolated compounds and whole extracts, has been made on a number of plant remedies (46). Between 1991-1994 a number of women on a slimming preparation in Belgium, were treated for renal failure. The slimming product contained the Chinese herbs, *Guang Fang Ji* (Radix Aristolochia Fangchi), and *Hang Fang Ji* (Radix Stephania tetrandra). Investigators reported that the plants, were used in inappropriate doses, for purposes for which they were never used before, especially in combination with mainstream drugs (46). Further, there is always a significant differences between plant species, which may appear to be identical.

The possibility of toxicity from Hypoxoside and Rooperol preparations, and extracts underscores the requirement of long term research in primates, and not rodents, rabbits or dogs, to resolve the crucial issue of toxicity (43, 44). Better understanding of their pharmacokinetics, toxicity profiles, and therapeutic windows, will enable researchers to focus on their promising therapeutic benefits. These benefits are likely to include not only immunomodulation, and cancer therapy but also other unexplored possibilities like antimicrobial activity. All plants with a long history of traditional use are currently being screened for antimicrobial activity but very little has been done for *H. hemerocallidea.*

Growing antibiotic resistance, and emerging infectious pathogens

Only seventy years ago in the 1930s, half a century after Robert Koch's landmark discovery of the TB bacillus, the introduction of the sulfonamides marked the beginning of the dramatic antibiotic era (6, 47). The discovery of a sulphonamide antibiotic, M&B693 at the Bayer Chemical Company in Germany, was made by Gerhard Domagk, a German scientist after extensive research on dyes (6). Before then, all infections, including TB and streptococcal pneumonia, were associated with high fatality rates over 27 %. The discovery lowered the mortality rate to 8 % (6). Rapid advances followed, leading to the introduction of penicillin at the end of the Second World War, in 1945 and the first TB drug, streptomycin, in the early 1950s (47, 48).

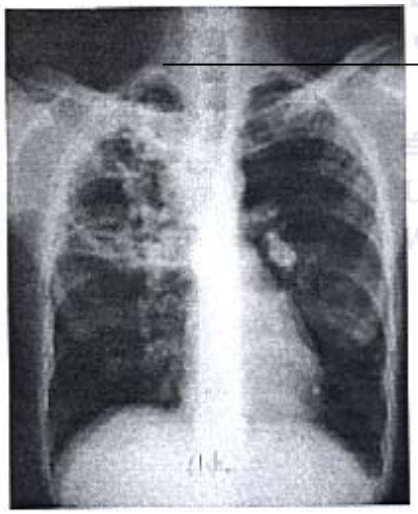
This was a major landmark in the previously bleak history of tuberculosis. But, the initial success achieved with streptomycin was quickly followed by the development of resistance, and disease relapse. Streptomycin is markedly bactericidal on actively dividing mycobacteria both *in vitro*, and *in vivo*, but only in the extracellular environment (47). The rapid development of mycobacterial resistance to streptomycin was halted by the introduction of a second highly bactericidal drug, isoniazid in 1952 (47, 48). From then on, the two drugs formed the backbone of TB treatment regimens for the next two decades, until the discovery of Rifampicin in the 1970s, which replaced the more toxic streptomycin (47, 48, Figure 3).

Today, despite major advances in conventional medicine the growing problem of bacterial resistance has set back the clock on treatable infectious diseases. Emergent multidrug-resistant TB (MDR-TB) strains are now a real threat to human health (49-55, Table 2).

TB: A perpetual plague

Mycobacterial DNA has been detected in the lungs of Egyptian mummies dating back to 1550 B.C. (50). For centuries the greatly feared disease, referred to as the white plague, remained incurable till the middle of the twentieth century. Prior to this, TB probably the oldest of human diseases, had remained an incurable mystery. The tubercle bacillus was first described by Robert Kock in his landmark lecture in 1882, in which he described it as a fungus (47). It took another twenty years for scientists to isolate the TB bacillus.

In 1905, a TB strain called H37 was isolated from a TB patient, and was thereafter used in *in vitro*, and animal research. The live Bacillus Calmette and Guerin (BCG) vaccine was one of the many results of this research (47). Researchers noted that some strains of H37 were non-pathogenic, while others like the H37Rv were more virulent (47).



Chest radiograph of active pulmonary tuberculosis

Apical cavities, a classical feature of advanced tuberculosis. As Caseous, necrotic lung tissue is expelled via the bronchi, in the form of purulent sputum, large cavities form. The exposed cavity wall then has access to more oxygen, and increased oxygen tension, which encourages faster bacillary growth, and multiplication (51). This classical picture of pulmonary tuberculosis is not seen in TB presenting in advanced HIV disease, and AIDS. Therefore TB in AIDS is often missed, or diagnosed too late (52, 53).

First line TB drugs	**One RHZE tablet = (R 150 mg, H 75 mg, Z 400 mg, E 275 mg)**	**Second-line TB drugs**
1. Isoniazid 2. Rifampicin 3. Pyrazinamide 4. Ethambutol 5. Streptomycin 6. Amikacin 7. Rifabutin	Directly observed treatment (DOT) was introduced by The World Health Organisation (WHO) in 1994, as a counter measure against the global spread of TB. RHZE, are the first line drugs used in the first-two Months of intensive treatment (48, 50, 54). A 70 kg adult needs 4 tablets of RHZE, 5 times weekly, for two months. Patients should be smear-negative at the end of 8 weeks. Only RH is continued for the second phase of 4 months (48, 50, 54). Strict compliance is a necessity to prevent the emergence of microbial resistance.	Cycloserine Ethionamide Ciprofloxacin Capreomycin Levofloxacin Moxifloxacin ofloxacin

(RHZE) R-Rifampicin, H-Isoniazid, Z-Pyrazinamide, E-Ethambutol

Figure 3. The current treatment of tuberculosis

By the early 1980s, tuberculosis was on the decline in most countries around the world, and most TB control programmes were beginning to shut down (47, 55). Beginning in the early eighties, this favorable trend was gradually reversed following HIV infection, and resulted in TB becoming the leading opportunistic infection in HIV patients (49, 52, 55, 56).

Latest figures indicate that there are 1-2 billion people carrying latent TB infections around the world, and approximately 16 million are carrying active disease (48, 56, 57). It is not surprising therefore, that the resurgence of TB has been more dramatic in communities where the prevalence of latent TB infection was already high.

Tuberculosis was not a serious problem in Southern Africa before European colonization. The disease is reported to have spread rapidly during the 19th century, and became a notifiable disease in South Africa for the first time in 1921 (47). The incidence of TB grew rapidly from the initial recorded rate of 43/100,000 in 1921 to 365/100,000 by 1958.

With the introduction of curative treatment, and effective preventive programs the TB incidence rate had fallen to 162/ 100,000 by 1986 (47). Thereafter a rapid reversal followed in the wake of the HIV epidemic, and today the latest figures stand at 562/100,000 in the Western Cape (47). In the background to the two parallel TB, and HIV epidemics there is another ominously silent epidemic emerging as MDR-TB, and lately, extremely resistant tuberculosis. The latest statistics indicate a MDR-TB prevalence rate of approximately 1-2.5 % in South Africa (Table 2). Another disturbing statistic is the TB/HIV co-infection rate of over 55 % which indicates that TB drug-resistance is very likely to rise in the HIV population, especially in communities where treatment adherence is difficult to maintain (49, Table 2).

Table 2. The prevalence of drug resistant TB presented as a percentage of tested TB isolates by country from 1996 to 1999.

Country	Overall resistance rate	Resistance to two drugs	Multidrug resistance
Uruguay	4.6	0.4	0.2
Botswana	7.7	2.5	2.0
South Africa	10.2	3.5	2.5
Uganda	22.1	8.1	0.8
India	24.1	13.4	7.1
Estonia	40.8	26.8	15.1

Data obtained from WHO/ International union against tuberculosis, and lung diseases (IUATLD). *Manson's tropical disease,* 21st ed., Saunders 2003 (11)

Similar reversals in declining trends in TB statistics have been reported from industrialized countries. In the United States, a steady downward trend in the incidence of TB was observed between the sixties and the late eighties, followed by an upward trend in the late eighties (55).

These deleterious changes in the global epidemiology of TB came about because of the following reasons:

1. The rapid emergence of a population of people highly susceptible to opportunistic infections including TB,
2. The emergence of strains of tuberculosis resistant to current TB drugs,
3. The inability of TB control programs to trace and treat active TB cases promptly and adequately,
4. The lack of alternative, cheap and effective drugs to treat both MDR, and latent TB,
5. Lack of an effective vaccine for TB,
6. Growing poverty, overcrowding, and the displacement of people by war,
7. The ease of world travel and faster disease transmission.

Tuberculosis and HIV co-infection

TB is the only opportunistic infection that occurs throughout HIV infection, irrespective of CD 4^+ T-lymphocyte counts (14, 49, 52). It is the most prevalent HIV co-infection, and the leading killer of HIV-infected individuals in sub-Saharan Africa. Up to 60-70 % of all HIV cases in sub-Saharan Africa are co-infected with TB (49, 52, 57).

The description, and mapping of the genome of the H37Rv tuberculosis strain in 1998 was a landmark step in the understanding and research on *Mycobacterium tuberculosis* (58). It has enabled scientists to study specific therapeutic strategies targeting the bacillus' biochemical pathways, and virulence genes. Only five years previously, in 1993, and only ten years into the HIV epidemic WHO had declared TB a global emergency (48, 58) Highly efficient resistance determinants were found in the TB genome, including genes for beta-lactamases, and ABC super-family efflux pump systems (58).

These genetic features, combined with a highly hydrophobic, and impenetrable cell wall, make the tubercle bacillus a formidable pathogen. TB researchers have always been frustrated with the slow growth of the bacillus, its ill-understood dormant state, the lack of suitable animal models, and poor funding.

Table 3: The immune response to TB co-infection with HIV

Cytokines and cells	Normal subjects	TB patients	HIV/TB	Advanced HIV
CD4+ cells/ mm^3	759 ± 244	569 ± 239	201 ± 162	130 ± 152
TNF-alpha (pg/ml)	231 ± 284	370 ± 453	232 ± 385	21 ± 42
IFN–gamma(pg/ml)	7.5 ± 14	10 ± 16	2.2 ± 6	01 ± 0.1
IL-12 (pg/ml)	0.6 ± 1.5	12.5 ± 10	4 ± 5.5	2.2 ± 5
IL-10 (pg/ml)		171 ± 253	160 ± 575	

*The cytokine profiles in supernatants of peripheral blood mononuclear cells (PBMC) cultured, and stimulated *in vitro* with mycobacterial antigens (PPD), and heat-killed *M. tuberculosis*, were measured by ELISA. Patients co-infected with both infections showed depressed production of the key Th1 cytokines IL-12, and IFN-gamma. There is a progressive decline in the $CD4^+$ T-lymphocyte profile, which contributes to immunodeficiency.
Data adapted from the 2002 9th conference on retroviruses, and opportunistic infections. Swaminathans *et al*, session 82: 627-w (53).
HIV- Human Immunodeficiency virus. PPD-Purified protein derivative, ELISA- Enzyme-linked immunosorbent assay. IL-interleukin.

Ironically, the most deleterious effects of TB on the host immune system occur at relatively high lymphocyte cells counts, before full blown AIDS ensues. In the initial stages, lymphocytes, and antigen presenting cells (APCs) are caught in a sustained state of hyperactivation which makes them highly vulnerable to HIV infection (52). Aggressive TB infections induce a shift from protective CMI to the less protective Th2, humoral cytokine profile. This shift accelerates HIV viral replication, and disease progression (14, 52, Table 3).

The worldwide expansion of an immuno-compromised population:

The prevalence of HIV/ AIDs in a population can be used as a surrogate marker to determine, and predict the incidence of TB in the same population. Our understanding of the immuno-pathogenesis of both disease processes will contribute significantly to the management of both diseases, and the discovery of novel immune therapies, or alternative treatment strategies. The vicious cycle shared by the two parallel epidemics can only be broken by effective prevention, and treatment of both diseases.

Therefore, new therapeutic approaches which are desperately needed will most likely involve conventional drugs as well as novel adjunctive therapies. Since the immune system has been shown to play a significant role in the pathogenesis of both diseases, it will have to be altered, modulated, or stimulated in various ways to combat HIV, and TB infections.

The immune system is an evolutionary defense system that has contributed to the survival of different species in changing harsh environments. It consists of the non-specific innate system, and the more specific acquired system made up of cell-mediated immunity (CMI) and, humoral immunity (36, 59). However, over the years, the use of immunosupressant drugs in transplant medicine, chronic inflammatory diseases, and autoimmune disease has contributed significantly to the growing population of infection-prone people. In addition, Third World populations are often malnourished, carry chronic helmintic infections, and are prone to frequent migrations in war situations, and overcrowding. It is not surprising, therefore, that the incidence of AIDS, and TB has risen so sharply in recent years. Further, recent surveys, in 2005 by the Center for Disease Control (CDC), revealed a total of 1,407 known human pathogens, 177 of which are emerging infections (10, 11). The bulk of these diseases affect human populations residing in developing countries.

It is not surprising therefore that cytokines have come under intense investigation in the search for novel immunomodulatory therapies including growth factors, monoclonal antibodies (MAB), and vaccines (60-63).

Cytokine biology

Cytokines are a family of small glycoproteins of molecular mass 15,000-25,000 Da, consisting of 145-166 amino acids (60-61). They are secreted by activated lymphocytes, macrophages and fibroblasts in response to antigenic stimuli, or synergistic cytokine stimulation between cells of the immune system. Their influence in disease microenvironments range from autocrine, and paracrine activity, to systemic hormone-like effects (62). In the initial stages of acute infections tumor necrosis-factor (TNF), interleukin (IL)-6, and IL-4 induce liver cells to produce acute-phase proteins, and the same cytokines reach the hypothalamus to influence temperature control (62, 63).

It is now possible to study specific cytokines like interferon (IFN)-gamma, as surrogate markers in disease processes (62, Table 3). Cytokine profiles indicate which patients have had previous exposure to TB, those with active disease, those defaulting from treatment, and those who are immunocompromised (14, 52, Table 3). Using IL-10, a regulatory anti-inflammatory cytokine, it is possible to identify who is likely to progress to active TB, and to predict disease outcomes (64). Interferon (IFN) gamma and IL-12 are key cytokine in CMI, and reliable prognostic indicators in both HIV, and TB disease (65, Table 3).

Cytokines for cell mediated immunity

The interferons (IFNs), first discovered in the 1950s, were the first cytokines to reach the market (60, 61). Three different IFNs have been identified so far: IFN-alpha, or Intron-A, IFN-beta or Betaseron, and IFN- gamma or Actimmune. The three are currently being used to treat diseases ranging from leukaemia to Kaposi's sarcoma, and viral hepatitis (60).

IFN-gamma, or immune interferon, is a key cytokine in CMI. It is secreted by activated $CD4^+$ T-lymphocytes, CD 8^+ T-lymphocytes, and natural killer cells. It is the most potent macrophage activating factor, inducing nitric oxide synthase (iNOS) within macrophages, and promoting intracellular pathogen killing (36, 60, 66). It up-regulates the respiratory burst in neutrophils in acute inflammatory processes, and effectively accentuates the shift towards Th1 cytokine profiles, and CMI. It achieves this by increasing cellular expression of MHC class II on CD4+ lymphocytes, and other antigen-presenting cells (APCs) as well as MHC class I on CD8+ cells (66).

IL-12 is a potent ThI cytokine that induces activated T-lymphocytes, and NK-cells to secrete IFN-gamma (65). Its receptors are mainly expressed on T-lymphocytes, NK cells, and some B cell subsets. It supports CMI by enhancing IFN-gamma output, and perpetuating IFN-gamma activity on macrophages and other APCs. It enhances the attack on virally infected cells, tumor cells, and allograft tissue by promoting the activity of NK cells, and cytotoxic $CD8^+$ T-lymphocytes (65). According to recent research, its main activities are enhancing helper-T lymphocyte activity, inducing IFN-gamma secretion, and facilitating NK-cell activity (65).

Since IFN-gamma is a powerful stimulus for macrophage activation, and intracellular killing, IL-12 must be a vital part of cell mediated immunity. Together, the combination of IL-12, IL-2, and TNF-alpha, leads to the secretion of large amounts of IFN-gamma contributing to effective formation of granulomata, fibrosis, and the containment of primary TB infections in lung tissue (65). IFN –gamma is, therefore, an ideal surrogate marker for *in vitro,* and *in vivo* studies of cell-mediated immunity. We used IFN-gamma as a marker in our immunology experiment to study immune cell responses to external stimuli in whole blood.

Cytokines for humoral immunity

Tumor necrosis factor (TNF) is an acute phase cytokine, a principal responder to gram negative bacterial infections, and has systemic effects responsible for fever, septic shock and cachexia (62, 63, 66). Its caters for a wide range of immune activities, so it cannot be effectively classified into specific cytokine subsets, because its effects overlap the two cytokine profiles. It can exhibit both immunostimulatory, as well as immunosuppressive activity, probably to counter the potentially destructive effects of pro-inflammatory cytokines (63, 66). It was originally referred to as cachectin as a result of its association with the severe wasting observed in cancer patients, advanced TB, and AIDs (66). However, high levels of TNF during the course of any infection are associated with poor outcomes (63, Table-3).

Since the humoral arm of the immune system is not effectively protective, as compared to the cellular arm, its dominance of the immune response is regarded as unsatisfactory, and less effective. It relies heavily on $CD4^+$ helper lymphocytes, without which full B-cell activation cannot be achieved, and antibody switching cannot take place (66). IL-10 is the prototypical Th2 cytokine that up-regulates the Th1 cytokine profile, and antagonizes the Th1 cytokines.

It blocks cytokines secretion from macrophages, and blocks their cytokine communication with activated T- cells (64). It achieves this by down-regulation of MHC class II molecules, and by blocking the secondary co–stimulatory molecules, B7-1 and B7-2, between macrophages and T-cells (64).

IL-10, is therefore a potent antagonist to CMI. It plays an effective role in protecting normal tissue from the destructive side-effects of CMI, such as the destruction of lung tissues, and cavity formation in pulmonary TB (64). Therefore, it's significant presence in HIV correlated TB may explain the relative absence of TB-cavities in advanced HIV disease (51, 52). However, the predominance of IL-10 has been linked to active TB, and HIV disease progression making it a poor prognostic marker (Table 3).

It is this exciting background of molecular immunology that has inspired scientific research seeking new immunomodulatory interventions to promote CMI, especially in TB and HIV pathology. Th1 IFN-gamma-dependent responses play a central role in controlling TB infections. Therefore this fact supports our use of IFN-gamma responses as markers of CMI.

Study objectives

In this study we focused on *Hypoxis hemerocallidea* as a promising candidate source for immunomodulatory, and antibiotic lead molecules. Hypoxoside, a novel molecule with well-documented cytotoxic activity, has some documented immunomodulatory, and anti-inflammatory activity, but has not been investigated for antibacterial activity, and neither do we know enough about Betasitosterol.

The main objectives of this study therefore were:

1. To investigate *H. hemerocallidea* as a natural antimycobacterial phytotherapy.

2. To assess *H. hemerocallidea's* ethanol extract, hypoxoside, and betasitosterol as immune modulation phytotherapies.

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Chapter 2

An assessment of *Hypoxis hemerocallidea* extracts,

and actives

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An assessment of *Hypoxis hemerocallidea* extracts, and actives as natural antibiotic phytotherapies

2.1 Abstract

In South Africa, the crude aqueous extract from *Hypoxis hemerocallidea* is used by Acquired Immunodeficiency Syndrome (AIDs) patients to treat opportunistic infections, such as tuberculosis. The rapid emergence of multidrug-resistant (MDR) tuberculosis, and extreme drug resistant (XDR) tuberculosis, in recent years, is a major threat to human health. The treatment of TB, nosocomial bacterial infections, and fungal infections is now a clinical challenge, especially in the immuno-compromised individual. There is a dire need for novel antibiotic alternatives with phytotherapies and plant-derived compounds as potentially promising alternatives. The main objective of this study was to investigate the antimycobacterial activity of *Hypoxis hemerocallidea,* a South African medicinal plant, using *Mycobacterium smegmatis* (Ms).

Crude standardized methanol, ethanol, water, and chloroform extracts were tested in serial dilutions of 10 %, 25 %, and 50 % against *Ms,* using the Kirby-Bauer disc diffusion method. Two *Hypoxis hemerocallidea*-derived compounds, Hypoxoside and Betasitosterol (BSS) at 5 mg / disk, were also tested against *Ms*. Each extract was tested in four sets of identical culture plates, with each one bearing the 3 test concentrations (50 μl each) on separate sterile disks, a positive control disk (Ciprofloxacin 5 μg), and one negative control disk impregnated with 50 μl of solvent. The methanol, and ethanol extracts stored at 4^{O} C, and tested at intervals against *Ms*, reached maximum potency at 8 weeks, and declined thereafter. At 8 weeks, the 50 % methanol extract was the most potent with an average 7. 3 mm zone ($P \leq 0.0004$), and the ethanol 50 % extract a 3. 4 mm zone ($P \leq 0.001$). Hypoxoside, at 5 mg/ disk, demonstrated 6.6 mm zones ($P \leq 0.0003$). BSS, the aqueous, and chloroform extracts demonstrated no activity, and no synergistic, or additive antimycobacterial activity was observed between hypoxoside, and BSS.

The activity of the methanol, and ethanol extracts was confirmed on Thin Layer Chromatography (TLC), and Bioautography where a clear zone of inhibition, over the active compound (Rf value 0.17), was observed. The methanol, and ethanol extracts' TLC fingerprints revealed at least 7 different compounds.

Conclusions: *Hypoxis hemerocallidea* is active against *Ms,* and its potency is positively related to its concentration. Its active compound Hypoxoside, also has activity against *Ms*. These outcomes warrant further research into *Hypoxis hemerocallidea's* potential anti-mycobacterial phytotherapy.

Key words: Hypoxis, Mycobacterium tuberculosis, AIDs, anti-mycobacterial, hypoxoside, betasitosterol, phytotherapy.

2.2: Introduction

Plant-derived compounds are attracting keen scientific interest as sources of safe, and effective substitutes for synthetic antimicrobials. Originally antibiotics were defined as naturally occurring chemical substances with the capacity to selectively inhibit microbial growth, with minimal damage to host cells (1, 2). This definition has been extended to cover man-made molecules synthesized as new chemical entities, or as chemically modified structures and analogues of original natural molecules. The first report of antimicrobial activity was in 1877, by Louis Pasteur, who while studying anthrax in animals noted they were protected from infection by an unknown substance (1). This observation marked the beginning of a scientific search for antimicrobial agents for the treatment, and prevention of infection.

In the last two decades scientific interest in herbal medicines has risen to the challenge of surging public demand for plant-derived medicines, which remain unregulated (3-6). Ethno-botanical use by indigenous populations, although crude and unregulated, provides valuable leads to promising drug candidates (4, 5). Scientists have turned to this source for badly needed antibiotics to combat the relentless threat of rising bacterial resistance (7, 8, 25). In South Africa, the use of over the counter medications (OTCs) totals over R2 billion annually. Over 70 % of this total is spent on plant-derived pharmaceuticals alone, which excludes herbal remedies sold by informal street vendors, and those dispensed by traditional healers. According to Brandt *et al* (1995) 60-80 % of South Africans use herbal remedies obtained from informal sources, before consulting medical practitioners (3, 25). This unprecedented demand for herbal remedies continues to grow with the HIV and TB epidemics.

In the United States, the market for dietary supplements and herbal remedies doubled between 1996 and 1998 to a total of $ 12 billion annually, and has since then risen steadily to beyond $ 14 billion (4, 6). Overall, the World Health Organisation (WHO) estimates that 80 % of the world's population relies on regular use of plant-derived medicines. The growing demand has inspired scientists, and clinicians to study the pharmacology of these preparations to better understand their efficacy, and safety.

The regular use of a plant for acute inflammatory illnesses, and febrile or infectious illness is an indication that it may contain antibacterial, anti-inflammatory or immuno-modulatory compounds (6, 7, 9, 10). *Hypoxis hemerocallidea* is such a plant, whose popularity in South Africa has been the result of the reportedly beneficial use of its crude aqueous extracts by HIV and AIDs patients (10).

Hypoxis hemerocallidea, a member of the *Hypoxidaceae* a lily family, grows in various geographical regions of Southern Africa (11). It is originally known as *Hypoxis rooperii*, the Yellow star, *Inkomfe* in Zulu, and popularly known as magic 'muti', or African potato to street vendors (11, 12). Common *Hypoxis* species in South Africa include *H. angustifolia, H. colchicifolia, H. obtusa, H. rigidura* and *H. hemerocallidea* (11-13). Although most species have been used for medicinal purposes, *H. hemerocallidea* is the most sought after species. Crude aqueous extracts from the rhizome, have been used for generations to treat the common cold, urinary tract infections, prostate diseases, gastrointestinal complaints, as well as mental disorders (12). In recent years, its extensive use has provoked scientific controversy because of reported toxicity, and potential interaction with mainstream medications (10).

Collectively, most of its traditional uses are strongly suggestive of the presence of antimicrobial compounds in the underground rhizome from *H. hemerocallidea.* Despite over 50 years of research, this has not been fully investigated (7, 13). Additional evidence comes from the use of its macerated rhizome as wound dressing, the aqueous extract for urinary tract infections, and multiple AIDS related opportunistic infections.

Its two main active compounds Betasitosterol (BSS), and Hypoxoside have been widely researched, but not specifically for antimicrobial activity (7, 13).

Betasitosterol, a ubiquitous plant sterol with a cholesterol-like structure, is not unique to *Hypoxis* species. It was first isolated from *H. hemerocallidea* in the late 1950s, and identified as the active compound against benign prostatic hypertrophy (14). In Germany, where herbal medicines are strictly regulated, BSS has been proved to be effective in the treatment of Benign Prostatic Hypertrophy (13, 15).

The dicatechol molecule, hypoxoside, was first isolated from the corms of *Hypoxis obtusa* by Marini-Bettolo in 1982, and from *H. hemerocallidae* (*H. rooperii*) at the University of Natal in 1984 (13). Hypoxoside is a precursor for a unique molecule named Rooperol, which has proved to be highly cytotoxic against most human cancer cell lines including lung cancer, cancer of the cervix, malignant melanoma, and colon carcinoma (16). Hypoxoside is not unique to *H. hemerocallidea*, and has been found in *H. rigidura, H. latifolia, H. acuminata, H. nitida, and H. angustifolia* (13).

The ominous re-emergence of tuberculosis (TB) as a threat to human health, the evolution of the TB bacillus into extremely resistant strains, and the slow pace of TB drug discovery indicate the possibility of totally drug-resistant TB in the future (17-19).

The spectacular performance of antimicrobial drugs has not been free of challenges. Almost from the beginning, the greatest predicament was the evolutionary development of resistance by common bacterial pathogens to every new antibiotic, including anti-mycobacterial drugs (8, 19). Since then, antimicrobial resistance has grown steadily to a point today where we are threatened by extremely-drug resistant pathogens including multidrug-resistant staphylococci, klebsiella, and pseudomonas species (19).

The TB bacillus is an aerobic, non-motile bacillus that grows very slowly both *in vitro,* and *in vivo*. Its thick waxy cell wall, enables it to survive in hostile conditions including the toxic, bactericidal environment within human macrophages (19, 20). It has the unusual characteristic of dormancy which has rendered it extremely difficult to eradicate, hence its latent infection of a third of the world's population (19). The growing population of immunocompromised people, and the mismanagement of TB control programmes in developing countries, has created TB 'hot zones' around the world.

Hot zones are geographical areas where the prevalence of multi-drug resistant (MDR) tuberculosis exceeds 5 % of reported TB cases (18, 19). Hot zones are indicators of poor performance by TB control programmes (19). Such zones have the alarming potential to amplify their resistance rates, especially with poor patient adherence to treatment, and create even more resistant strains of TB (19). This ominous trend is strongly related to HIV epidemiology, and the failed management of HIV infection. The eventual treatment of MDR-TB consists of very expensive drugs like Ethionamide, Thiacetazone, Capreomycin, and Cycloserine, which are not readily available in sub-Saharan Africa (18, 20, 21).

Against this frightening background, it is imperative that novel TB drugs must be found. Plants are a promising source. The history of *Hypoxis hemerocallidea* makes it an ideal candidate for further antimicrobial investigation.

Study aims and objectives

Therefore, our main goals in this study were the following:

1. To evaluate the antimycobacterial activity of *Hypoxis hemerocallidea* phytotherapy.
2. To assess which of the *Hypoxis hemerocallidea* aqueous, or organic solvent extracts are better antibiotic dosage forms.
3. To investigate the antimycobacterial activity of *Hypoxis*-derived Betasitosterol, and Hypoxoside, as the main actives from *Hypoxis hemerocallidea*.

2.3: Materials and methods

2.3.1 Plant material and extract preparation.

The fresh underground rhizomes of *Hypoxis hemerocallidae* were used in this experiment. They were harvested from wild species in the countryside outside the University of Natal, Durban, South Africa, during the winter of 2005. The department of Botany at the University of the Western Cape, Cape Town, identified the plants, and rhizomes. A voucher specimen number 6741 was stored at the university herbarium.

Methanol extracts

The fresh rhizomes were washed in distilled water, dried and cut into pieces, which were then macerated in a blender. 50 g of plant material was then soaked in 100 ml of methanol to make a 50 % extract. It was allowed to stand at room temperature for 24 hours in a clean beaker, and vacuum filtered, under sterile conditions, the following day. The filtrate was stored in sterile screw-capped amber glass bottles at 4° C. Serial dilutions were then prepared for the 25 %, and 10 % extracts. Each extract was filter sterilized and stored separately in labeled, sterile glass amber bottles at 4° C. Sterile 8 mm diameter disks were then impregnated with 50μl of each extract, and dried at 37° C for 1 hour before the experiment.

The ethanol, and chloroform extracts were prepared in a similar manner to the methanol extract with each in serial dilutions of 10 %, 25 %, and 50 %. Hot distilled water was used to processing the aqueous extract in a similar manner, to the same serial dilutions.

Hypoxoside and betasitosterol

Samples of *Hypoxis*-derived betasitosterol, and hypoxoside were obtained from Essential Sterolin products, Halfway House, in Johannesburg. Hypoxoside was dissolved in ethanol at a 10 % (100 mg/ml = 5mg/ 50 μl/ disk) concentration for this experiment, was vortexed and filter sterilized. The sample was stored in a sterile amber bottle at 4° C. Betasitosterol was dissolved at the same concentration in ethanol for this experiment.

50 μl of each solution was transferred onto sterile disks. The four solvents methanol, ethanol, water, and chloroform served as negative controls. Similar discs for Ciprofloxacin, a broad-spectrum antibiotic with activity against mycobacteria, served as reference positive controls at 5 μg per disk.

Mycobacterium smegmatis

Mycobacterium smegmatis, obtained from the department of Medical Bioscience at the University of the Western Cape, was used as a surrogate for *Mycobacterium tuberculosis* (MTB). *M. Smegmatis* is a suitable surrogate because it is less pathogenic, and relatively fast growing with a cell wall similar to MTB.

2.3.2 The Kirby-Bauer disc diffusion method

The Kirby-Bauer disk diffusion method was used in this study to determine the sensitivity of growing mycobacteria exposed to a diffusion gradient of different concentrations of plant extracts, impregnated unto sterile discs.

Colonies of *M. smegmatis* were transferred from agar plates, and inoculated into tubes of nutrient broth, and allowed to grow at 37^{o} C, for 30 minutes. Using sterile swabs, the mycobacterium was streaked evenly unto sterile plates for even growth on 7H11 medium. This spread-plate technique provided an even lawn of growth unto which sterile 8 mm disks, impregnated with 50 μl of extract, were transferred. Each plate carried one positive disk (Ciprofloxacin at 5 μg/disk), one negative control (solvent), and three discs impregnated with the extract's serial dilutions. A total of 5 disks per plate. Each set of identical discs was repeated on four different plates. The disks were incubated at 37^{o} C respectively, for 72 hours. All work was performed under sterile conditions.

Hypoxoside, and betasitosterol were placed unto different culture plates each bearing disks impregnated with 50 % methanol, and ethanol extracts for comparison. Ciprofloxacin was used as a positive control, and ethanol as a negative control.

The Kirby-Bauer procedure was repeated for all extract concentrations at intervals, through out a twenty-week period of study to assess the effects of age on *hypoxis* extracts. Extract samples were stored at 4^{o} C through out this period.

2.3.3: Thin layer chromatography

Thin layer chromatography was used as a separation method for compounds from *Hypoxis hemerocallidea* extracts. Two fluorescent 20 x 20 cm Silica gel plates were spotted, at 1.5 cm from the bottom, with 20 μl of the four different extracts of 50 % methanol, ethanol, chloroform, and aqueous extract. After drying, the 2 plates were immersed in a solution of methanol (30ml): ethyl acetate (120 ml) (1:4) in a sealed glass TLC chamber. It was left to stand for 1 hour. The two plates were removed and dried again, and the different spots, or fingerprints, were visualized under UV light at 254-365 nm for quenching, and fluorescent zones. The plate was then sprayed with a solution of 3 g vanillin: 30-ml ethanol: 5ml sulfuric acid, and heated to reveal the positions of different separated compounds.

Bioautography was performed on the second TLC plate to assess the anti-microbial activity of compounds isolated from extracts. A millimeter-thin layer of H7110 medium, inoculated with *Mycobacterium smegmatis,* was laid over the plate as an agar overlay. The different compounds, concentrated at specific spots during the TLC, diffused from the plate into the culture medium laid over the plate. After solidification of medium, the plate was incubated at 37^{o} C for 72 hours.

To visualize the zone of inhibition, a tetrazolium dye (MTT) was then sprayed over the TLC plate to reveal clear zones where growing bacteria were killed by the active compound. Over the rest of the plate, living bacteria produced dehydrogenase enzymes that converted tetrazolium salts to an intense coloured formazan, seen as a purple color. The clear zone of inhibition was clearly visible as a pale area against the purple background.

2.4 Results

After 72 hours' incubation, clear zones of inhibition (mm) against *Mycobacterium smegmatis* cultures were measured (Table 1).

2.4.1 Statistical analyses

Statistical analyses to compare the zones of inhibition was done by conducting a *t*-test, with the MedCalc version 7.1(2000) statistical programme. Means ± SEM (standard error of the mean) were considered significant at $P \leq 0.05$.

2.4.2 *Hypoxis hemerocallidea* extracts

Fresh and aged extracts

The serially diluted extracts of *Hypoxis hemerocallidea* differed in their activity against *M. smegmatis*. Our initial antimycobacterial assessment of fresh crude *Hypoxis hemerocallidea* extracts (day 2), with the disk diffusion method against *M. smegmatis,* demonstrated very weak activity observed only from the 50 % methanol extract (1.8 mm zones) and the 25 % methanol extract (0.9 mm zones). There was no activity at all from the 10 % methanol extract, ethanol extracts, and the water and chloroform extracts (Table 1).

Aged extracts

It was observed that the antimicrobial activity of the methanol, and ethanol extracts improved steadily in the first 8 weeks, to reach a peaked at eight weeks (Table 1, Figure 2). At 8 weeks, the 50 % methanol extract exhibited the highest activity with a fourfold increase in potency against *Mycobacterium smegmatis,* and reached a mean of 6.8 mm ($P \leq 0.001$) (Table 1, Figures 1, 2 and 3).

Similar results were observed with the 50 % ethanol extract at 8 weeks. The 50 % ethanol extract at 8 weeks with 3.4 mm zones, was comparable with the 25 % methanol extract at 3.8 mm (Table 1).

Table 1. The activity of *Hypoxis hemerocallidea* extracts, and compounds, against *Mycobacterium smegmatis*.

Extracts, controls	Mean zones of inhibition in mm					*P* values
	Week 1	Week 3	Week 8	Week 17	Week 21	
Methanol 10%	0 ± 0.25	0.2 ± 0.25	0.8 ± 0.4	0.8 ± 0.64	0.2 ± 0.25	N/S
Methanol 25%	0.9 ± 0.24	1.4± 0.48	3.8±0.34	2.2± 0.68	1.8± 0.9	N/S
Methanol 50%	1.8 ± 0.4	3 ± 0.9	*6.8 ± 1. 01	3.9 ± 0.3	3.5 ± 0.84	*$P \leq$ 0.0004
Ethanol 10%	0	0	0.9 ± 0.48	0.3 ± 0.25	0.4 ± 0.75	N/S
Ethanol 25%	0	0.4 ± 0.48	2.2 ± 0.73	1.6 ± 0.48	1.4 ± 0.49	N/S
Ethanol 50%	0	1.3 ± 0.40	3.4 ± 0.48	2.9 ± 1.14	1.9 ± 0. 25	N/S
Aqueous 10, 25, 50 %	0	0	0	0	0	NS
Chloroform 10, 25, 50 %	0	0	0	0	0	NS
Ciprofloxacin (+) control	15.5 ± 1.4	16.9 ± 2.16	16.3 ± 0.62	16.1 ± I.87	16.9 ± 1.82	N/S
Hypoxoside (tested once)	6.8 ±0. 24	5.5 ± 0.76	* 6.6 ± 0.18	5.8 ± 0.47	6 ± 0.80	*$P \leq$ 0.001
Betasitosterol (tested once)	0	0	0	0	0	NS
Solvent controls (-)	0	0	0	0	0	NS

The antimicrobial activity of extracts was compared with the plant-derived active compounds, Hypoxoside and Betasitosterol, from *Hypoxis hemerocallidea*. Negative controls consisted of methanol, ethanol, chloroform, and distilled water. Ciprofloxacin was used as a positive control.
NS-not significant

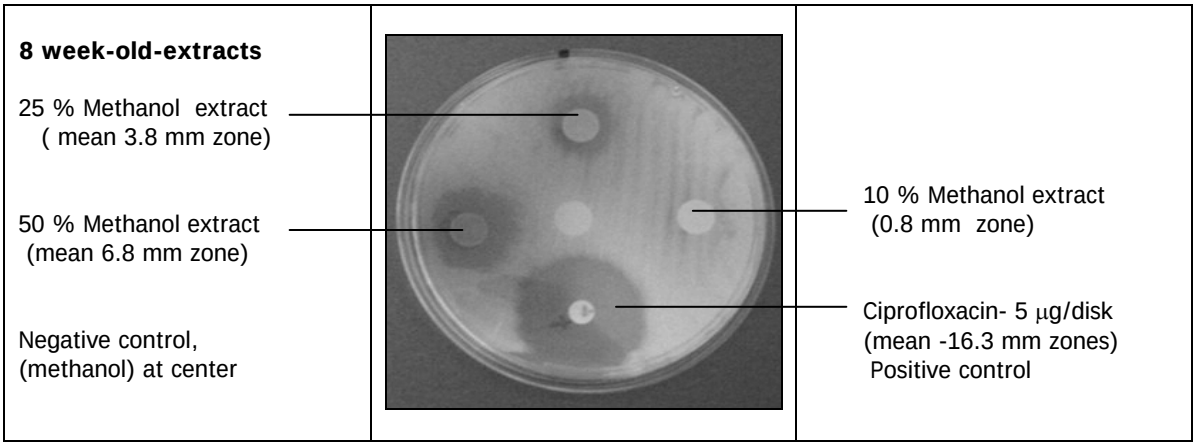

Figure 1. The activity of the 8-week-old methanol extract of *Hypoxis hemerocallidea* against *M. smegmatis.*

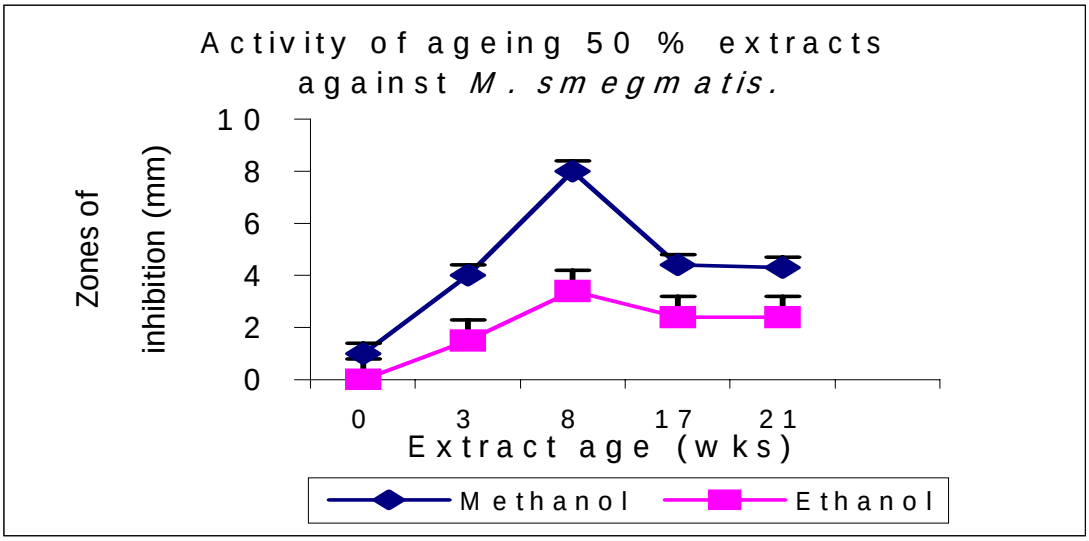

Figure 2. The steady maturation of active extracts up to the 8th week.

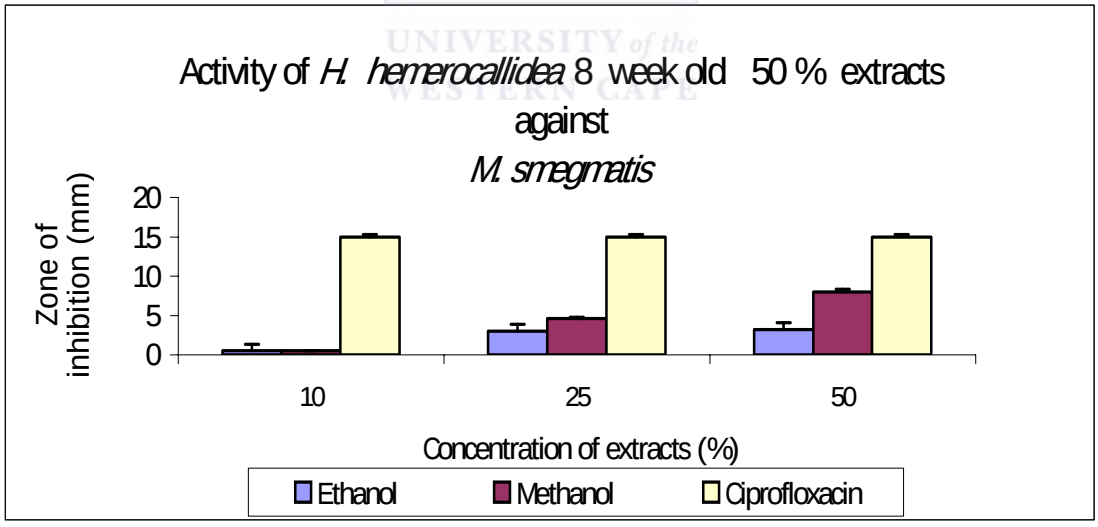

Figure 3. The positive relationship between extract concentration, and activity during the 8th week.

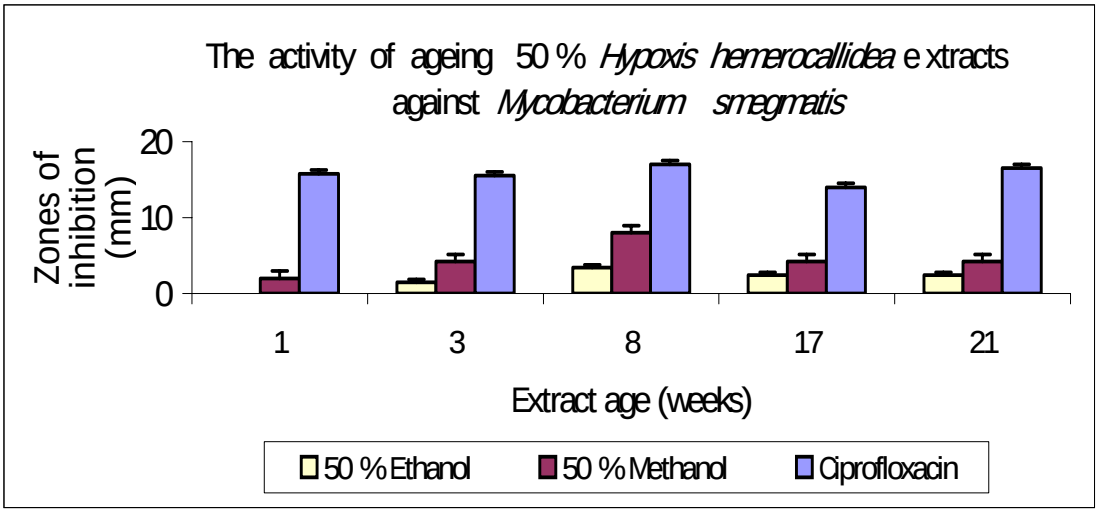

Figure 4. The progressive potency of the 50 % methanol, and 50 % ethanol extracts against *Mycobacterium smegmatis*, over a 21-week period. There was no activity from the ethanol extract during the first week. Significant differences in activity were noted.

Although the 50 % methanol extract remained consistently more potent than the ethanol extract, steady deterioration in all extracts' potency was observed after the eighth week. By the 21st week the inhibition zones for the 50 % methanol extract had deteriorated to an average of 3.5 mm and the ethanol to 1.9 mm (Table 1, Figures 2 and 4).

Both the methanol, and ethanol extracts remained moderately active against *Mycobacterium smegmatis*, beyond the 21st week. Although all extracts were stored at 4° C through out this experiment, no improvement was observed in the aqueous, and chloroform extracts.

2.4.3 Hypoxoside and betasitosterol

Hypoxis-derived Hypoxoside, and Betasitosterol in 10 % concentrations in ethanol, were tested separately against *Mycobacterium smegmatis*, and also in combination. Only 10 % Hypoxoside exhibited activity against *Mycobacterium smegmatis* (6.6 mm) with potency comparable to the 8- week old 50 % methanol extract (6.8 mm), and to Ciprofloxacin ($P \leq 0.001$) (Table 1, Figures 5 and 6). The Betasitosterol had no activity at all.

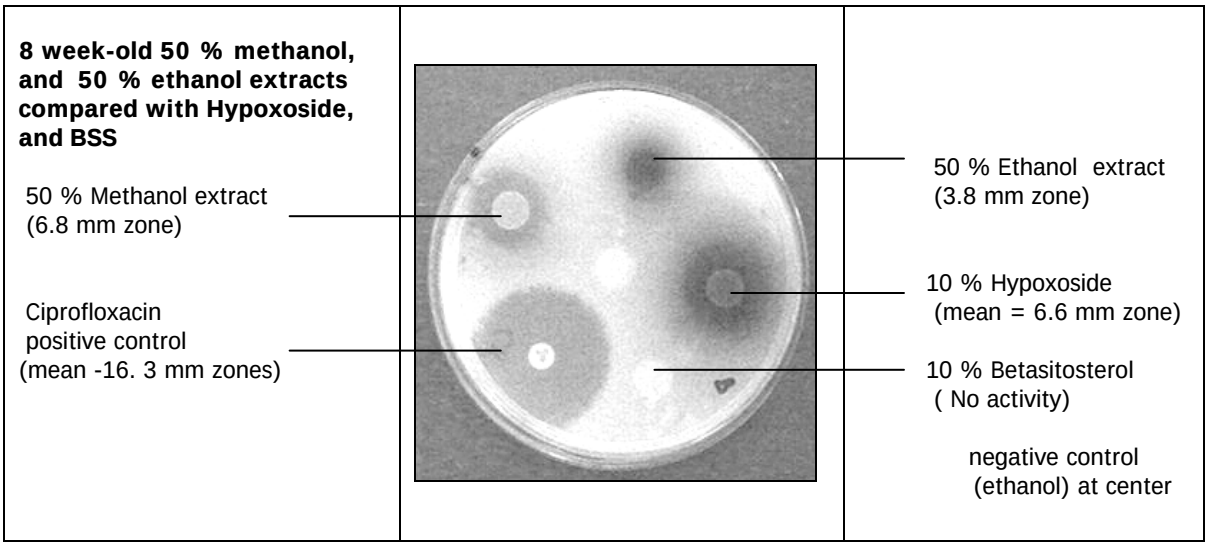

Figure 5. The activity of 8-week-old 50 % methanol, and ethanol extracts against *M. smegmatis*, compared to 10 % Hypoxoside, and Betasitosterol.

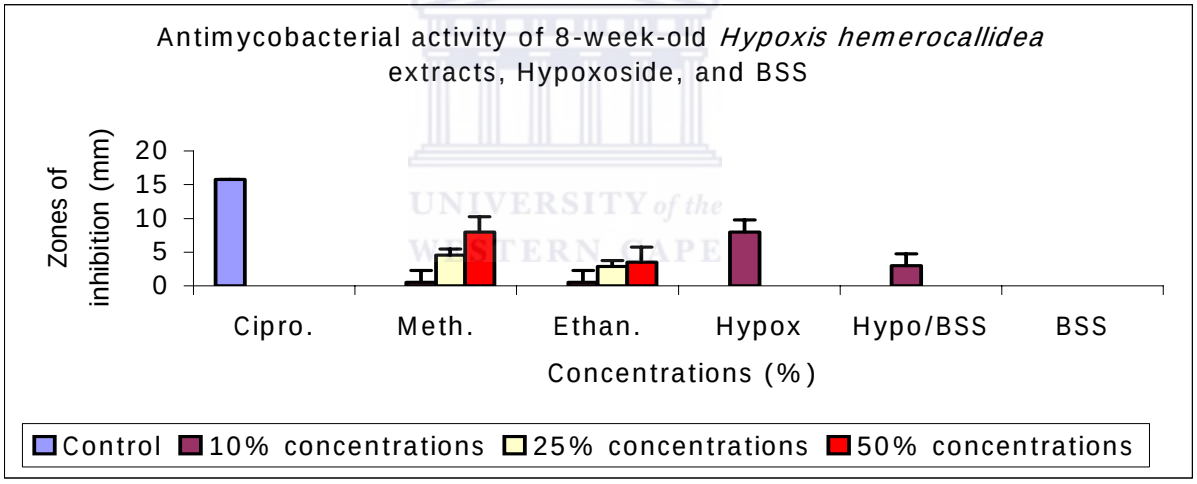

Figure 6. Antimycobacterial activity of 25 % and 50 % methanol and ethanol extracts, Hypoxoside and BSS. Hypoxoside combined with BSS was observed for possible synergistic activity. There was no activity from BBS, and it appeared to inhibit the activity of Hypoxoside.

The combination of 10 % Hypoxoside, and 10 % Betasitosterol on the same discs was used to assess any synergistic, or additive activity between both compounds. No evidence of additive, or synergistic acitivity as observed (Figure 6). In stead the Betasitosterol appeared to have an inhibitory effect on the antimycobacterial activity of Hypoxoside.

2.4.4: Thin layer chromatography and bio-autography

On silica gel thin-layer chromatograms, the methanolic extract of *Hypoxis hemerocallidea* was separated into six spots with different Rf values (0.89, 0.78, 0.56, 0.40, 0.28, 0.17).

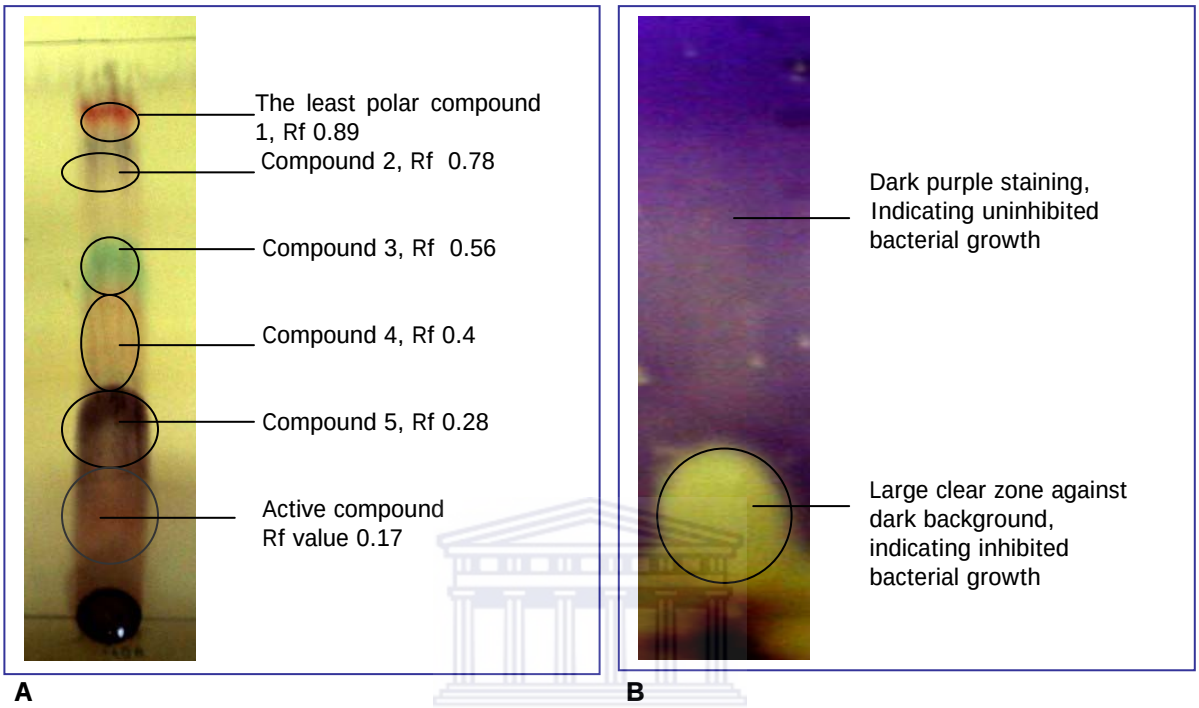

Figure 7 (A). Thin Layer Chromatogram of the 8 week old 50 % methanol extract of *Hypoxis hemerocallidea*. The TLC plate was developed in a solvent system of methanol: ethyl acetate (1:4) and, sprayed with Vanillin, 3g/ Ethanol, 30 ml/ Sulfuric acid, 5ml. It shows 6 distinct spots depicting different compounds with different Rf values. **(B)** Identical bioautogram plate after incubation at 37 ^{0} C for 72 hours, with an overlaid inoculum of *Mycobacterium smegmatis* in 7H11 medium. spraying with MTT, revealed a large, clear zone of inhibition with an Rf factor of 0.17.

Initially, only four spots seen as fluorescent zones, were visualized under UV light at 254-365 nm. With vanillin-sulfuric acid-methanol spray, and heating, six spots became visible in different colors, (Figure 7A). They were all inactive except the spot at Rf 0.17, that demonstrated a strong inhibitory activity against *M. smegmatis,* observed as a large clear zone against the dark purple back ground on the bioautogram (Figure 7B). An MTT spray, converted to a purple formazale dye by the microorganism, highlighted this clear area.

2.5: Discussion

This is the first time that the crude extracts of *H. hemerocallidea*, and its active compounds, have been specifically evaluated for anti-mycobacterial activity. The two active compounds, Hypoxoside and Betasitosterol, were each tested separately against *M. smegmatis*. The crude extracts were used as representation of the two compounds in their natural combination.

The results of this antimicrobial activity study indicate 'dose'-dependent levels of antimycobacterial activity from both the methanol and ethanol extracts. The extracts' activity, greatly affected by maturity, was highest during the 8th week. The highest potency against *M. smegmatis*, observed from the 50 % methanol extract during the 8th week ($P \leq 0.0004$), was observed to decline there after. A similar trend was observed with the 50 % ethanol extract, which although weaker than methanol, was most potent during its 8th week. The activity of the 50 % methanol, 50 % ethanol, and 25 % methanol extracts were found to reach peak activity during the 8th week. Although these extracts were tested against a non-pathogenic mycobacterium, its cell wall and biochemistry is similar to *Mycobacterium tuberculosis* (20). Therefore these are major findings, being reported for the first time.

These observations indicate that the nature, and potency of the crude extracts is constantly changing with time depending not only on the interaction, and deterioration of the active compounds, but also with environmental conditions, like temperature. Further, the same findings indicate that other, yet unexplored active compounds against different pathogenic bacteria, may still be found with further testing of aged extracts. Thin Layer Chromatography of the methanol and ethanol extracts indicated the presence of multiple compounds, with one spot at Rf 0.17 effectively inhibitory to the growth of *M. smegmatis*. This spot might be a combination of yet unknown compounds.

Although we demonstrated no *in vitro* activity in the aqueous extracts, this alone may not fully negate their medicinal efficacy. Hydrophobic compounds from *Hypoxis hemerocallidea* may still be released from carrier molecules in the acidic stomach environment, or by intestinal flora in the small, and large intestines. Hypoxoside is likewise degraded in the small, and large intestines to release a more potent molecule, Rooperol (16).

Traditional healers use crude aqueous extracts, whose efficacy has come under great scrutiny by medical practitioners concerned about interactions with conventional medicines (10). Reports of Hypoxis toxicity have heightened the controversy about the use of such remedies (10). The fact that up to 80 % of the population, in both rural and urban areas, may be using these extracts is a matter for serious concern (3-5, 10).

Betasitosterol is a plant derived lipid molecule that is lipophilic, and relatively insoluble in water. Based in a 10 % ethanol extract, Betasitosterol demonstrated no activity against *M. smegmatis*. This may be due to the fact that Betasitosterol might be insoluble in ethanol. However, only one solvent (ethanol) was used for both Betasitosterol and Hypoxoside.

Hypoxoside, at a 10 % concentration, showed significant activity against *M. smegmatis*, comparable to that of the 50 % methanol extract. Although the exact mode of action of Hypoxoside against the mycobacterium is unknown, part of its activity may be attributed to its high cytotoxicity directed particularly against the thick waxy mycobacterial cell wall (16). This activity may be very similar to the cytotoxic effects demonstrated by Hypoxoside against melanoma cancer cells *in vitro* (16). A progressive disintegration of the cancer cell wall, exposed to Hypoxoside, was observed under a microscope over a twenty-four hour period.

The anti-mycobacterial activity of the 50 % methanol, and ethanol extracts, confirmed on TLC and Bioautography, clearly indicates the presence of a compound, or compounds in *H. hemerocallidea* that has significant potential as an antimycobacterial phytotherapy. Although the exact identity of the active compound is unknown, it's interesting to note that Hypoxoside, which is a precursor for Rooperol, demonstrated significant activity against *M. smegmatis*. The identities of the 6 compounds separated on TLC remains to be confirmed. The large number of compounds, within actives, indicates the very likely presence of synergism, or additive behaviour shared between them (22).

The short comings of our assessment included the limited number of solvents for the extraction of a wide range of compounds. Lack of knowledge about the chemical properties of the unknown compounds limited the selection of suitable solvents for their extraction. The finding of age-related extract potency warrants further research into aged extracts' activity against pathogenic mycobacteria, and fungal infections. High Performance Liquid Chromatography (HPLC) will assist in confirming the absence or presence of unknown compounds within, especially the water and chloroform extracts, as well as the changing compositions of compounds within ageing extracts.

Future research is therefore needed to explore the full anti-mycobacterial activity of both Betasitosterol and Hypoxoside, within appropriate solvents, and the crude extracts over a longer life-span. To exploit the potential benefits of synergism, more compounds need to be isolated from *Hypoxis hemerocallidea*, and their medicinal properties evaluated. Isolation of the anti-mycobacterial compound, or compounds may then be possible, and will facilitate further research against *Mycobacterium tuberculosis,* and *Mycobacterium avium-intracellulare.*

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Chapter three

An assessment of *Hypoxis hemerocallidea* ethanol extract,

and actives as immune modulation phytotherapies

Chapter three

An assessment of *Hypoxis hemerocallidea* ethanol extract, Hypoxoside and Betasitosterol as immune modulation phytotherapies

3.1. Abstract

The main objective of this study was to investigate the immune modulating effects of an ethanol extract of *Hypoxis hemerocallidea,* and its two active compounds Hypoxoside, and Betasitosterol (BSS). Hypoxoside and BSS were separately dissolved in ethanol, to make up 50 mg /ml of Hypoxoside and 100 mg /ml of BSS. The extract, and active compounds, were incubated with diluted whole blood samples over 3-5 days. Interferon (IFN)-gamma, the prototypical cytokine for the protective Th1 cytokine profile, was used as a bio-marker for cell-mediated immunity. Supernatants were analyzed, for IFN-gamma expression, using colorimetric ELISA technology.

An *in vitro* whole blood assay was conducted with diluted blood (1:9) samples from 6 young, healthy volunteers. Diluted blood (180 μl) was incubated with (10 μl) serially diluted 12-week 25 % ethanol extract, hypoxoside, and BSS. All cultures were run in duplicate. Serially diluted parallel cultures were further stimulated with (10 μl) of phytohemagglutinin (PHA) at 25 μg/ml, or Purified Protein Derivative (PPD) at 10 μg /ml. PHA, a plant- derived mitogen with non-specific stimulation of immune cells, or PPD a *Mycobacterium tuberculosis* derived protein, were used as extra stimuli. After 3 days incubation, supernatants from PHA cultures were harvested along with parallel extract, BSS, and Hypoxoside cultures. The PPD related cultures were harvested on day 5.

The results of this study indicated that *Hypoxis hemerocallidea* has moderate immunostimulatory activity, as indicated by cellular expression of IFN-gamma, when exposed to its ethanol extract, and compounds. The extract, and both compounds demonstrated moderate effects on IFN-gamma expression, which was up-regulated by PHA and PPD. Hypoxoside, despite its high cytotoxicity, had significant immunostimulatory activity at its highest concentration (50 mg/ml) on day 3 ($P \leq 0.05$). BSS on both days 3 and 5, has moderate effects, which was in agreement with previous research. BSS was inhibitory at its highest concentration. Only the ethanol extract, containing both active compounds, demonstrated a steady, dose-dependent rise in IFN-gamma expression with PHA stimulation. This interesting outcome could be indicative of synergistic activity between the two active compounds. The extract's rising trend with IFN-gamma expression was inhibited at its highest concentration (25 %), with both PHA ($P \leq 0.05$) and PPD. The activation of immune cells appeared to rendered them more vulnerable to cytotoxic effects from Hypoxoside ($P \leq 0.05$), and ethanol extract ($P \leq 0.05$). Our results confirm the immunostimulatory characteristics of *Hypoxis hemerocallidea* ethanol extract and the evaluated compounds.

Key words: *Hypoxis, Hypoxoside, Betasitosterol, immunostimulation, tuberculosis, synergistic, interferon-gamma.*

3.2 Introduction

The last two decades have seen enormous advances in the fields of immunology and biotechnology. Out of our broad understanding of the immune system has come the exciting ability to evaluate immune activation, immune deficiency, autoimmunity and allergy. It is now possible to characterise, and quantify cytokines, and specific antibodies, as well as evaluate lymphocyte phenotypes (1). The manipulation of molecular immune functions *in vitro* has contributed tremendously towards the development of novel therapeutic interventions (1).

At molecular level, it has become possible to study the effects of novel molecules on their target cells using blood assays, or cell cultures. The *in vitro* manipulation of cellular cytokines presents an unprecedented opportunity to study disease processes in order to create novel therapies. This wide range of possibilities has put *in vitro* immunomodulation at the forefront of biotechnological research (1). The potential market is so huge that immunomodulatory products currently make up the largest biotechnology-derived therapeutic agents in the process of pharmaceutical research (1).

Currently consumers are turning to alternative therapies, including crude herbal medicines and pharmaceutical preparations (2-4). Biotechnological advances have made the study of herbal medicines more thorough, easier, and faster. Cytokine biology is increasingly being employed in the study of these medicines effects on the immune function. Cytokines, like IL-2, are already being used as immunomodulatory therapies, as prognostic indicators, and also as bio-markers of immune function (1). In this experiment we used interferon (IFN)-Gamma expression as a bio-marker of immune function in order to assess the effects of *Hypoxis hemerocallidea*'s ethanol extract, and active compounds on immune cells *in vitro*.

According to previous researchers, *Hypoxis hemerocallidea* contains two major active molecules, Betasitosterol (BSS) and Hypoxoside (5-7). In the last decade it has come under increasing scrutiny, and criticism regarding its reported toxicity (2, 5). However, it remains a promising source of novel bioactive compounds.

BSS is a ubiquitous phytosterol which cannot be synthesised in the human body, but has a structure very similar to cholesterol (4, 8). Because of this structure, BSS has the ability to competitively inhibit cholesterol absorption within intestinal epithelia, and has been reported to effectively lower serum cholesterol levels, a beneficial cardio-protective effect (8, 9). Only 5 % of the oral intake of phytosterols reaches the blood circulation in humans due to its expulsion by efflux pumps in the intestinal epithelial cells and, similar pumps within hepatocytes which efficiently expel plant-derived phytosterols into bile canaliculi (8, 9). Since phytosterols cannot be synthesised in the body, their levels in the blood are 800-1000 times less than those of endogenous cholesterol (6, 8). However, even at these minute levels, phytosterols including BSS, can mimic human estrogens, and interact with estrogen receptors (ERs), resulting into various levels of endocrine disruption (10).

Interest in BSS has focused on its immunomodulatory and anti-inflammatory effects. Although BSS and Hypoxoside have not been investigated together, most of their activities overlap (11-13). Both plants have anti-inflammatory activity that has not been fully exploited for therapeutic purposes, and BSS remains an effective clinical treatment for Benign Prostatic Hypertrophy (14).

According to Bouic *et al* (1999) BSS has lympho-proliferative effects, especially on $CD4^+$ T-lymphocyte subsets both *in vitro,* and *in vivo* (7, 11). T Lymphocytes are the prototypic cells of cell mediated immunity, which, when activated secrete IFN-gamma, interleukin (IL) –2 and IL-12, which are prototypic Th1 cytokines (15).

HIV infection is characterised by prolonged clinical latency, defective immunity and continuous viral replication, within cells of the immune system. The progressive decline in CD4 + T-lymphocytes is the hallmark of HIV infection, and the subsequent immunodeficiency that follows the infection (15, 16, 17). It highlights the urgent need for immunostimulants with the specific capability of inducing $CD4^+$ and $CD8^+$ T-cell proliferation (11, 12, 18). Therefore, further investigations into the immunomodulatory activity of *Hypoxis hemerocallidea*, especially in HIV infection, are necessary for us to further understand, and exploit its beneficial effects (3, 12).

Mycobacterium tuberculosis (TB) infection is closely linked to HIV infection in sub-Saharan Africa, and synergistic activity shared between the two diseases against the host immune system has been shown to lead to profound cytokine alteration including further decline in cell-mediated immunity. Individuals with no previous exposure to mycobacterial infections lack the appropriate memory T-lymphocytes required to mount a proper immune response to TB infection (16, 17). Similarly, those with genetic anomalies in interferon (IFN)-gamma expression cannot fend off the infection (17).

The question being asked is how can immunology contribute to the control of not only TB but also HIV infection? (19). The search for an effective vaccine, which began with Robert Kock in 1882, has been on going for over a hundred years (20). BCG introduced by Calmette and Guerine in 1929 has been in use for over 80 years, and after over 3 billion administrations, it is still in use today despite its disputed efficacy (20). The human population, therefore, remains unprotected and increasingly vulnerable to the new, more aggressive multi-drug resistant (MDR) forms of TB and extremely-drug-resistant (XDR)-TB.

Plant–derived immunomodulators, with their effects on cytokine biology in disease, will therefore play a more prominent role in the control of intracellular infections like TB and HIV. Researchers have developed 'cytokine shells' to increase cytokine half-lives and improve target specificity (21). By sustaining $CD4^+$ lymphocyte counts, immune intervention will prevent HIV disease progression to profound immunodeficiency and AIDs, and will effectively break the vicious cycle between TB and HIV infection.

As an alternative source, plants with long histories of traditional use are being explored for chemo-protective, and immunomodulatory phytotherapies. In Southern Africa, *Hypoxis hemerocallidea*, the African potato, is one of these promising plants.

Study objectives

Using diluted whole blood in *in vitro* assays, and IFN-gamma expression as a biomarker of cell-mediated immune response and T-cell activation, our main objectives were the following:

1. To assess *Hypoxis hemerocallidea* ethanol extract as an immune modulation phytotherapy.
2. To determine which of the *Hypoxis hemerocallidea* actives, Hypoxoside or Betasitosterol is an immune modulation phytotherapy.
3. To evaluate synergism between the two actives.

3.3 Materials and methods

RPMI-1640 medium, and PHA were obtained from Sigma Aldrich Ltd, Cape Town. RPMI-1640 was used as culture medium throughout this experiment. PHA, a plant-derived mitogen, was used as a non-specific stimulus for immune cell activation, and proliferation. The more specific Purified Protein Derivative (PPD), a mycobacterium derived protein, was used as an antigenic stimulus for immune cells. It was kindly provided by The Department of Biochemistry, University of Stellenbosch, Cape Town. Betasitosterol (BSS), and Hypoxoside, *Hypoxis hemerocallidae*–derived compounds, were obtained from Essential Sterolin Products in Midrand, Johannesburg in 200 mg vials. The ELISA and cytoTox 96 kits purchased from Sigma Aldrich, both came with the complete manufacturers instructions. All wash solutions, buffers, color substrates, and stop solution, were prepared according to the manufacturer's instructions.

3.3.1 Preparation of Hypoxoside, Betasitosterol (BSS) and 25 % ethanol extract

Hypoxoside was dissolved in ethanol at a concentration of 5 g /100ml (50 mg/ml) and filter sterilized. Four serial dilutions were made out (to a dilution factor of 8) for 50 mg/ml, 6.3 mg/ml, 0.8 mg/ml and 0.09 mg/ml. BSS was dissolved in ethanol to a concentration of 10g % (100mg/ml) and filter sterilized. Serial dilutions were made out to 100 mg/ml, 0.6 mg/ml, 0.3 mg/ml and 0.03 mg/ml.

A standardized ethanol extract was prepared from a fresh *Hypoxis hemerocallidea* underground rhizome, harvested during winter 2005 from Natal South Africa. A voucher specimen No. 6741 was registered with The Department of Botany at The University of the Western Cape. 25g of fresh, macerated rhizome was soaked in 100 ml of ethanol over night. It was then filtered and diluted into 4 serial dilutions of 25 %, 3.125 %, 0.39 % and 0.04 % for this experiment.

3.3.2 Whole blood assay

Brief histories were taken from 6 volunteers to confirm they were in good general health, had had no recent medication, were non-smokers, and were between the ages of 20-25.
We had no confirmed knowledge of our volunteers medical histories, especially concerning previous exposure to mycobacterial infections, including TB. The procedure of veno-puncture was approved by the university ethics committee. 3 ml of fresh blood was taken, under sterile conditions, from the cubital veins into sterile, heparinised EDTA tubes, and diluted (1:9) with RPMI-1640 medium. All work was performed under sterile conditions within a laminar flow.

All wells were filled to a uniform volume of 200 μl / well, and duplicate samples were set up for BSS, Hypoxoside, and extract. The BSS samples consisted of 4 wells, each with 180 μl of diluted blood, 10 μl of BSS (100, 0.6, 0.3, 0.03 mg / ml), and 10 μl of RPMI-1640 medium to make up 200 μl. A 5^{th} well, as control, contained 180 μl of diluted blood, and 20 μl of medium. Similar sets, and dilutions, were set up for Hypoxoside, and extract.

Similar sets of 4 wells were set up for PHA with 180 μl of diluted blood, 10 μl of BSS, and 10 μl of PHA. The control consisted of 180 μl of blood, 10 μl of PHA, and 10 μl of medium. Likewise the PPD set consisted of 4 wells with 180 μl of blood, 10 μl of BSS, and 10 μl of PPD. The control consisted of 180 μl of blood, 10 μl of PPD, and 10 μl of medium.
This procedure, with duplicate sets, was repeated for Hypoxoside and extract in their respective dilutions, and similar controls.

All samples were incubated at 37^0 C with 5 % carbon dioxide. On day three, supernatants from the PHA wells were harvested and stored at -20^0 C. The PPD samples were harvested on day 5 and stored at $-2O^0$ C.

3.3.3 IFN-gamma measurement-The sandwich ELISA

A sandwich ELISA kit (DuoSet Elisa) was used in this experiment to measure human IFN-gamma concentrations in culture supernatant. DuoSet is an Enzyme Linked ImmunoSorbent Assay designed to detect and quantify IFN-gamma captured in a specific antibody-antigen interaction.

Mouse anti-human IFN-gamma capture antibody, 50 μl, was immobilized as a coating to 96-well micro plates at a concentration of 4.0 μg/ml in PBS. Plates were incubated overnight at room temperature, and then washed in wash buffer to remove non-specifically bound material. Unoccupied sites were blocked with 300 μl of block buffer (1 % BSA, 0.05 % NaN_3 in PBS) per well, and incubated at room temp for 1 hr. A through wash technique (0.05 % Tween-20 in PBS) was used between all steps.

Cell culture supernatants were then added to the trays at 50 μl per well and incubated at room temperature for 2 hours. Then, 50 μl of detection goat anti-human IFN-gamma antibody, in reagent diluent at concentrations of 200 ng/ml, was then added to wells for a 2-hour incubation period. Different monoclonal antibodies, capture and detection antibodies were used in this assay to avoid overlapping of epitopes bound by each antibody. This was to enable simultaneous binding of both antibodies to antigen (human IFN-gamma) without obscuring each other's epitopes. 50 μl of streptavidin conjugated to horseradish –peroxidase (HRP) enzyme was added to the wells for 20 min, and incubated in the dark. A colour substrate, which turns blue after 20 minutes incubation in the dark, was then added. Without washing, 50 μl of stop (2 N H_2SO_4) solution was added to each well. The optical density was determined immediately in a microplate reader at 450nm. The levels of IFN-gamma are in direct proportion to color intensity. All steps in this ELISA were performed according to the manufacturer's protocol.

3.3.4 Cytotoxicity assay

The CytoTox 96 assay is a colorimetric assay, which quantitatively measures lactate dehydrogenase (LDH) in culture supernatants. LDH is a cytosolic enzyme which can only be measured in culture supernatants after cell lysis.

Whole blood (dilute at 1:9 with RPMI-1640) was placed in 96 well plates (180 μl / well). 10 μl from each serial dilution of hypoxoside (100 mg/ ml, 0.6 mg/ ml, 0.3 mg/ml, and 0.03 mg/ml) was incubated with the blood. Diluted ethanol extract (25 %, 3.13 %, 0.39 % and 0.04 %) at 10 μl/well was added to a similar series of wells, and each series was run in duplicate. All cultures were incubated at 37^0 C, with 5 % CO_2, for 4 hours. After the incubation period, 50 μl supernatants from each well were transferred to fresh plates, and incubated with Tetrazolium salt for 30 minutes, in the dark.

Positive controls, in duplicate, consisted of 180 μl of diluted blood with medium. 10 μl of standardized Lysis solution was added to these wells, 45 minutes before the four-hour incubation period expired. Negative controls remained as plain diluted blood with medium alone. 50 μl supernatant from all wells were harvested and transferred to fresh 96 well plates, and incubated with a tetrazolium salt for 30 minutes. Stop solution was added after 30 minutes, and colour absorbency read off a colorimetric reader at 450 nm.
The concentration of red color is directly proportional to the number of cells lysed.

3.3.5 Statistical analysis

The results of IFN-gamma were statistically analyzed using Microsoft Excel package and the Wilcoxon Rank Test. *P* –values equal to or less than 0.07 ($P \leq 0.05$) were regarded as significant.

3.4 Results
Standard curve

Table 1. Standard curve for the evaluation of IFN-gamma in whole blood assay supernatant

Standard IFN	OD	OD	Average	STDev	COV
1000	0.58	0.56	0.57	0.325	0.32
500	0.38	0.38	0.38	0.143	0.143
250	0.21	0.24	0.23	0.051	0.051
125	0.15	0.16	0.15	0.024	0.024
62	0.12	0.11	0.12	0.014	0.014
31	0.09	0.09	0.09	0.008	0.008
15	0.08	0.08	0.09	0.007	0.007
0	0.07	0.09	0.08	0.007	0.007

OD-Optical density. COV-Coefficient of variance. STDev- Standard deviation
Interferon (IFN)–gamma in pg/ml.

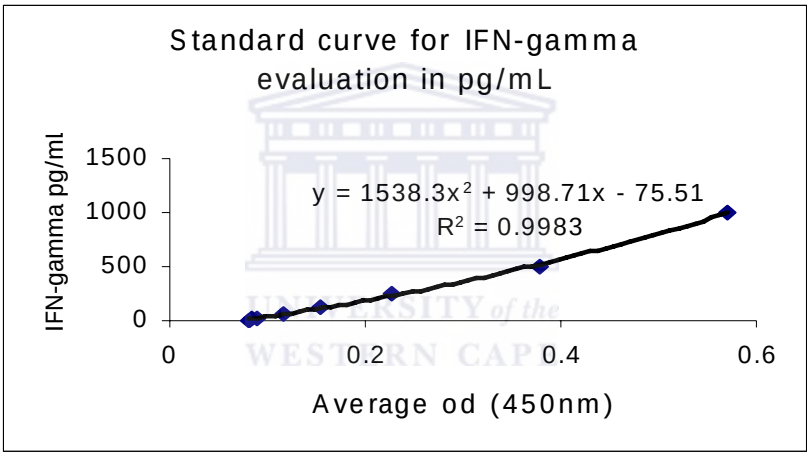
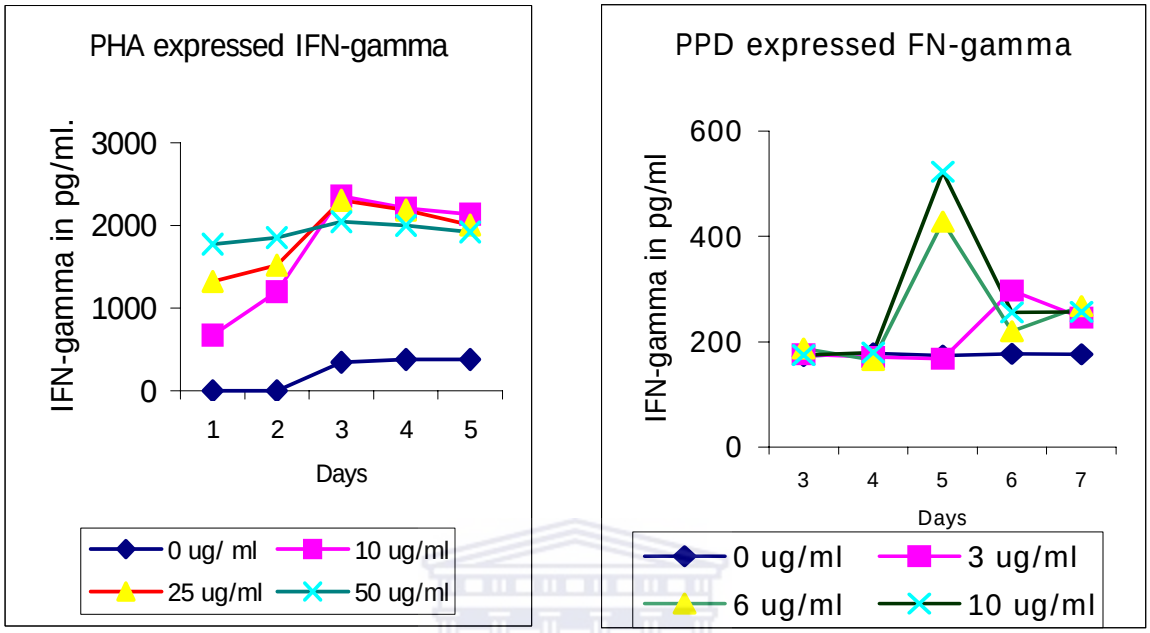


Figure 1. Standard curve. A curve was generated using eight different concentrations, in duplicate, which were analyzed with the same ELISA (DuoSet kit) employed for our IFN-gamma samples. Optical densities were read off a colorimeter at 450 nm. The concentrations of IFN –gamma in pg/ml were plotted against the optical densities obtained. A correlation coefficient of 0.99 was obtained.



A **B**

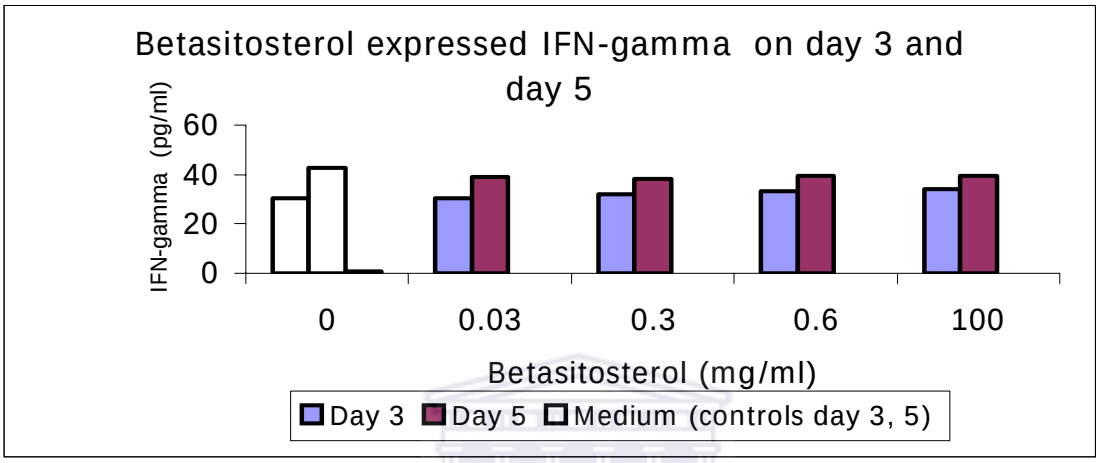
Figure 2. **(A)** The effects of the non-specific, mitogen stimulus, phytohemagglutinin (PHA) on IFN-gamma expression from cells of the immune system, in whole blood (diluted 1:9) assay. These results, generated from one volunteer's blood, are typical of all samples from other volunteers. All specimens were run in duplicate, and incubated at 37 ^{0} C with 5 % CO_2, in a humidified chamber. The expression of IFN-gamma was assessed at different concentrations of PHA, as shown above, over a period of 5 days. The peak expression of IFN-gamma was seen on day 3, at the PHA concentration of 25 μg/ml. This was the concentration selected for the PHA stock solution used in this assay. Similarly, all PHA related supernatants were harvested on day 3. PHA is a lectin, isolated from red kidney beans, which binds $CD4^{+}$ T-cell surface molecules including the T-cell receptor. This results into non-specific stimulation of the T-cell population of the immune system, mainly because it doesn't require major histocompatibility complex (MHC) involvement.

(B) A similar assay was performed using the mycobacterium-derived protein antigen, PPD. The maximum expression of IFN-gamma was seen on day 5 at the PPD concentration of 10 μg/ml. The stock solution for PPD was therefore prepared at a concentration of 10 μg/ml, and all PPD related samples were harvested on day 5. Purified Protein Derivative (PPD) is a *Mycobacterium tuberculosis* related protein antigen, which causes a recall response, and antigen-specific T-cell proliferation only in those previously exposed to TB (19, 23).

Results of whole blood assay

Betasitosterol expressed IFN-gamma

We used whole blood cultures to study the effects of *Hypoxis hemerocallidea* on immune cells in whole blood, with IFN-gamma as a biomarker. The effect of Betasitosterol (BSS) on IFN-gamma expression from whole blood cultures is shown in Figure. 3.



A

Figure 3. Betasitosterol expressed interferon-gamma, day 3 and 5.

* 0- indicates unstimulated, diluted blood in culture medium (control).

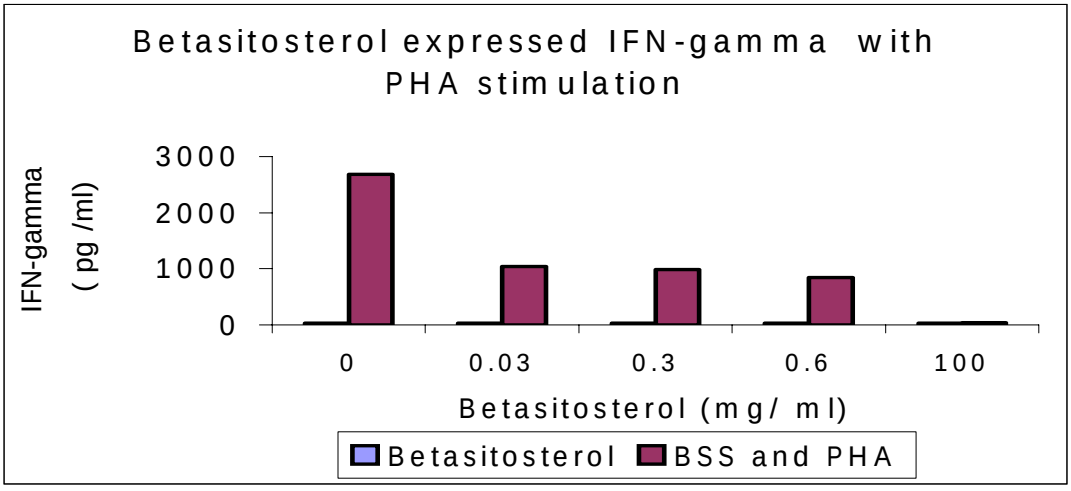


Figure 4. Betasitosterol expressed interferon-gamma, with mitogen stimulation, day 3.

* 0- indicates control, diluted blood in culture medium, and PHA.

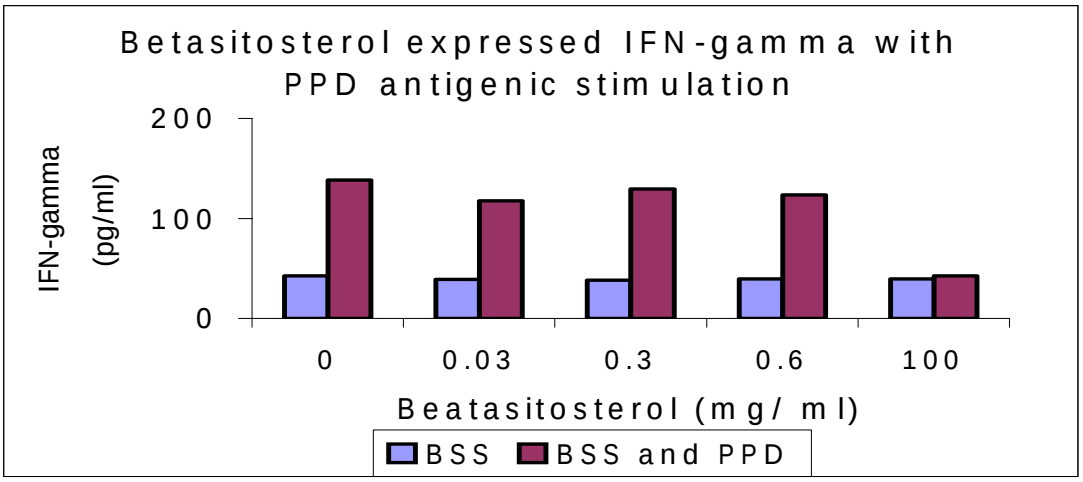


Figure 5. Betasitosterol expressed interferon-gamma, with PPD stimulation, day 5.
***** 0- indicates control, diluted blood in culture medium with PPD.

Unstimulated cells exposed to culture medium only, and used as controls, produced an average 30 pg/ml of IFN-gamma on day 3 with a slight rise to a maximum of 42.6 pg /ml of IFN-gamma on day 5 (Figure 3). Whole blood cultures incubated with only BSS responded with a low expression of IFN-gamma ranging from 33.9 pg/ml on day 3, to 39.5 pg/ml ($P \leq 0.07$) on day 5. Therefore, the response to BSS was seen to be insignificant. However, samples cultured with a combination of BSS and PHA showed a dramatic 300-fold increase in IFN-gamma expression (Figure 4). The maximum expression of IFN-gamma, after PHA stimulation, was seen at the lowest BSS concentration of 0.03 mg/ml with a maximum expression of 1038 pg/ml of IFN-gamma. However, this was still below that of the positive control (PHA), of 2686.11 pg/ml on day 3. In addition, the highest concentrations of BSS at a 100 mg/ml were highly inhibitory to IFN-gamma expression in all samples.

The antigenic stimulation with PPD on whole blood cultures with BSS caused a moderate 3-fold amplification (117.5 pg/ml) of IFN-gamma expression, which declined back to baseline levels at the highest concentration of BSS (Figure 5).

Hypoxoside expressed IFN-gamma

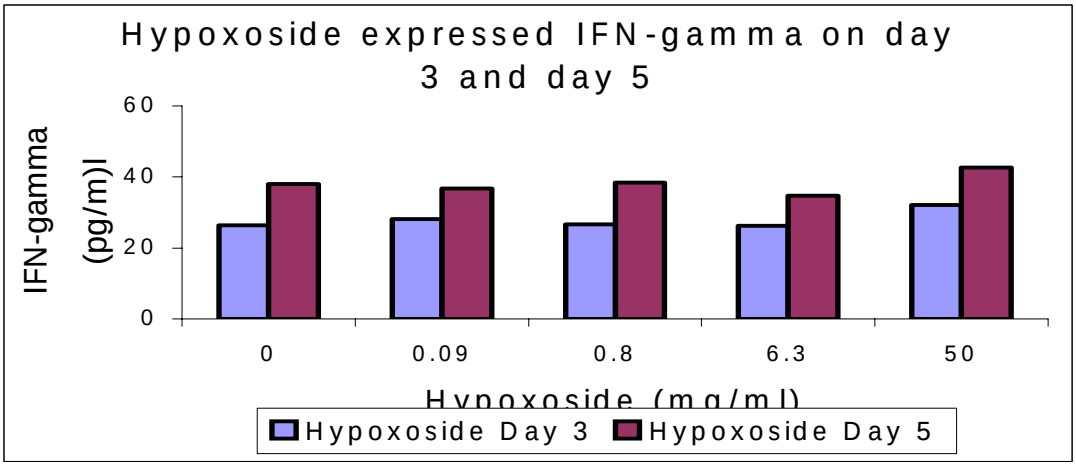


Figure 6. Hypoxoside induced interferon-gamma, day 3, and 5.
* 0- indicates unstimulated, diluted blood in medium.

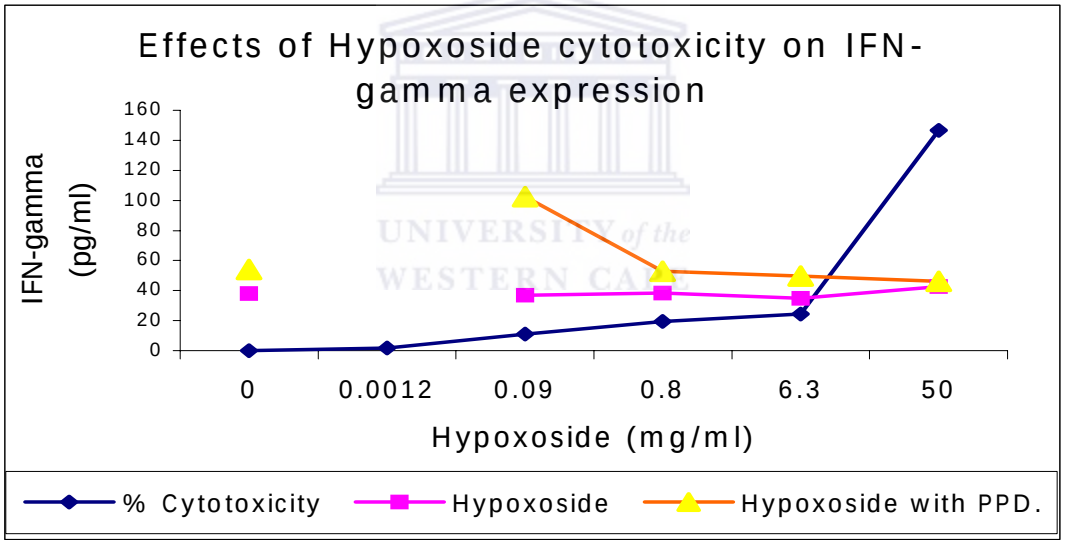


Figure 7. The effects of Hypoxoside cytotoxicity on interferon-gamma expression. The cytotoxicity study had more serial dilutions (0.0012) than our whole blood assay for Hypoxoside.
* 0- Controls, diluted blood plus medium, diluted blood plus PPD and Hypoxoside

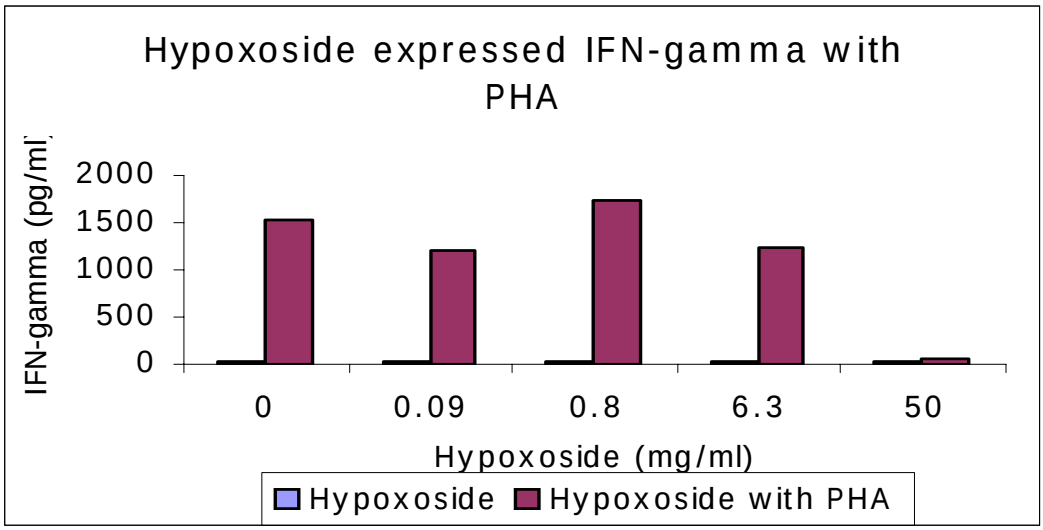


FIGURE 8. Effects of PHA stimulation on Hypoxoside expressed IFN-gamma.
* 0- indicates control- diluted blood with PHA.

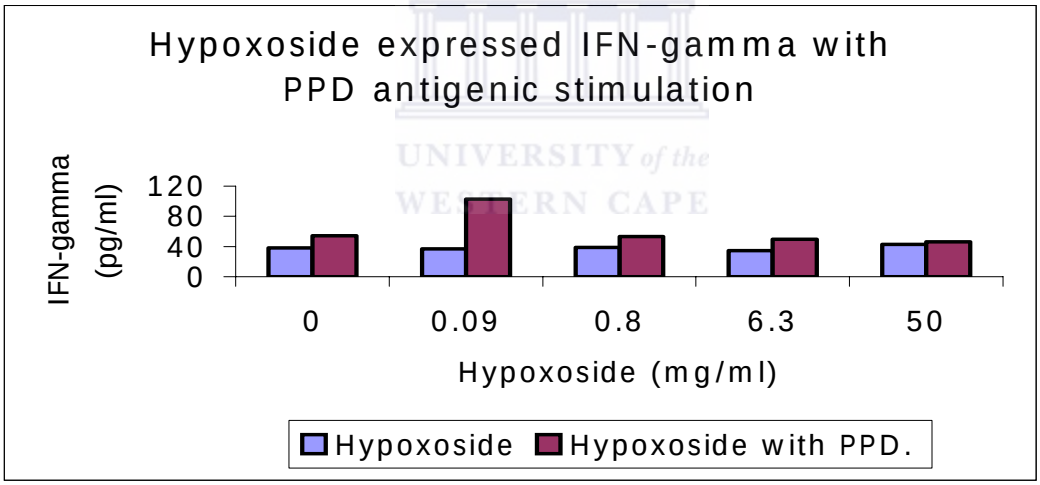


Figure 9. Effects of PPD on Hypoxoside expressed IFN-gamma.
* 0-Control- diluted blood with PPD.

Hypoxoside had moderate expression of IFN-gamma, on its own (Figure 6). Its expression of IFN-gamma remained the same, despite differences in concentration, except for the highest expression which occurred at the highest concentration ($P \leq 0.05$). The additional stimulus of PPD had very moderate effect, observed at all concentrations of Hypoxoside (Figure 9). With PHA stimulation, a significant rise in IFN-gamma was observed at lower concentrations of Hypoxoside, followed by a significant decline at 50 mg/ml ($P \leq 0.05$) (Figure 8). It was also noted that the cytotoxic effects of Hypoxoside had a detrimental effect on its IFN-gamma induction, especially at its highest concentration (Figure 7).

However, although IFN-gamma secretion was low with PPD stimulation, an isolated high peak (1735 pg/ml) was noted at the very low concentrations of 0.09 mg/ml of Hypoxoside (Figure 9). Overall Hypoxoside appeared to have an inhibitory effect on PPD induced expression of IFN-gamma.

20 week-old, 25 % ethanol extract expressed IFN-gamma

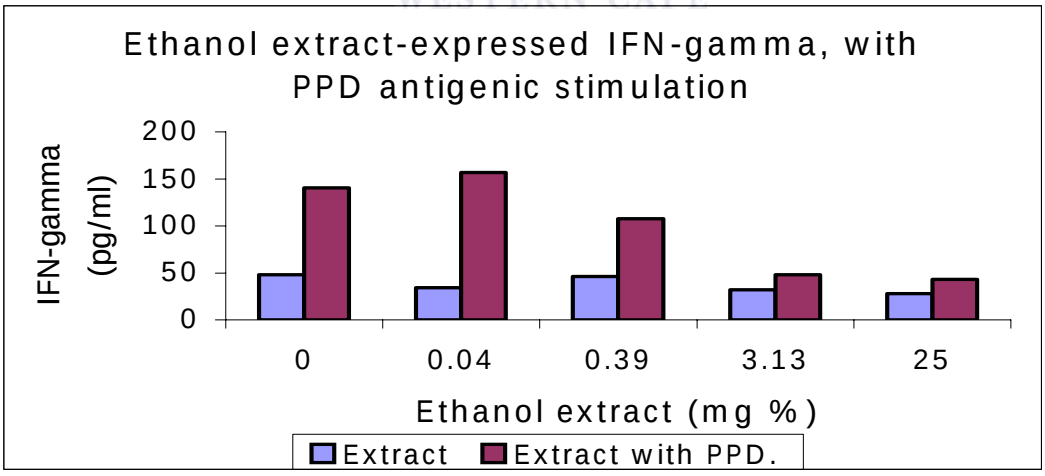


Figure 10 . Effects of PPD on ethanol extract expressed IFN-gamma.
* 0- indicates control- diluted blood in medium with PPD.

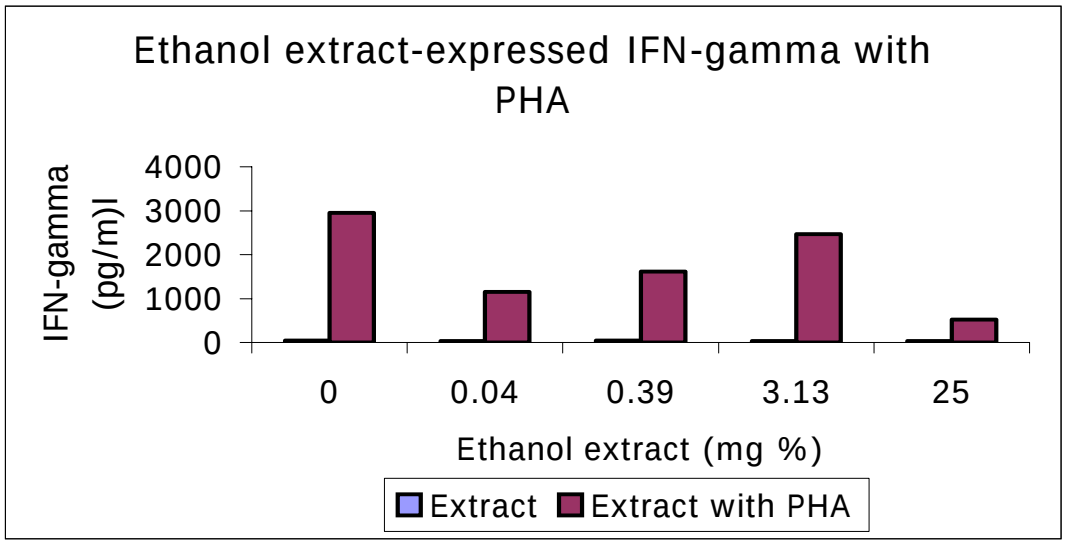


Figure 11. Effect of PHA on ethanol extract expressed IFN-gamma
* 0- indicates control- diluted blood in medium with PHA

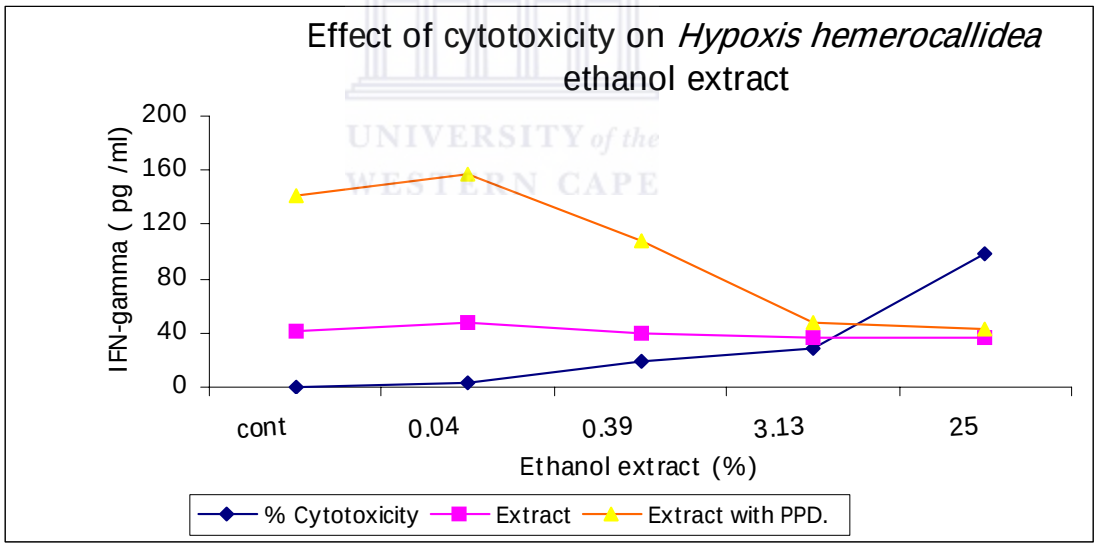


Figure 12. The effects of extract cytotoxicity on its IFN-gamma expression. The ethanol extract from *Hypoxis hemerocallidea*, although less cytotoxic than Hypoxoside, was demonstrated to be almost 100% cytotoxic at 25 % concentration when incubated with diluted whole blood *in vitro* for 4 hours

The ethanol extract, like the two active compounds, had a very moderate expression of IFN-Gamma. Like wise, PPD had very moderate effects, and only at the lowest extract concentrations (Figure 10). Only, the PHA had a strong effect on IFN-gamma expression with extract. More interesting, was the steady rise in IFN-gamma expression observed against the rising cytotoxicity of the extract [Figure 11, Table 13 (Appendix)]. A sharp decline in IFN-gamma expression was observed at the highest concentration of extract ($P \leq 0.05$). In contrast, the PPD expressed IFN-gamma was observed to decline with rising cytotoxicity levels especially at the highest concentration of extract ($P \leq 0.07$, Figure 12).

3.5 Discussion

In this experiment, we had no prior knowledge of the state of activation of the immune cells, or the genetic backgrounds of our volunteers. All volunteers were assumed to be in good health.

The expression of IFN-gamma is a reliable bio-marker of effective cell-mediated immunity (19). The vigorous cellular response induced by IFN-gamma from sensitized macrophages, and T-lymphocytes, is vital in the containment of intracellular pathogens like tuberculosis. The expression of IFN-gamma, in response to a stimulus, is therefore largely dependent on the state of activation of the immune system, and the presence of circulating memory $CD4^+$ T-lymphocytes for that specific stimulus (15).

The moderate IFN-gamma expression observed with BSS agrees with previous findings in normal subjects, since normal digestion, and absorption allow only 5 % of the daily intake to reach the circulation, for the sole purpose of maintenance of the immune system (4, 11). Further, BSS as a steroid molecule is a poor immunogen, which is unlikely to provoke full activation of immune cells (15). Therefore, BSS expressed not more than 40 pg\ml of IFN-gamma.

BSS, with a steroid structure, has the inherent ability to mimic endogenous steroids (9, 10). Steroids are known to be powerful immuno-suppressants, with the ability to cause profound lymphopenia, especially on the $CD4^+$ subset (22). Steroids significantly inhibit T-lymphocyte activation by rendering them unresponsive to IL-1, and IL-2. This is achieved by blocking T-lymphocyte co-stimulatory molecules like B7, CD28 and intracellular adhesion molecules (ICAM-1), which complete the signaling between antigen presenting cells (APCs), and T-cells (22). This is the most likely explanation for the inhibitory effects observed at the highest concentrations of BSS, especially after PHA, and PPD stimulation.

PHA in a plant-derived protein, or lectin, from red kidney beans. PHA behaves as a mitogen, taking resting CD4+ lymphocytes from the resting stages into the S-phase of the cell cycle. The exact mechanism of its activity is not well understood, but it's believed to be phagocytosed by B cells, and presented to T-lymphocytes in association with the Major Histocompatibility (MHC) class II molecule. Mitogens provoke a much faster response from the immune system than pathogenic stimuli, therefore a vigorous cellular response was observed with PHA.

PPD is a mycobacterium-derived protein. Like all immunogenic proteins, after being phagocytosed by monocytes and macrophages, PPD is degraded into peptide fragments (9, 19). The peptide fragments, presented in association with MHC II by macrophages to $CD4^+$ T-lymphocyte receptors, induce lymphocyte activation, and cytokine secretion. Our blood donors, without prior TB exposure, were unlikely to express high outputs of IFN-gamma in response to PPD stimulation, because they lacked the necessary memory cells for a vigorous response to PPD, a mycobacterial antigen. Further, previous research carried out with Rooperol, a product of Hypoxoside, indicated some inhibitory activity on macrophage activity (23).

Hypoxoside demonstrated a moderate, but uniform immunostimulatory effect which appeared to be consistent irrespective of concentration, or cytotoxicity. An interesting outcome was its IFN-gamma expression for days 3, and 5. Its highest output occurred at its highest concentration on both days ($P \leq 0.05$), and its inhibitory effects only became apparent after PHA stimulation, again at its highest concentration. With PPD, high cellular expression of IFN-gamma was only observed at very weak concentrations of Hypoxoside (0.09 mg/ml), indicative of a possible therapeutic window.

Although the exact mode of activity of Hypoxoside on immune cells is not known at this stage, these findings are highly significant because they indicate immunostimulatory activity which appears to be dependent on concentration of Hypoxoside, the state of activation of the immune cells, and cytotoxicity which appears to be negated by unknown mechanisms.

Raising the state of immune cell activation, with PHA, appears to have rendered them more vulnerable to cytotoxic effects at high concentrations of Hypoxoside ($P \leq 0.05$). This may indicate that Hypoxoside is more likely to be cytotoxic within hyper-activated immune systems.

The ethanol extract was found to be moderately immunostimulatory. However, immune cells demonstrated different outcomes with the ethanol extract, presumed to contain both compounds, especially with PHA stimulation. With PHA, the extract yielded the highest expression of IFN-gamma, 2000 pg/ml, at an extract concentration of 3.13 %. An interesting phenomenon was observed where IFN-gamma output rose in direct proportion to extract concentration, and only declined at the highest concentration of extract. The presence of unknown compounds, or synergism between BSS, and Hypoxoside in the extract might explain the steady rise of IFN-gamma. This was the only steady dose-related rise in IFN-gamma output noted in this experiment, which was reversed at the highest extract concentrations ($P \leq 0.05$). The inhibitory effect, related to rising concentrations of isolated active compounds, appeared to have been abolished with the extract. Therefore, highly activated immune cells may be protected by unknown mechanisms within the extract itself, from its cytotoxic effects.

Therefore, possibility of synergism or additive activity, between BSS and Hypoxoside, should be seriously considered regarding its protective activity. Both active compounds need to be analyzed further, especially at those specific concentrations where they display maximum stimulation of IFN-gamma.

In spite of high toxicity displayed by Hypoxoside, and the crude ethanol extract as well as the inhibitory activity seen at high concentrations of extract and active compounds, there is still significant immunostimulatory activity noted at specific concentrations of extract and both compounds. It remains to further exploit these windows of opportunity to gain therapeutic benefit from a combination of both compounds, which appear to be synergistic in their effects on immune cells.

This finding, that both compounds have immunostimulatory activity, represents an opportunity to exploit the combined synergism between BSS and Hypoxoside, for therapeutic benefit. Because of its cytotoxic effects, it is imperative that further research be done to investigate therapeutic windows observed at its lower concentrations to avoid the cytotoxic effects observed at higher concentrations.

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Chapter four

Conclusion

Key findings of *Hypoxis hemerocallidea* extracts, and actives as natural antibiotic and immune modulation phytotherapies.

Chapter 4

Table 1. A summary of the most important findings of this investigation

Aims	Outcomes
Antimycobacterial assessment	
1. To evaluate antimycobacterial activity of *Hypoxis hemerocallidea*.	Both the methanol and ethanol extracts from *Hypoxis hemerocallidea* demonstrated anti mycobacterial activity, in a dose-dependent manner.
2. To assess which of the aqueous and organic extracts of *Hypoxis hemerocallidea* are better antibiotic dosage forms.	The 8th week, 50 % methanol extract showed the most potent antimycobacterial activity.
3. To investigate the antimycobacterial activity of *Hypoxis*-derived Betasitosterol, and Hypoxoside as the main active compounds from *Hypoxis hemerocallidea*.	Only Hypoxoside, at a concentration of 10 % demonstrated significant antimycobacterial activity.
Immune modulation assessment	
1. Too assess the effects of the compounds, hypoxoside and Betasitosterol, from *Hypoxis hemerocallidea* on human immune cells.	Betasitosterol had a moderate effect on IFN-gamma expression without exogenous stimulation. Hypoxoside had an even weaker effect on IFN-gamma expression, without exogenous stimulation, compared to Betasitosterol.
2. To assess the effects of *Hypoxis hemerocallidea* 25 % ethanol extract on human immune cells.	The 25 % ethanol extract, without exogenous stimulation, had an effect on IFN-gamma expression 3-4 times higher than Betasitosterol, especially on day 5.
Cytotoxicity assessment	
1. To assess Hypoxoside, and the ethanol extract for cytotoxic effects	Hypoxoside was more cytotoxic than the extract.
2. To assess the effects of cytotoxicity on immunomodulatory activity	Although the activity of Hypoxoside decreased with increasing cytotoxicity, the extract's immunomodulatory activity was not significantly deterred by rising cytotoxicity.
To find evidence of synergism	
To find any evidence of synergistic activity, between Hypoxoside and Betasitosterol, within the 25 % ethanol extract.	The better performance of the whole extract, with and without PHA, compared to the two isolated compounds, is highly suggestive of synergism, or additive immunomodulatory activity between the two.

In spite of the availability of curative drugs, a vaccine and public health surveillance measures, tuberculosis (TB) remains a major killer around the globe (1). The emergence of resistant TB strains is an ominous sign of the impending era of untreatable TB. There is therefore, a palpable urgency to identify novel antimicrobials against TB. While medicinal plants remain a promising source of novel therapeutic compounds, they are threatened with extinction in different parts of the world. The procrastination and neglect which prevailed in TB research in the last 50 years, and the emergence of the *Human immunodeficiency virus* (HIV) provided the TB bacillus the opportunity to evolve into formidable, resistant strains (2, 12, 17, 18). Meanwhile, since Rifampicin was discovered, there hasn't been a single new first-line TB drug in the last 40 years (2).

The first objective of our study, therefore, was to assess *Hypoxis hemerocallidea*, specifically for antibacterial activity using a wide range of extracts from its underground rhizome. To make this assessment more through, the extracts were allowed to mature over 21 weeks and the potency of each one was re-assessed at intervals. The results indicated that the methanol, and ethanol extracts, stored at 4^0 C, needed at least 8 weeks to mature to their full potency against *Mycobacterium smegmatis*. The most concentrated methanol (50 %) extract was found to be the most potent against *Mycobacterium smegmatis*. Both the methanol and ethanol extracts demonstrated a strong correlation between their concentrations and potency, in the first 8 weeks.

The only *Hypoxis*-derived active compound that demonstrated significant activity against *M. smegmatis* was Hypoxoside at a 10 % concentration in ethanol with potency comparable to that of the 8^{th} week 50 % methanol extract.

The lack of activity from Betasitosterol, like the water and chloroform extracts, was most likely due to the lack of proper solvent, and especially for those still unknown compounds visualized on the Thin Layer Chromatogram (TLC).

These results, although obtained *in vitro* against a non-pathogenic mycobacterium, indicate that the rhizome of *Hypoxis hemerocallidea* does contain a compound, or compounds capable of overcoming the highly resistant mycobacterial cell wall.

It is also possible that part of this activity could be attributable to the high cytotoxicity observed from the ethanol extract, and Hypoxoside (3). Or, it could be due to some yet unknown mechanism of activity on the mycobacterial cell wall by unknown compounds. The exact identity of the unknown compound or compounds remains to be investigated, and the possibility of synergism between them cannot be excluded at this stage (4).

The presence of multiple compounds was confirmed on the TLC, of the ethanol, and methanol extracts. However, it is the crude aqueous extract which is extensively used in ethno-botanical preparations. Its also possible that minute quantities of active compounds, present in the extract may be adequate to achieve some therapeutic benefits. This is more likely to be the case with phytosterols, where only minute concentrations have been found adequate to sustain immune function (5), and provoke $CD4^+$ T-lymphocyte proliferation (6). We have also noted, in this experiment, that very minute concentrations of both Hypoxoside, and Betasitosterol are more likely to be immunostimulatory, rather than immunosuppressive, or toxic. This is in agreement with previous studies on the therapeutic windows of potential toxic active compounds (7). It is therefore not surprising that *H. hemerocallidea*'s crude aqueous extracts has proved to be so popular with HIV, and AIDS sufferers, especially those without access to conventional medicine (8).

It can also be hypothesized that inactive compounds in the aqueous extract, once ingested may be broken down either in the acidic stomach, or digested in the gastro-intestinal tract into more active forms. This has been the finding with Rooperol, which is released from Hypoxoside by bacterial enzymes in the small and large intestine (9). These findings, and observations, give some credibility to the popular use of the aqueous extract, which continues to be highly controversial (8, 10). In spite of the cytotoxicity observed here, with the ethanol extract and Hypoxoside, the minuteness of the concentrations of active compounds in the crude aqueous extract may explain the relative absence of fatal toxicity related to popular use of the aqueous extract in Southern Africa (5).

Our second major objective was to explore the immune modulating effects of *Hypoxis hemerocallidea's* ethanol extract as well its two major active compounds, Betasitosterol and Hypoxoside. Using IFN-gamma expression, as a biomarker for the protective Th1 cytokine profile and cell-mediated immunity, we demonstrated that *Hypoxis hemerocallidea* does have unusual immune-modulatory properties.

Both the active compounds, Betasitosterol and Hypoxoside, were found to be moderate immunostimulants, and this agreed with previous research (5). Mitogen stimulation from phytohemagglutinin (PHA), and antigenic stimulation from Purified Protein Derivative (PPD), enhanced their immunostimulatory activity. Their moderate effect on IFN-gamma expression from our volunteers confirmed their lack of previous exposure to mycobacterial infections, and therefore absence of memory T-cells to provoke a vigorous cell-mediated response (CMI) to PPD. Although PPD induced IFN-gamma expression its not a direct correlate of specific immunity against TB, our findings indicated lack of immuno-protection, and therefore vulnerability to TB in our volunteer group (11, 12).

Betasitosterol, like the extract, was inhibitory to IFN-gamma expression at its highest concentration. The only exception was Hypoxoside, which demonstrated better expression of IFN-gamma at its highest concentration, especially in the presence of mitogen stimulation [Table 6 and 8 (Appendix)]. These findings indicate that the different compounds and extract have very different therapeutic windows which need further assessment, if they are to be exploited for synergistic activity.

The ethanol extract demonstrated dose-dependent immunostimulation, especially after mitogen stimulation, where IFN-gamma expression rose steadily with rising concentrations, in spite of its rising cytotoxicity [Table 10 and 12 (Appendix)]. This was most likely a direct result of some yet unexplained form of protective synergism, or additive behaviour shared between the active compounds. Synergism, considered to be a great asset of phytotherapy, is the potentiation of beneficial effects shared between active compounds (4).

Again, this kind of collective, protective activity is likely to explain the absence of toxicity, or gross side effects in users of the crude aqueous extract. Synergism and additive behaviour adds credibility to the unexplained efficacy of crude aqueous extracts.

Even more interesting, is the finding that the immuno-protective effect, observed with the extract appears to be more potent when immune cells are highly activated. This is highly significant in HIV pathology where the immune system is in a perpetual state of high activation (13, 14).

In HIV infection there is a progressive decline of $CD4^+$ T-lymphocytes, associated with dramatic loss of cell-mediated immunity (CMI), poor cytotoxic T-lymphocyte responses as well as poor humoral responses (13, 14). Since IFN-gamma, the prototypic cytokine for the Th1 profile is mainly secreted by $CD4^+$ T lympocytes, and $CD8^+$ T lymphocytes, a decrease in Th1 cytokines precedes the onset of AIDS. It has been observed in animal, and humans that the absence of IFN-gamma increases the risk of mycobacterial infections, and is associated with disseminated I infections. AIDS sufferers are therefore poised to benefit greatly from further research into *Hypoxis*. All its features, from CD4 T-lymphocyte proliferation (5, 6, 15), cytotoxicity against cancer cell lines (9), promotion of CMI as indicated by IFN-gamma expression, antimycobacterial activity, and finally immuno-protective activity indicate in this experiment, hold great promise for the future management of HIV, and resistant TB co-infections.

Since the two compounds from *Hypoxis hemerocallidea* appear to have different mechanisms of immunostimulation, at different concentrations, their synergistic effects need to be explored further to determine therapeutic windows and the specific concentrations of maximum benefit.

Although not fully understood yet, even the immunosuppressive effects, at higher concentrations can be exploited in the future for therapeutic benefits in the management of allergic and inflammatory conditions.

Future research should explore the therapeutic effects of *Hypoxis hemerocallidea* extract, as well as Betasitosterol and Hypoxoside on immune cells and cytokine profiles from the following:

1. Patients with latent tuberculosis,
2. Patients with active tuberculosis,
3. Patients with latent HIV infection,
4. Patients with full-blown AIDs,
5. And patients co-infected with both latent and active infections.

This study therefore highlights the potential benefits to disease prevention, and treatment from *Hypoxis hemerocallidea*. Our results suggest that full immunomodulatory benefits from *Hypoxis hemerocallidea* will most likely be realized from the exploitation of synergy between its active compounds.

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Appendices

Raw data of experiments

Appendices

1. Betasitosterol induced IFN-gamma in whole blood supernatant from 6 volunteers - Day 3

	mg/ml	1	2	3	4	5	6	Average	Median	ST
trol	Medium 0	27.39	33.47	38.72	40.25	23.80	22.01	30.94	30.43	7.7
S	0.03	24.03	63.55	45.04	42.13	25.68	25.44	37.65	33.91	15
S	0.3	23.57	57.75	40.87	39.33	27.14	21.79	35.08	33.23	13
S	0.6	24.73	41.18	39.33	42.45	22.89	20.93	31.92	32.03	10
S	100	24.26	55.88	41.18	34.32	26.65	24.26	34.43	30.48	12

PHA (25 μg/ml) =0.25 μg/10 μl/ well

2. Betasitosterol induced IFN-gamma, (with PHA stimulation) in whole blood supernatant from 6 volunteers - Day 3

	mg/ml	1	2	3	4	5	6	Average	Median	ST
trol	PHA	48.06	2955.23	1632.63	6838.71	5462.43	2416.99	3225.67	2686.11	250
PHA	0.03	26.90	5046.50	1699.50	43.73	29.95	31.01	1146.27	37.37	202
PHA	0.3	24.73	130.76	592.49	7022.15	5902.70	1089.61	2460.41	841.05	314
PHA	0.6	201.53	99.46	1193.32	7147.23	5447.29	776.64	2477.58	984.98	303
PHA	100	27.64	836.17	1241.54	5722.99	6376.41	282.75	2414.58	1038.85	285

Phytohemagglutinin (PHA), Betasitosterol (BSS)

3. Betasitosterol induced IFN-gamma in whole blood supernatant from 6 volunteers - Day 5

	mg/ml	1	2	3	4	5	6	Average	Median	ST
trol	Medium 0	163.22	40.87	86.40	44.39	30.74	24.97	65.10	42.63	52
S	0.03	46.04	41.81	37.22	42.13	32.64	28.15	38.00	39.52	6.
S	0.3	41.81	37.22	44.39	43.41	28.91	26.16	36.98	39.52	7.
S	0.6	36.92	44.06	39.63	47.04	30.74	27.64	37.67	38.28	7.
S	100	40.87	47.04	36.92	43.09	31.28	27.14	37.72	38.90	7.

Purified Protein Derivative- PPD (10 μg/ml) = 0.1 μg/10 μl / well

4. Betasitosterol induced IFN-gamma (with PPD stimulation) in whole blood supernatant from 6 volunteers-Day 5

	mg/ml	1	2	3	4	5	6	Average	Median	ST
trol	PPD	119.47	58.13	43.41	157.45	1018.20	490.39	314.51	138.46	381
PPD	0.03	127.32	96.97	39.33	45.70	30.74	33.19	62.21	42.51	40
PPD	0.3	129.03	117.82	40.25	270.05	364.15	37.81	159.85	123.43	130
PPD	0.6	126.18	67.99	45.04	766.70	590.01	132.50	288.07	129.34	309
PPD	100	65.55	143.79	34.32	91.13	1072.79	519.07	321.11	117.46	408

Hypoxoside

5. Hypoxoside induced IFN-gamma in whole blood supernatant from 6 volunteers - Day 3

oxoside in mg/ml		1	2	3	4	5	6	Average	Median	STD
rol	Medium	28.15	24.73	34.89	36.63	24.50	23.12	28.67	26.44	5.7
p	0.09	28.91	32.37	44.06	38.72	31.55	31.82	34.57	32.09	5.6
p	0.8	26.90	21.79	35.46	33.47	24.97	25.68	28.04	26.29	5.2
p	6.3	28.15	25.20	32.37	34.89	22.67	24.50	27.96	26.67	4.8
p	50	26.16	25.68	33.19	34.60	23.80	30.21	28.94	28.19	4.3

Hyp = Hypoxoside PHA (25 μg/ml) =0.25 μg/10 μl/ well

6. Hypoxoside induced IFN-gamma, with PHA stimulation, in whole blood supernatant from 6 volunteers-Day 3

rol	PHA	42.77	1395.75	1630.56	5634.18	1434.26	6137.27	2712.46	1532.41	252
PHA	0.09	75.16	36.04	144.39	39.63	68.40	35.17	66.47	54.02	41.
PHA	0.8	97.47	27.64	1371.00	2251.22	1071.11	6936.53	1959.16	1221.05	257
PHA	6.3	86.87	1092.99	1642.99	4134.61	1826.33	3952.18	2122.66	1734.66	160
PHA	50	27.39	63.15	1765.57	3773.86	657.63	5079.32	1894.49	1211.60	210

7. Hypoxoside induced IFN-gamma in whole blood supernatant from 6 volunteers – Day 5

rol	Medium	38.11	37.81	39.02	42.77	34.03	28.40	36.69	37.96	4.9
p	0.09	36.92	51.18	41.81	43.41	34.03	45.70	42.18	42.61	6.3
p	0.8	33.47	36.04	41.81	39.02	25.68	28.15	34.03	34.76	6.2
p	6.3	48.06	40.25	36.63	45.04	26.41	28.40	37.46	38.44	8.1
p	50	37.81	40.25	35.75	44.39	30.48	27.39	36.01	36.78	6.2

PPD (10 μg/ml) = 0.1 μg/10 μl / well

8. Hypoxoside induced IFN-gamma, with PPD stimulation, in whole blood supernatant from 6 volunteers- Day 5

rol	PPD	37.81	40.56	40.25	67.58	1288.87	118.37	265.57	54.07	502
PPD	0.09	37.22	43.09	49.43	54.41	37.51	51.89	45.59	46.26	7.4
PPD	0.8	44.06	37.81	77.34	48.06	51.54	54.05	52.14	49.80	13.
PPD	6.3	35.46	38.11	39.94	65.95	167.78	130.18	79.57	52.94	56.
PPD	50	35.46	72.59	39.63	132.50	395.97	441.91	186.34	102.54	184

PHA-phytohemagglutinin, PPD – Purified Protein Derivative

25% *Hypoxis hemerocallidea* Ethanol extract

Table 9. *Hypoxis hemerocallidea,* 20-week-old ethanol extract, induced IFN-gamma in whole blood supernatant from 6 volunteers-Day 3

		1	2	3	4	5	6	Average	Median	ST
ntrol	0	67.17	23.35	677.44	57.00	39.33	25.20	148.25	48.16	259
tract	0.04	29.17	26.16	26.41	39.94	1051.11	27.14	199.99	28.16	410
tract	0.39	29.43	25.20	61.58	42.77	34.60	24.50	36.35	32.02	14
tract	3.13	66.36	24.26	190.16	58.13	34.03	26.65	66.60	46.08	62
tract	25	32.92	22.89	312.62	44.39	35.46	25.44	78.95	34.19	114

Ext = Extract PHA (25 μg/ml) =0.25 μg/10 μl/ well

10. *Hypoxis hemerocallidea* , 20-week-old ethanol extract, induced IFN-gamma (with PHA stimulation) in whole blood supernatant from eers Day 3

ntrol	0	5115.90	72.59	3615.06	1391.92	11315.25	2275.59	3964.38	2945.32	400
PHA	0.04	3101.81	219.96	252.78	724.81	3948.96	316.23	1427.43	520.52	165
PHA	0.39	4217.38	23.12	3465.70	1451.76	3611.98	993.85	2293.97	2458.73	169
PHA	3.13	5676.57	23.35	260.93	1337.09	3910.42	1888.12	2182.75	1612.60	220
PHA	25	5364.41	23.80	591.25	1397.66	5002.92	894.85	2212.48	1146.25	234

Table 11. *Hypoxis hemerocallidea,* 20-week-old ethanol extract, induced IFN-gamma in whole blood supernatant from 6 volunteers-Day 5

ntrol	0	34.32	38.41	465.83	44.06	49.09	31.55	110.54	41.24	174
tract	0.04	26.41	39.02	460.34	33.47	43.09	34.03	106.06	36.53	173
tract	0.39	36.63	82.72	475.80	36.92	34.89	31.82	116.46	36.77	171
tract	3.13	35.46	104.03	465.83	43.09	32.37	26.65	117.90	39.28	172
tract	25	46.70	146.83	510.95	41.50	47.04	25.92	136.49	46.87	188

PPD (10 μg/ml) = 0.1 μg/10 μl / well

12. *Hypoxis hemerocallidea,* 20-week-old ethanol extract, induced IFN-gamma (with PPD stimulation) in whole blood supernatant from eers Day 5

ntrol	0	259.29	42.13	488.13	39.63	90.17	190.86	185.04	140.52	172
PPD	0.04	26.65	40.56	463.63	45.37	47.38	40.56	110.69	42.96	173
PPD	0.39	29.69	117.82	476.91	34.89	39.33	56.63	125.88	47.98	174
PPD	3.13	68.81	143.79	479.15	36.92	71.32	294.87	182.47	107.55	172
PPD	25	167.13	146.22	474.69	37.81	107.65	498.34	238.64	156.68	197

Table 13. Results of cytotoxicity assay to assess toxicity of hypoxoside and 20-week-old 25 % ethanol extract on whole blood

CytoTox 96- Cytotoxicity whole blood assay							
Control + Lysing solution	**max LDH release**	Assay in triplicate			**Average**	**Adjusted**	**% Cytotoxicity**
		OD	**OD**	**OD**			
0.85	1.801	1.69	2.12	2.351	2.05	1.25	95.63
0.832	1.524	1.529	1.535	1.83	1.63	0.83	63.27
0.735	1.588	0.938	1.041	1.461	1.15	0.34	26.13
0.806	1.305	0.993	1.077	1.068	1.05	0.24	18.42
Control - 0 RPMI medium		0.763	0.941	0.655	0.79	-0.02	0.00
0		0.79	0.977	0.618	0.80	-0.01	0.00
0		0.442	0.842	0.71	0.66	-0.14	0.00
0		0.8798	1.057	0.925	0.95	0.15	0.00
Hypoxoside mg/ml							
	50	2.397	2.819	2.937	2.72	1.91	146.51
	6.3	1.274	1.109	0.988	1.12	0.32	24.37
	0.8	1.085	0.879	1.217	1.06	0.25	19.51
	0.09	1.01	0.934	0.907	0.95	0.14	11.09
	0.0012	0.916	0.925	0.641	0.83	0.02	1.66
Ethanol extract in g %							
	25	2.21	2.18	1.906	2.10	1.29	99.08
	3.13	1.347	1.137	1.061	1.18	0.38	28.81
	0.39	0.68	1.127	1.354	1.05	0.25	19.00
	0.04	0.725	0.787	1.024	0.85	0.04	3.04
	0.006	0.862	0.961	0.644	0.82	0.02	1.28
	0.0008	0.889	0.976	0.586	0.82	0.01	0.87

OD- Optical density read from colorimetric reader at 450 nm

LDH-Lactate dehydrogenase- (cytosolic enzyme) Roswell Park Memorial Institute (RPMI) culture medium