


The immune-modulating activity of *Sutherlandia frutescens*

By

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The logo of the University of the Western Cape, featuring a classical building with columns and a pediment, with the text 'UNIVERSITY of the WESTERN CAPE' below it.

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Human whole blood culture

Sutherlandia frutescens

T_{H1}/T_{H2}



Abbreviations

AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
COX-2	Cyclooxygenase 2
DAS	Double antigen sandwich
DC	Dendritic cells
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme linked immune-sorbent assay
GABA	γ -aminobutyric acid
GO	Government organization
GIT	Gastro-intestinal tract
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
IFN γ	Inteferon-gamma
Ig	Immunoglobulin
IL-1 β	Interleukin-1 β
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-13	Interleukin-13
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MASP	MBL-associated serine protease
MBL	Mannose binding lectin
MHC	Major histocompatibility complex
N	Mean
NGO	Non government organization
NK	Natural killer
NO	Nitrous oxide
PHA	Phytohaemagglutinin
ROS	Reactive oxygen species
TB	Tuberculosis
TCR	T cell receptor
TLRs	Toll like receptors
TNF	Tumour necrosing factor
SD	Standard deviation
UNICEF	United nations children's fund
UTI	Urinary tract infection
WHO	World health organization



Latin Abbreviations

α (*alpha*)-

β (*beta*)-

γ (*gamma*)-

et al (*et alii*)-

etc (*et cetera*)-

and others



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Abstract

Sutherlandia frutescens is a traditional herbal remedy commonly used in South Africa. It is used to treat conditions like colds, influenza, fever, diabetes mellitus, liver conditions, rheumatism, urinary tract infection (UTI), stress and anxiety, treatment and prevention of cancer, treatment of wasting in cancer and patients suffering from acquired immune deficiency syndrome (AIDS). *Sutherlandia frutescens* can also be used topically to treat burns, wounds and inflammatory skin conditions.

Due to its versatility, it is a popular herb of choice for scientific scrutiny. *Sutherlandia frutescens* contains three main constituents, L-canavanine, pinitol and γ -aminobutyric acid (GABA). Found in the leaf and seeds, L-canavanine is responsible for the antiviral, anti-cancer and anti-inflammatory properties ascribed to *Sutherlandia frutescens*. Pinitol has been recognized for its anti-diabetic and immune modulating effects. GABA is a non essential amino acid and neurotransmitter with anti-anxiety effects. It has therefore been indicated for hypertension. There are many studies validating the medicinal value of *Sutherlandia frutescens* on a range of illness conditions, such as viral disease, cancer, inflammation and diabetes mellitus. Focus was placed on the biological activity of *Sutherlandia frutescens* on the immune system by using whole blood cells.

The aim of this study was to investigate the effects of *Sutherlandia frutescens* on the inflammatory response and T cell differentiation *in vitro* using cytokines as biomarkers. Whole blood cells containing various concentrations of *Sutherlandia frutescens* were stimulated *in vitro* with either Lipopolysaccharide (LPS) or Phytohaemagglutinin (PHA). Results show that *Sutherlandia frutescens* is not toxic at any of the concentrations tested. The addition of *Sutherlandia frutescens* at high concentrations to the stimulated whole

blood cell cultures reflects a significant down regulation of Interleukin(IL) 6 and IL-10 compared to the control ($P < 0.05$) hence suppressed the inflammatory and humoral immune response. Results obtained for Inteferon-gamma ($IFN\gamma$) shows that *Sutherlandia frutescens* is donor specific as it reflects both up and down regulation in the release of $IFN\gamma$ at the concentrations tested. The *in vitro* data generated by this study supports the use of *Sutherlandia frutescens* in the management of inflammatory conditions and allergies such as asthma. However the effects of *Sutherlandia frutescens* on cell mediated immunity was found to be donor specific. Further investigation of *Sutherlandia frutescens* on cellular immunity is advised.



Declaration

I, Najwa Kisten, declare that the thesis entitled “The immune-modulating activity of *Sutherlandia frutescens*” is my work and has not been submitted before for any degree or examination in any other university, and that all the sources of my information have been indicated and acknowledged by complete references.



Najwa Kisten

May 2010

Signed:.....

Acknowledgments

In the name of God, the Most Beneficent, the Most Merciful

I make *shukr* to my Creator for granting me the inner strength and ability to fulfill my goals. I feel privileged to be chosen to make a contribution to the science world.

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Chapter I: The biological activity of *Sutherlandia frutescens*

1.1 Introduction

Sutherlandia frutescens is commonly used as a traditional herbal remedy in South Africa. It is a well known multipurpose medicinal plant (Fernandes, et al., 2004; Ojewole, 2008). Widely known as cancer bush, *Sutherlandia frutescens* has been used in traditional medical systems of different cultural groups such as Zulu, Xhosa, Sotho, Khoi-San and Cape Dutch for a wide variety of illness conditions (van Wyk and Albrecht, 2008).

This versatile medicinal herb is indigenous to Africa. It is found in the southern parts of Namibia, the extreme southeastern corner of Botswana, the western, central and eastern parts of South Africa and most of Lesotho. *Sutherlandia tomentosa*, a second species, has a restricted distribution and is localized along the Cape coast (van Wyk and Albrecht, 2008). Both species are used in traditional medicine.

Because of the wide distribution range of *Sutherlandia frutescens*, it is known by several vernacular names in different languages. In English, it is known as *cancer bush*, *sutherlandia*, *balloon pea* and *turkey flower*. In Afrikaans, it is known as *kankerbos*, *gansies*, *grootgansies*, *wildekeur(tjie)*, *keurtjie*, *rooikeurtjie*, *kalkoen(tjie)bos*, *kalkoenblom*, *belbos*, *kalkoenbelletjie*, *klapperbos*, *bitterbos*, *eentjies* and *hoenderbelletjie*. In German, it is known as *blasenstrauch*, *krebsbusch* and *sutherlandia*. In Sesotho, it is known as *musa-pelo*, *'musa-pelo-oa-noka* and *motlepelo*. In Setswana, it is known as *phetola*. In Zulu, it is known as *insiswa*, and in Xhosa, it is known as *unwele* (van Wyk and Albrecht, 2008).

Recent taxonomic data suggest that *Sutherlandia frutescens* is synonymous to *Lessertia frutescens* (Matsabisa, 2006; van Wyk, 2008). There are countless regional forms, genotypes and chemotypes represented by the species of *Sutherlandia frutescens* (van Wyk, 2008).

Sutherlandia frutescens comprises of six taxa all of which are endemic to southern Africa (Moshe, et al., 1998). Habitat, orientation of the fruit stipe, the shape of the pod and the shape and pubescence of the leaflets are the taxonomically significant characters used to distinguish the taxa. Moshe, et al. (1998) used horizontal starch gel electrophoresis to examine the genetic diversity of *Sutherlandia frutescens* var. *incana*, *Sutherlandia microphylla*, *Sutherlandia speciosa*, *Sutherlandia humilis*, *Sutherlandia montana* and *Sutherlandia frutescens*.

Sutherlandia frutescens, (figure 1.1 and 1.2) is a perennial shrub which grows up to 2m in height (van Wyk and Wink, 2004). It is a flowering shrub of the pea family (*Fabaceae/Leguminosa*) (Sia, 2004). The stems are prostrate to erect with compound pinnate leaves and leaflets that are oblong to linear elliptic. The leaves are three or more times longer than wide, slightly to densely hairy and silvery in appearance. The flowers are bright red and the fruit resembles inflated bladder-like, papery pods (Gericke, et al., 2001; van Wyk and Albrecht, 2008). The seeds are black, flattened and approximately 3mm in diameter (van Wyk and Albrecht, 2008).



Figure 1.1: Photograph showing the leaves and flowers of *Sutherlandia frutescens* (Matsabisa, 2006)



Figure 1.2: Line drawing depicting *Sutherlandia frutescens* (Matsabisa, 2006)

1.2 Medicinal uses of *Sutherlandia frutescens*

Sutherlandia frutescens is commonly used as a medicinal plant in South Africa (Fernandes, et al., 2004; Mills, et al., 2005; Ojewole, 2004; van Wyk and Albrecht, 2008). The aerial parts of *Sutherlandia frutescens* are used in traditional medicine for the management, treatment and/or control of various human ailments in various systems (Ojewole, 2008). The systems affected, along with the anecdotal and traditional uses of *Sutherlandia frutescens* are summarized in Table 1.

Table 1: Alphabetical list of human systems affected by the anecdotal and/or traditional uses of *Sutherlandia frutescens*

System affected	Anecdotal and/or traditional uses
Cardio-vascular system	Heart failure, piles, rheumatism, varicose veins (Gericke, et al., 2001, van Wyk and Albrecht, 2008).
Central nervous system	Ailments of the eye, convulsions, depression, irritability and anxiety, Mental and emotional stress, shock, trauma (Gericke, et al., 2001; Mills, et al., 2005).
Endocrine System	Adaptogen, diabetes, menopausal symptoms (Gericke, et al., 2001; van Wyk and Albrecht 2008; van Wyk and Wink 2004).
Gastrointestinal tract	Constipation, diarrhoea, dysentery, gastritis, indigestion, oesophagitis, liver conditions, peptic ulcers, poor appetite, reflux (van Wyk and Albrecht 2008; van Wyk and Wink 2004).
Immune system	Cancer tonic, chickenpox, fever, malignant tumors, wasting in AIDS and cancer patients (Gericke, et al., 2001; Mills, et al., 2005; van Wyk and Albrecht 2008).
Integumentary system	Inflammatory skin conditions, topical burns, wounds (Gericke, et al., 2001; van Wyk and Wink 2004).
Musculo-skeletal system	Backache, gout, rheumatoid arthritis, osteoarthritis (Mills, et al., 2005).
Respiratory system	Asthma, bronchitis, cold, cough, Influenza, tuberculosis (Gericke, et al., 2001; Mills, et al., 2005; van Wyk and Albrecht 2008; van Wyk and Wink 2004).
Urogenital tract	Cystitis, gonorrhoea (van Wyk and Albrecht 2008; van Wyk and Wink 2004).

1.3 Dosage

The therapeutic dose of *Sutherlandia frutescens* is 9mg/kg of body weight (Mills, et al., 2005; Seier, et al., 2002). The dried, ground herb is infused in one litre of boiling water and cooled. The infusion is then strained and taken in half tea cup doses (90ml) three times daily (Matsabisa, 2006; Mills, et al., 2005). Children 6-12 years: one quarter tea cup (45ml) three times daily (Matsabisa, 2006). A detailed safety study was conducted by the Medical Research Council of South Africa with the aim of investigating the possible toxicity of *Sutherlandia microphylla* leaf powder in vervet monkeys. Results revealed that *Sutherlandia microphylla* was not associated with toxic or other side effects at one, three and nine times the recommended dose which is equivalent to 9, 27 and 81mg/kg of body weight respectively (Seier, et al., 2002).

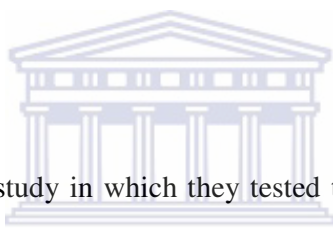
1.4 Constituents of *Sutherlandia frutescens*

1.4.1 Free amino acids

The leaves of *Sutherlandia frutescens* contain high levels of free and protein bound amino acids (Moshe, 1998; van Wyk, 2008). Analysis of the leaves of commercial *Sutherlandia frutescens* grown at different localities showed high levels of the free amino acids asparagine (1.6-35.0 mg/g), proline (0.7-7.5mg/g) and arginine (0.5-6.7mg/g) (van Wyk and Albrecht, 2008). The presence of arginine is of particular importance because of its biological activities.

Arginine is considered a non-essential amino acid as the body produces it naturally (Boom, et al., 2008). Free arginine is an important signaling molecule in both animals and plants. In plants, arginine is converted to nitrous oxide (NO) by a group of enzymes called nitric oxide synthases. NO can also be synthesized from nitrite using nitrate reductase (Bethke, et al.,

2004). NO is an important signaling molecule that acts on many tissues to regulate a wide range of physiological and cellular processes. The endogenous production of NO plays a vital role in influencing physiological processes (Abramson, 2005) such as vasodilation. NO production is suggested to be beneficial in the treatment or management of illness conditions that improve with vasodilation (Boom, et al., 2008). Increasing evidence indicates that NO also plays a complex role in modulating the inflammatory response (Abramson, 2005). Arginine is also active against viral infections, including Coxsackie virus due to its contribution to NO synthesis. However, increased NO production can lead to NO-induced cytotoxicity by oxidative injury, resulting in cellular and organ dysfunctions. Thus, arginine may be beneficial or harmful to the body and the question of its safety could be related to the dose administered (Sia, 2004).



Goret, et al. (2008) designed a study in which they tested the hypothesis that a decrease in arginine bioavailability could be involved in hypoxia induced pulmonary hypertension. This is said to be associated with an impairment of NO mediated vasorelaxation in the pulmonary circulation that is not prevented by exercise. These results confirmed that there is a potential beneficial effect of exercise on NO mediated pulmonary artery vasorelaxation, as it is partly blunted by the harmful effects of hypoxia on arginine bioavailability (Goret, et al., 2008).

1.4.2 L-canavanine and GABA

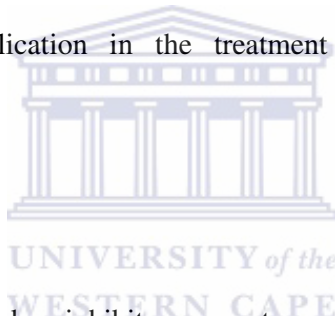
1.4.2.1 L-canavanine

L-canavanine is a common compound recorded in members of the legume family (van Wyk and Albrecht, 2008). It is one of three principle constituents of *Sutherlandia frutescens* (Mills, et al., 2005). High levels of L-canavanine in *Sutherlandia frutescens* leaves were discovered by Moshe (1998). The levels of L-canavanine in the leaves vary from 0.42mg/g

to 14.5 mg/g (Moshe, 1998) and about 1.3-3.1 mg/g dry weight in commercial preparations of *Sutherlandia frutescens* (van Wyk, 2008).

L-canavanine is a compound that may be responsible for the anti-cancer and anti-inflammatory properties ascribed to *Sutherlandia frutescens*. L-canavanine also has an anti-cancer capacity that has been found to abate pancreatic cancer (Sia, 2004). Its major metabolite, canaline is being developed as a potentially new anti-cancer drug for pancreatic cancer (Reid, et al., 2006). L-canavanine is reported to have anti-viral activities against influenza and retroviruses including HIV (Mills, et al., 2005).

L-canavanine is able to prevent the arginine-derived synthesis of NO (Anfossi, et al., 1999). It is a potent arginine antagonist and is also a selective inhibitor of inducible NO synthase. It therefore has a possible application in the treatment of septic shock and chronic inflammation.



1.4.2.2 GABA

GABA is both an amino acid and an inhibitory neurotransmitter that acts outside the central nervous system (Sia, 2004; Mills, et al., 2005). It is a non-protein amino acid that is biologically important (Chemistry 108B, 2005) as it is responsible for most of the brain's fast inhibitory transmission (Jocham and Ullsperger, 2008). According to van Wyk and Albrecht (2008) commercial samples of *Sutherlandia frutescens* contain 0.23-0.85mg/g of GABA.

The presence of GABA could partly account for *Sutherlandia frutescens*' use in the treatment of stress and anxiety (van Wyk and Albrecht, 2008). Stress related ailments are known to be linked to the endocrine system. It was suggested by Tai, et al. (2004) that GABA could play a role in elevating the mood of patients suffering from a chronic illness. Prevoo, et al. (2008) recently showed that *Sutherlandia frutescens* may reduce the adrenal P450 enzyme, and thus

indicate a possible mechanism by which symptoms of stress and glucocorticoid levels are reduced.

1.4.3 Pinitol

Moshe (1998) reported a level of pinitol of up to 14 mg/g per dry leaves. Pinitol is one of three principle constituents of *Sutherlandia frutescens*. It is a type of sugar found in many legumes and is classified as chiro-inositol (Mills, et al., 2005). It has been recognized for its anti-diabetic and immune modulating effects by interacting with dendritic cell maturation (Catelani, et al., 2008). Pinitol exerts an insulin like effect resulting in a decreased level of blood glucose, hence an increased availability of glucose for cell metabolism (van Wyk and Albrecht, 2008). Kim, et al. (2007) evaluated the effect of pinitol therapy in type 2 diabetic patients who were poorly controlled with hypoglycemic drugs. After a 12 week pinitol treatment (20 mg/kg daily), fasting glucose and post prandial glucose levels were significantly decreased, indicating that pinitol treatment can alter glucose metabolism in type 2 diabetic patients.

Pinitol has been described as being active against acute edema in rat models and reducing the production of pro-inflammatory cytokines, such as TNF- α and IL - 1 β (Ojewole, 2004).

1.4.4 Flavonoids

In an attempt to provide chemical markers for the aerial parts of *Sutherlandia frutescens*, a phytochemical investigation done by Avula, et al. (2010) lead to the identification of four flavonoids, sutherlandin A, sutherlandin B, sutherlandin C and sutherlandin D (Avula, et al., 2010). Flavonoids occur both in the free state and as glycosides. These group of constituents are the largest group of naturally occurring phenols (Evans, 2002). Flavonoids are known for its anti-inflammatory and anti-allergic effects, for anti-thrombotic and vaso-protective

properties, for inhibition of tumour promotion and as a protective for the gastric mucosa (Evans, 2002).

1.4.5 Triterpenoid saponins and other compounds

A minimum of 56 different triterpene glycosides has been found in *Sutherlandia frutescens* (van Wyk and Albrecht, 2008). *Sutherlandia frutescens* leaves also contain high levels of unidentified polysaccharides (van Wyk and Albrecht, 2008).

1.5 Studies on *Sutherlandia frutescens*

1.5.1 Epilepsy/convulsions

Aerial parts of *Sutherlandia frutescens* are used in South African traditional medicine for various illnesses including childhood convulsion and epilepsy. Ojewole (2008) conducted a study in which he examined the anti-convulsant property of *Sutherlandia frutescens* against drug induced seizures in mice. Phenobarbitoin and Diazepam were used as reference anti-convulsant drugs. When compared to the reference anti-convulsant drugs, *Sutherlandia frutescens* shoot aqueous extract, delayed the onset of certain drug induced seizures significantly. The findings of this *in vivo* study confirms that *Sutherlandia frutescens* shoot aqueous extract has anti-convulsant properties, and may therefore be used as a natural supplementary remedy in the management, control and/or treatment of convulsions (Ojewole, 2008).

1.5.2 Stress

A study to investigate the influence of *Sutherlandia frutescens* on the biosynthesis of the glucocorticosteroid associated with the symptoms of chronic stress was conducted by Prevo,

et al. (2008). The results showed a possible mechanism by which *Sutherlandia frutescens* reduces glucocorticoid levels and alleviates symptoms associated with stress.

1.5.3 Diabetes mellitus

Sutherlandia frutescens is widely used for the treatment of type 2 diabetes mellitus. It has gained recognition as a potential hypoglycaemic agent. Chadwick, et al. (2007) investigated the hypoglycaemic effects of *Sutherlandia frutescens* in diabetic rats. Wistar rats received a diet specifically designed to induce obesity, insulin resistance and lead to a typical prediabetic state. The rats were then divided into 3 groups. The first group received Metformin. Metformin is a drug administered to hyperglycaemic individuals. The second group of rats received an infusion made with the leaves of *Sutherlandia frutescens*, and the third group of rats served as a control. After 8 weeks of medicinal compliance, the rats receiving *Sutherlandia frutescens* in their drinking water displayed normal insulin levels. There was a marked increase in glucose uptake into muscles and adipose tissue and a significant decrease in intestinal glucose uptake. The results obtained from this experiment indicate that *Sutherlandia frutescens* extract has beneficial effects on type 2 diabetes.

1.5.4 Cancer

Sutherlandia frutescens is an old South African herbal remedy which is also traditionally used in the treatment of internal cancers, hence the herbs' common name 'cancer bush'. Extract produced from the leaves have anti-proliferative effects on cancer cells (Sia, 2004). A study by Tai, et al. (2004) showed that *Sutherlandia frutescens* has an anti-proliferative effect on several human tumor cell lines. Crude aqueous whole plant extract of *Sutherlandia frutescens* induce cytotoxicity in cervical carcinoma cells (Chinkwo, 2005) and a significant decrease of up to 50% of malignant cell numbers of human breast adenocarcinoma were

observed after 24 hour exposure to 1.5mg/ml extract (Stander, et al., 2007). The aqueous extract of *Sutherlandia frutescens* inhibits the growth of the estrogen dependant cancer cell lines. Ethanolic extracts of commercially prepared *Sutherlandia frutescens* (tablet and powder) have been reported to inhibit proliferation of human breast cancer cells (Steenkamp and Gouws, 2006).

1.5.5 HIV/AIDS

It is stated that in Africa, herbal medicine is often used as primary treatment for HIV/AIDS or HIV related symptoms such as dermatological conditions, nausea, insomnia and weakness (Mills, et al., 2005). Recent anecdotes suggest that *Sutherlandia frutescens* improves the quality of life of HIV/AIDS patients by counteracting cachexia (van Wyk, 2008).

Oosthuizen and van De Venter (2005) screened extracts of *Sutherlandia frutescens* and *Lobostemon trigonus* to detect if the extracts inhibited the immunodeficiency virus (HIV). The study showed that *Sutherlandia frutescens* extract contains compounds that inhibit HIV target enzymes (Oosthuizen and van de Venter, 2005). According to a study by Brown, et al. (2008), *Hypoxis hemerocallidea* and L-canavanine (amino acid found in *Sutherlandia frutescens*) increases the bioavailability of Nevirapine, an anti-retroviral drug. This study indicates that lower doses of Nevirapine can potentially be used if given in conjunction with *Sutherlandia frutescens* due to the *Sutherlandia frutescens* induced increase in Nevirapine bioavailability.

1.5.6 Inflammation

The pathogenesis of some inflammatory conditions is due to reactive oxygen species derived from phagocytes such as hydrogen peroxide and superoxide radicals. According to Fernandes, et al. (2004), the anti-inflammatory properties of *Sutherlandia frutescens* can be

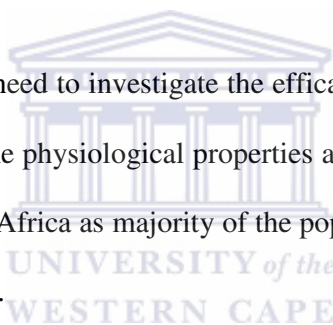
linked to its antioxidant activity. However, the study by Tai, et al. (2004) showed no significant antioxidant activity. According to Kundu, et al. (2005), the anti-inflammatory property of *Sutherlandia Frutescens* could be attributed to its ability to inhibit COX-2 expression. COX-2 is a rate limiting enzyme involved in inflammation. According to Sia (2004) pinitol reduces the production of pro-inflammatory cytokines such as IFN γ hence decrease inflammation. According to Anfossi, et al. (1999), the anti-inflammatory properties of *Sutherlandia frutescens* can be linked to L-canavanine because it has been noted as a potent arginine antagonist. L-canavanine inhibits the enzyme NO synthase. Inhibition of this enzyme results in vasoconstriction.

1.6 Research problem

Towards the end of the 19th century, health care systems throughout the world were in turmoil. The existing trend within the healthcare system was geared towards providing expensive treatment for a selected few sick individuals, as oppose to basic health care for many. This trend of inequality was found in both developed and developing countries. In response to this world wide outcry, an international conference on primary health care was held by World Health Organization (WHO) in collaboration with the United Nations Children's Fund (UNICEF). The conference was attended by 134 nations including government (GO) and non-government organizations (NGO). At this conference, the philosophy of primary health care was endorsed and was seen as a means of achieving "universally available health care" (Interdisciplinary teaching and learning, 2010). According the WHO website, 80% of Africa's population relies on traditional herbal medicine for primary health care. The WHO explains some of the challenges facing the use of traditional herbal medicine with the main challenge being the lack of scientific data to

prove its safety and efficacy. It is generally assumed by the layman that herbal medicine, being natural, is safe and free from side effects and contra-indications without realizing that if the medication is taken incorrectly or co-administered with other drugs, adverse reactions can occur. It is for this reason that the necessary information and training with regard to herbs and herbal products is of vital importance. Methods of researching a herb is complex due to its numerous phytochemicals. This requires the use of expensive methods and equipment yet it is pertinent to the growth and development of the use of traditional herbal medicine in primary health care. Dr Manto Tshabalala-Msimang, the former South African minister of health, issued a draft policy pertaining to the incorporation of African Herbal Medicine into the healthcare system. This was noted in the July 2008 edition of the *Government Gazette*.

In summary, there is a growing need to investigate the efficacy of commonly used traditional herbal medicine by evaluating the physiological properties and scientifically proving its uses. This is particularly important in Africa as majority of the population rely on traditional herbal medicine for primary health care.



1.7 Concluding remarks

There are many studies validating the medicinal value of *Sutherlandia frutescens* on a range of illness conditions, such as viral disease, cancer, inflammation and diabetes many of which require either the stimulation or inhibition of certain immune pathways. Scientific data relating to the mechanism whereby *Sutherlandia frutescens* acts on the immune system have not been comprehensively documented (Mills, et al., 2005). Due to this, the following study was undertaken to investigate the role of *Sutherlandia frutescens*, variety *microphylla*, on the immune system.

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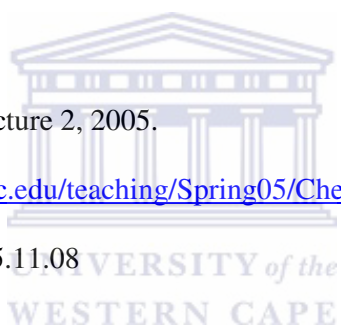
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Chapter 2: The immune system

2.1 Introduction

The immune system of vertebrates consists of multiple physical, chemical and cellular components, which in combination provides protection to the body from possible invasion by pathogens (Helbert, 2006; Nairn and Helbert, 2005; Paul, 2008). The main function of the immune system is self/non-self discrimination. The ability to differentiate between the self and non-self is necessary to protect the organism from invading pathogens. Pathogens can replicate intra-cellularly (viruses, some bacteria and parasites) or extra-cellularly (most bacteria, fungi and parasites). Due to this, different components of the immune system have evolved to protect the organism against various pathogens (Meyer, 2009). The immune components are classified as innate or adaptive. Both components have co-operative mechanisms that work synergistically to eradicate foreign substances (Edgar, 2006).

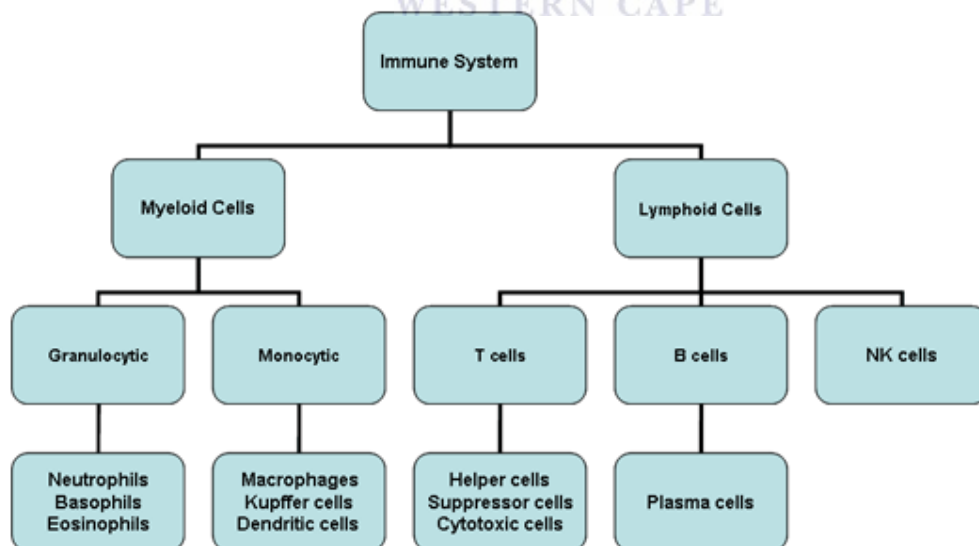


Figure 2.1: Graphical representation of the cells of the immune system (Meyer, 2009).

Cells of the immune system (Figure 2.1) originates in the bone marrow, and includes myeloid progenitor (stem) cells which gives rise to neutrophils, basophils, eosinophils, macrophages and dendritic cells whereas the lymphoid progenitor (stem) cells gives rise to B lymphocytes, T lymphocytes and NK cells (Meyer, 2009).

2.2 Innate immunity

The initial immune response is known as the innate immune system (Griffin, et al., 2003). It is not directed towards specific foreign particles but against general pathogens that enters the body (Timmis, et al., 2008). It plays a crucial role in limiting the early replication and spread of pathogens (Le Bon and Tough, 2002). Cells of the innate immune system trigger the early responses to infection. This response is a powerful nonspecific defense that prevents or limits infection by most potentially pathogenic microorganisms (Paul, 2008). Although the innate immune system plays a role in all tissues and organs, its function is more dominant in areas that are exposed to the external environment such as the skin, digestive, respiratory and genitourinary tracts. Innate immune defenses are based on cells that are permanently located in a specified area of the body and on the migration of additional cells to the site of infection where it is needed (Paul, 2008). Components of the innate immune system include anatomical barriers, secretory molecules and cellular components (Meyer, 2009).

2.2.1 Anatomical barriers

Intact skin and mucous membranes serves as barriers to invading pathogens. The closely packed epithelial cells prevent the entry of pathogens into the body (Helbert, 2006). Desquamation of skin epithelium also aids in the removal of bacteria and other infectious agents that adhered to the epithelial surface. Cilia present on epithelium to increase surface area or aid in peristalsis ensures the lumen of the respiratory tract, as well as the

gastrointestinal tract is free from microorganisms. Epithelial cells not only serve as a physical barrier but also secrete antimicrobial peptides that act directly on invading pathogens as well as upregulate cytokines and chemokines. These cytokines and chemokines attract additional T and B cells to the site of infection (Meyer, 2009).

2.2.2 Secretory molecules

Other first line defense mechanisms provided by the body are secretions. The fatty acids in sweat are bacteriostatic (Meyer, 2009). The mucus secreted by the mucus membranes in the respiratory tract traps foreign particles while the cilia lining wafts the trapped foreign matter out of the body (Helbert, 2006). Secretions are also used as a flushing tool, e.g. the washing effect of tears and saliva. Other mechanisms of expulsion of foreign matter involve urination, defecation and emesis (Magcwebeba, 2008). The lysozymes and phospholipase in tears, saliva and nasal secretions can break down the cell wall of bacteria and destabilize bacterial membranes (Meyer, 2009). The low pH of sweat and hydrochloric acid in gastric secretions provide additional anti-microbial protection (Edgar, 2006; Meyer, 2009). Defensins, a low molecular weight protein found in the lung and gastrointestinal tract have anti-microbial activity. Surfactants in the lung act as opsonins (Meyer, 2009).

2.2.3 Cellular components

The combined effect of all the first line defense mechanisms makes it challenging for pathogens to enter and invade the host's body (Helbert, 2006). The normal flora of the skin and in the gastrointestinal tract can ward off pathogens by competing for nutrients or attachment to cell surfaces or by secreting toxic substances (Meyer, 2009). However, these basic defenses are prone to damage, e.g. injury. When there is a breach in this barrier, pathogens can enter and invade the body (Helbert, 2006) activating the second part of the first

line of defense. This line of defense is mediated through phagocytosis along with inflammation and cell lysis.

2.2.3.1 Phagocytosis

Phagocytosis, driven by the production of nitric oxide (NO) (Bourgeon, et al., 2007), is an important phylogenetic practice of innate immunity. It is a process in which leukocytes and other phagocytic cells ingest particles whose size exceeds 1 μ m. Apart from phagocytosis, phagocytic cells can also recognize and remove cells that underwent programmed cell death (apoptosis) (Henson and Hume, 2006). Apoptotic cells do not induce the expression of inflammatory cytokines in phagocytic cells. On the contrary, it elicits a direct anti-inflammatory effect (M'ajai, et al., 2006). There are four principles that govern the cell death recognition model for the immune system. Firstly, only antigens that shed from apoptotic or necrotic cells can present to naïve T cells. Secondly, only apoptotic or necrotic cells can attract phagocytic cells. Thirdly, macrophages or dendritic cells (DC) localized in non-lymphoid tissue phagocytose dying/dead cells; migrate to lymphoid tissues and present antigen to naïve T cells found in that area. Lastly, tolerance or adaptive responses are not dependent on whether the antigens are self or non-self, but on the ways of cell death during antigen presentation (Sun, 2008).

Two essential immune functions are accomplished when pathogens are ingested by phagocytic cells. Firstly, they initiate a microbial death pathway by routing ingested pathogens to hydrochloric rich organelles such as lysosomes. Secondly, DC (phagocytic leukocytes) utilizes phagocytosis to direct antigens to both major histocompatibility complex I and II compartments (Greenberg and Grinstein, 2002).

Agents that penetrate the epithelium are met with macrophages and other phagocytic cells possessing “microbial sensors” that recognizes key molecular characteristics of pathogens.

These “pattern recognition receptors” include many families of molecules, of which the most intensively studied are the toll like receptors (TLRs). TLRs are composed of at least 10 members which are widely distributed between inflammatory cells and are able to recognize a distinct substance associated with microbial agents: TLR4 recognizes LPS; TLR3, double stranded RNA and TLR9, unmethylated CpG-containing DNA (Paul, 2008; Trinchieri, 2003). TLRs stimulate phagocytic cells to produce pro-inflammatory cytokines such as TNF, IL-1 β and IL-6 (Hendricks, 2008). These cytokines stimulates the macrophages and neutrophils, as well as initiate inflammation.

The influx of phagocytic cells to the site of activity allows many bacteria to be recognized, ingested and destroyed. Some bacteria have a polysaccharide capsule that allows them to resist direct engulfment by phagocytes. These bacteria become susceptible to phagocytosis only when they are coated with antibody that engages the Fc γ receptors on phagocytic cells triggering engulfment and destruction of the bacterium. The process of coating the microorganism with molecules that allows its destruction by phagocytes is called opsonization. The internalization and destruction of microorganisms are enhanced by the interaction between the molecules coating an opsonized microorganism and specific receptors on the phagocyte surface. When the antibody coated pathogen binds to Fc γ receptors, the phagocyte surface extends around the surface of the microorganism. Engulfment of the microorganism leads to the formation of an acidified cytoplasmic vesicle known as a phagosome. The phagosome then fuses with one or more lysosomes to generate a phagolysosome. The release of lysosomal enzymes into the phagosome destroys the bacterium (Janeway and Travis, 1994).

Phagocytes can also damage pathogens by an oxidative mechanism with the production of reactive oxygen radicals (Paul, 2008). The most important of these reactive oxygen radicals, which are directly toxic to the bacterium, are hydrogen peroxide (H₂O₂), superoxides anion

(O₂) and nitric oxide (NO). The production of these compounds is induced by the binding of aggregated antibodies to Fcγ receptors (Janeway and Travis, 1994).

The elimination of a pathogen from a system involves the activation and recruitment of phagocytic cells to a site of infection where phagocytosis is initiated. This influx of cells to the affected area and the release of bactericidal substances results in inflammation (Magwebeba, 2008).

2.2.3.2 Inflammation

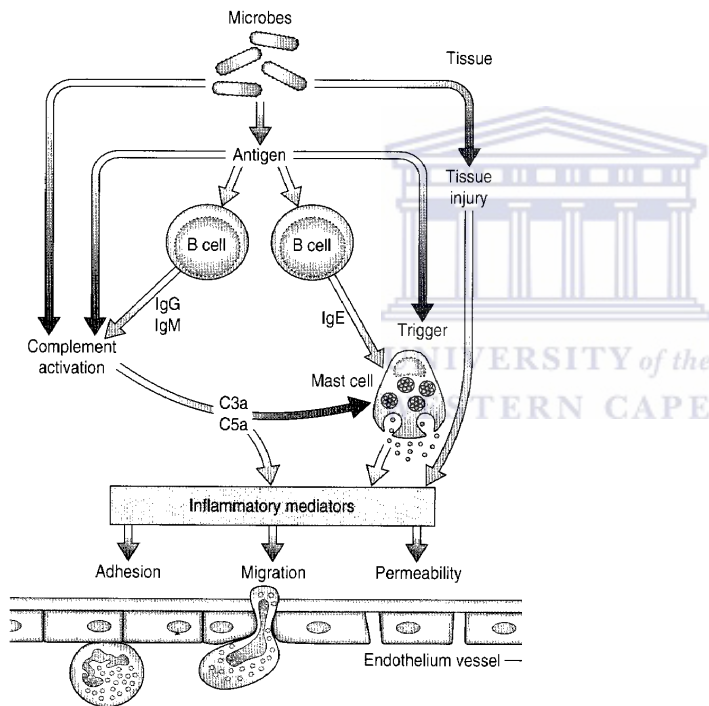
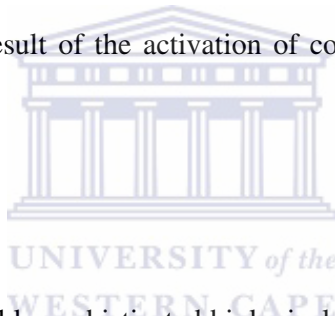


Figure 2.2: The immune system in acute inflammation. The interaction of a range of innate and adaptive components contributing to acute the inflammatory processes (Edgar, 2006).

Inflammation describes the body's immediate response to infection or damage (Edgar, 2006; Griffin, et al., 2003; Janeway and Travis, 1994). It was originally defined by four Latin words *dolor*, *rubor*, *calor* and *tumor* translated as pain, redness, heat and swelling

respectively (Janeway and Travis, 1994). It is triggered by a range of stimuli including chemical or thermal damage and infection. Preformed mediators are released in response to a breach in the first line of defense. This results in immediate aggregation of platelets which is associated with the release of serotonin. This promotes vasoconstriction, further platelet aggregation and the formation of a platelet plug. Other preformed mediators released include histamine, heparin, lysosomal enzymes and proteases, neutrophil chemotactic factor and eosinophil chemotactic factor. These factors are responsible for vasodilation, i.e. increase in blood flow to the site of injury and the recruitment of specific inflammatory cells to the area (Edgar, 2006). This increase in vascular permeability gives rise to the four clinical signs of inflammation; i.e. pain, redness, heat and swelling which is crucial to the early inflammatory response and is initiated as a result of the activation of complement (Edgar, 2006; Meyer, 2009).



2.2.3.3 Complement

The complement system is a highly sophisticated biological reaction system that plays a vital role in both innate and adaptive immunity in jawed vertebrates and serves as an important interface between these two systems (Fujita, et al., 2004; Holers and Kulik, 2007). Complement can be activated by interactions between the antibody and antigen (Nairin and Helbert, 2005). The complement system has a series of serum proteins circulating in the bloodstream (Edgar, 2006; Magcwebeba, 2008; Meyer, 2009). These serum proteins are produced by a variety of cells including, hepatocytes, macrophages and gut epithelial cells. Some complement proteins bind to immunoglobulins (Ig) or to membrane component cells while others are proenzymes that when activated, cleave one or more other complement proteins. When cleaved, some complement proteins yields fragments that activate cells, increase vascular permeability or opsonize bacteria. Complement activation can be divided

into four pathways; the classical pathway, the lectin pathway, the alternative pathway and the membrane attack (lytic) pathway. The classical and alternative pathways lead to the activation of C5 convertase and result in the production of C5b. C5b plays an important role in the activation of the membrane attack pathway (Meyer, 2009).

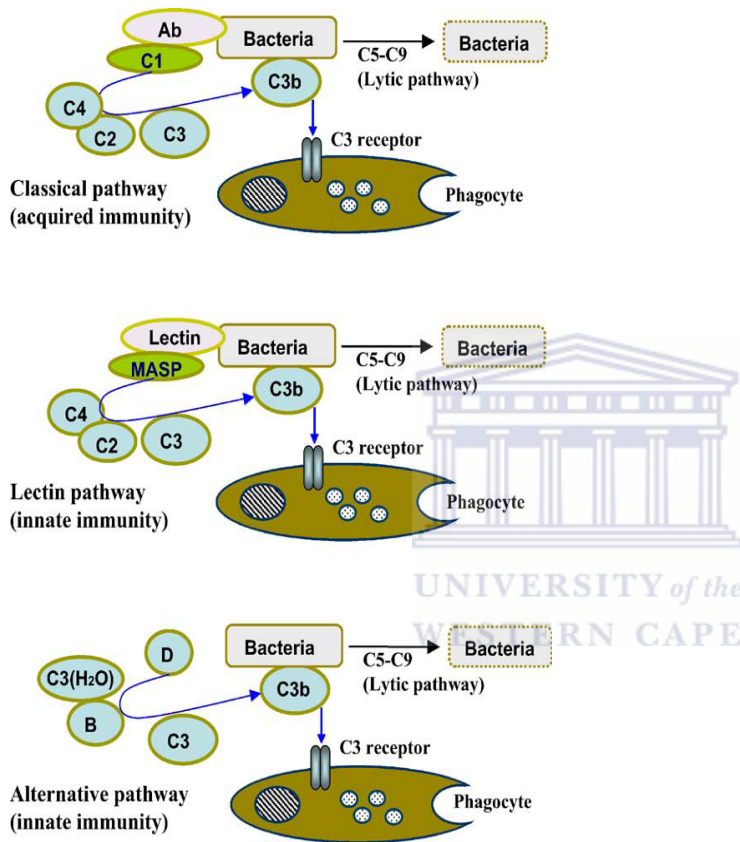


Figure 2.3: The activation of the classical, lectin, and alternative pathways (Fujita, et al., 2004). The three pathways converge at the cleavage of C3 and the formation of a C5 convertase, which triggers the final lytic pathway of a C5 convertase, binding lectin-associated serine protease; MBL, mannose-binding lectin (Edgar, 2006).

2.2.3.3.1 The classical pathway

The classical pathway is activated by the formation of soluble antigen-antibody combinations. The components of the classical pathway are activated in the following sequence: C1, C4 followed by C2. Activation involves the cleavage of each molecule which forms an enzyme complex that activates the next molecule and releases molecular fragments. The formation of the classical pathway C3 convertase (C4b2a) results in cleavage of C3 and the initiation of the terminal pathway. The cleavage products include C4a which is chemoattractants for phagocytes and cause degranulation of mast cells. Activation of the classical pathway recruits other inflammatory cells to the area of activation (Edgar, 2006).

2.2.3.3.2 The lectin pathway

The lectin pathway is very similar to the classical pathway. The binding of mannose binding lectin (MBL) to bacterial surfaces with mannose-containing polysaccharides (mannans) initiates the process. Binding of MBL to a pathogen results in the association of two serine proteases, MASP-1 (MBL-associated serine proteases) and MASP-2. MASP-1 and MASP-2 are comparable to C1r and C1s respectively, and MBL is comparable to C1q. Construction of the MBL/MASP-1/MASP-2 tri-molecular complex results in the activation of the MASPs, hence cleavage of C4 into C4a and C4b. The C4b fragment binds to the pathogen membrane and the C4a fragment is released. Stimulated MASPs also cleave C2 into C2a and C2b. C2a binds to the pathogen membrane in association with C4b and C2b is released. The resulting C4bC2a complex is a C3 convertase, which cleaves C3 into C3a and C3b. C3b binds to the pathogen membrane in association with C4b and C2a and C3a is released. The resulting C4bC2aC3b is a C5 convertase. The generation of C5 convertase is the end of the lectin pathway (Meyer, 2009).

2.2.3.3.3 The alternate pathway

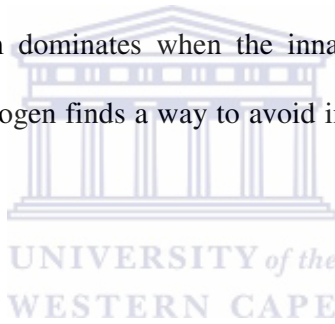
Another kind of C3 convertase can be formed by the stabilization of a different group of proteins (includes C3b – a product of the classical pathway). A degree of C3 conversion can occur, in the absence of classical pathway activation, due to spontaneous hydrolysis enhanced by proteases, e.g. plasmin, or other inflammatory products. The low level of C3 conversion makes it possible for the alternative pathway to operate without activation of earlier components of the classical pathway. C3b binds pathogen membranes. The complex C3b forms with factor B in the presence of the protease factor D, i.e. C3bBb, dissociates rapidly unless stabilizing factors are present. These factors include microbial polysaccharides such as endotoxin, zymosan, sialic acid-deficient erythrocytes, nephritic factor, aggregated forms of IgA and some subclasses of IgG. The inhibitory factor H constantly acts to separate C3bBb but the affinity of C3b is significantly reduced in the presence of stabilizing factors. This then acts on C3. C4 and C3 are structurally homologous as are C2 and factor B. It is thus clear that the alternative pathway takes the form of feedback mechanism that operates whenever C3b is formed, and is sustained when factors are present to stabilize the assembly of its C3 convertase (Reeves and Todd, 2002).

2.2.3.3.4 The membrane attack (lytic) pathway

C5 convertase from the classical (C4b2a3b), lectin (C4b2a3b) or alternative (C3bBb3b) pathway cleaves C5 into C5a and C5b. C5a remains in the fluid phase and the C5b swiftly links with C6 and C7 and is included into the membrane. C8 then binds, followed by several molecules of C9. The C9 molecules form an aperture in the membrane through which the cellular contents escapes and lysis occurs. The complex consisting of C5bC6C7C8C9 is referred to as the MAC. C5a generated in the lytic pathway has numerous effective biological activities. It is the most potent anaphylotoxin, and it is a chemotactic factor for

neutrophils. It also stimulates the respiratory burst and inflammatory cytokine production by macrophages. C5a activities are regulated by inactivation by carboxypeptidase B (C3-INNA). Some of the C5bC6C7 complex formed can detach from the membrane and enter the fluid phase. If it attaches to other nearby cells, cell lysis can occur. Protein S binds to soluble C5bC6C7 thus preventing damage to bystanding cells (Meyer, 2009).

The innate and adaptive systems are interconnected (Nairin and Helbert, 2005). Phagocytosis serves a dual role: as an innate immune effector as well as a link between the innate and acquired immune response (Greenberg and Grinstein, 2002). Innate immune activation triggers and paves the way for an adaptive immune response by antigen-specific T and B lymphocytes. Adaptive system dominates when the innate system fails to eradicate the invading pathogen or if the pathogen finds a way to avoid interaction with the innate system (Nairin and Helbert, 2005).



2.3 Adaptive immunity

Adaptive immunity has evolved to provide a broader base for recognition of self from non-self in vertebrates (Bonilla and Oettgen 2010; Nairin and Helbert, 2005). This is accomplished by creating a defense system of recognition molecules that interact with foreign antigens which consist of T and B cells (Holers and Kulik, 2007). The ability of vertebrates to generate an anticipatory defense system against foreign molecules is increased in the germline that encodes proteins that has binding sites and can function as receptors. The products of these gene duplications is the gene families that encode the antigen-recognition molecules (antibodies, T cell receptors, MHC proteins) (Nairin and Helbert, 2005) and this is a key feature of the adaptive immune system (Helbert, 2006). Another key feature of adaptive immunity is the generation of immunologic memory. Long lived memory of T and

B cells are established after the first encounter with antigen which yields a more rapid and robust protective response in subsequent encounters with the same antigen (Bonilla and Oettgen, 2010).

2.3.1 B cells

The differentiation of B cells is initiated by IL-6 (Hendricks, 2008). B cells are lymphocytes that play a major role in humoral immunity (Pawelec and Larbi, 2008). The principle function of B cells is to create antibodies against antigens. It thus has a crucial role in antigen presentation and cytokine secretion (Nashi, et al., 2010). Humoral immunity also contributes to natural resistance and host defense against certain fungi e.g. *Candida albicans* (Casadevall, et al., 2002).

B cells are presented as antigen presenting cells (APCs) and express peptides and MHC II on their surface (Dorner, et al., 2009). These peptides arise as a result of processed antigen that was engulfed after binding to the B-cell surface immunoglobulin receptor. T cells can activate B cells for differentiation into memory or plasma cells. B cells and DC express many of the same costimulatory molecules such as CD40, B7-1 (CD80), and B7-2 (CD86). Activated B cells enter one of two pathways. They can either become short-lived plasma cells or they enter a follicle to establish a germinal centre. In the germinal centre, B cells can evolve from the production of IgM and IgD to other isotypes such as IgG, IgA and IgE. This process is called class-switching and can occur through a mechanism of gene rearrangement analogous to the process of TCR and B cell receptor gene segment rearrangement (Bonilla and Oettgen, 2010).

2.3.2 T cell

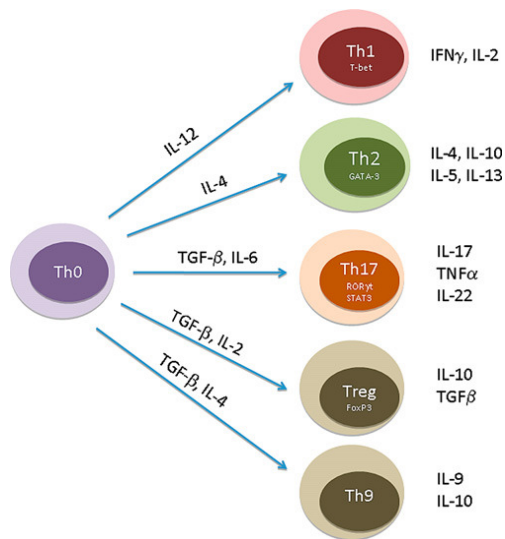
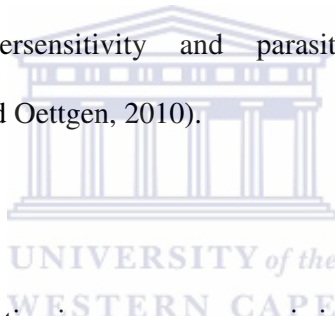


Figure 2.4: Expansion of antigen specific naïve T_H0 cells on interaction with APC expressing MHC class II/peptide complexes. Depending on the type of APC and the cytokine (arrows) at the site of antigen encounter, T_H0 cells can move along one of several differentiation pathways (Bonilla and Oettgen, 2010).

T cells regulate immune responses by limiting tissue damage due to autoreactive or excessive inflammatory immune response. It is a major component of the adaptive immune system (Rabb, 2002) and can directly eliminate pathogens by killing infected target cells and can function as helper cells (involving direct cellular contact), signal cytokines to enhance B and T cell responses, as well as activate mononuclear phagocytes. T cell antigen receptors can only recognize antigens when it is presented on the surface of another cell by MHC molecules (Nairin and Helbert, 2005). The T cells that recognizes antigen peptides derived from the extracellular environment are called T helper (T_H) cells (Helbert, 2006). T_H cells produce a range of cytokines on activation. There are two main categories of T_H cells, namely T_H1 and T_H2. Each of these cells produces a mutually exclusive panel of cytokines.

T_H1 cells differentiate from naïve T_H0 precursors under the influence of IL-12 (Figure 2.4). These cells produce IFN γ and IL-2. Cytokines from T_H1 cells drive cell mediated responses, activates mononuclear phagocytes, NK cells and cytotoxic T cells for killing intracellular microbes and virally infected target cells. Much attention has been given to evaluating the contribution of the various T-cell subsets to immunity to intracellular parasites and bacteria. As previously established, CD4 lymphocytes may control the parasite/bacterial load, however, increasing evidence is accumulating on the role played by CD8 and T lymphocytes in reducing the growth of these microorganisms (Milon and Liou, 1993).

T_H2 cells produce IL-4, IL-5, IL-10 and IL-13. Their development is initiated by IL-4 and the transcription factor GATA-3. Cytokines released from T_H2 enhances antibody production, aspects of hypersensitivity and parasite-induced immune responses (eosinophilopoiesis) (Bonilla and Oettgen, 2010).



2.4 Immune homeostasis

A balance in the innate and adaptive immune responses is important in maintaining optimum health. Even though these two systems are important in protecting the body from invading pathogens, unregulated over activity can lead to pathology (Magcwebeba, 2008). Persistent and dysregulated inflammation combined with an exaggerated immune response is a contributing factor to cystic fibrosis lung disease (Fayon, 2006). Chronic inflammation, which results from innate immune signals, such as the activation of TLRs, and adaptive signals including T_H1 cytokine release, can lead to the development of Chron's disease (Arranz, et al., 2008) and is an important risk factor for the development of cancer (Kundu and Surh, 2008; McKay, et al., 2008). Unregulated chronic inflammation can result in necrosis of surrounding tissue due to prolonged release of reactive oxygen species (ROS). It is suggested that chronic immune activation plays a central role in the pathogenesis of

immunodeficiency, CD4+ depletion and T cell energy. These are all characteristic of HIV-1 infection (Leng, et al., 2002). The expression of several pro-inflammatory cytokines governs the expression of COX-2 which has been implicated in inflammation, fibrogenesis and carcinogenesis (Baskaran, et al., 2009). Dominant T_H1 immune response suppresses humoral immunity increasing the susceptibility of the host to bacterial and parasitic infections. The host's susceptibility to viral infections is increased when there is a suppression of T_H1 and/or dominating T_H2 immune response. The susceptibility of the host to extracellular infections is increased with the suppression of the T_H2 immune response e.g. the development of opportunistic infections in individuals with HIV/AIDS (Magcwebeba, 2008).

2.5 Concluding remarks

Sutherlandia frutescens is an old Cape remedy used for various ailments. According to tradition, the virtues of this plant extends to include cold, influenza, chicken pox, inflammation, arthritis, etc. (van Wyk, et al., 2009). Some of *Sutherlandia frutescens* medicinal properties are due to its effects on the immune system. This would include either the stimulation or suppression of certain immune pathways decreasing the susceptibility of the host to intracellular and/or extracellular pathogens. The aim of this study is to monitor the effects of *Sutherlandia frutescens* on inflammation, cellular immunity and humoral immunity.

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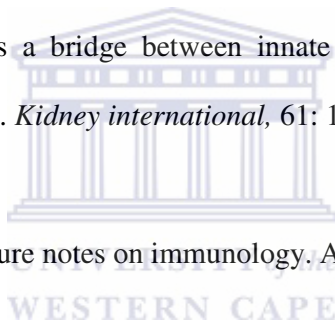
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Chapter 3: Validation of whole blood cell assays to monitor immune function

3.1 Introduction

3.1.1 In vitro and in vivo methods

To investigate the immune system thoroughly, both *in vitro* and *in vivo* experiments are necessary. *In vitro* experiments play an important role in monitoring certain biomarkers of the immune system in the presence or absence of endotoxin. However, data collected from *in vitro* experiments may lead to incorrect beliefs regarding the effects on the immune system as the microenvironment of mammals cannot be mimicked. Investigation of both innate and acquired immunity in human subjects is limited by ethical and moral concerns “to do no harm” (Sulliman and Wang, 2006). Therefore, evaluation of the human immune system must be completed *in vitro* first to establish a base line before *in vivo* studies can be employed. Even though much can be learnt from *in vitro* studies, these model studies may not be able to achieve the precise complexity which includes mimicking the microenvironment of intact mammals. After completing an *in vitro* experiment, it is recommended that the experiment is repeated in an animal model to ensure that the data gathered has *in vivo* relevance. Animal models are useful to test various hypotheses *in vivo*, however, there are factors such as the complexity of the animal system in which the study is conducted, that need to be taken into account before extracting information from experimental data employing higher eukaryotes and applying this data to the human physiology (Sulliman and Wang, 2006).

Due to this study being the first of its kind for *Sutherlandia frutescens*, *in vitro* methods were employed. Running *in vitro* assays using whole blood cells was preferred as they mimic the *in vivo* environment more accurately than isolated monocytes with reference to the immune system (Pool and Bouic, 2001).

3.1.2 *Sutherlandia frutescence* and the immune system

The ability to differentiate between self and non self to protect the host from pathogenic micro organisms is vital (Sulliman and Wang, 2006; Meyer, 2009). Pathogens can replicate intracellularly or extracellularly. Due to this, different aspects of the human immune system are evolving to protect the host against pathogenic invasion (Meyer, 2009). The immune defense system of human hosts mainly includes inflammation, humoral and cellular immunity. Inflammation describes the host's immediate response to infection or damage (Griffin, et al., 2003; Edgar, 2006; Janeway and Travis, 1994). Humoral immunity contributes to the natural resistance of the host to extracellular pathogens such as fungi, parasites and most bacteria (Casadevall, et al., 2002). Cellular immunity activates mononuclear phagocytes, natural killer (NK) cells and cytotoxic T cells for killing intracellular microbes and virally infected target cells (Milon and Liou, 1993).

To study the human immune response to *Sutherlandia frutescence*, the *in vitro* effects on human whole blood cells requires careful monitoring. Most studies done on the immune response have used the *in vitro* whole blood culture system (Pike, 1975).

Before the immune activity of *Sutherlandia frutescence* can be evaluated, assay systems to monitor cytotoxicity, inflammation, cellular immunity and humoral immunity must be validated.

Toxicity can be measured by assessing cellular damage. Damage to cells causes the leakage of lactate dehydrogenase (LDH). The amount of LDH released from the cells into the culture medium indicates the toxicity of a substance.

Inflammation illustrates the body's immediate response to harmful stimuli (Griffin, et al., 2000). The use of immune biomarkers in molecular epidemiology is rapidly expanding the field of research (Duramad, et al., 2007). Biomarkers can be defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological response to a therapeutic intervention" (O'Hara Jr., et al., 2006). Biomarkers closely linked to the mechanism of a pharmaceutical action can be used to measure the potency of that agent. IL-6 is a cytokine which has pro-inflammatory actions hence the synthesis of IL-6 by whole blood cells was used as a biomarker for inflammation. Humoral immunity is responsible for the elimination of extracellular pathogens such as bacteria and toxic molecules which is brought about by the production of antibodies (Griffin, et al., 2003; Medeiros, et al., 2009). Cytokines released from T_H2 cells enhances the production of antibodies. T_H2 cells produce IL-4, IL-5, IL-10 and IL-13 (Bonilla and Oettgen, 2010). The synthesis of IL-10 by whole blood cells was used as a biomarker to test humoral immune activity. Cellular immunity is responsible for the protection against intracellular pathogens (Griffin, et al., 2003). The differentiation of T_H1 cells from naïve T_H0 cells are influenced by various cytokines. T_H1 cells produce INF γ and IL-2. These cytokines facilitate cell mediated immune responses (Milon and Liou, 1993). The synthesis of INF γ by whole blood cells was used as a biomarker to test cellular immune activity.

The aim of this study was to validate the biomarker assays that will be used in the research project.

3.2 Methods

3.2.1 Blood collection

The blood obtained was collected by the doctor/nurse at the campus clinic. Blood from 3 healthy male volunteers, not on any medication was used. Blood samples were collected by venipuncture directly into heparinised vacuum tubes. The blood was stored at room temperature and used within 4 hours of collection. Preparation of the whole blood cell cultures were done under sterile conditions in a laminar flow cabinet. Approval for the study was received from the University of the Western Cape's ethics committee and an informed consent was obtained from all participants.

3.2.1.1 Whole blood cells stimulated with Lipopolysaccharide (LPS)

Stimulated whole blood cultures contained 1 volume of 10 µg/ml LPS in dimethyl sulfoxide (DMSO), 10 volumes of blood and 89 volumes of RPMI-1640 medium. Unstimulated whole blood contained 10 volumes of blood and 89 volumes of RPMI-1640 and 1 volume DMSO. The (stimulated and unstimulated) blood (200µl/well) was incubated at 37 °C for 24 hours. Culture supernatants were then collected and assayed for LDH and IL-6.

3.2.1.2 Whole blood cells stimulated with phytohaemagglutinin (PHA)

Stimulated whole blood cultures contained 10 volumes of blood, 89 volumes of RPMI-1640 medium and 1 volume of 1.6µg/ml PHA in RPMI. The final concentration of PHA in the stimulated blood mixture was 1.6µg/ml. Unstimulated whole blood contained 10 volumes of blood and 90 volumes of RPMI. Stimulated and unstimulated blood (200µl/well) cultures were incubated for 48 hours. Culture supernatants were then used for IL-10 and INF γ assays.

3.2.2 Cytotoxicity

Culture supernatants were collected after the incubation with and without immune stimulation. LDH was measured using a Cytotoxicity Detection kit (Biovision, USA). The kit includes all the components required for the assay. Total cellular LDH were obtained by lysing diluted whole blood with the detergent Triton X100. The lysed cells were used to determine total cellular LDH. Cell culture supernatants and the lysed cells were assayed on a 96 well plate (Nunc-Immuno plate, MaxiSorp). 100µl of the kit reaction mixture was added to the respective wells and incubated for approximately 15 minutes. The absorbance of the reaction mixtures were measured at 492nm using a plate spectrophotometer at various time intervals. Cytotoxicity was expressed as the amount of LDH in supernatant as % of total cellular LDH.



3.2.3 Cytokine analysis

Double antigen sandwich enzyme linked immune sorbent assays (DAS ELISAs) (e-Bioscience, Germany) were used to measure cytokine release from the supernatants of the whole blood cells cultures. Nunc maxisorp (Nunc™, Denmark) plates were used for the assays. This kit contained all the reagents, buffers and diluents needed for performing quantitative ELISAs. The ELISAs were carried out according to the manufacturer's instructions. In summary: 96 well plates were coated with primary antibody against the respective cytokine and incubated overnight at 4 °C. After incubation, the plates were washed with sterile phosphate buffered saline containing 0.05% Tween-20. Non-specific binding sites were then blocked with assay diluent for 1 hour at ambient temperature after which the wells received either recombinant human cytokine standards or sample. The plate was sealed and incubated for 2 hours at ambient temperature on a shaker. After incubation the wells were washed. The wells then received Biotin-conjugated antibody against the

respective cytokine. The plate was incubated for 1 hour at ambient temperature on a shaker followed by washing as before. The wells then received Avidin-HRP conjugate. The plate was then incubated for 30 minutes at ambient temperature on a shaker. After the last wash, the bound peroxidase was monitored by addition of Tetramethylbenzidine solution (substrate solution) to each well, after which the plate was incubated for approximately 15 minutes. The reaction was stopped by adding 2M H₂SO₄ to each well. The absorbance was read at 450nm on an ELISA plate reader. Excel was used to generate a standard curve for each ELISA plate which was used to determine the cytokine concentrations of the culture supernatants.

3.3 Results and discussion

3.3.1 Cytotoxicity

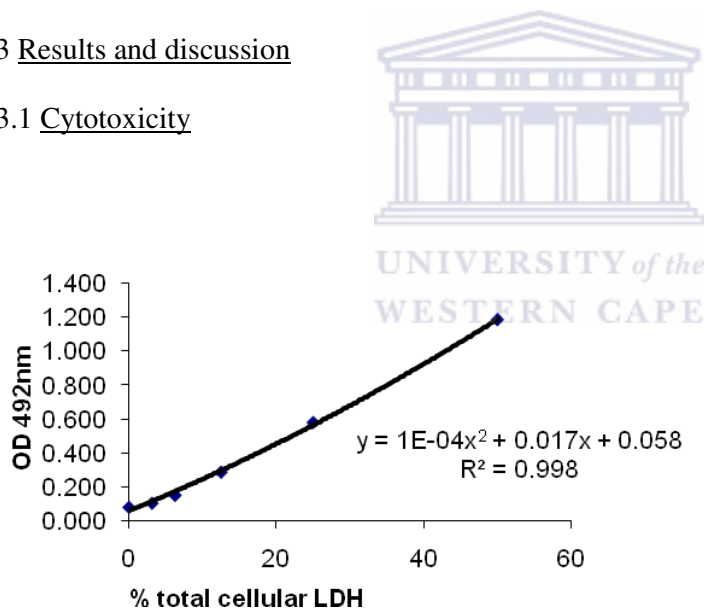


Figure 3.1: Standard curve for cytotoxicity. A graphical representation of the absorbance of the reaction mixtures measured at 492nm at various total cell lysate concentrations after 10 minutes of incubation. Data are presented as the mean n=2. n is the number of replicates used in the experiment.

The total LDH from the blood cultures was considered to be 100% toxicity (after being released into the medium). A standard curve was constructed from dilutions of the 100% toxicity sample. There exist a good correlation between the average absorbance and the LDH level ($R^2=0.998$) as reflected in Figure 3.1.

3.3.2 Inflammatory activity

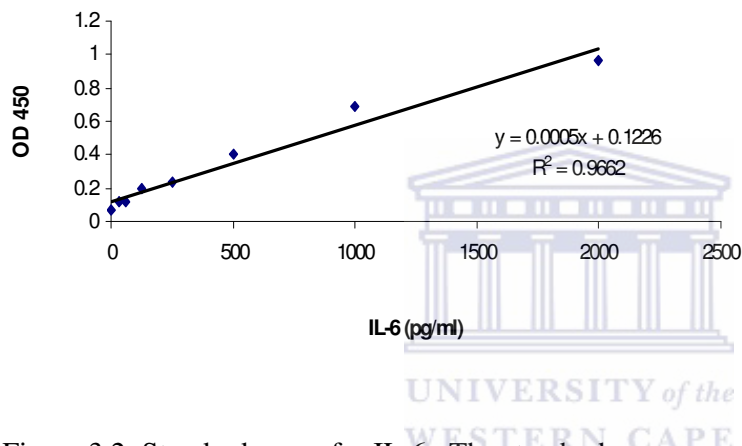


Figure 3.2: Standard curve for IL-6. The standard curve was assayed by the DAS ELISA according to the instructions of the manufacturer. Data are presented as the mean $n=2$. n is the number of replicates used in the experiment.

Supernatants from LPS stimulated cultures were analyzed for inflammatory activity using IL-6 as a biomarker. Figure 3.2 represents the standard curve obtained using the IL-6 ELISA kit. There exist a good correlation in the release of IL-6 ($R^2=0.966$). The IL-6 released at $0\mu\text{g/ml}$ for stimulated whole blood cell cultures was $8793.7\pm 914.6\text{pg/ml}$ while the IL-6 released by the unstimulated blood was $116.5\pm 31.39\text{pg/ml}$. This data shows that there is a statistically significant difference ($P\leq 0.001$) between the IL-6 released from the stimulated and

unstimulated whole blood cell cultures showing that this biomarker assay can be used to monitor inflammatory activity.

3.3.3 Humoral immunity

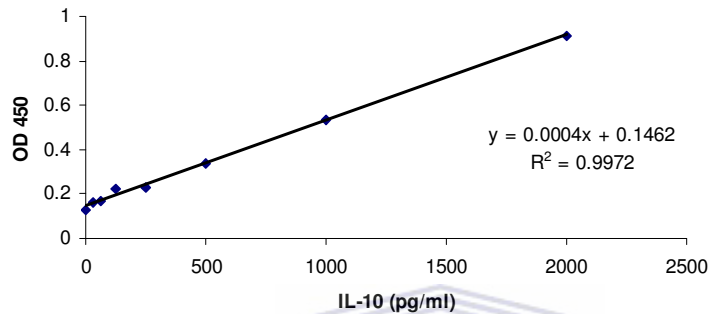


Figure 3.3: Standard curve for IL-10. The standard curve was assayed by the DAS ELISA according to the instructions of the manufacturer. Data are presented as the mean $n=2$. n is the number of replicates used in the experiment.

Supernatants from PHA stimulated cultures were analyzed for humoral immune activity using IL-10 as a biomarker. Figure 3.3 represents the standard curve obtained using the IL-10 ELISA kit. There exist a good correlation in the release of IL-10 ($R^2=0.997$). The IL-10 released at $0\mu\text{g/ml}$ for stimulated whole blood cell cultures was $22.6\pm 3.7\text{pg/ml}$ while the IL-10 released by the unstimulated blood was $2.6\pm 11.9\text{pg/ml}$. This data shows that there is a statistically significant difference ($P=0.003$) between the IL-10 released from the stimulated and unstimulated whole blood cell cultures showing that this biomarker assay can be used to monitor humoral immune activity.

3.3.4 Cellular immunity

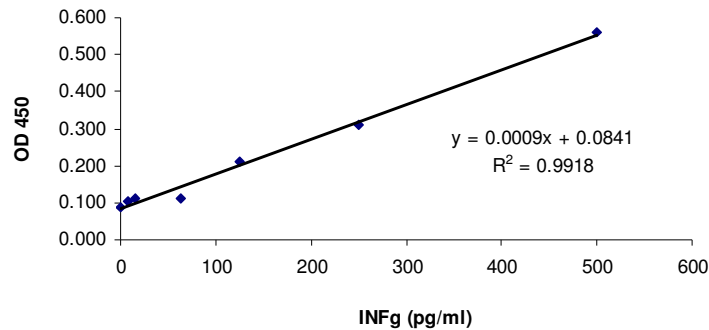


Figure 3.4: Standard curve for INF γ . The standard curve was assayed by the DAS ELISA according to the instructions of the manufacturer. Data are presented as the mean $n=2$. n is the number of replicates used in the experiment.

Supernatants from PHA stimulated cultures were analyzed for cellular immune activity using INF γ as a biomarker. Figure 3.4 represents the standard curve obtained using the INF γ ELISA kit. There exist a good correlation in the release of INF γ ($R^2=0.992$). The release of INF γ at $0\mu\text{g/ml}$ for stimulated whole blood cell cultures was $891.1\pm 661.0\text{pg/ml}$ while the INF γ released by unstimulated blood was $11.1\pm 5.8\text{pg/ml}$. This data shows that there is a statistically significant difference ($P=0.009$) between the INF γ secretions of the stimulated and unstimulated whole blood cell cultures indicating that this biomarker assay can be used to monitor cellular immune activity.

3.4 Concluding remarks

Increasing emphasis is being placed on biomarkers as indicators for certain inflammatory and auto-immune diseases such as rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus. Expression of cell surface markers and cytokines produced by T and B lymphocytes can lead to a more detailed description of disease activity in patients and serve as indicators of the patient's response to treatment (O'Hara Jr., et al., 2006). It is for this reason that cytokines are commonly used as diagnostic biomarker to monitor the effects of a pharmaceutical on immune pathways (Fiala and Veerhuis, 2010). The *in vitro* cell culture assay is based on the fact that mitogens can stimulate whole blood cells. Studies have shown that the amount of cytokines released by cultured cells is directly proportional to the endotoxin concentration of the culture medium (Pool, et al., 1998). Running *in vitro* assays via whole blood cell cultures is preferred as it represents the *in vivo* environment more accurately than isolated monocytes in terms of the immune system (Pool and Bouic, 2001).

The above data shows that mitogen activated whole blood cultures can be used as an effective method to monitor immune pathways as there is a good correlation in the release of the cytokines tested.

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Chapter 4: The screening of *Sutherlandia frutescens* for immune modulating activity using *in vitro* whole blood culture assays

3.1 Introduction

Sutherlandia frutescens is a perennial, flowering shrub of the pea family (*Fabaceae/Leguminosa*) (Sia, 2004). It is a commonly used, versatile medicinal herb that is indigenous to Africa (Fernandes, et al., 2004; Ojewole, 2008). Widely known as cancer bush, *Sutherlandia frutescens* has been used in the traditional medical systems of different cultural groups in Africa such as Zulu, Xhosa, Sotho, Khoi-San and Cape Dutch. Traditionally, *Sutherlandia frutescens* has been used for a variety of illness conditions (van Wyk and Albrecht, 2008).

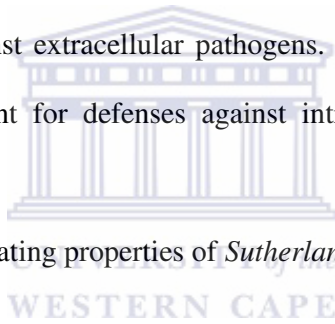
The plant is used by many cultural groups for fever, poor appetite, indigestion, gastritis, oesophagitis, peptic ulcers, dysentery, cancer tonic (prevention and treatment), diabetes, cold, influenza, cough, asthma, chronic bronchitis, kidney and liver conditions, rheumatism, heart failure, urinary tract infections and stress and anxiety (van Wyk and Albrecht, 2008). According to *Medicinal Plants of the World* by van Wyk and Wink (2004), it is also used as a bitter tonic, an adaptogen, appetite enhancer, tuberculosis remedy, immune stimulating properties, treatment for wasting in cancer and AIDS patients and topically used to treat burns, wounds and inflammatory skin conditions. *Sutherlandia frutescens* has also been used for chronic fatigue syndrome, rheumatoid arthritis, osteoarthritis, menopausal symptoms and clinical depression (Mills, et al., 2005).

The medicinal properties of *Sutherlandia frutescens* are linked to its constituents. The constituents of *Sutherlandia frutescens* is divided into six groups, namely free amino acids, L-canavanine and GABA, pinnitol, flavonoids, triterpenoid saponins and other compounds (van Wyk and Albrecht, 2008; van Wyk, et al., 2009). There are three principle constituents

of *Sutherlandia frutescens* that contributes to its efficacy. These constituents are L-canavanine, GABA and pinnitol (Mills, et al., 2005).

Scientific data on the effects of *Sutherlandia frutescens* on the immune system has not been well documented (Mills, et al., 2005). Signaling pathways within the immune system is initiated via cytokines. Cytokines are proteins that functions as intracellular mediators. They are produced by leukocytes and act on target tissues resulting in multiple biological actions (Kapsimalis, et al., 2008). Interleukin (IL) 6 is a multi-potential cytokine. It is known to play a role in inflammation through the activation of T cells and differentiation of B cells. It is therefore commonly used as a biomarker for inflammation (Heyen, et al., 2000). IL-10 and INF γ are biomarkers used to determine T cell activity. IL-10 upregulates humoral immunity and thus defends the host against extracellular pathogens. INF γ upregulates cell mediated immunity and is very important for defenses against intracellular pathogens and cancer (Fuchs, et al., 1988).

In this study, the immune modulating properties of *Sutherlandia frutescens* are evaluated.



3.2 Methods

3.2.1 Preparation of *Sutherlandia frutescens*

A 20% (w/v) extract of *Sutherlandia frutescens* were prepared in 94.4% ethanol by Parceval (Pty) Ltd pharmaceuticals (South Africa). The leaves of the plant were crushed (sieve size~ 2-3 mm) after which it was mixed with 94.4% ethanol at 20g/100ml ethanol. The mixture was incubated overnight then pressed to separate the leaves from the tincture. The tincture was then filtered to remove excess debris and stored and -4°C. The samples for immune assays were prepared by air drying 5ml of the ethanol extract. The dried extract was then reconstituted with 2ml DMSO to give a final concentration of 5g leave extract/ml DMSO.

3.2.2 Blood collection

The blood obtained was collected by the doctor/nurse at the campus clinic. Blood from 3 healthy male volunteers, not on any medication was used. Blood samples were collected by venipuncture directly into heparinised vacuum tubes. The blood was stored at room temperature and used within 4 hours of collection. Preparation of the whole blood cell cultures were done under sterile conditions in a laminar flow cabinet. Approval for the study was received from the University of the Western Cape's ethics committee and an informed consent was obtained from all participants.

3.2.3 Whole blood cells stimulated with Lipopolysaccharide (LPS)

Stimulated whole blood cultures contained 1 volume of 10 µg/ml LPS in dimethyl sulfoxide (DMSO), 10 volumes of blood and 89 volumes of RPMI-1640 medium. Unstimulated whole blood contained 10 volumes of blood and 89 volumes of RPMI-1640 and 1 volume DMSO. The (stimulated and unstimulated) blood (200µl/well) was incubated at 37 °C for 24 hours. Culture supernatants were then collected and assayed for LDH and IL-6.

3.2.4 Whole blood cells stimulated with phytohaemagglutinin (PHA)

Stimulated whole blood cultures contained 10 volumes of blood, 89 volumes of RPMI-1640 medium and 1 volume of 16µg/ml PHA in RPMI. The final concentration of PHA in the stimulated blood mixture was 16µg/ml. Unstimulated whole blood contained 10 volumes of blood and 90 volumes of RPMI. Stimulated and unstimulated blood (200 µl/well) cultures were incubated for 48 hours. Culture supernatants were then used for IL-10 and INF γ assays.

3.2.5 Cell culture

Sutherlandia frutescens was diluted with DMSO. The diluted *Sutherlandia frutescens* (2µl/well) was added to wells of a 96 well plate. Stimulated or unstimulated whole blood (200µl/well) was added to wells containing *Sutherlandia frutescens* extract. Control cultures contained 2µl DMSO instead of *Sutherlandia frutescens* extract. The 96 well plate was sealed with Platemax cyclerseal sealing film and thereafter incubated at 37 °C. Plates containing LPS and PHA stimulated blood were incubated for 24 and 48 hours respectively. Assays were done in triplicate.

3.2.6 Cytotoxicity

Culture supernatants were collected after the incubation with and without immune stimulation. LDH was measured using a Cytotoxicity Detection kit (Biovision, USA). The kit includes all the components required for the assay. Total cellular LDH were obtained by lysing diluted whole blood with the detergent Triton X100. The lysed cells were used to determine total cellular LDH. Cell culture supernatants and the lysed cells were assayed on a 96 well plate (Nunc-Immuno plate, MaxiSorp). 100µl of the kit reaction mixture was added to respected wells and incubated for approximately 15 minutes. The absorbance of the reaction mixtures were measured at 492nm using a plate spectrophotometer at various time intervals. Cytotoxicity was expressed as the amount of LDH in supernatant as % of total cellular LDH.

3.2.7 Cytokine analysis

Double antigen sandwich enzyme linked immune sorbent assay (DAS ELISAs) (e-Bioscience, Germany) was used to measure cytokine release from the supernatants of the whole blood cells cultures. Nunc maxisorp (NuncTM, Denmark) plates were used for the assays. This kit contained all the reagents, buffers and diluents needed for performing quantitative ELISAs. The ELISAs were carried out according to the manufacturer's instructions. In summary: 96 well plates were coated with primary antibody against the respective cytokine and incubated overnight at 4 °C. After incubation, the plates were washed with autoclaved phosphate buffered saline containing 0.05% Tween-20. Non-specific binding sites were then blocked with assay diluent for 1 hour at ambient temperature after which the wells received either recombinant human cytokine standards or sample. The plate was sealed and incubated for 2 hours at ambient temperature on a shaker. After incubation the wells were washed. The wells then received Biotin-conjugated antibody against the respective cytokine. The plate was incubated for 1 hour at ambient temperature on a shaker followed by washing as before. The wells then received Avidin-HRP conjugate. The plate was then incubated for 30 minutes at ambient temperature on a shaker. After the last wash, the bound peroxidase was monitored by addition of Tetramethylbenzidine solution (substrate solution) to each well, after which the plate was incubated for approximately 15 minutes. The reaction was stopped by adding 2M H₂SO₄ to each well. The absorbance was read at 450nm on an ELISA plate reader. Excel was used to generate a standard curve for each ELISA plate which was used to determine the cytokine concentrations of the culture supernatants.

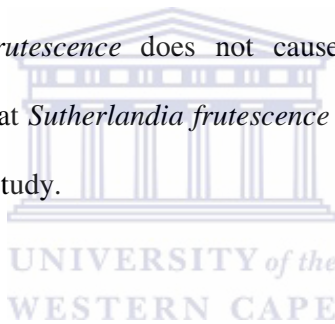
3.2.7 Statistical analysis

Experiments were performed three times in duplicate. All data is presented as a mean \pm standard deviation (SD). Data was statistically analyzed via one-way ANOVA ($P < 0.05$) and regression analysis.

3.3 Results

3.3.1 Cytotoxicity

The cytotoxicity of *Sutherlandia frutescence* was determined by evaluating the Lactate dehydrogenase (LDH) activity in cell culture supernatants. The toxicity *Sutherlandia frutescens* was tested at concentrations ranging from 0 μ g/ml - 5000 μ g/ml. Results obtained concludes that *Sutherlandia frutescence* does not cause LDH leakage at any of the concentration. This indicates that *Sutherlandia frutescence* is not toxic to the cells at any of the concentrations used for this study.



3.3.2 The inflammatory activity of *Sutherlandia frutescens*

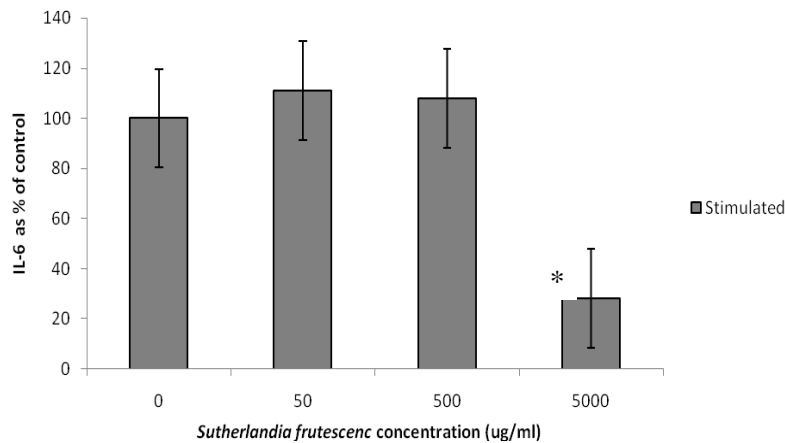


Figure 4.1: The inflammatory activity of *Sutherlandia frutescens*. Induction of IL-6 (pg/ml) of whole blood cultures *in-vitro* by LPS, in the presence of various concentrations of *Sutherlandia frutescens*. An asterisk (*) designates the significant difference ($P < 0.05$) to the control. Data are presented as the mean $n=6$. n is the number of replicates used in the experiment. All three donors were assayed simultaneously in duplicate to reduce the variables and to ensure accuracy.

The addition of *Sutherlandia frutescens* to stimulated blood has no effect on the release of IL-6 at concentrations between $0\mu\text{g/ml}$ - $500\mu\text{g/ml}$ (Figure 4.1). However, there is a significant decrease ($P < 0.05$) in the release of IL-6 at $5000\mu\text{g/ml}$. The addition of *Sutherlandia frutescens* at $5000\mu\text{g/ml}$ to the stimulated whole blood cell cultures resulted in a significant lower release of IL-6 as compared to the control ($P < 0.05$). The addition of *Sutherlandia frutescens* to unstimulated whole blood cell cultures had no effect on IL-6 concentrations. Total IL-6 produced by unstimulated cultures was $<10\%$ of the level produced by stimulated cultures.

3.3.3 The effects of *Sutherlandia frutescens* on humoral immunity

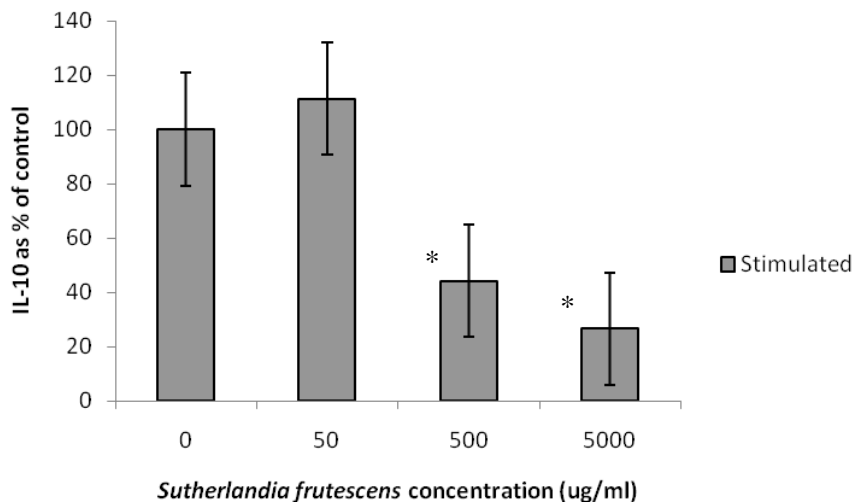


Figure 4.2: The effects of *Sutherlandia frutescens* on the release of IL-10 secreted by PHA stimulated whole blood cells. An asterisk (*) designates the significant difference ($P < 0.05$) to the control. Data are presented as the mean $n=6$. n is the number of replicates used in the experiment. All three donors were assayed simultaneously in duplicate to reduce the variables and to ensure accuracy.

The addition of *Sutherlandia frutescens* to stimulated blood has no effect on the release of IL-10 at the concentration of 50 μ g/ml. However, there is a significant difference ($P < 0.05$) in the release of IL-10 at 500 μ g/ml and 5000 μ g/ml as depicted in Figure 4.2. The addition of *Sutherlandia frutescens* at concentrations of 500 μ g/ml and 5000 μ g/ml to stimulated whole blood cell cultures resulted in a significantly lower release of IL-10 compared to that of the control ($P < 0.05$). The addition of *Sutherlandia frutescens* to unstimulated blood had no effect on IL-10 concentrations. Total IL-10 produced by unstimulated cultures were $< 10\%$ of the level produced by stimulated cultures.

3.3.4 The effects of *Sutherlandia frutescens* on cellular immunity

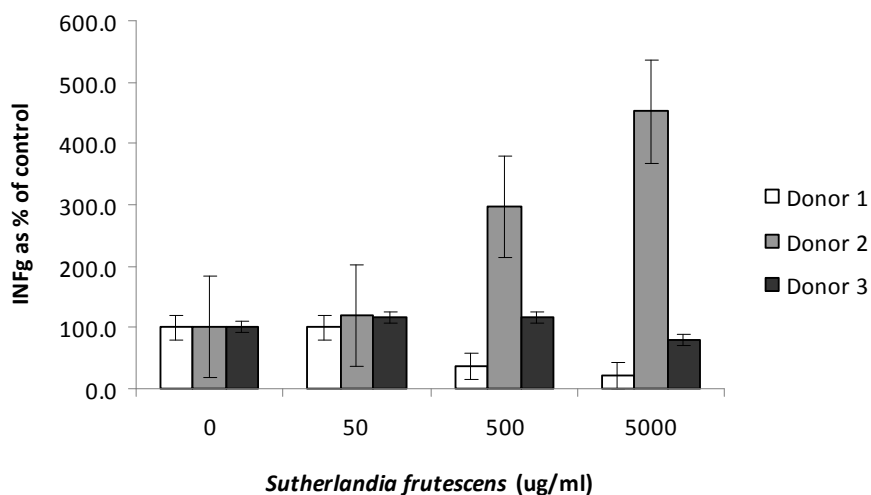


Figure 4.3: The effects of *Sutherlandia frutescens* on the release of INF γ secreted by PHA stimulated whole blood cells of three donors. Data are presented as the mean $n=2$. n is the number of replicates used in the experiment. All three donors were assayed simultaneously in duplicate to reduce the variables and to ensure accuracy.

It was observed (Figure 4.3) that there is a wide variation in the release of INF γ at the various concentrations of *Sutherlandia frutescens* between the 3 donors tested. In donor 1, the addition of *Sutherlandia frutescens* at concentrations 500 μ g/ml and 5000 μ g/ml suppressed the release of INF γ . In donor 2, the release of INF γ was increased at concentrations 500 μ g/ml and 5000 μ g/ml and in donor 3 there was no effect on the release of INF γ .

Due to the different responses by the above donors, whole blood cells from an additional 3 donors were collected and assayed. Smaller increments of *Sutherlandia frutescens* concentrations were used to assess the release of INF γ more accurately. The results obtained were as follows:

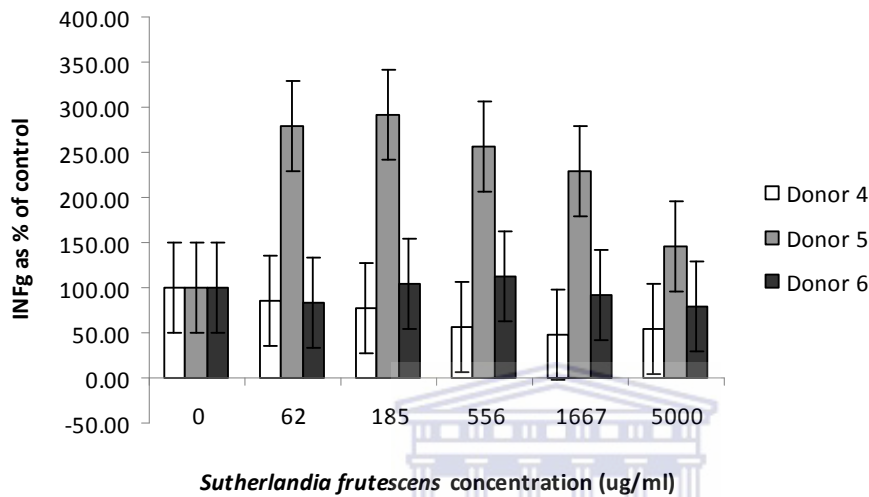


Figure 4.4: The effects of *Sutherlandia frutescens* on the release of INF γ secreted by PHA stimulated whole blood cells of three donors. Data are presented as the mean $n=3$. n is the number of replicates used in the experiment.

These assays tested the release of INF γ at concentrations ranging from $0\mu\text{g/ml}$ – $5000\mu\text{g/ml}$ in increments of 3, i.e. 3 fold dilution. The assays were conducted in triplicate for accuracy and consistency. The data above (figure 4.4) depicts an average of INF γ released as a percentage of the control. It was observed that there is a wide variation in the release of INF γ at the various concentrations of *Sutherlandia frutescens* between the above 3 donors tested (Figure 4.4). Data collected for donor 4 shows a slight suppression in the release of INF γ . Donor 5 showed a marked increase in the release of INF γ at all the concentrations of *Sutherlandia frutescens* tested. In donor 6 there was neither an increase nor decrease in the

release of INF γ . Despite the larger donor sampling pool, the wide variation in the release of INF γ at all the concentrations tested was again noted indicating donor specific variations to *Sutherlandia frutescens*.

3.4 Discussion

The immune system's main function is to protect the host from infection. Sometimes the immune response to pathogens such as measles or small pox may be so powerful that the initial infection provides a life long protection. Sometimes the immune response is so weak that infection usually results in long term infectious disease or the individual becomes a "healthy carrier" of the antigen (Sun, 2008).

The aim of this study was to evaluate the effects of *Sutherlandia frutescens* on the immune pathways as this herb is used by many cultural groups for a wide range of illness conditions (table 1). It is used in conditions which require either the suppression or stimulation of certain immune pathways. *Sutherlandia frutescens* was screened for toxicity at various concentrations. Results showed that *Sutherlandia frutescens* was non toxic at all the concentrations tested.

Various concentrations of *Sutherlandia frutescens* extract were screened for its effects on inflammation and T cell activity by using *in vitro* human whole blood cultures.

Inflammation is an intricate biological response of the host's immune system to harmful stimuli (Medeiros, et al., 2009). IL-6 is a pleiotropic cytokine which has a pro-inflammatory action (Ahmed and Ivashkiv, 2000). IL-6 was used as a biomarker to determine the effect of *Sutherlandia frutescens* extract on inflammation.

Whole blood cell cultures were stimulated with the extract as explained in the methods section. Exposure to a pharmaceutical may initiate either an elevation or suppression in the levels of IL-6 released.

The supernatant of the stimulated and unstimulated whole blood cell cultures incubated with *Sutherlandia frutescens* were screened using the whole blood cells from 3 healthy male donors simultaneously and duplicated for consistency.

Sutherlandia frutescens at high concentrations inhibits IL-6 indicating that the extract has anti-inflammatory properties. The anti-inflammatory properties of *Sutherlandia frutescens* validate its uses as an anti-inflammatory agent. It is reported to have a possible application in the treatment of septic shock and chronic inflammation. The anti-inflammatory properties have been linked to flavonoids sutherlandin A-D, L-canavanine and pinitol (Anfossi, et al., 1999; Ojewole, 2004; Avula, et al., 2010). Flavonoids are known for its anti-inflammatory effect (Evans, 2002). L-canavanine prevents the arginine derived synthesis of nitric oxide (NO). L-canavanine is an arginine antagonist that selectively inhibits NO synthase causing vasoconstriction hence contributes to *Sutherlandia frutescens* anti-inflammatory action (Anfossi, et al., 1999; Abramson, 2005). Pinnitol decreases the production of pro-inflammatory cytokines (Ojewole, 2004). The proven anti-inflammatory action of *Sutherlandia frutescens* validates its anecdotal use in the treatment of oesophagitis, gastritis, dysentery, inflammatory skin conditions, rheumatoid arthritis, osteoarthritis, asthma, bronchitis and cystitis (Albrecht, 2008; Gericke, et al., 2001; van Wyk and Albrecht 2008; van Wyk and Wink 2004). *Sutherlandia frutescens* can also be used in the management of cardiovascular diseases as enhanced inflammation is involved in the development and progression of atherosclerosis (Gokkusu, et al., 2010).

The cytokines IL-10 and INF γ were used as biomarkers to determine the effect of *Sutherlandia frutescens* on T cell activity. IL-10 directs the differentiation of T_{H2} cells which are responsible for strong antibody production, eosinophil activation, and inhibition of several macrophage functions. It thus provides phagocyte-independent protective responses. INF γ coordinates a diverse array of cellular programs including the differentiation of T cells (T₀) into T_{H1} cells. T_{H1} cells mainly develop during infections caused by intracellular bacteria and may trigger phagocyte-mediated host defense (Romagnani, 1999).

Whole blood cell cultures were stimulated with PHA and incubated with *Sutherlandia frutescens* as explained in the methods section. The results obtained indicate that *Sutherlandia frutescens* hinders the differentiation of T_{H2} cells at higher concentrations, hence suppresses the phagocyte independent host response. *Sutherlandia frutescens* is commonly used in the management of asthma. Asthma can be defined as the increased irritability of the bronchial tree resulting in narrowing of the airways (Underwood, 2004). Asthma is a common clinical manifestation of an allergy. An immunological pathway driven by T_{H2} cells such as the production of eosinophils aids this immune response (Arshad, 2005). Eosinophils are non-dividing immune cells that originate in the bone marrow (DiScipio and Schraufstatter, 2007). It functions as effector cells in the pathogenesis of allergic inflammatory reactions such as bronchial asthma (Chihara, et al., 1999). The data collected validates the anecdotal use of *Sutherlandia frutescens* in the management of asthma as the suppression of T_{H2} differentiation at high concentrations results in a decrease in eosinophil activation. Even though the results obtained validate the use of *Sutherlandia frutescens* in the management of asthma, it would not be effective in eliminating extra-cellular bacteria such as *Salmonella enterica* and *Listeria monocytogenes* which are food borne pathogens with clinical features including gastroenteritis and septicemia (Fablet, et al., 2006; Braun and Cossart, 2000).

The data collected shows a good correlation between the effects of *Sutherlandia frutescens* on the release of IL-6 and IL-10 at the higher concentrations tested. It can be noted that *Sutherlandia frutescens* down regulates the release of both cytokines, however, IL-10 is suppressed at $\geq 500\mu\text{l/ml}$ whereas the suppressing effect of *Sutherlandia frutescens* on the release of IL-6 takes place at $\geq 5000\mu\text{l/ml}$. This indicates that the T_{H2} suppression by *Sutherlandia frutescens* takes place at lower concentrations than the anti inflammatory effect.

Sutherlandia frutescens is commonly used to treat many viral infections such as chickenpox, influenza and gonorrhoea. The anti-viral properties have been linked to L-canavanine and is effective in the management of influenza and retroviruses including HIV (Mills, et al., 2005). $INF\gamma$ coordinates the differentiation of T_{H0} cells into T_{H1} cells. T_{H1} cells drives the cell mediated immune response which is mainly involved with infections caused by intra-cellular bacteria and cancer (Romagnani, 1999; Milon and Liou, 1993).

The effects of *Sutherlandia frutescens* on the release of $INF\gamma$ are depicted above in Figures 4.3 and 4.4 respectively. It is noted that the results obtained from the donors varied considerably. In some donors the release of $INF\gamma$ was less than that of the control. In some donors the release of $INF\gamma$ was markedly higher than that of the control and in other donors, there were neither an increase nor decrease in the release of $INF\gamma$. This shows that *Sutherlandia frutescens*, at the concentrations tested, has individual specific effects on the differentiation of T cells (T_0) into T_{H1} cells.

The results obtained for $INF\gamma$ can serve to motivate that science cannot always conclusively validate the exact mechanism of allelopathy hence the evolution of technology is an ongoing process.

3.5 Concluding remarks

The current study indicates the suppression of the inflammatory and phagocyte independent host's response at high concentrations of *Sutherlandia frutescens* with no effect on the release of IL-6 and IL-10 at the lower concentrations. *Sutherlandia frutescens* was found to be donor specific for INF γ as it acted in both inhibitory and stimulatory manners on the phagocyte-mediated host response. This is illustrated with the up and down regulation of INF γ released in the stimulated whole blood cells of the 6 donors tested. A possible reason for the lack of scientific data available regarding *Sutherlandia frutescens* and the immune system as mentioned by Mills, et al. (2005) may be linked to variation in data regarding the release of INF γ as this is a cytokine involved in cellular immunity. Cellular immunity is responsible for activating mononuclear phagocytes, NK cells and cytotoxic T cells for killing intra-cellular pathogens and cancer (Milon and Liou, 1993). The results indicate that *Sutherlandia frutescens* may in fact upregulate the T_{H1} process in some donors and this will help these donors fight intra-cellular pathogens and cancer. However, *Sutherlandia frutescens* is contraindicated in the treatment of intra-cellular pathogens and cancer where INF γ is down regulated. Further investigation of the effects of *Sutherlandia frutescens* on cellular immunity on a larger sample pool is therefore recommended.

3.6 Reference

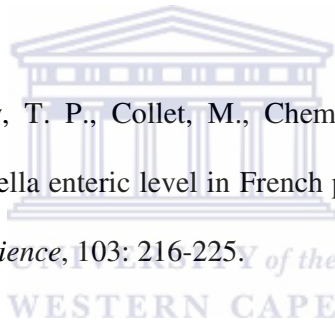
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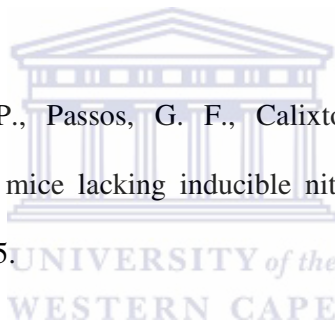
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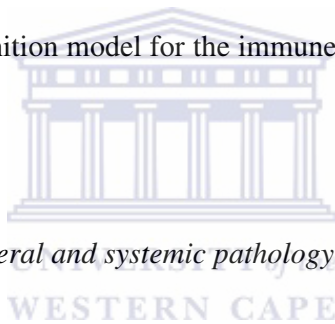


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Chapter 5: Summary, Concluding remarks and Recommendations

4.1 Summary

Human beings live in an environment surrounded by microorganisms – viruses, bacteria, fungi and parasites, many of which can be pathogenic if the host's body is not physiologically prepared to initiate and drive responses against these pathogens. An efficient immune system is vital in preventing systemic infections which can be fatal. Cytokines are low-molecular weight proteins that are essential in driving immune responses. They act through binding to specific cell-surface receptors initiating specific cellular functions or suppressing certain cellular activities. Interleukin (IL)-6 is linked to the innate immunity and is responsible for inducing the initial acute inflammatory response brought about by a breach in the first line of immune defenses. Antigens are passed on to the adaptive immunity by antigen presenting cells (APC). Interferon gamma ($INF\gamma$) is the cytokine that initiates the differentiation of naïve T_H0 cells to T_H1 . This drives the cell mediated immunity which activates mononuclear phagocytes, NK cells and cytotoxic T cells for killing intracellular microbes such as virally infected target cells and cancers. On the other hand, the development of T_H2 cells produces IL-4, IL-5, IL-10 and IL-13. These cytokines increase antibody production, aspects of hypersensitivity, parasite induced immune responses and humoral immunity.

Pharmaceuticals can play an active role in modulating immune responses. It can up regulate, down regulate, inhibit or have no effect on certain pathways. Limited literature on the biological activities of herbs exists with even less literature on indigenous South African herbs. Past studies on indigenous South African herbs focused mainly on the pharmacokinetics of the herb's secondary metabolites using HPLC and GC-MS. No previous studies examining the immunological effects of *Sutherlandia frutescens* has been done despite its wide range of anecdotal uses. The aim of this study was to evaluate the immune

modulating activity of *Sutherlandia frutescens* using *in vitro* methods. The study focused on monitoring the release of the main cytokines involved in inflammation and T cell differentiation.

Results were consistent with some of *Sutherlandia frutescens* anecdotal uses such as bronchial asthma, bronchitis, oesophagitis, gastritis, dysentery, inflammatory skin conditions, rheumatoid arthritis, osteoarthritis and cystitis. These conditions require a decrease in the release of IL-6 and/or IL-10 which are the cytokines involved in inflammation and humoral immunity respectively.

The effects of *Sutherlandia frutescens* on cellular immunity has proven to be donor specific as the herb acted in both stimulatory and inhibitory manners on the phagocyte mediated host response. The lack of scientific data on the biological effects of *Sutherlandia frutescens* on the immune system may be linked to its donor specific effects on cellular immunity.

4.2 Concluding remarks

The effect of herbal medicine on the immune system can be screened effectively using *in vitro* whole blood cell cultures. *Sutherlandia frutescens* was not toxic at any of the concentrations used in this study. *In vitro* data generated by this study support *Sutherlandia frutescens* use in the treatment of inflammatory conditions and allergies such as bronchial asthma. In terms of cellular immunity, *Sutherlandia frutescens* was found to be donor specific. This study provides scientific evidence validating some of the anecdotal uses of *Sutherlandia frutescens* requiring the suppression of certain immune pathways.

4.3 Recommendations

This study is the first of its kind monitoring the activity of *Sutherlandia frutescens* on inflammation and T cell differentiation. Methods to improve this work may include isolating specific compounds in *Sutherlandia frutescens* such as L-canavanine, to clarify its effect on cellular immunity. This compound is said to be responsible for *Sutherlandia frutescens* antiviral activities against retroviruses including HIV. As the management of HIV is a major concern for the health sector of South Africa, validating the use of *Sutherlandia frutescens* against retroviruses would be beneficial. Further investigations exploring the effects of *Sutherlandia frutescens* on immune pathways in an *in vivo* system may be required to validate the results obtained in this *in vitro* study. Water or low-medium alcoholic extractions of *Sutherlandia frutescens* is more commonly used anecdotally. Therefore, when preparing the herb sample, a dosage form closer to what is commonly used by the general public represents the ideal.

