An assessment of medicinal hemp plant extracts as natural antibiotic and immune modulation phytotherapies

By

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A thesis in partial fulfillment of the requirements for the degree of Magister Scientiae in the South African Herbal Science and Medicine Institute, Faculty of Natural Sciences, at the University of the Western Cape.

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South African Herbal Science and Medicine Institute

November 2005

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Acknowledgements

I am grateful to all for making this study a success:

- God, for giving me the wisdom and strength to complete this study.
- My husband, Bradley, the journey has finally come to an end. Words cannot convey my gratitude for your love, continuing support and words of wisdom throughout my study.
- My parents, thank you for always being an encouragement and for the support in furthering my higher education.
- My supervisor, Prof. Quinton Johnson, thank you for your continuous guidance and valuable input throughout my study.
- Dr. Joanita Adams, for my training in immunology. Thank you for your perpetual eagerness to assist and sharing your invaluable expertise.
- Mr. André Braaf, thank you for all your help, humour and kind words of inspiration.
- Dr. Jeremy Klaasen, Dr. Edmund Pool, Mr. Lilburne Cyster and Mr. Vaughan Gabrielse who were always available to offer their assistance.
- Sisters Engelbrecht and Prince from the UWC Campus Health Clinic who kindly assisted in drawing blood.
- The blood donors who so eagerly assisted in donating blood for my experiments.
- The NRF, for the financial support of my study.
- The South African Herbal Science and Medicine Institute for providing me with laboratory space and time off from my position, to complete this research project.

Declaration

I declare that **"An assessment of medicinal hemp plant extracts as natural antibiotic and immune modulation phytotherapies"** is my own work, only submitted to the University of the Western Cape, with all research resources used in this project duly acknowledged by means of complete references.

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Olivia Case
November 2005

Dedication

To my husband, Bradley, and parents, Clive and Elvra, thank you for teaching me the value of hard work and perseverance. Your motivation, love and support have inspired me to complete this study successfully.

Chapter One

Overview

An assessment of medicinal hemp plant extracts as natural antibiotic and immune modulation phytotherapies

Chapter One

Overview

Medicinal Plants – History and context

South Africa is exceptionally rich in plant diversity with some 21 000 species of flowering plants – almost one tenth of the world's higher plants – of which 80 % are endemic (Fennel, Light, Sparg, Stafford and Van Staden, 2004). It is estimated that 27 million South Africans utilize traditional herbal medicines from more than 1020 plant species (Stafford, Jager and Van Staden, 2004). Many plants have been used for different purposes, such as food, drugs and perfumery. Yet, there has been limited research and support for the systematic understanding and development of the enormous wealth of medicinal plants in our country.

People who use traditional remedies may not understand the scientific rationale behind their medicines, but they know from personal experience that some medicinal plants can be highly effective if used at therapeutic doses (Van Wyk, Gericke and Van Oudtshoorn, 2000). As plants were tried and tested, the knowledge was spread and shared amongst people, some of which is still in practice today. Factors such as urbanization and acculturation have lead to an increase in the use of orthodox or western medicine compared to that of traditional plant use. The medicinal knowledge of the Griquas, Outeniquas and Namas is already very much remnant, and urgent, focused programmes are needed to rescue this knowledge (Gericke, 1996).

In terms of world health, traditional medicinal plants continue to play a central role in the health care systems of large proportions of the world's population (Akerele, 1988). The World Health Organization (WHO) reported that 25 - 50 % of modern medicines are made from plants first used traditionally (WHO, 2003). Indeed, increasingly, more pharmacognostic investigations of plants are carried out to find novel drugs or templates for the development of new therapeutic agents (Beringer, 1999).

Natural antibiotic properties of plant medicine

In many parts of the world, there is a rich tradition in the use of herbal medicine for the treatment of many infectious diseases. Antimicrobial drugs have proved remarkably effective for the control of bacterial infections. However, there is an increased attention on extracts and biologically active compounds isolated from plant species used in herbal medicine due to the side effects and the resistance that pathogenic micro-organisms build against the antibiotics (Essawi and Srour, 1999). According to Eloff (1998), the amount of resistant strains of microbial pathogens is increasing since penicillin resistant and multiresistant pneumococci caused a major problem in South African hospitals in 1977. The potential problem of emerging resistance to antimicrobial agents requires careful monitoring.

Antimicrobial studies have shown that gram-negative bacteria show a higher resistance to plant extracts than gram-positive bacteria (Palambo and Semple, 2001; Kudi *et al*., 1999; Paz, 1995; Vlietinck, 1995). This can be as a result of the variation in the cell wall structures of gram-positive and gram-negative bacteria (Palambo *et al*., 2001). More

specifically, gram-negative bacteria have an outer membrane that is composed of high density lipopolysaccharides that serves as a barrier to many environmental substances including antibiotics (Palambo *et al*., 2001). New compounds inhibiting microorganisms such as benzoin and emetine have been isolated from plants (Cox, 1994). Eloff (1999) stated that the antimicrobial compounds from plants may inhibit bacteria by a different mechanism than the presently used antibiotics and may have clinical value in the treatment of resistant microbial strains.

There is a continuous and urgent need to develop new antibiotic and immune modulating compounds with diverse chemical structures and novel mechanisms of action, because there has also been an alarming increase in the incidence of new and re-emerging infectious diseases (Rojas, Bustmante, Bauer, Fernandez and Alban, 2003). The South African flora offers great possibilities for the discovery of new compounds with important medicinal applications in combating infection and strengthening the immune system. This is indeed very important because *Candida albicans, Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli* are amongst the examples of some important human pathogens that developed resistance to antimicrobials (Barbour, 2004). The microorganisms examined in this study include *Staphylococcus aureus, Pseudomonas aeruginosa* and *Mycobacterium smegmatis*.

Staphylococcus aureus

Staphylococcus aureus is a bacterium which is a common coloniser of human skin and mucosa. *S. aureus* can cause disease, particularly, if there is an opportunity for the bacteria to enter the body. Prescott (1996) states that *S. aureus* is the most important human staphylococcal pathogen and causes boils, abscesses, wound infections, pneumonia, toxic shock syndrome amongst other diseases.

S. aureus is also a pathogen frequently reported to produce food poisoning, which leads to cramps and severe vomiting. Most strains of this bacterium are sensitive to many antibiotics, and infections can be effectively treated. Some *S. aureus* bacteria are resistant to the antibiotic methicillin, termed methicillin-resistant *S. aureus* (MRSA). Since its emergence in 1961, MRSA has spread to nearly every continent, reaching near epidemic proportions in some countries and becoming one of the most common pathogens in hospitals worldwide (Abbas, Lichtman and Pober, 2004).

Pseudomonas aeruginosa

Pseudomonas aeruginosa is an opportunistic pathogen, meaning that it exploits some break in the host defenses to initiate an infection. It is a common environmental microorganism present in water and soil and is notorious for its resistance to antibiotics and is, therefore, a particularly dangerous and dreaded pathogen (Prescott *et al*., 1996). The bacterium is naturally resistant to many antibiotics due to the impermeability characteristics of the outer membrane. Moreover, its tendency to colonize surfaces in a

biofilm form makes the cells impervious to therapeutic concentrations of antibiotics (Craig, 1997).

Mycobacterium smegmatis

Mycobacterium smegmatis is a non-pathogenic surrogate of *M. tuberculosis* and is not deemed an important human pathogen. *M. smegmatis* is more amenable to experimentation unlike *M. tuberculosis,* which is a slow grower and genetically less tractable (Wang, Elchert, Hui, Takemoto, Bensaci, Wennergren, Chang, Rai and Chang, 2004). *M. smegmatis* shares several clinically important properties that characterize *M. tuberculosis*, including similar resistance to certain macrolide drugs. *M. tuberculosis* is pathogenic and not easily manipulated experimentally, therefore, *M. smegmatis* is typically used to investigate issues related to the biology and pathogenesis of the former. According to Stephan (2004), *M. tuberculosis* is a major global health problem and causes about two million deaths per year (Sharma, Al-Azem, Hershfield and Kabani, 2003).

Synergism of antimicrobial compounds

As certain antibiotic treatments lead to the development of multiresistant organisms, it is now standard clinical practice to use a combination of two or more antibiotics with different mechanisms of action in an attempt to prevent the development of antibiotic resistance and improve the outcome of therapy (Beringer, 1999). Craig (1977), states that infections caused by *Pseudomonas aeruginosa* are often difficult to treat and in an

attempt to achieve an optimal outcome, clinicians would use combinations of antibiotic therapies with the aim of achieving antibiotic synergy.

Extract stability

Plant materials supplied by street markets are usually spoilt due to a lack of storage facilities and trading infrastructure (Mander 1997; Stafford *et al*., 2004). Plant material is exposed to microbial and insect attack as well as the effects of temperature, light and gases. According to Stafford *et al.* (2004), the informal markets are generally situated close to pedestrian and motor vehicle traffic, which places plant material in close contact with various kinds of pollution and therefore impacting on the potency of plant remedies. This poses a great concern as many users of traditional plant remedies purchase material from these markets.

Another concern is that consumers store plant material for long periods after purchase therefore, using dried material instead of fresh plants. Many active compounds are volatile and would not be active in plant remedies after extensive storage periods. The determination of the increase or decrease of plant activity over time will allow herb sellers to make informed decisions regarding the prescription of stored plant material. Griggs, Manandhar, Towers and Taylor (2001), states that the disposal of unstable plants will contribute to the efficacy of herbal medicines, whereas plants that have stable biological activity can safely be stored for longer periods of time, which will be beneficial both to the healer (financially) and to the environment, since fewer plants will have to be harvested.

Sansevieria Species

Extracts from indigenous medicinal hemp plants from the genus *Sansevieria*, were investigated in this study for their natural antibiotic and immune modulatory value. *Sansevieria* species are commonly known as piles root, bowstring hemp and also aambeiwortel in Afrikaans. Its association with *C. sativa* or hemp is mainly the fibrous leaves which produce good quality fibres. *Sansevieria* plants are tough and semi-succulent with erect, hard, mottled leaves arising from thick fleshy rhizomes. *Sansevieria* species are popular garden plants and also used indoors due to their ability to flourish under low light conditions. *S. aethiopica, S. hyacinthoides* and *S. personii* are amongst the common species in southern Africa (Van Wyk *et al*., 2000).

The medicinal uses of *Sansevieria* species include treatment for ear infection, earache, toothache, hemorrhoids, ulcers and intestinal worms (Van Wyk *et al.*, 2000). Traditionally, in treating earaches and hemorrhoids, the leaves are heated and the warm juice is squeezed onto the affected area. In the case of internal treatments, the heated leaves are chewed and the juices swallowed. Van Wyk *et al*. (2000) state that *Sansevieria* consists of many sapogenins, of which Ruscogenin (Fig.1) is the most common, and is commercially used as anti-inflammatory agent and venotonic.

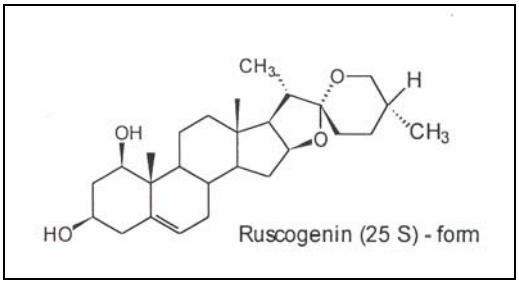

Fig. 1: Chemical structure of the sapogenin, Ruscogenin.

Cannabis sativa

Extracts from the exotic hemp plant, *Cannabis sativa*, were evaluated as controls in this investigation. *Cannabis sativa* is an erect herb commonly known as hemp or marijuana or by vernacular names such as grass, weed and pot. This ancient crop plant is suspected to be indigenous to temperate Asia and has been domesticated since the early history of mankind (Van Wyk and Wink, 2004). Hemp has one major disadvantage: it is associated with the use of illegal narcotics and therefore, it is categorized as illegal in many countries.

Struik, Amaducci, Bullard, Stutterhaim, Venturi and Cromack (2000), states that fiber hemp is grown for a multitude of end products that is useful to mankind, thus, being an excellent model crop for the establishment of multi-output systems through stepwise breakdown of biomass into several useful components. The end products are derived from the cannabinoids, seeds, fiber and the wooden core. The hemp plant is complex; it synthesizes about 460 chemicals of which about 60 have the 21-carbon structure of the cannabinoids (Ames, 1996). Δ^9-Tetrahydrocannabinol (Δ^9 –THC), cannabinol and cannabidiol (Fig. 2) are the major constituents of the *Cannabis sativa* plant.

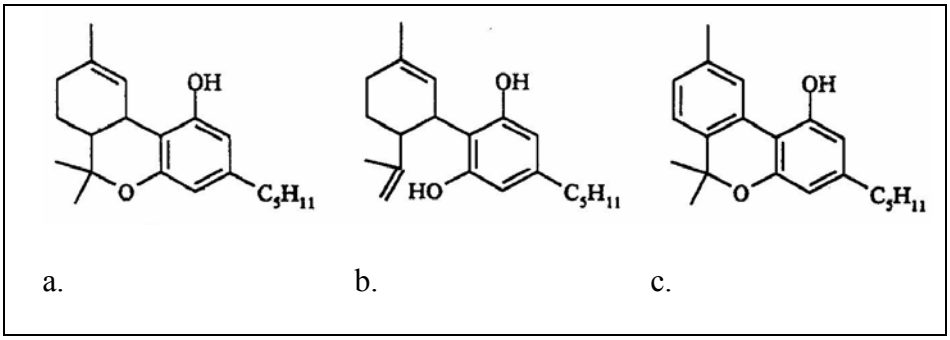

Fig. 2 a-c: Chemical structures of cannabinoids: a, Δ^9 –THC; b, cannabidiol; c, cannabinol.

Cannabis has been recognized for centuries as a valuable therapeutic agent. Mechtler, Bailer and De Heuber (2003), reported that folk uses include treatment of insomnia, inflammation, various psychoses, digestive disorders, depression, rheumatism, migraine, appetite disorders and it has also been employed by women to facilitate childbirth, stimulate lactation, and relieve menstrual cramping. Wilson (1995) states that *Cannabis* is also used in traditional remedies to relieve pain and muscle spasms, to prevent seizures and to aid sleep. *Cannabis* use may also alleviate nausea, though it often triggers nausea in new users, and it can boost appetite (Van Wyk *et al*., 2004). Recent research is confirming the role of cannabis as a useful therapeutic agent in chronic conditions such as AIDS and multiple schlerosis (Williamson, 2001). The drug is best known for its effects on the mind: it is an intoxicant that makes people feel happy and relaxed, and over the past century, its recreational use has become increasingly popular.

Cannabis sativa is traditionally used as an anti-asthmatic treatment. Asthma is one of the most common chronic health problems and is characterized by airway hyper responsiveness, airway inflammation and airway remodelling (Diamant, 2005). According to Craig (1997), inflammation in the airways induces twitchiness of the airways to a number of specific stimuli known as the airway hyper responsiveness, which causes the signs and symptoms of asthma. The immune system can become sensitised after exposure to an allergen and produce antibodies to allergen-specific proteins, thus causing an inflammatory response that leads to airway hyper-reactivity (Breyse, 2005).

Allergens in homes are an established risk factor for asthma. Earlier studies done by Busse, Calhoun and Sedgwick (1993), states that the eosinophil is an important cell in asthma, including pro-inflammatory cytokines generated from activated lung mononuclear cells. The consequence of this multiple cell, multiple pro-inflammatory product interaction is the establishment of a self-perpetuating, redundant process by which asthma severity increases (Busse *et al*., 1993). However, recent studies by Brightling, Bradding, Symon, Holgate, Wardlaw and Pavord (2002), state that the mast cell instead of the eosinophil is causative of airway hyper-reactivity in asthma. Allergens and other stimuli activate the mast cell and cause generation of a complex of autacoids including histamine, cytokines and enzymes such as tryptase (Williams and Galli, 2000).

Asthma is currently treated using two main strategies: the use of bronchodilators to reverse broncho-constriction and the use of steroids to target inflammation (Peachell, 2005). However, these treatments inadequately target the mast cell. A study done by Peachell (2005), regarding approaches to asthma treatments, suggests anti-IgE therapy, adenosine receptor antagonists and phosphor-diesterase inhibitors. Regarding anti-IgE therapy, it is stated that antigen-antibody interactions are the most commonly recognized mechanism of mast cell activation (Peachell, 2005).

Hemp fiber crops are one of the world's oldest non-food crops and is very high yielding compared to many other crops. The renewed interest in hemp is due to its large plasticity and its being an attractive non-food crop, amongst other reasons. The large plasticity allows it to be grown under a wide variety of agro-economical conditions which

contribute to economic, environmental, agronomic and social sustainability of arable farming (Struik *et al*., 1999).

Moreover, hemp is an attractive non-food crop, which produces a wide variety of renewable resources, in a way that is much more efficient than with other non-food crops. Hemp crop cultivation is not difficult. According to Van der Werf (1994), little or no biocide is needed, it suppresses weeds efficiently and requires minimal fertilizer or crop rotation. It has been a breeding goal for years to cultivate hemp plants with a low stimulant level, therefore, making it unsuitable for drug use. Mechtler *et al.* (2003), states that the presence of the psychoactive components in the hemp plant has no adverse effects on the fibre quality.

The whole *C. sativa* seed has long been used as a source of food and oil, however, its potential health contribution has never gained much attention. Hempseed, in addition to its nutritional value, demonstrated positive health benefits, including the lowering of cholesterol and high blood pressure (Sacilik, Öztürk and Keskin, 2003). Hemp seed contains approximately 20-25% protein, 20-30% carbohydrates, 25-35% oil and 10-15% insoluble fiber and a rich array of minerals (Deferne and Pate, 1996).

According to Oomah, Busson, Godfrey and Drover (2002), the hempseed oil has been suggested to be perfectly balanced in regards to the ratio (3:1) of the two essential polyunsaturated fatty acids (linoleic and linolenic acids) for human nutrition. Moreover, due to the presence of γ-linolenic acid, it is ideal as an ingredient for light body oils and

lipid-enriched creams, known for their high penetration into the skin (Rausch, 1995). The numerous uses of the seed can benefit itself to the development of numerous products for the food, cosmetic, therapeutic, functional food and nutraceutical industries.

Natural immune modulatory characteristics of plant medicine

The immune system

The immune system is the basic defense system of the body against pathogens and other foreign substances. Its function is to prevent foreign substances from invading the body and causing ailments. Inflammation is a response to infection, antigen challenge or tissue injury and leads to eradication of microbes or irritants and potentiates tissue repair (Sherwood and Kinsky, 2004). Inflammation is divided into two categories namely acute and chronic, which is on the basis of timing and pathophysiological features. Acute inflammation has a relatively short duration and is characterized by vasodilation, the exudation of protein-rich fluid and a migration of cells into the injury site and activation of coagulation cascade (Sherwood *et al*., 2004). Chronic inflammation persists over longer periods in which active inflammation, tissue destruction and attempts of tissue repair occur simultaneously (Prescott *et al*, 1996).

There are two types of immune responses to antigen exposure, which is the innate, and the adaptive or acquired defense system. The innate immune system is the defense system that an individual is born with and includes barriers such as the skin and mucosa and also cells such as neutrophils and phagocytes. Phagocytosis represents an important innate defense mechanism against ingested particulates including whole pathogenic microorganisms (Tiwari, Rastogi, Singh, Safari and Vyas, 2004). The specialized cells that are capable of phagocytosis include blood monocytes, neutrophils and tissue macrophages.

Macrophages participate in various processes which include tissue remodeling during embryogenesis, wound repair, removal of damaged or senescent cells subsequent to injury or infection, haemopoiesis and homeostasis (Hong, Jeong, Chung, Kim, Chae, Shin, Seo and Kim, 2005). Once the particulate materials, including antigens and pathogens, are ingested into phagosomes, the phagosomes fuse with lysosomes and the ingested antigens or pathogens are then digested (antigens are molecules that the immune system can recognize as foreign and react against and can be proteins, nucleic acids, polysaccharides, *etc*.). Inside the cells, small pieces of these antigens bind to a special class of proteins known as the major histocompatibility complex (MHC) molecules (Prescott *et al*., 1996). The MHC is a host of receptors that bind to molecules that confer tolerance or alerts the body that the material is foreign. The innate immune response also serves to activate and amplify acquired immunity. Acquired or adaptive immunity refers to the type of specific immunity a host develops after exposure to a suitable antigen, or after transfer of antibodies or lymphocytes from an immune donor (Prescott *et al*., 1996).

Cytokines

Cytokines are polypeptides with a low molecular weight that ranges between 15-25 kDa and are important mediators of inflammation, immunity, proteolysis, cell recruitment and proliferation (Firestein, 1995). Cytokines refer to proteins released by one cell population that function as intercellular mediators in response to infections and injuries (Prescott *et al*., 1996). They can be classified as Th0 (T-helper 0), Th1 and Th2 depending on their cellular origin (Sherwood *et al*, 2004).

T Cells

T cells play an important role in immune functions as they act both as effectors (cytotoxic T-cells or Tc-cells) and regulators (T-helper and T-suppressor cells). Hong *et al.* (2005), states that Tc cells can kill virus-infected cells and cells undergoing malignant transformation. Tc-cell activation is dependant on antigen challenge and signals sent from activated Th-cells. According to Riddell, Murata, Bryant and Warren (2002), Th-cells mediate the link between antigen-presenting and the triggering of other cellular (which include natural and lymphokine-activated killer cells, macrophages, granulocytes) and humoral (B-cell-produced antibodies) components of the immune response.

Th cells are divided into two subsets, namely Th-1 and Th-2. These cells are distinguished by the cytokines they secrete. Th-1 lymphocytes produce interleukin-2, Interferon Gamma (IFN- ɣ) and tumor necrosis factor (TNF-ß), which promote cell-mediated immunity. Th-2 lymphocytes produce IL-4, IL-5, IL-6, IL-10, and IL-13 which promote antibody-mediated immune response (Carter and Dutton, 1996).

Interleukin 6

This study focuses on interleukin 6 (IL-6) as a biomarker for inflammation, when human cells are exposed to medicinal hemp phytotherapies. Interleukin-6 (IL-6) is an 26-kDa immune protein with many previous names, including 26-kD protein, interferon-ß2 (IFN-ß2), B-cell stimulatory factor-2 (BSF-2), hepatocyte stimulating factor (HSF), cytotoxic T-cell differentiation factor (CDF), interleukin-HP1 (IL-HP1), monocyte-granulocyte inducer type 2 (MGI-2), and hybridoma/ plasmacytoma growth factor (HPGF/HGF), and given its final distinction as IL-6 in December of 1988 (Riddell *et al*., 2002). IL-6 is a monomer of 184 amino acids produced by T-cells, macrophages, and endothelial cells found on a single gene located at 7p21 (Van Snick, 1990).

It is produced by a variety of cells, including macrophages, fibroblasts, epithelial and endothelial cells (Riddell *et al*., 2002). IL-6 is released in response to infection, burns, trauma and injury. Its functions range from acute-phase protein induction to B-and T-cell growth and differentiation. Riddell *et al.* (2002), states that apart from its participation in acute phase responses, IL-6 also participates in the growth of endometrial tissue, bone formation and synthesis of hormones from various glands including the pituitary gland. IL-6 can have a direct effect on cells, can mediate the effects of other cytokines, can be coagonistic or antagonistic in conjunction with other cytokines, and interact with glucocorticoids (Carter *et al*., 2002). This protein is often linked with the so-called interleukin-6-type cytokines: IL-11, leukemia inhibitory factor (LIF), oncostatin M (OM), ciliary neurotrophic factor (CNTF), and cardiotrophin-1 (CT-1) (Van Snick, 1990).

IL-6 is released in response to IL-1 and TNF-ß (Gomaraschi, 2005). The IL-6 receptor is found on many cell surfaces, including resting normal T-cells, activated normal B-cells, myeloid cell lines, hepatoma cell lines, myeloma cell lines, and on Epstein-Barr virus (EBV) modified B-cells, in which it promotes proliferation (Van Snick, 1990). IL-6 can lead to the transcription of a wide variety of proteins through all three major signal transduction pathways; protein kinase C, cAMP/protein kinase A, and the calcium release pathway (Hong *et al*., 2005). IL-6 has various molecular forms and each molecule has a different function when secreted by different cells in distinct situations (Van Snick, 1990).

IL-6: The Acute Phase

IL-6 stimulates the acute-phase reaction, which enhances the innate immune system and protects against tissue damage (Sherwood *et al*., 2004). It results in the release of certain proteins, known as acute-phase proteins, into the blood plasma by liver cells and the decrease in rate of synthesis of other proteins. Acute phase proteins mimic antibodies but have a very broad specificity (Firestein, 1995). IL-6 increases the synthesis of the two major acute-phase proteins, c-reactive protein (CRP), which increases the rate of phagocytosis of bacteria, and serum amyloid A (SAA) by regulating changes in the gene transcription rate of these proteins. In the same way, it also increases the synthesis of fibrinogen, an important clotting agent. Albumin and transferrin levels are decreased in the presence of IL-6 (Sherwood *et al.*, 2004). The acute phase local reaction leads to a systemic reaction which includes fever, increased erythrocyte sedimentation rate,

increased secretion of glucocorticoids, and the activation of the complement and clotting cascades (Castell, Gomez-Lechon, David, Fabra, Trullenque and Heinrich, 1990).

IL6: B-Cells

Interleukin-6 is also very important in the stimulation of differentiation and proliferation of B-cells (Firestein, 1995). Its most noted effect is found in the induction of permanent differentiation of B-cells into plasma cells, antibody producing cells (Carter *et al*., 2002). IL-6 enhances the release of antibodies by acting as a growth factor for already differentiated plasma cells. It stimulates mostly the release of IgG and IgA antibodies from these cells (Kishimoto, 1998).

IL-6: T-Cells

Interleukin-6 is especially important in the early stages of T-cell differentiation. In this phase, it reinforces the effect of IL-2 and promotes the differentiation of CD4 cells into Th-2 cells (Riddell *et al*., 2002). It controls the growth and proliferation of early progenitor cells in the thymus and bone marrow and is later important in both T-cell and Natural Killer (NK) cell activation (Kishimoto, 1998). The molecular form of IL-6 responsible for T-cell activation is released by monocytes. It augments the early events of activation, especially in CD3 T-cell receptor-mediated proliferation (Firestein, 1995). IL-6 also functions as the required second signal in both antigen- or mitogen-activated T-cells (Carter *et al*., 2002). This protein holds a very important role in the life of NK cells. It is first an activator and later stimulates them to perform a more effective lysis of a

pathogen. IL-6 provides support for continued development throughout the life of a natural killer cell (Riddell *et al.*, 2002).

Lyson and McCann (1999), states that uncontrolled or defective IL-6 has severe effects on the immune system, including a major decrease in the acute phase immune reaction and in the production of IgA antibodies. The over expression of the IL-6 gene can lead to the substantial polyclonal proliferation of plasma cells. Lack of regulation of the gene can lead to autoimmune disease and many lymphoid malignancies, including multiple myeloma (Kishimoto, 1998). An uncontrolled or defective production of this protein most often leads to disease and is involved in the pathogenesis of many diseases and autoimmune disorders, such as liver autoimmune disease (Lyson *et al*., 1999).

Study Aims and Objectives

1. To evaluate the antibacterial efficacy of medicinal hemp plant extracts.
    a. To determine the antibacterial effects of indigenous *Sansevieria* species and exotic *Cannabis sativa* phytotherapy varieties;
    b. To assess which of the aqueous or organic solvent extracts of *Sansevieria* species and *Cannabis sativa* varieties, are better dosage forms of antibiotic phytotherapies.

2. To evaluate the immune-modulating effects of medicinal hemp plant extracts.
    a. To determine the effect of *Sansevieria* species and *Cannabis sativa* varieties on human immune cells;
    b. To assess the effect of *Sansevieria* species and *Cannabis sativa* varieties, as therapeutic and prophylactic remedies.

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Chapter Two

An assessment of medicinal hemp plant extracts

as natural antibiotic phytotherapies

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An assessment of medicinal hemp plant extracts as antibiotic phytotherapies

2.1 Abstract

The objective of this study was to evaluate the antibacterial effects of the indigenous hemp species of *Sansevieria,* in comparison to varieties of the exotic hemp plant, *Cannabis sativa*. These hemp phytotherapies have been widely used to combat infections. 50 % Methanol and aqueous extracts of *Sansevieria* species (8 mg/ml) and *C. sativa* cultivars (81 mg/ml) were prepared and tested for effectiveness. Susceptibility was tested *in vitro* by a disk diffusion assay on three microorganisms, which included *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 27853) and *Mycobacterium smegmatis.* Ciprofloxacin (5 μg/disk) served as a positive control, whereas aqueous and methanol disks (50 μl) served as negative controls. No inhibition was observed when bacteria were exposed to the aqueous extracts of indigenous and exotic hemp phytotherapies ($P \geq 0.05$). However, methanol extracts of both hemp varieties inhibited *S. aureus* and *M. smegmatis.* Methanolic extracts of *S. personii* showed significantly greater inhibition of these bacteria (3 - 5 mm), compared to the other indigenous hemp species ($P \leq 0.05$). Moreover, the methanolic extracts of the Uniko-B cultivar of *C. sativa* showed significantly greater inhibition of these bacteria (3 - 4 mm), compared to other exotic hemp extracts ($P \leq 0.05$). *S. personii* had the best minimum inhibitory concentration against *S. aureus* (1 mg/ml) and *M. smegmatis* (4 mg/ml) compared to all other indigenous and exotic medicinal hemp extracts. Furthermore, the indigenous and exotic hemp extracts did not inhibit the growth of the gram-negative bacteria, *P. aeruginosa.* Moreover, the stability experiments indicated that after one-year storage, the medicinal hemp extracts exhibited reduced, but continued natural antibiotic activity. The variation in inhibition efficacy could be due to the variation of active compounds within species or varieties of indigenous and exotic medicinal hemp extracts, which was observed using HPLC analysis. This proof-of-concept investigation indicates that the indigenous *S. personii* and the exotic Uniko-B variety of *C. sativa* are the most effective natural antibiotic phytotherapies tested in this study.

Key words: *Sansevieria, Cannabis sativa*, bacteria, disk diffusion, HPLC, natural antibiotic phytotherapies.

2.2 Introduction

The use of complementary and alternative medicine (CAM) has been on the increase extensively over the past 15 years (Romero, Chopin, Buck, Martinez, Garcia and Bixby, 2005). In many parts of the world, medicinal plants have been used for its antibacterial, antifungal and antiviral activities for hundreds of years (Barbour, 2004; Ali-Shtayh 1998; Yasunaka, 2005). In South Africa, many populations still utilize traditional medicine for their psychological and physical health requirements (Rabe and Van Staden, 1997). However, medicinal plant remedies have not been comprehensively investigated, and should be studied for its safety and efficacy (Fransworth and Soejarto, 1991; Eloff, 1998). To this end, numerous assays are available to evaluate the safety and efficacy of promising natural products, firstly *in vitro* and later *in vivo* (Fennell, Lindsey, McGraw, Sparg, Stafford, Elgorashi, Grace and Van Staden, 2004).

The indiscriminate use of antibiotics has resulted in many bacterial pathogens rapidly becoming resistant to a number of the originally discovered antimicrobial drugs (Barbour *et al*., 2004). The antimicrobial compounds found in plants may prevent bacterial infections by different mechanisms than the commercial antibiotics and therefore may have clinical value in treating resistant microorganism strains (Eloff, 1999). There is, thus, a continuous search for new antibiotics, and medicinal plants may offer a new source of antibacterial agents.

Combinations of two or more antibiotics with different mechanisms of action are sometimes tested in an attempt to improve efficacy through synergy and prevent the development of antibiotic resistance (Beringer, 1999). Many potential antimicrobial

compounds are not singularly effective, and in combination with another compound, could possibly improve antimicrobial efficacy through synergism. Moreover, the increased cost of new and more effective antimicrobial remedies (Salie, Eagles and Leng, 1996) together with their side effects and lack of healthcare facilities in some rural areas (Griggs, Manandhar, Towers and Taylor, 2001), makes the search for safer, more effective and affordable alternative remedies imperative.

The majority of medicinal plant consumers purchase herbal material from markets. Stafford, Jager and Van Staden (2004), state that approximately 450 plant species are sold in large volumes from herbal trade markets. These markets generally have poor physical conditions and infrastructures with a lack of storage facilities resulting in spoilage of plant material (Stafford *et al*., 2004; Mander, 1997). These poor conditions lead to wastage and a decrease in the quality of plant materials. Fennell *et al*. (2004), state that Africa is fairly behind with regard to the control and development of the medicinal plant industry, and researchers are, thus, focussing on several aspects needed for the development of the medicinal plant trade in Africa.

Members of the *Sansevieria* genus are commonly referred to as bowstring hemp and piles root in English and ‘aambeiwortel’ or ‘skoonma-se-tong’ in Afrikaans (Van Wyk and Wink, 2002). *Sanseveria* has undergone many changes with regard to classification. Initially, this genera was classified as part of the Liliaceae Family (Watt and Breyer-Brandwijk, 1962), whereas later documentation categorised it under the Agavaceae Family (Mimaki, Inoue, Kuroda and Sashida 1996). The most recent classifications state that *Sansevieria* forms part of the Dracanaceae Family (Germishuizen and Meyer, 2003). These plants are popular garden and indoor plants

due to their ability to flourish under low light conditions and little growth attention (Van Wyk *et al*., 2002).

The medicinal uses of *Sansevieria* species include treatment for abdominal pains, earache, diarrhoea and haemorrhoids (Watt *et al*., 1962). Traditionally, in treating earaches and haemorrhoids, the leaves are heated and the warm juice is squeezed onto the affected area. *Sansevieria* plays a major role in the fibre industries as it is a source of leaf fibre for making strong cords. According to Van Wyk *et al*. (2002), *Sansevieria* species consists of many sapogenins, of which ruscogenin is the most common, and is commercially used as an anti-inflammatory agent and venotonic.

Cannabis sativa, also commonly known as fibre hemp, is amongst the medicinal plants that have been domesticated since the early history of mankind and recognised as a valuable therapeutic agent (Van Wyk *et al*., 2002). There are two variations of *C. sativa*: one contains low levels of Δ^9-THC (tetrahydrocannabinol), which is the psychoactive compound, and high levels of cannabidiol, whereas the other has low concentrations of cannabidiol and high concentrations of Δ^9-THC (Gambaro, Dell'Acqua, Faré, Rino, Froldi, Saligra and Tassoni, 2002). The first-mentioned is mainly used in the production of fibre, whereas the latter is used for its euphoric effects. For this study, however, the focus is on the fibre hemp crops, which consists of minimal psychoactive compounds.

Folk uses of *C. sativa* include insomnia, inflammation, glaucoma, rheumatism, asthma, menstrual cramping and appetite disorders amongst others (Clarke and Pate, 1994). *C. sativa* or fibre hemp is grown for a multitude of end-products derived from

the cannabinoids, seed, fibre and wooden core (Struik *et al*., 2000). The disadvantage of *C. sativa* is that it is associated with the use of illegal narcotics and thus classified as illegal in many countries.

The objective of this study was to screen for the presence of natural antibiotic activities of three indigenous *Sansevieria* species compared to five exotic *Cannabis sativa* varieties. The three *Sansevieria* species included *S. aethiopica, S. hyacinthoides* and *S. personii.* The five *Cannabis* species included two French, one Yugoslavian, one Hungarian and one Uniko-B variety.

Study Aims and Objectives

1. To evaluate the antimicrobial efficacy of medicinal hemp plant extracts:
    a. To determine the antibacterial effects of indigenous *Sansevieria* species and exotic *Cannabis sativa* phytotherapy varieties.
    b. To assess whether aqueous or organic solvent extracts of *Sansevieria* species and *Cannabis sativa* varieties, are better dosage forms of antibiotic phytotherapies.

2.3 Materials and Methods

2.3.1 Plant Material Collection

The *Sansevieria aethiopica, S. personii* and *S. hyacinthoides* were obtained from the South African National Botanical Institute (SANBI) at Kirstenbosch Gardens, Cape Town. Five cultivars of *Cannabis sativa* was collected from the Elsenburg Agricultural Research Centre (Stellenbosch) which include two French cultivars (F33 and F77), one Yugoslavian, one Hungarian cultivar and one Uniko-B cultivar. Plant specimens were identified by botanical experts. Voucher specimens were deposited in the Department of Botany's Herbarium at the University of the Western Cape.

2.3.2 Plant Extraction

2.3.2.1 Methanol extract

Leaves of the indigenous and exotic hemp species were washed with distilled water to remove excess soil. The freshly collected leaves were finely grounded and soaked in methanol overnight. The crude extracts were filtered with Whatman No.1 filter paper and thereafter, concentrated by means of a rotary evaporator (Buchi Rotavapor R-200) at a temperature of 40 °C to allow optimal separation of methanol and active compounds. This process was repeated four times to ensure maximum yield of extracted compounds. Since the fresh plant material contain water, the extracts were further concentrated to dryness by means of a freeze-dryer to extract any excess water from the sample. The obtained samples of *Sansevieria* and *Cannabis* species were stored in a 4 °C cooler room and redissolved in methanol to desired concentrations (8 mg/ml and 81 mg/ml stock solutions respectively) for future use. The stock solutions are both equivalent to 50 %.

2.3.2.2 Aqueous extract

The plant material was washed of excess soil with distilled water. The leaf material was finely grounded followed by an infusion in hot (95 ˚C) distilled water. The sample was left overnight under refrigeration (4 ˚C) to prevent any possible contamination of microorganisms. The crude extracts were filtered with Whatman No.1 filter paper and subsequently frozen at –70 ˚C. The frozen extracts of *Sansevieria* and *Cannabis* species were then freeze-dried to a powder, which was redissolved in distilled water to desired concentrations (8 mg/ml and 81 mg/ml stock solutions respectively) for experiments. These stock solutions are both equivalent to 50 %. The remaining unused plant material was dried at 40˚C and stored in brown paper bags at room temperature for future use in stability experiments.

2.3.3 Microorganisms and growth media

The microorganism strains tested include *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 27853) and *Mycobacterium smegmatis*. The cultures were provided by the Department of Medical Biosciences at the University of the Western Cape, South Africa. *M. smegmatis*, obtained from Tygerberg Medical School, was used instead of *M. tuberculosis* as the latter is highly pathogenic and air-borne. The cultures were inoculated in a nutrient agar broth medium and streaked by means of the sterilised swab method onto agar plates that were stored in a cold room at 4 ºC. The microorganisms were cultured on nutrient agar (Biolab, Merck) plates, except for *M. smegmatis* that was cultured on Mycobacteria 7H11 (Difco, Becton Dickinson) agar plates.

2.3.4 Antimicrobial analysis – disk diffusion assay

The agar plate disk diffusion assay was employed in triplicate for the determination of *in vitro* antimicrobial activities of the 50 % aqueous and methanolic hemp plant extracts. The following bacteria were tested: *Staphylococcus aureus, Pseudomonas aeruginosa* and *Mycobacterium smegmatis*. The base plates were prepared by pouring agar into sterile petri dishes (9 cm) which was allowed to set. Molten nutrient agar broth (Biolab, Merck) held at 4 °C was inoculated with the test organism and transferred onto the agar base plates, using the sterile swab technique, forming a homogenous top layer. Pure colonies of each of the different test organism were sub-cultured regularly to ensure fresh microorganism cultures.

Non-sterile filter paper discs (9 mm; Schleicher and Schuell) were autoclaved at 120 °C in a Speedy Autoclave (Lasec, South Africa) for 20 minutes and subsequently impregnated with 50 µl methanol and aqueous extracts respectively. After the application, the methanol disks were allowed to dry for 30 minutes, whereas the aqueous disks were left to dry overnight, due to water evaporating at a slower rate than methanol. 50 µl Methanol and distilled water disks served as negative controls. Ciprofloxacin (5 µg/disk) antibiotic disks (Mastdiscs, Mast Diagnostics, UK) were used as positive controls. Each plate consisted of the following disks: three plant extracts, one negative and one positive control disk, which were placed at equidistance on labelled inoculated agar plates. The inverted agar plates were incubated at 37 °C for 24 hours except for *M. smegmatis* that requires a growth period of 48 hours. The plates were evaluated after each incubation period by measuring the diameter (mm) of the clear, growth-free zones around the disks to estimate the inhibition efficacy.

2.3.5 Minimum inhibitory concentrations

The minimum inhibitory concentrations (MIC) were determined by preparing serial dilutions of the methanolic extracts of indigenous and exotic hemp phytotherapies. The MIC values were defined as the concentration that inhibited bacterial growth at 1 mm, since this is the smallest inhibitory zone that can be detected *in vitro* by the disk diffusion assay method.

2.3.6 High Performance Liquid Chromatography (HPLC)

The methanol extracts were filtered through a 0.45 µm syringe prior to analysis. The spectra were generated on a Beckman System Gold HPLC, consisting of a double pump Programmable Solvent Module 126, Diode Array Detector Module model 168, with 32 Karat Gold software, supplied by Beckman; Column C_{18} Bondapak 5µm and dimensions 250mm x 4.6 mm (Luna-Separations). Ambient temperature was maintained at 23 °C.

Mobile phase, solvent A: methanol (MeOH), solvent B: 0.1 % acetic acid (CH3COOH); **Mode**: gradient, increasing the organic phase MeOH from 20 – 90% over 18 minutes; flow rate: 1 ml/min; reference standard: rutin (2.5 g dissolved in 10 ml MeOH); **Sample**: 50% Methanol extracts of *Sansevieria* and *Cannabis sativa* varieties; **Injected volume**: 20 µl. The run time was 25 minutes. The wavelength scanning range was 200 – 600nm at 2nm/step.

2.3.7 Statistical analysis

The inhibition zones were statistically analysed by conducting an unpaired *t*-test using the MedCalc (version 7.1, 2000) statistical programme. Means ± SEM (standard error of the mean) were considered significant at $P < 0.05$.

2.4 Results

Antibacterial analysis of three *Sansevieria* species and five *Cannabis sativa* varieties;

Sansevieria species:
1. *S. hyacinthoides*
2. *S. personii*
3. *S. aethiopica*

Cannabis sativa varieties: (as referenced in results)
1 - Yugoslavian (Novasadska)
2 - French (F77)
3 - French (F33)
4 - Hungarian (Kompolti)
5 – Uniko-B

Table 1: Shows the equivalence between plant % and mg/ml for *Sansevieria* and *Cannabis* species.

	Sansevieria spp.	*Cannabis* spp.
%	**mg/ml**	**mg/ml**
50	8	81
25	4	41
12.5	2	20
6.25	1	10

2.4.1 Minimum Inhibitory Concentrations

Table 2: The minimum inhibitory concentrations (MIC) of methanolic extracts of *Sansevieria* species and *Cannabis sativa* cultivars.

Hemp extract	Extract concentrations (mg/ml)		Zone of inhibition (mm)
	S. aureus	*M. smegmatis*	
<u>*Sansevieria* species</u>			
S. hyacinthoides	8	8	1
S. personii	1	4	1
S. aethiopica	2	8	1
<u>*Cannabis* varieties</u>			
Yogoslavian (1)	41	81	1
French F77 (2)	41	81	1
French F33 (3)	20	81	1
Hungarian (4)	41	41	1
Uniko B (5)	10	20	1

The MIC value is defined as the lowest concentration of an extract, which visibly inhibits the growth of the micro-organism after incubation. *S. personii* had the lowest minimum inhibitory concentration against *S. aureus* and *M. smegmatis* compared to the other hemp phytotherapies.

2.4.2 Antibacterial analyses against *Staphylococcus aureus*

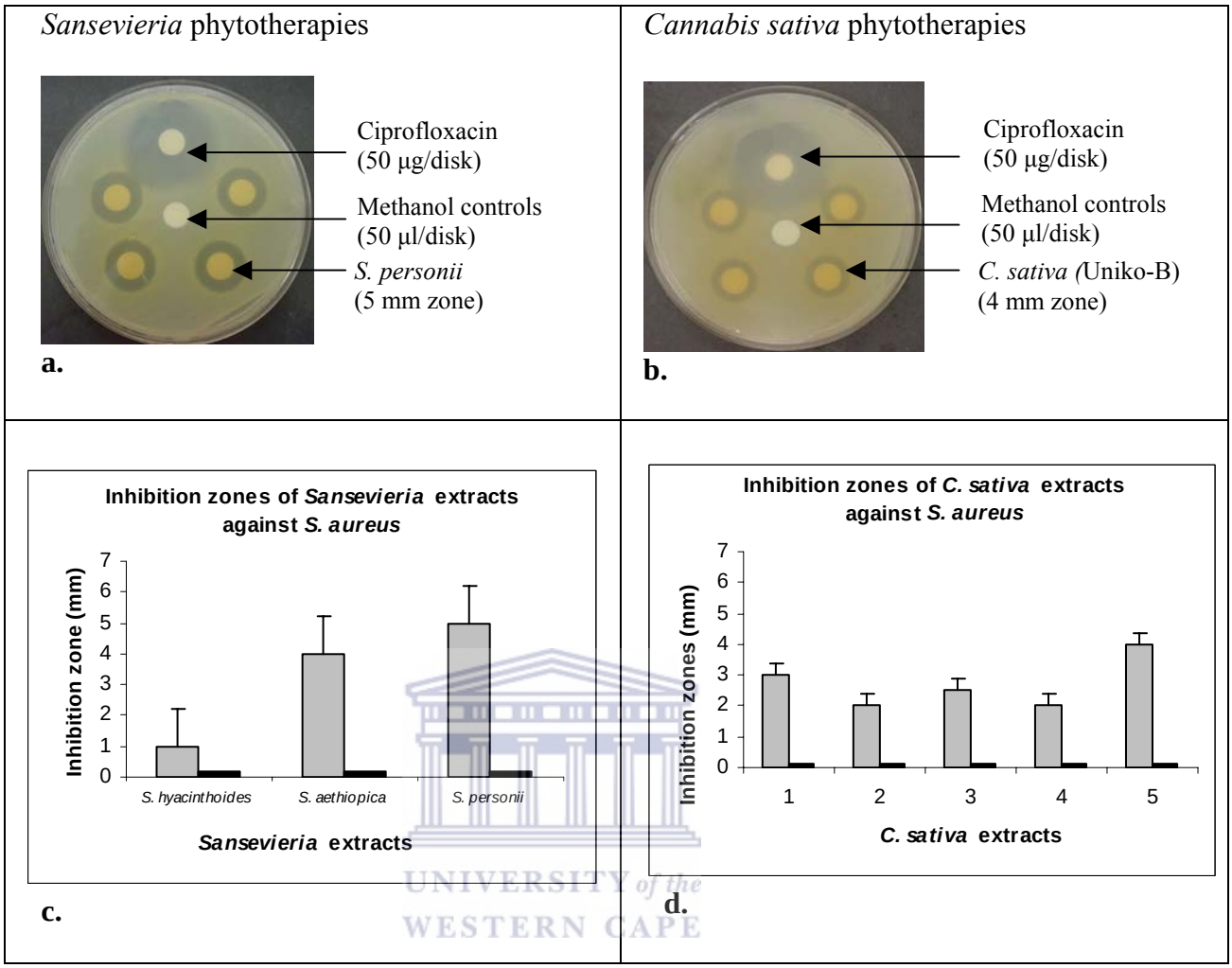


Figure 1a-d: The effects of 50 % aqueous and methanol medicinal hemp phytotherapies on *S. aureus*:

Key: ■ = aqueous; □ = methanol

a: *S. aureus* plate with 50 % *S. personii* extracts (50 % extract = 8 mg/ml);
b: *S. aureus* plate with 50 % *C. sativa* Uniko-B extracts (50 % extract = 81mg/ml);
c: Inhibition efficacy of *Sansevieria* species against *S. aureus*;
d: Inhibition efficacy of *C. sativa* cultivars against *S. aureus.*

Inhibition zones (9 mm) were observed in the positive control, Ciprofloxacin (50µg/disk). The methanol negative control disks showed no inhibition against *S. aureus* and thus did not affect the pathogen as an extract solvent. *S. personii* (5 mm) and Uniko-B cultivar (4 mm) of *C. sativa* showed the most effective inhibition ($P \leq 0.05$) against *S. aureus*.

2.4.3 Antibacterial analyses against *Mycobacterium smegmatis*

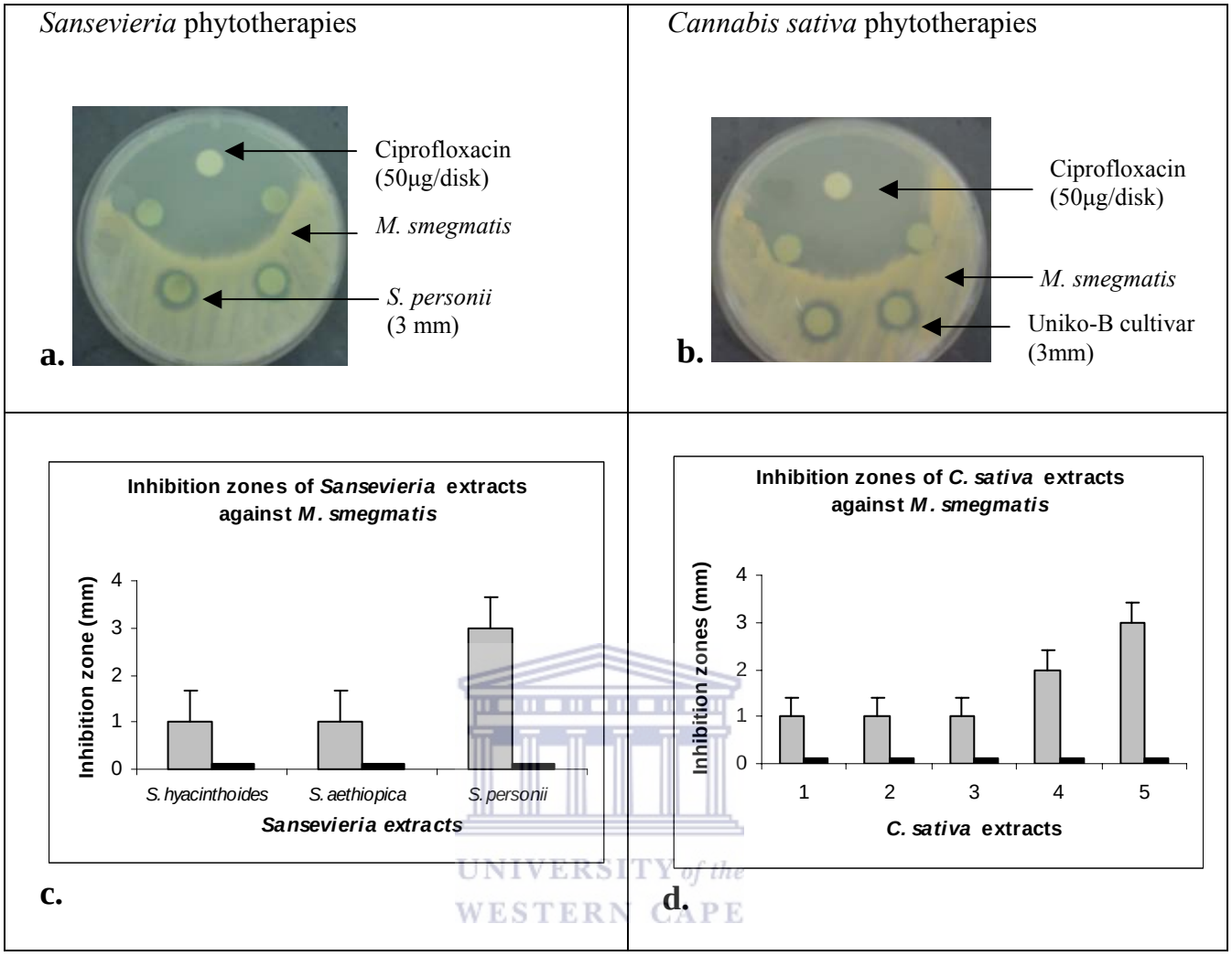


Figure 2a-d: The effects of 50 % aqueous and methanol medicinal hemp phytotherapies on *M. smegmatis*:

Key: ■ = aqueous; □ = methanol

a: *M. smegmatis* plate with *S. personii* extracts (50 % extract = 8 mg/ml);
b: *M. smegmatis* plate with *C. sativa* Uniko-B extracts (50 % extract = 81 mg/ml);
c: Inhibition efficacy of *Sansevieria* species against *M. smegmatis*;
d: Inhibition efficacy of *C. sativa* varieties against *M. smegmatis.*

Ciprofloxacin (50 µg/disk) showed high efficacy against *M. smegmatis* (3 cm). Ciprofloxacin exhibited significantly greater efficacy ($P \leq 0.05$) compared to *Sansevieria* and *Cannabis* species. No inhibition was observed in the methanol negative control disks. *S. personii* (3 mm) and the Uniko-B variety of *C. sativa* (3 mm) showed the most effective inhibition against *M. smegmatis*, compared to the other medicinal hemp extracts ($P \leq 0.05$).

2.4.4 Stability experiments

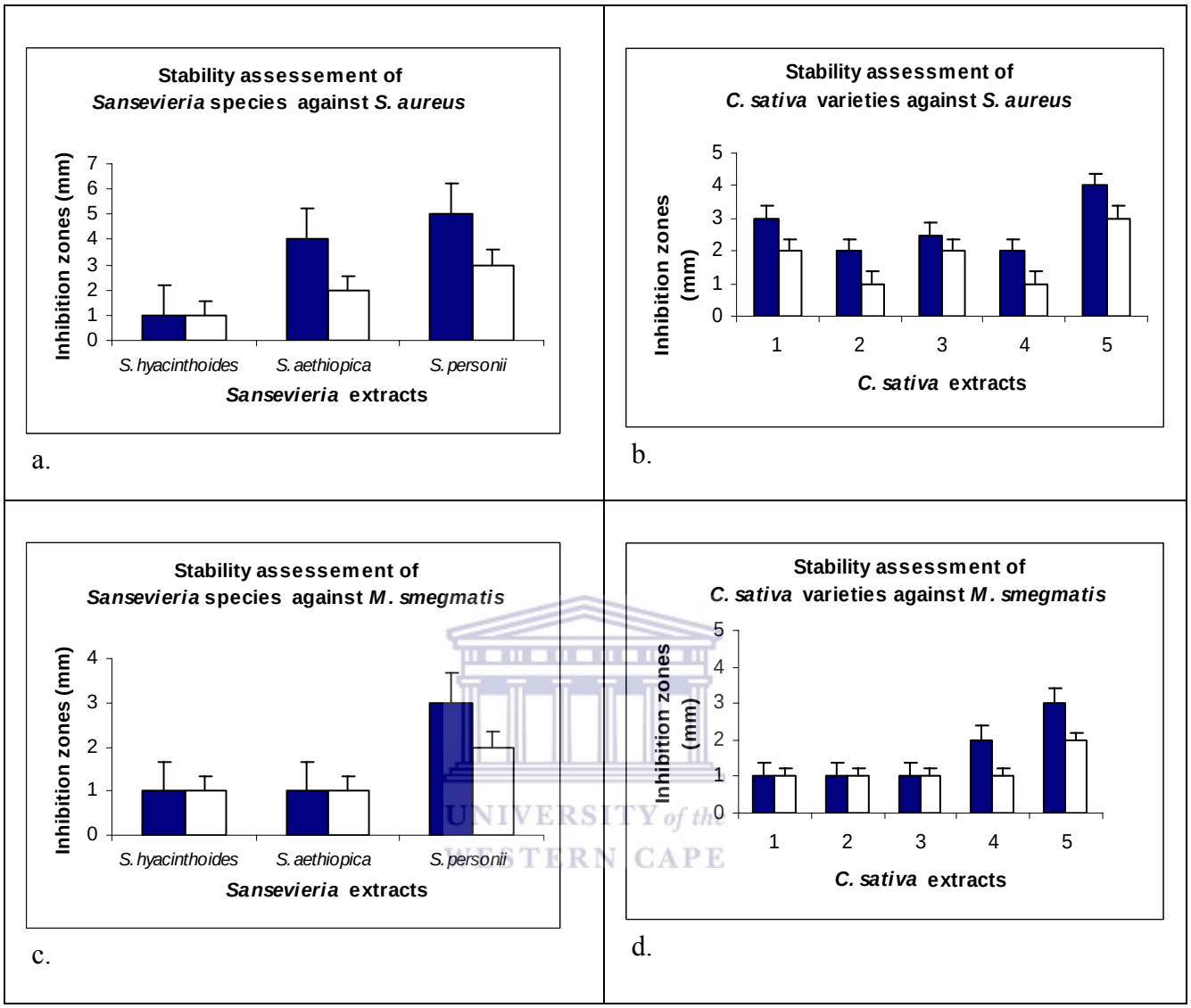


Fig. 3 a-d: The original 50 % methanol hemp extract efficacy compared to the effects of the hemp phytotherapies post one year storage.

Key: ■ = original efficacy; □ = efficacy post one year storage

a. *S. personii* against *S. aureus*;

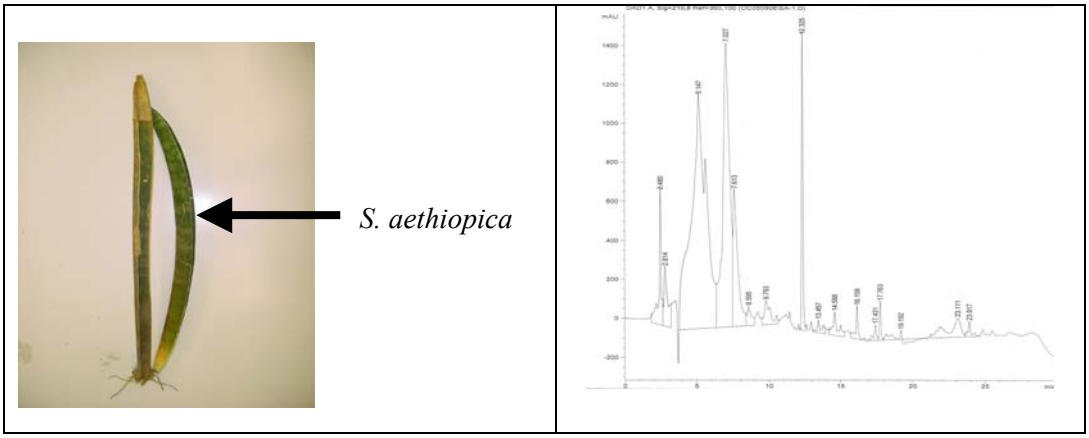
b. *C. sativa* against *S. aureus*;

c. *S. personii* against *M. smegmatis;*

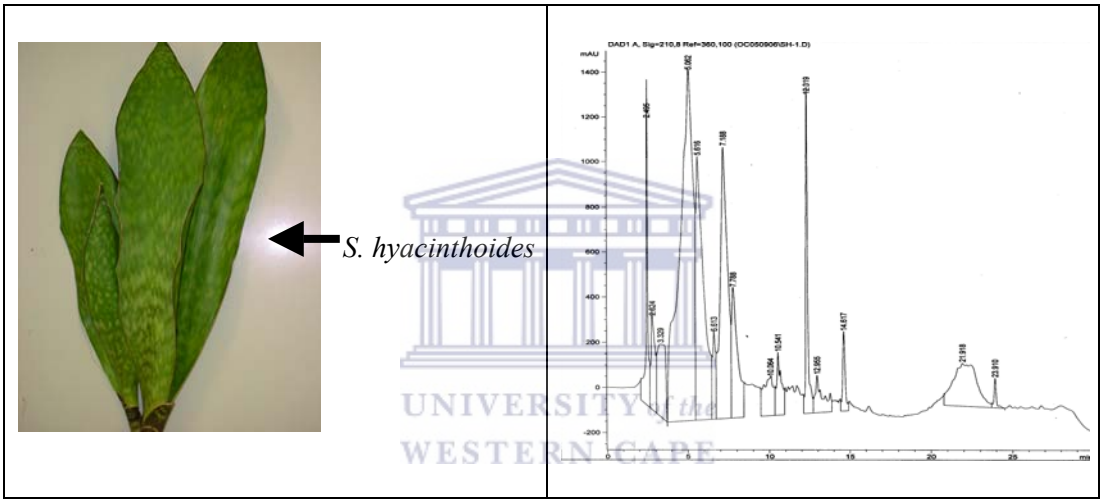
d. *C. sativa* against *M. smegmatis.*

The stability tests were done before and after the *Sansevieria* and *Cannabis* varieties were stored for one year. Figure 3 a-d shows that the various medicinal hemp phytotherapies inhibited growth after the storage period.

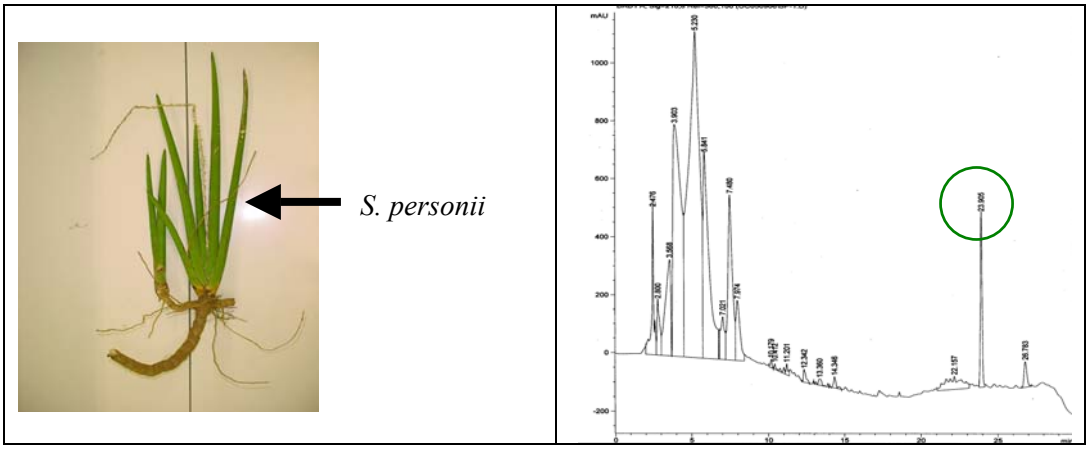
2.4.5 HPLC Results



a.



b.



c.

Figure 4 a – c: HPLC chromatograms of: a - *S. aethiopica*, b – *S. hyacinthoides* and c – *S. personii*

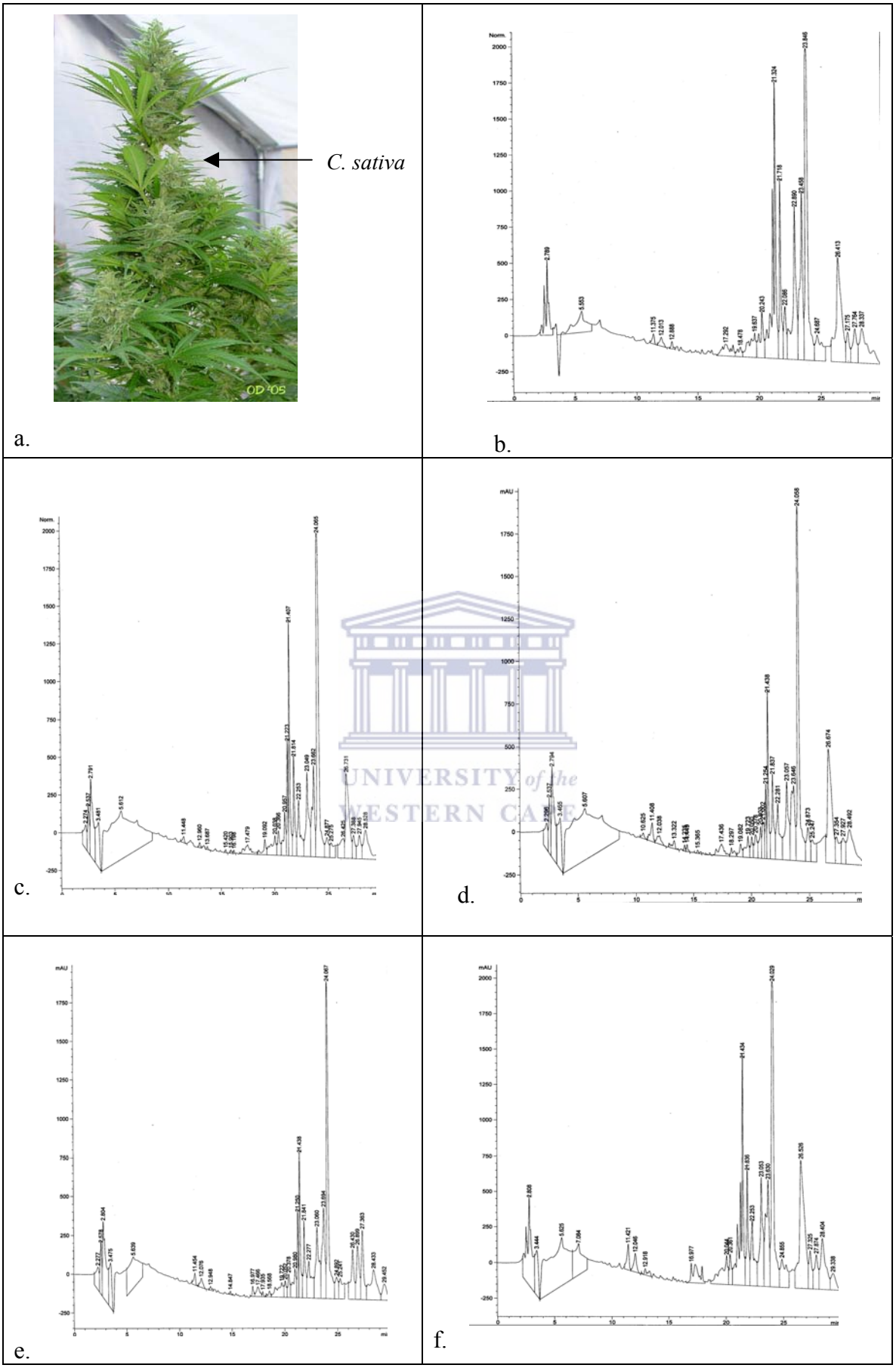


Fig. 5 a – f: HPLC chromatograms of *Cannabis sativa* varieties; a – Image, b - C1, C - C2, d - C3, e – C4, f – C5.

2.5 Discussion

In the constant effort to improve the efficacy and ethics of modern medical practice, researchers are increasingly focussing on folk medicine as a source of new drugs (Haslam, Lilley, YaChai, Martin and Magnolato, 1999). The development of resistance by a pathogen to many of the commonly used antibiotics provides a reason for further attempts to search for new antimicrobial agents to combat infections and overcome the problems of resistance and side effects of the currently available antimicrobial agents (Essawi, 1999). Hence, this *in vitro* study aimed at screening the antibacterial activities of varieties of indigenous *Sansevieria* species and exotic *Cannabis sativa* extracts, and determining whether their use in folkloric medicine is justified.

Aqueous extracts of medicinal hemp varieties had no effect on inhibiting pathogen development in this *in vitro* study. This is in keeping with previous studies that suggest this might have resulted from the lack of solubility of the active constituents in aqueous solutions, or perhaps, the hot water denatured such constituents (Eloff, 1998). However, this investigation showed that methanolic extracts of medicinal hemp species have variable antibacterial value (Fig. 1 and 2). Methanol is understood to extracts more compounds from plant fractions compared to aqueous extracts (Eloff, 1998). Methanol extracts from plants that are typically used as anti-infectives, typically inhibits the growth of gram-positive bacteria more readily compared to its efficacy against gram-negative bacteria (Ali-Shtayeh *et al*., 1998). This pattern is supported by the findings of this study.

All three methanolic extracts of *Sansevieria* species showed antibacterial activity against *S. aureus* and *M. smegamatis,* with the exception against *Pseudomonas aeruginosa*. The lack of efficacy against *P. aeruginosa* might reflect the absence of antibacterial compounds in the extracts to combat these specific microorganisms, or the inability of the stereo-specific chemical actives to inhibit gram-negative bacteria. Gram-negative bacteria consist of a multi-layered cell wall, bounded by an outer membrane that is composed of high-density lipopolysaccharides that serves as a barrier to many environmental substances including antibiotics (Palambo, 2001), whereas the cell wall of gram-positive bacteria consists of a single layer (Ali-Shtayeh *et al*., 1998).

S. personii was the most active antibacterial indigenous hemp species ($P \leq 0.05$), whereas the overall least active plant was *S. hyacinthoides* (Fig. 1 and 2). *S. hyacinthoides* showed constant zones of 1 mm, whereas the *S. aethiopica* showed inhibition ranging from 1 mm to 4 mm. All *Cannabis sativa* cultivars showed activity against S. *aureus* and *M. smegmatis* (Fig. 1 and 2). The Uniko-B cultivar produced statistically greater inhibitions ($P \leq 0.05$) against *S. aureus* (4 mm) and *M. smegmatis* (3 mm) (Fig. 1, 2), compared to the other cultivars. The French (F77) and Hungarian (Kompolti) cultivars both produced average inhibition of 2 mm (Figure 1) against *S. aureus*. The French (F77) cultivar inhibited growth ranging from 1-2 mm, whereas the Kompolti cultivar produced constant inhibition of 2 mm (Figure 1, 2). The F33 French cultivar produced constant inhibition of 2.5 mm against *S. aureus* (Figure 1).

Commercial antibiotics appeared to be effective in limiting the growth of these pathogens. Although the positive control, Ciprofloxacin, showed a significantly greater (P=0.01) inhibition to both *S. aureus* (3 cm) and *M. smegmatis* (9 cm) than the hemp extracts, it should be noted that this pharmaceutical drug is purified, whereas the hemp extracts, may contain compounds that masks the potency of the phytotherapies (Eloff, 1998). Moreover, the methanol negative control disks showed no inhibition against the pathogens and thus did not affect the experiment as an extract solvent.

The minimum inhibitory concentration (MIC) values refer to the lowest concentration of the extracts, which inhibits the growth of the micro-organisms. The *Sansevieria* extracts had lower MIC values compared to the exotic C*annabis sativa* cultivars. Moreover, *S. personii* had the best overall MIC against *S. aureus* and *M. smegmatis* compared to all other indigenous and exotic medicinal hemp extracts (Table 2).

The results of the antibacterial stability tests show minimal variation (Figure 3a, b). The active ingredients in *Sansevieria* and *Cannabis* species appear to be stable and thus, storing this plant in a dry form in the dark at room temperature should be sufficient to ensure little or no change in biological activity. Silva *et al*. (1998) demonstrated that in some instances ultraviolet radiation might produce chemical reactions that give rise to compound artefacts. Correct storage methods are important to ensure the viability of plant materials after long storage periods. Some plants used for their antibacterial properties seem not to be adversely affected by short-term storage, and in fact, may improve with age (Stafford, 2004).

The HPLC chromatograms (Fig. 4 and 5) show that there is some variation within the chemical structures of the species, which could be linked to the variation in inhibition. The variations in retention times of the compounds in the various extracts indicate that different compounds are present in the different extracts of the hemp phytotherapies. Within the *Sansevieria* species, we note according to the antibacterial results, that *S. personii* is the most effective of the three species (Figures 1 and 2). This finding is all the more interesting, given that the HPLC fingerprint of *S. personii* (Figure 4c) shows a peak at 23.905 min., which is not present in the chromatograms of *S. aethiopica* (Fig. 4a) and *S. hyacinthoides (*Fig. 4b). The active compound presumed to be responsible could be ruscogenin, the saponin present in *Sansevieria* species (Van Wyk, 2002). Further chemical analysis should be done to identify the specific chemical compounds within these species.

There is no notable variation between the HPLC fingerprints of the *Cannabis sativa* cultivars (Fig. 5). The variation between the cultivars that caused the difference in the antibacterial effects might not be explained by the HPLC analysis alone, but further analytical systems could be used in future experiments for improved understanding. These technologies may well include preparative TLC, SPE-NMR and MS-MS.

In conclusion, the indigenous and exotic hemp varieties investigated, exhibited selective antibacterial activity to varying degrees. Furthermore, this study showed that methanolic extracts showed to be more effective than aqueous extracts. More specifically, the indigenous variety of *S. personii* and the exotic Uniko-B cultivar of *C. sativa*, were the most efficacious phytotherapies. Moreover, *S. personii* was the most powerful herbal antibiotic of all the medicinal hemp varieties given its excellent

MIC profile and unique chemical fingerprint. These phytotherapies may, therefore, provide new leads in the ongoing search for novel antibacterial drugs.

2.6 References

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Chapter Three

An assessment of medicinal hemp plant extracts

as natural immune modulation phytotherapies

Chapter 3

An assessment of medicinal hemp plant extracts as immune modulation phytotherapies

3.1 Abstract

The objective of this study was to investigate the immune modulation effects of the indigenous *Sansevieria personii* and the exotic Uniko-B cultivar of *Cannabis sativa,* as the best performing natural antibiotics identified from our first study. IL-6 was used as a biomarker. Serially diluted hot and cold 200 % extracts of *S. personii* (0.03 mg/ml – 32 mg/ml) and *C. sativa* (0.30 mg/ml – 325 mg/ml) were initially tested to evaluate the impact of various doses of the phytotherapies on human cells. Cell lysis occurred at concentrations of 32 and 16 mg/ml of *S. personii* and at 325 mg/ml of *C. sativa*, indicating that these high concentrations are unfavorable to the cells. An *in vitro* assay was performed in which whole blood cultured cells were treated with different concentrations of plant extracts and endotoxin, which served as an inflammatory stimulant. Six healthy volunteers, consisting of one male and five females ranging between 20-30 years, were selected as blood donors for this study, which received ethical approval from the University of the Western Cape. The outcomes of unstimulated, therapeutic and prophylactic experiments were analyzed using colorimetric ELISA technology. The results of this study revealed that both *S. personii* and *C. sativa* phytotherapies modulate IL-6 production even in the absence of a stimulant. Hot *S. personii* extracts produced a significant anti-inflammatory effect at 0.3 mg/ml ($P \leq 0.03$); 0.5 mg/ml ($P \leq 0.03$) and 2 mg/ml ($P \leq 0.03$), whilst the same effect for *C. sativa* was only observed at 0.1 mg/ml ($P \leq 0.03$). On the other hand, hot *S. personii* extracts were pro-inflammatory at 1 mg/ml ($P \leq 0.05$), whilst *C. sativa* induced similar effects at 1.1 mg/ml ($P \leq 0.05$) and 5 mg/ml ($P \leq 0.03$). In unstimulated cells, hot extracts of *S. personii* are mostly anti-inflammatory, compared to cold extracts, which are pro-inflammatory. This is different from *C. sativa*, which is largely pro-inflammatory as a hot or cold herbal remedy. Moreover, *S. personii* and *C. sativa* extracts are mostly significant anti-inflammatory agents when used therapeutically. Similarly, hot extracts of *S. personii* and *C. sativa* are anti-inflammatory prophylactic remedies, whilst cold extracts are pro-inflammatory in nature.

Key words: *Sansevieria personii*, immune-modulation*, Cannabis sativa,* IL-6, ELISA

3.2 Introduction

Herbal-based treatments have become an essential constituent of standard healthcare, based on a combination of traditional usage and ongoing scientific research (Tiwari, Rastogi, Singh, Saraf and Vyas, 2004). The modulation of immune response with the aid of various bioactives in order to alleviate certain diseases is an active area of interest. Apart from being specifically stimulatory or suppressive, certain agents have been shown to possess activity to normalize or modulate pathophysiological processes and are referred to as immune-modulatory agents (Wagner, 1983).

An inflammatory cascade involves macrophages and neutrophils, which secrete a range of mediators including cytokines, which are responsible for the initiation, progression and persistence of acute or chronic state of inflammation (Lefkowitz, Gelderman, Fuhrmann, Graham, Starnes, Lefkowitz, Bollen and Moguilevsky, 1999). The activation of macrophages is a fundamental event in the innate immunity for the initiation and propagation of defensive reactions against pathogens. Macrophages release interleukin (IL)-1, IL-6 and other inflammatory mediators when stimulated by pathological stimuli or injury (Wu, Weng, Wang and Lian, 2004).

In this investigation, we assessed the immune modulatory activity of *S. personii* and *Cannabis sativa* Uniko-B cultivar that showed the most potent effects as natural antibiotics in an earlier part of this study, using IL-6 as a biomarker. IL-6 is a cytokine involved in inflammation, which acts as a B-cell growth factor, thereby promoting antibody formation and release (Barton, 1996). These hemp phytotherapies are traditionally used to combat infections and inflammatory conditions.

S. personii is commonly known as mother-in-laws tongue, piles root, aambeiwortel and also bowstring hemp. *Sansevieria* is traditionally used in treatments of ear infection, toothache, hemorrhoids, ulcers and intestinal worms (Van Wyk and Wink, 2002). In the treatment of earache and hemorrhoids, the leaves are heated and the juices are squeezed onto the affected areas. For internal treatments the heated leaves are chewed and the juices swallowed. Sapogenins are the main active compound in the *Sansevieria* species, of which ruscogenin is the most popular and is commercially available as an anti-inflammatory treatment and venotonic (Van Wyk *et al*., 2004).

Cannabis sativa was chosen as the exotic hemp species to compare to the indigenous *Sansevieria* species. Van Wyk, Van Oudtshoorn and Gericke (2000), state that *C. sativa* is indigenous to temperate Asia and has been domesticated since the early history of humankind. *C. sativa* or fiber hemp is cultivated for a variety of end products ranging from cannabinoids, seed oil, fiber and wooden core (Struik, Amaducci, Bullard, Stutterhaim, Venturi and Cromack, 2000). Traditional medicinal uses of *C. sativa* include asthma, depression, snakebite, glaucoma, appetite stimulation and numerous other conditions (Van Wyk and Gericke 2000).

Study aims and objectives

This proof-of-concept study was to determine the efficacy of *S. personii* and *C. sativa* (Uniko-B cultivar) as inflammatory response modulators, using IL-6 as a biomarker. The aim was to evaluate the immune-modulating effect of medicinal hemp plant extracts, by investigating:

a. The effect of *Sansevieria* species and *C. sativa* varieties on human immune cells.
b. The effect of *Sansevieria* species and *C. sativa* varieties, as therapeutic and prophylactic remedies.

3.3 Materials and Methods

3.3.1 Plant Material Collection

The S*ansevieria personii* and the Uniko-B cultivar of *Cannabis sativa* showed most effective results as antimicrobial agents compared to the other species and were subsequently chosen as candidates for the immunomodulatory experiments. *Sansevieria personii* was obtained from the South African National Botanical Institute (SANBI) at Kirstenbosch Gardens. *Cannabis sativa* (Uniko-B cultivar) was collected from the Elsenburg Agricultural Research Centre. Plant specimens were identified by a botanical expert. Voucher specimens were deposited in the Department of Botany, Herbarium, University of the Western Cape, Bellville, Cape Town.

3.3.2 Preparation of plant extracts

Aqueous extracts were prepared with pyrogen-free (SABAX) water (Adcock Ingram), as methanol is toxic to cells and thus, not a favourable solvent for the immunology experiments. The fresh plant material was washed with distilled water, followed by a second wash with pyrogen-free water. 50 g *S. personii* was chopped fine in a blender and 50 g *C. sativa* was grounded to a fine powder.

200% hot and cold pyrogen-free water extracts were made of both *S. personii* and *C. sativa* (Uniko-B cultivar), to establish the initial trend of IL-6 modulation. For the hot extracts, the desired amount of pyrogen-free water was placed in a beaker, which was subsequently submerged in a water-bath at 95°C for 10 minutes. The cold extracts were made with pyrogen-free water at room temperature of 22°C. The water was added to the plant material and infused overnight.

The extracts were filtered using Whatman filter paper no.1, and the clear filtrates were freeze dried to a powder over three days. The final powder extracts were then redissolved in pyrogen-free water to concentrations of 200%, which is equivalent to 32 mg/ml of *Sansevieria* and 325 mg/ml for *Cannabis* species (Table 1). Aliquots of each plant extract was stored in labeled sterile eppendorfs and kept in the freezer at -20°C, until analysis.

Table 1: Method of plant extraction for *S. personii* and *C. sativa*.

*50 g *S. personii* to 100 ml water (50%)	**50 g *C. sativa* to 200 ml water (25 %)
↓	↓
Filtered 40 ml of 50 %	Filtered 135 ml of 25 %
↓	↓
Freeze-dried to a powder (0.32g)	Freeze-dried to a powder (5.49g)
↓	↓
Redissolved in SABAX water to 200 % (added 10 ml water to 0.32g)	Redissolved in SABAX water to 200 % (added 16.87 ml to 5.49 g)
↓	↓
STOCK SOLUTION: 32 mg/ml = 200%	**STOCK SOLUTION: 325 mg/ml = 200%**

* *S. personii*: 100 ml of water was the minimum required to create a solution

** *C. sativa*: 200 ml of water was the minimum required to create a solution

3.3.3. Whole Blood Culture Assay

This study received ethical clearance from the university. Blood was collected from six healthy donors under good clinical practice, by means of venous puncture, in 7ml sodium heparin tubes at the UWC Campus Clinic. The exclusion factors included smoking, drug use, alcohol use and any known disease or infection at time of recruitment. The sodium heparin tubes were endotoxin tested. The donors consisted of one male and five females ranging between the ages of 20-30 years old. The blood was immediately processed in the tissue culture laboratory, where cell culture was undertaken under sterile conditions in the laminar flow. Only pyrogen-free tips and eppendorfs were used in this study. EVV Endotoxin (Charles River, ENDOSAFE) derived from *Escherichia coli* was used in these experiments to simulate an infection to cells. The stock solution was at 2 000 EU/ml (endotoxin units per milliliter), however 20 EU/ml was used in the experiments.

Blood was diluted to 1:9 with RPMI-1640 (Roswell Park Memorial Institute) medium and 180 µl was pipetted into sterile 96-well flat bottom plates (Corning Costar, Cambridge, MA). 20 µl of serially diluted filter sterilized (0.22 µm) extract, from 200 % to 0.02 %, was then added in duplicate to wells with blood, serving as stimulated cells. The plant extracts (20 µl) thus made up 10 % of the total well volume of 200 µl. Unstimulated wells containing 20 µl of only RPMI medium in duplicate served as the negative control. Wells prepared with 20 µl endotoxin (20 EU/ml) and 20 µl pyrogen-free water served as controls. The total volume per well was thus 200 µl. The blood culture plate was maintained in a Thermo humidified incubator at 37 °C in 5% CO_2 for 18 hours. The supernatant fluid was harvested after 18 hours as required for testing of

IL-6 production. The harvesting was done cautiously to prevent mixing with sedimented red blood cells.

The serially diluted concentrations for *Sansevieria* species (32.00 mg/ml – 0.03 mg/ml) and *C. sativa* varieties (325 mg/ml – 0.30 mg/ml) were used in the initial experiments to establish the trend of IL-6 modulation by the extracts. Four favorable concentrations of both hot and cold extracts were subsequently identified for the experiments done on volunteers. *S. personii* concentrations of 0.3 mg/ml, 0.5 mg/ml, 1 mg/ml, and 2 mg/ml were used for experiments done on volunteers whereas *C. sativa* concentrations of 0.1 mg/ml, 1.1 mg/ml, 3 mg/ml and 5 mg/ml showed the desired trend. The whole blood assays for the six volunteers focused on the following three experiments which included hot and cold extracts only, therapeutic and prophylactic evaluations:

Hot and cold plant extracts only – 180 µl of blood mixture and 20 µl hot and cold plant extracts were added to wells simultaneously and harvested after 18 hours. Control wells contained 180 µl blood mixture and 20 µl RPMI medium, thus unstimulated.

Therapeutic experiment – 10 µl hot and cold plant extracts distinctively and 10 µl endotoxin was added simultaneously to 180 µl of blood mixture. This was incubated for 18 hours and harvested. Control wells contained 180 µl blood mixture and 10 µl endotoxin (20 EU/ml).

Prophylactic experiment – 180 µl of blood mixture was added to wells, immediately followed by 10 µl of hot or cold plant extracts. This mixture was incubated for 24 hours. 10 µl of endotoxin was then added to all wells and incubated for an additional 18 hours, after which the supernatants were harvested. Control wells contained 180 µl blood mixture and 10 µl endotoxin (20 EU/ml).

3.3.4 Cytokine measurements – Enzyme-linked Immunosorbent Assay (ELISA)

Cytokine concentrations in the supernatant fluids were determined by ELISA kits from R&D Systems (Minneapolis, USA) consisting of capture and detection antibodies, IL-6 standard and streptavidin horseradish-peroxidase. A sandwich ELISA was completed to measure natural and recombinant human Interleukin-6 (IL-6) in the cell cultures. Assays were performed according to the manufacturer's instructions.

A 96-well plate (Nunc Maxisorb, Amersham) was coated with 50 µl of IL-6 capture antibody (mouse anti-human IL-6) diluted to a working concentration of 2 µg/ml in Phosphate Buffered Saline (PBS – 137 mM NaCl, 2.7 mM KCL, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and incubated overnight at room temperature. After incubation, the plate was washed thrice in PBS containing 0.05% Tween 20 (wash buffer), followed by the blocking of free antibody binding sites by adding 300 µl of blocking buffer (1% Bovine Serum Albumin (BSA) in PBS) to all wells.

After incubation of one hour the blocking buffer was discarded and the plate was washed thrice with wash buffer. 50 µl of serially diluted recombinant standard and 1:5 diluted plant extracts diluted in reagent diluent was added respectively in duplicate to wells and incubated for two hours. The plate was washed thrice after incubation, followed by adding 50 µl of detection antibody (goat anti-human IL-6) diluted to a working concentration of 200 ng/ml in reagent diluent, to wells and incubated for two hours. The detection antibody binds to the captured cytokine. After incubation, the unbound secondary antibody was washed thrice with wash buffer followed by adding 50 µl of streptavidin conjugated to horseradish-peroxidase. Incubation of 20 minutes under dark

conditions, was followed by washing the plates with wash buffer. 50 µl of substrate solution (R&D Systems, Minneapolis, USA) was added, which produced a blue colour as a result of peroxidase. 50 µl of stop solution (2 N H_2SO_4) was added after 20 minutes to stop the reaction. The colour changes from blue to yellow and the intensity of colour is proportional to the levels of IL-6 present in the samples. The optical density of each well was determined by a Titertek Multicsan microplate reader at 450nm.

3.3.5 Cytotoxicity assay

The CytoTox 96 Assay (Promega) was used to determine death of cell types in culture. Cell samples were lysed by adding 15 µl of Lysis 10X Solution (9% (v/v) Triton X-100 in water) per 100 µl of culture medium. This was then incubated at 37 °C for 45 minutes. 50 µl of the supernatants were then transferred to a fresh 96-well assay plate. 50 µl of Reconstituted Substrate mix was then added to the supernatants followed by dark incubation of 30 minutes at room temperature to allow the enzymatic reaction to proceed. 50 µl of Stop Solution is added to wells to stop the enzymatic assay. The plate was then read at 490 nm using a Titertek Multicsan microplate reader. The number of cells present is directly proportional to the absorbance values and was expressed in percentage.

3.3.7 Statistical analysis

IL-6 modulation by the hot and cold hemp extracts was statistically analysed by the Wilcoxon Signed Rank Test statistical package. The median values of the experiments were calculated using the Microsoft Excell package. A *P*-value equal to or less than 0.05 ($P \leq 0.05$) was regarded as significant.

3.4 Results

3.4.1 Phytotherapy extracts – Concentrations and Cytotoxicity

Table 2: Shows the equivalence between plant % and mg/ml for *Sansevieria* and *Cannabis* species.

	Sansevieria spp.	*Cannabis spp.*
%	**mg/ml**	**mg/ml**
200	32	325
100	16	163
50	8	81
25	4	41
12.5	2	20
6.25	1	10
3.12	0.50	5.00
1.56	0.30	3.00
0.78	0.13	1.10
0.39	0.06	0.10
0.195	0.03	0.30

A stock solution of 200 % was prepared of both plant extracts, which is equivalent to 32 mg/ml of *S. personii* and 325 mg/ml of *C. sativa* (Table 1). The variance in ratio is approximately ten-fold, which should be taken into account when interpreting the results.

Key: ☐ = Concentrations tested in this study.

Table 3: Cytotoxicity results of hemp phytotherapies

Hemp Extract	Concentration (mg/ml)	% Cytotoxicity	
		Hot	Cold
S. personii	**12.5**	27.12	22.68
	6.25	19.22	29.50
	3.12	23.76	31.45
	1.56	27.10	32.73
C. sativa	**3.12**	19.80	18.88
	1.56	21.38	19.61
	0.78	21.54	23.16
	0.39	31.10	23.82

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme which is present in most cells. In a cell culture, damage or rupture of cell cytoplasmic membranes results in the release of LDH from cells into the cell culture supernatant. Thus, quantitation of LDH in cell culture supernatants is a means used as an indicator of cell death. The amount of enzyme activity, measured as absorbance using a microplate reader at 490 nm, correlates to the number of damaged cells in the culture. Table 3 shows that minimal cytotoxicity (LDH released by plant sample/ LDH released by control x 100 = cytotoxicity %) occurred in the hemp phytotherapy concentrations used in this study. Moreover, the cytotoxicity % increased with a decrease in the hemp phytotherapy concentrations.

3.4.2 IL-6 and Endotoxin Standard Curves

Table 4: IL-6 Standard Curve Values

IL-6 Std	OD	OD	Average	STDev	COV
600	0.86	0.88	0.87	0.02	2.36
300	0.50	0.50	0.50	0.00	0.28
150	0.29	0.29	0.29	0.00	0.00
75	0.18	0.18	0.18	0.00	0.79
38	0.12	0.11	0.12	0.00	1.23
19	0.08	0.08	0.08	0.00	0.00
9	0.07	0.07	0.07	0.00	2.11
0	0.05	0.05	0.05	0.00	1.37

Samples were done in duplicate and read at 450nm optical density (OD). The averages, standard deviation (STDev) and coefficient of variance (COV) was calculated in Microsoft Excell. COV ranges between 0 and 10 are acceptable. IL-6 secretion by the whole blood cultures was measured and expressed as pg/ml. The data in this study are expressed as median values.

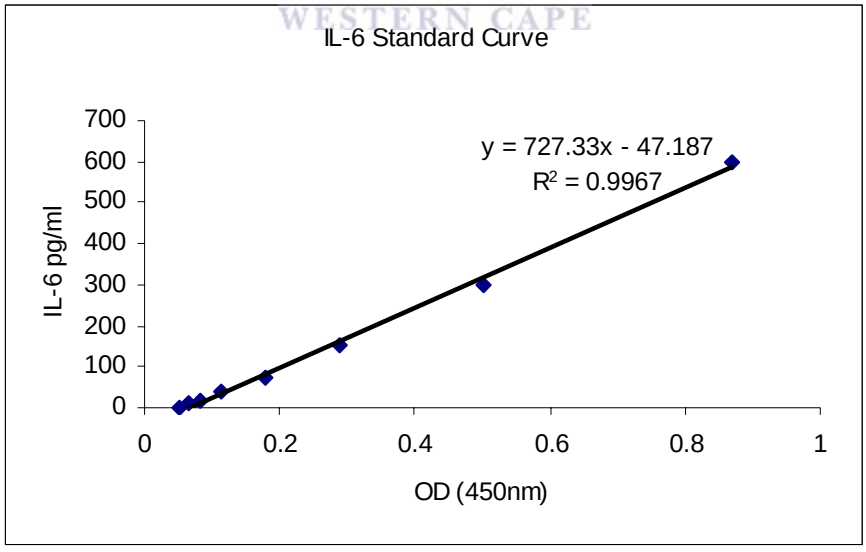


Figure 1: IL-6 Standard Curve used to determine the concentration of this cytokine in experimental human cells.

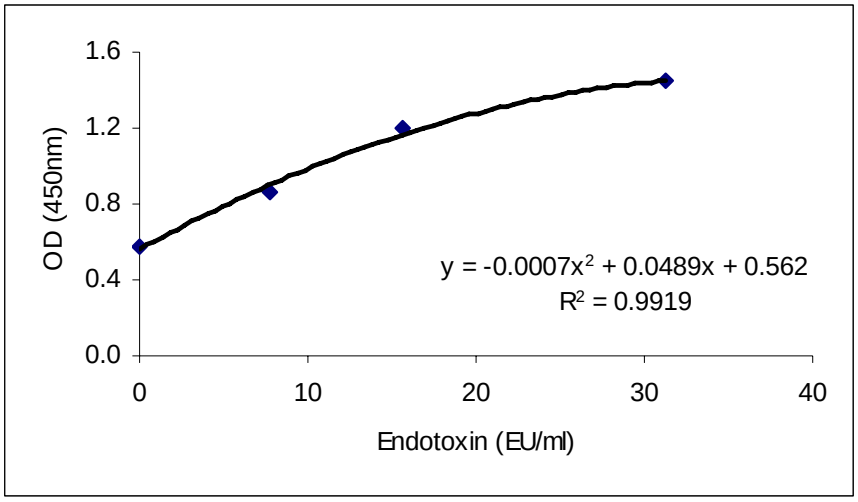
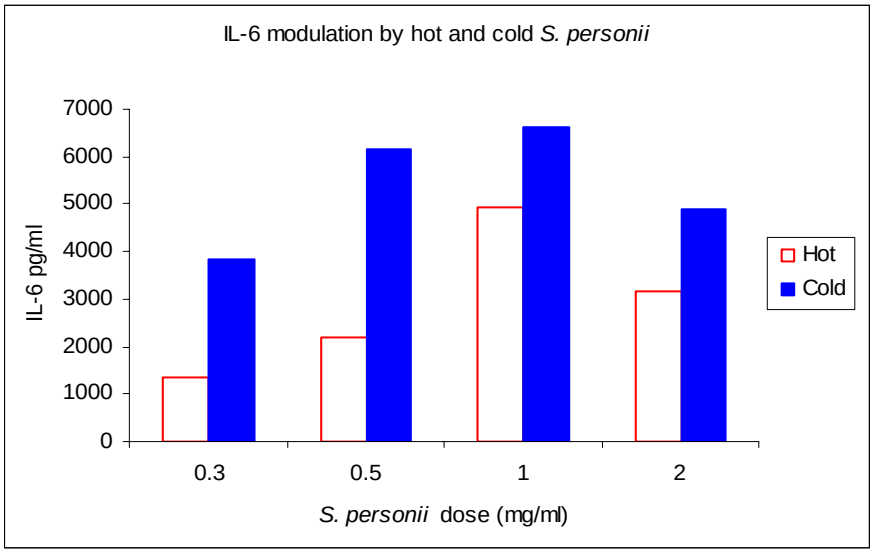


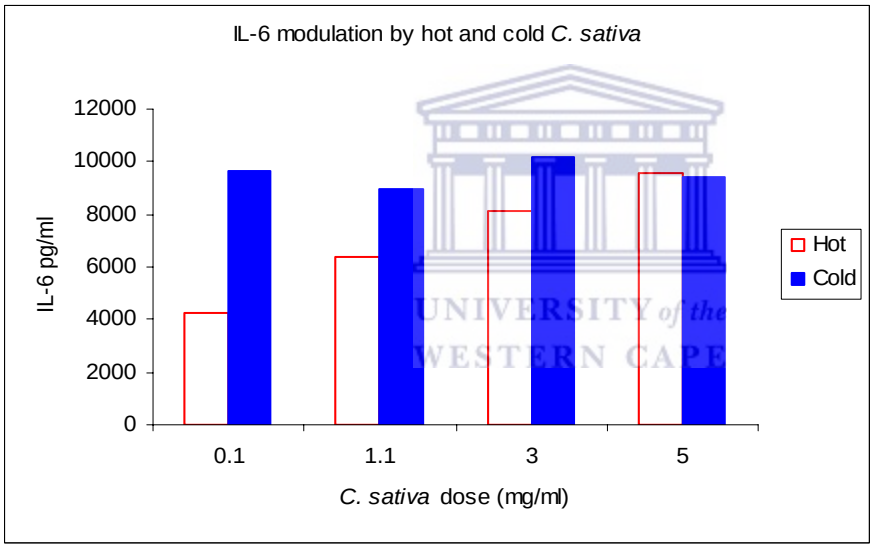
Fig. 2: The endotoxin standard curve from which the endotoxin recovery percentages were calculated (0 - 31.25 EU/ml).

The curve was calculated between 0 and 31.25 EU/ml, as it reached a plateau beyond this point. The formula was used to establish at which levels the hemp phytotherapy concentrations were pro- or anti-inflammatory. An endotoxin recovery above 100 % indicates a pro-inflammatory state, whereas below 100 %, an anti-inflammatory state is observed.

3.4.3 Hot versus cold *S. personii* and *C. sativa* extracts



a.



b.

Figure 3a-b: The modulation of IL-6 by plant extracts in unstimulated whole blood cell cultures. The unstimulated control cells, which were not exposed to endotoxin or plant extracts, produced 730 pg/ml of IL-6. The endotoxin (20 EU/ml) control produced 5 814 pg/ml of IL-6.

Table 5. The median IL-6 values (pg/ml), endotoxin recovery % and *P*-value of hot and cold *S. personii.*

	Hot		**Cold**		
Dose (mg/ml)	Median (a)	% Recovery	Median (b)	% Recovery	*P*-Value (ab)
S. personii (0.3)	1370	6.83	3843.78	125.54	0.03
C. sativa (0.1)	4267.67	71.70	9615.26	687.63	0.03
S. personii (0.5)	2208.28	30.27	6172.18	409.16	0.03
C. sativa (1.1)	6405.30	198.26	8964.04	355.68	0.05
S. personii (1)	4919.35	240.14	6640.057	537.67	0.05
C. sativa (3)	8105.93	374.65	10190.26	657.92	0.07
S. personii (2)	3160.52	76.27	4875.24	318.25	0.03
C. sativa (5)	9560.48	578.25	9380.297	640.41	0.03

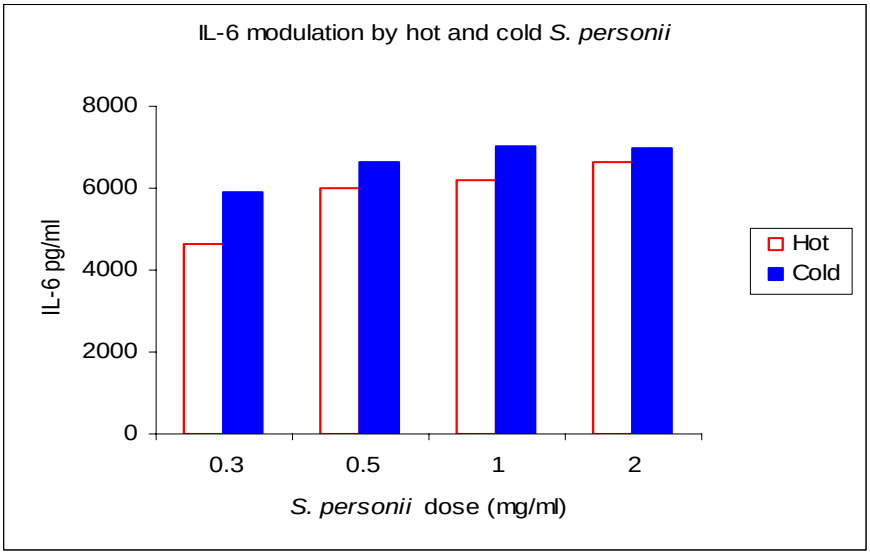
The effect of hemp extracts on IL-6 secretion by unstimulated whole blood cultures.

The serially diluted concentrations of *S. personii* (0.03 mg/ml - 32 mg/ml) and the Uniko-B variety of *C. sativa* (0.3 mg/ml - 325 mg/ml) were used in the initial tests to establish the dose-specific effects on IL-6 modulation. Cell lysis occurred at concentrations of 32 and 16 mg/ml of *S. personii* and at 325 mg/ml of *C. sativa*, thus indicating that these high concentrations are unfavorable to the cells. Four concentrations of *S. personii*: 2, 1, 0.50 and 0.30 mg/ml were selected for the immunology experiments. *C. sativa* was tested at

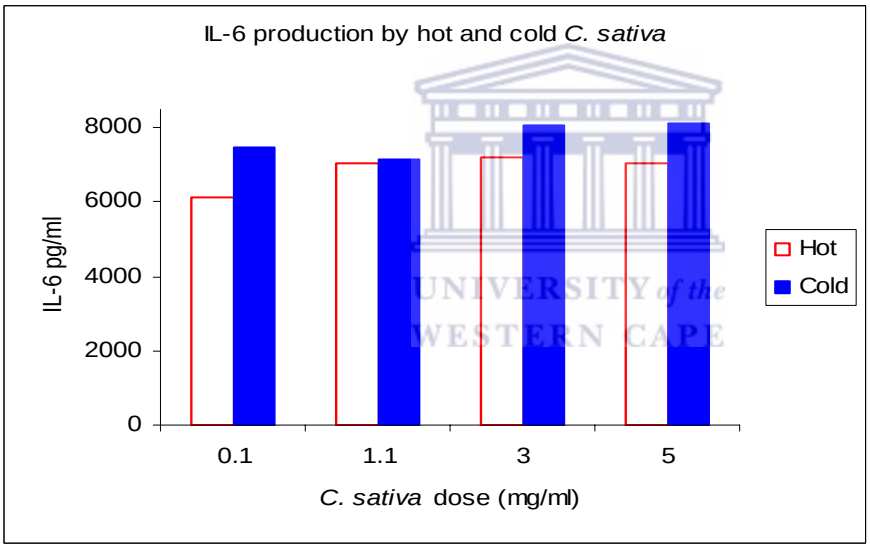
5, 3, 1.10 and 0.10 mg/ml. These extracts (20 µl) made up 10 % of the total well volume of 200 µl.

Figure 3a-b shows that both *S. personii* and *C. sativa* phytotherapies modulate IL-6 production in the absence of a stimulant. Figure 3a shows a significant peak ($P \leq 0.05$) of IL-6 production from the unstimulated state to concentrations of 1 mg/ml for both hot and cold extracts. A decrease in IL-6 production occurs between *S. personii* concentrations of 1 and 2 mg/ml ($P = 0.03$). Moreover, the cold *Sansevieria* extracts overall produced significantly higher levels ($P \leq 0.05$) of IL-6 in comparison to the hot extracts. At 1 mg/ml, cold *S. personii* produced significantly higher ($P \leq 0.05$) IL-6 compared to the hot extracts. According to the endotoxin % recovery (Table 5), it is noted that hot *S. personii* acts as pro-inflammatory measure at 1 mg/ml (Recovery = 240.14%), and an anti-inflammatory agent at concentrations of 0.3, 0.5 and 2 mg/ml (Recovery < 100%). The cold *S. personii* acts as pro-inflammatory agent (Recovery > 100%). *C. sativa* predominantly acts as a pro-inflammatory agent except at a hot concentration of 3 mg/ml (Recovery = 71.70 %).

3.4.4 Therapeutic Experiments



a.



b.

Figure 4a-b: The modulation of IL-6 by plant extracts in whole blood cell cultures stimulated with endotoxin and exposure to medicines. The endotoxin (20 EU/ml) control produced 5 814 pg/ml of IL-6.

Table 6. The median IL-6 values (pg/ml), endotoxin recovery % and *P*-value of hot and cold *S. personi* in therapeutic experiments.

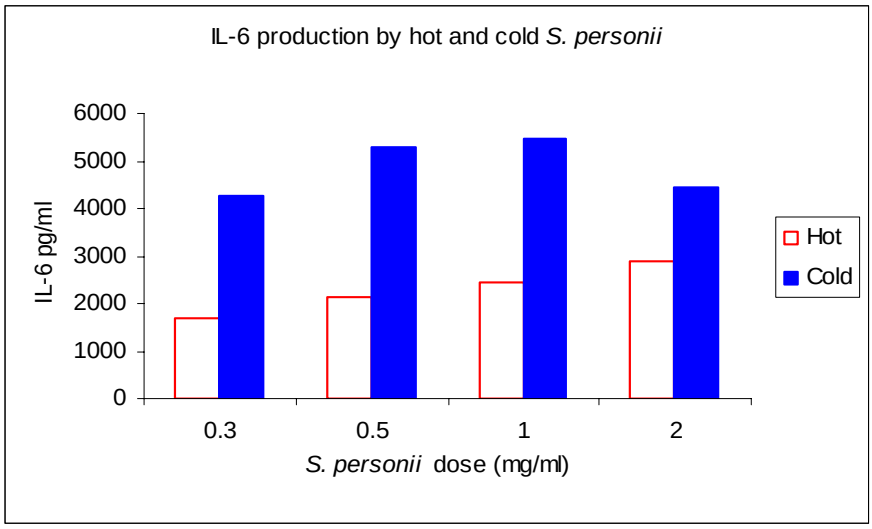
	Hot		**Cold**		
Dose (mg/ml)	Median (a)	% Recovery	Median (b)	% Recovery	*P*-Value (ab)
S. personii (0.3)	4627.47	31.66	5913.13	39.15	0.20
C. sativa (0.1)	6108.30	39.64	7455.55	71.76	0.07
S. personii (0.5)	6021.77	40.56	6618.51	57.02	0.03
C. sativa (1.1)	7024.05	44.56	7165.71	91.59	0.90
S. personii (1)	6171.35	55	7021.36	67.33	0.05
C. sativa (3)	7184.33	62.02	8068.97	119.79	0.03
S. personii (2)	6657.244	61.87	6977.35	63.35	0.03
C. sativa (5)	7028.17	56.40	8110.13	115.46	0.03

The effect of hemp extracts on IL-6 secretion by stimulated whole blood cultures – therapeutic experiments.

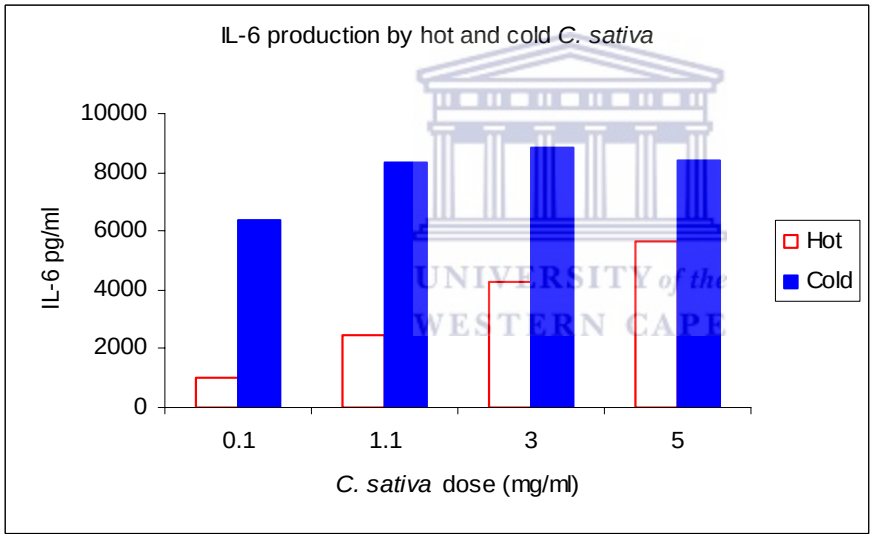
This experiment depicts the use of hemp extracts as therapeutic remedy. Extracts of indigenous and exotic hemp phytotherapies (10 µl) and endotoxin (10 µl) were added to whole blood cultures (180 µl) simultaneously. The control refers to whole blood cultures stimulated with 20 µl endotoxin (20 EU/ml) in the absence of phytotherapy.

Figure 4a and b show the IL-6 modulation when hemp varieties are used as therapeutic remedies. Cold *S. personii* showed a statistical increase (P = 0.03) from 0.3 to 0.5 mg/ml. The cold *S. personii* extracts was significantly higher ($P \leq 0.05$) compared to the hot extracts at concentrations of 0.5, 1 and 2 mg/ml, whereas at 0.3 mg/ml no significant variation occurred. Cold *C. sativa* showed a significant increase (P=0.0277) between 1.10 and 3 mg/ml. Moreover, the cold *C. sativa* extracts were significantly higher ($P \leq 0.05$) compared to the hot extracts at concentrations of 0.1, 1.1 and 5 mg/ml, whereas at 3 mg/ml (Fig. 4b) no significant difference ($P \geq 0.05$) was noted. The endotoxin recovery % (Table 6) results, shows that both therapeutic hemp phytotherapies generally act as anti-inflammatory agents.

3.4.5 Prophylactic Experiments



a.



b.

Figure 5a-b: The modulation of IL-6 by plant extracts in whole blood cell cultures stimulated with endotoxin after 24 hours of exposure to medicines. The endotoxin (20 EU/ml) control produced 3 601.02 pg/ml of IL-6.

Table 7. The median IL-6 values (pg/ml), endotoxin recovery % and *P*-value of hot and cold *S. personii* in prophylactic experiments.

	Hot		**Cold**		
Dose (mg/ml)	Median (a)	% Recovery	Median (b)	% Recovery	*P*-Value (ab)
S. personii (0.3)	1666.98	9.48	4245.38	154.16	0.03
C. sativa (0.1)	1040.54	13.68	6418.20	145.82	0.03
S. personii (0.5)	2118.192	17.59	5292.64	246.57	0.03
C. sativa (1.1)	2427.56	25.50	8297.87	268.20	0.03
S. personii (1)	2444.94	53.10	5452.95	301.18	0.05
C. sativa (3)	4283.83	84.13	8807.09	299.76	0.03
S. personii (2)	2903.94	63.56	4438.63	81.26	0.03
C. sativa (5)	5638.85	103.08	8433.49	336.97	0.07

The effect of hemp extracts on IL-6 secretion by endotoxin stimulated whole blood cultures after 24 hours – prophylactic experiments.

This experiment (Figure 5a-b) depicts the use of hemp extracts as a prophylactic remedy. Extracts of indigenous and exotic hemp phytotherapies (10 µl) were added to whole blood cultures after 24 hours of exposure to endotoxin (10 µl). The control refers to whole blood cultures stimulated with 20 µl endotoxin (20 EU/ml) in the absence of the hemp phytotherapies.

The hot *S. personii* extracts showed a significant decrease ($P = 0.03$) in IL-6 production between the endotoxin control and 0.30 mg/ml. The cold *S. personii* increased significantly between 0.30 and 0.50 mg/ml, whereas a decrease in IL-6 occurred between 1 and 2 mg/ml ($P = 0.04$). The cold *C. sativa* increased significantly ($P \leq 0.05$) from the endotoxin control to 3 mg/ml, where it reached a plateau in IL-6 production. The hot *C. sativa* showed an increase ($P \leq 0.05$) in IL-6 production with and increase in plant concentration. Furthermore, the cold extracts of both hemp phytotherapies produce higher levels of IL-6 compared to the hot extracts ($P \leq 0.05$).

The endotoxin recovery % for the prophylactic experiments (Table 7) shows that the inflammatory (pro- or anti-inflammatory) state is dose and plant specific. The hot *S. personii* and *C. sativa* extracts act as anti-inflammatory agents (recovery % < 100%), whereas the cold extracts predominantly act as pro-inflammatory agents (recovery % > 100%).

3.5 Discussion

The prime objective of this study was to investigate the immune modulatory effects of *Sansevieria personii* and *Cannabis sativa* (Uniko-B) by measuring their effects on interleukin-6 (IL-6) as an inflammatory biomarker. These hemp phytotherapies have been used for treatment of infections in particular, yet very little is known about its immune-modulating characteristics. There is a growing interest in identifying herbal immune modulators ever since their possible use in modern medicine was suggested (Lee, 1995).

The release of pro-inflammatory cytokines is essential for host survival from infection, and is also required for the repair of tissue injury (Hirazumi and Furusawa, 1999). These beneficial effects however, are critically dependant on the magnitude of the immune response, because large amounts of macrophage-derived pro-inflammatory cytokines can cause collateral damage to normal cells and are potentially lethal when the release is sufficient to cause systemic exposure (Okusawa, Kitahara, Kishimoto, Matsuda and Hirano, 1998).

Patwardan and Hooper (1992), states that the efficiency of the immune system may be influenced by various exogenous and endogenous factors, leading to either immune-suppression or immune-stimulation. The healthy state is believed to be based on a sophisticated fine-tuning of immune-regulatory mechanisms (Gauldie, Richards and Baumann, 1992). Immune-modulatory agents have been shown to possess activity to normalise or modulate pathophysiological processes (Wagner, 1983).

From this study, it is evident that both indigenous and exotic medicinal hemp extracts affect human cells by modulating the release of IL-6. Figure 3 to 5 shows that it is evident that the cold hemp phytotherapies overall exhibited a significantly higher ($P \leq 0.05$) production of IL-6 compared to the hot phytotherapies. According to the endotoxin recovery (240.14 %) (Table 5), it is noted that hot *S. personii* showed a distinctive peak in IL-6 production at a concentration of 1 mg/ml, thus suggesting when using *S. personii* as pro-inflammatory agent this should be a favourable concentration to get an optimal effect. *C. sativa* predominantly acts as a pro-inflammatory agent for both hot and cold infusions (Table 5).

It is indeed a significant finding that IL-6 was modulated in the experiments in the absence of an endotoxin stimulant, as most plant species only modulate IL-6 in the presence of an endotoxin or upon an infection. This might have resulted due to the presence of lectins in the plant material that reacts with surface-exposed carbohydrates, acting as an adjuvant. Lectins are non-enzymatic proteins that bind mono- and oligosaccharides reversibly and with high specificity (Gaidamashvili and Van Staden, 2002).

It is evident that the cold hemp phytotherapies overall exhibited a significantly higher ($P \leq 0.05$) production of IL-6 compared to the hot phytotherapies (Fig. 3-5), which are predominantly anti-inflammatory. This finding justifies the traditional method of administration which suggests that the hemp phytotherapies should be used after heating (Van Wyk *et al*., 2000), in order to obtain anti-inflammatory significance.

The therapeutic experiments (Fig. 4) consisted of endotoxin-infected blood cultures, which were treated with phytotherapies to simulate the response of hemp as a treatment in the presence of an infection. Both hot hemp extracts shows a production of IL-6 in the region of 6 000 to 7 000 pg/ml, predominantly showing no significant ($P \geq 0.05$) variation in the immune modulation (Fig. 4 a, b). The endotoxin recovery (Table 6) shows that the therapeutic method of administration exhibits an anti-inflammatory response (endotoxin recovery < 100 %).

The prophylactic whole blood cultures were initially exposed to the plant extracts followed by exposure to endotoxin stimulant after 24 hours of incubation. This study was performed to explore the function of *S. personii* and *C. sativa* as preventative measure to an infection or inflammation. The endotoxin recovery results indicate that both pro- and anti-inflammatory states are obtained and are plant-and dose-dependant (Table 7). The hot hemp extracts acts as anti-inflammatory agents, whereas the cold hemp species act as pro-inflammatory agents. Therefore, when an individual has a high fever, the hot concentrations should be taken, whereas individuals with an infection should take cold infusions to ensure killing off the infection.

The current study showed that the indigenous and exotic hemp phytotherapies have potent stimulant action on the IL-6 cytokines which compliments its traditional uses in folk remedies, and suggests it to be promising candidates for further studies. An important priority is the confirmation of the identity of the active compounds responsible for the cytokine stimulation and the precise mode of action, including the effect on other interleukins.

In addition, upon further investigation, the following considerations should be taken into account in this very important research area:

- More volunteers should be recruited in these studies,
- The mechanism in which the hemp species exerts its immune modulatory function should be investigated,
- A further advanced study should be pursued that specifically focuses on the formulation of appropriate therapeutic dosages of these hemp varieties, which may be used by the pharmaceutical industry.

3.4 References

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Chapter Four

Conclusion

Key findings of medicinal hemp plant extracts as natural antibiotic and immune modulation phytotherapies.

Chapter 4

Conclusion

Table 1: The following table contains a summary of the most important findings of this investigation.

AIMS	OUTCOMES
Antibacterial Evaluation	
• To determine the antibacterial effects of indigenous and exotic hemp.	• *S. personii* was the most powerful natural antibiotic against *S. aureus* and *M. smegmatis.* • Uniko-B cultivar of *C. sativa* was the most effective antibiotic of the exotic hemp variety.
• To assess whether aqueous or organic solvent extracts are the best dosage forms	• Aqueous extracts of medicinal hemp plants had no antibiotic effect. • Methanol extracts of medicinal hemp phytotherapies were the most effective dosage form.
Immune Modulation Assessment	
• The effect of hemp phytotherapies on human immune cells.	• Both indigenous and exotic hemp phytotherapies modulate IL-6 in the absence of an endotoxin stimulant.
• The effect of hemp phytotherapies as therapeutic and prophylactic remedies.	<u>Unstimulated remedy</u> • Hot *S. personii* extracts are anti-inflammatory, whereas cold extracts are pro-inflammatory. Unstimulated *C. sativa* is largely pro-inflammatory. <u>Therapeutic remedy</u> • *S. personii* and *C. sativa* are significant anti-inflammatory agents when used therapeutically. <u>Prophylactic remedy</u> • Hot hemp extracts are anti-inflammatory, whereas cold extracts are pro-inflammatory.

Medicinal plants were once the primary source of medicine in the world. Currently, natural products and their derivatives still play an integral role in the pharmaceutical industry. The increased cost of Western medicine and resistance of antibiotic treatments have caused a renewed interest in alternative therapies. Traditionally the hemp plants in this study have been used for infections and inflammatory disorders, ranging from earaches to asthma. The traditional method of administration for the indigenous *Sansevieria* species is by squeezing the heated leaf juice onto affected areas, whereas a warm water infusion is used in the case of the exotic *Cannabis sativa* varieties.

The first objective of this study was to evaluate the antibacterial efficacy of the indigenous and exotic hemp phytotherapies and to assess which organic solvent is a more effective. The antibacterial data of this study confirmed that the methanolic *Sansevieria* and *Cannabis* phytotherapies obtains antibacterial values to some extent. The investigation indicated that the *S. personii* and Uniko-B variety of *C. sativa* are the most effective natural antibiotic hemp phytotherapies. Moreover, *S personii* showed the lowest minimum inhibitory concentration against *Staphylococcus aureus* and *Mycobacterium smegmatis* compared to the other hemp varieties.

However, no effects of inhibition were exhibited by the aqueous extractions. This could be due to the insolubility of the active compounds in water or the hot water could have caused denaturation of the active compounds. It should be noted that this study was done *in vitro*, thus differing from the *in vivo* environment, which consists of functional cells and sophisticated systems that cannot be mimicked on a petri dish. Moreover,

considering that the aqueous hemp phytotherapies had no inhibition effect on the microbes, yet still act as immune modulators, does not cancel it out as an anti-infective, because the mode of action is different. It is for this reason that it is crucial to explore experimental analysis as different effects are obtained.

No inhibition was observed against the gram-negative, *Pseudomonas aeruginosa*, which might be due to the absence of antibacterial compounds in the hemp extracts to combat these specific microorganisms, or the inability of the actives to inhibit gram-negative bacteria. The cell walls of gram-positive bacteria consists of a single layer, whereas gram-negative bacteria consist of a multi-layered cell wall bounded by an outer membrane, composed of high-density lipopolysaccharides that serves as a barrier to many environmental substances including antibiotics (Palambo, 2001).

The hemp phytotherapies showed efficacy after a long period of storage. Herbal traders who sell these plant materials, could therefore store these materials for prolonged periods, given that conducive storage facilities are used. The storage facilities of traders are often exposed to unfavorable external factors, which might however, impact plant material in a way that minimize its shelf life. Further stability investigations should be done in which testing is done beyond the periods tested in this study, to further establish the effectiveness of these materials.

Upon further antibacterial investigation, the following considerations should be taken into account:

- Using a variety of extraction solvents,
- Applying various antibacterial methods,
- Chemical identification of the antibacterial ingredients using specialized analytical equipment.

The second objective was to evaluate the immune-modulating effects of these hemp extracts and more specifically as therapeutic and prophylactic remedies. The *S. personii* and Uniko-B cultivar of *C. sativa* were tested in this investigation as it proved to be the most efficacious antibacterial agents. The immune modulatory investigation showed that the indigenous and exotic hemp varieties enhance human immune health by modulating the production of IL-6 to combat infections. IL-6 was produced without a stimulant, which is a significant finding, as most phytotherapies only show an effect once exposed to an infection.

The cold hemp phytotherapies overall exhibited a significantly higher production of IL-6 compared to the hot phytotherapies, which are predominantly anti-inflammatory. This finding justifies the traditional method of administration, which suggests that the hemp phytotherapies should be used after heating (Van Wyk *et al.*, 2000), in order to obtain anti-inflammatory significance.

Moreover, the unstimulated hot *S. personii* showed a distinctive peak in IL-6 production at a concentration of 1 mg/ml, thus suggesting when using *S. personii* as pro-inflammatory agent, this should be a favourable concentration to get an optimal effect. Unstimulated *C. sativa* predominantly acts as a pro-inflammatory agent for both hot and cold infusions.

The therapeutic method of administration of both indigenous and exotic species leads to anti-inflammatory response. The prophylactic results indicate that both pro- and anti-inflammatory states are obtained and is plant and dose-dependant (Table 6). The hot hemp extracts acts as anti-inflammatory agents, whereas the cold hemp species act as pro-inflammatory agents. Therefore, when an individual has a high fever, the hot concentrations should be taken, whereas individuals with an infection should take cold infusions to ensure killing off the infection.

Furthermore, the following considerations should be taken into account when pursuing further studies in this very important research area:

- More volunteers should be recruited in future studies,
- The identity of the active compounds responsible for the cytokine stimulation and the precise mode of action,
- The mechanism in which the hemp species exert its immune modulatory function should be investigated,
- The effect of the hemp phytotherapies on other interleukins,

- A further advanced study should be pursued, which specifically focuses on the formulation of appropriate therapeutic dosages of these hemp varieties that may be used by the pharmaceutical industry.

To conclude, the data collected in this investigation indicates that both indigenous *Sansevieria* species and exotic *Cannabis sativa* species exhibit antibacterial and immune modulatory effects, which compliments their traditional use as folk remedies. Upon further investigation and research, these findings may be incorporated into the formulation of effective and complementary natural antibiotic and immune modulation phytotherapies.

Appendices

Raw data of Immunology Experiments

Appendices

Table 1: IL-6 modulation by hot *S. personii* of 6 volunteers

	DONORS								
mg/ml	1	2	3	4	5	6	Average	Median	STDev
0.00	562.51	1207.46	344.33	747.10	713.66	981.76	759.47	730.38	304.46
0.30	969.57	2181.21	530.66	1050.65	1689.36	4156.72	1763.03	1370.00	1308.88
0.50	2160.32	5485.28	1342.03	2256.25	2131.85	3144.25	2753.33	2208.28	1455.55
1.00	4527.06	6118.72	2187.07	4390.79	5311.64	6902.03	4906.22	4919.35	1638.35
2.00	2804.46	3067.85	1108.71	5530.07	3253.19	4278.76	3340.51	3160.52	1484.91

Table 2: IL-6 modulation by cold *S. personii* of 6 volunteers

	DONORS								
mg/ml	1	2	3	4	5	6	Average	Median	STDev
0.00	562.51	1207.46	344.33	747.10	713.66	981.76	759.47	730.38	304.46
0.30	2969.13	4218.55	1972.18	3811.86	3875.71	6276.91	3854.06	3843.79	1437.35
0.50	5941.11	7017.63	3477.17	4927.33	6403.26	6734.41	5750.15	6172.18	1332.80
1.00	6594.14	6685.98	4322.79	6057.61	7316.50	6702.22	6279.87	6640.06	1038.87
2.00	4868.22	4762.23	2587.35	6937.72	4882.26	5211.39	4874.86	4875.24	1386.66

Table 3: IL-6 modulation by hot *C. sativa* of 6 volunteers

	DONORS								
mg/ml	1	2	3	4	5	6	Average	Median	STDev
0.00	562.51	1207.46	344.33	747.10	713.66	981.76	759.47	730.38	304.46
0.10	2086.50	8384.80	1094.33	1853.96	6448.86	7642.57	4585.17	4267.68	3260.28
1.10	4767.20	10266.56	2787.49	4990.97	7819.63	10365.60	6832.91	6405.30	3139.07
3.00	6631.01	10171.55	4340.22	7174.61	9037.26	11041.66	8066.05	8105.93	2488.63
5.00	9393.53	9727.45	4635.80	8244.58	10624.87	10522.60	8858.14	9560.49	2242.26

Table 4: IL-6 modulation by cold *C. sativa* of 6 volunteers

	DONORS								
mg/ml	1	2	3	4	5	6	Average	Median	STDev
0.00	562.51	1207.46	344.33	747.10	713.66	981.76	759.47	730.38	304.46
0.10	9986.27	11455.43	5411.02	7605.65	11534.17	9244.25	9206.13	9615.26	2368.69
1.10	6477.41	11622.36	6248.60	8228.03	12677.71	9700.06	9159.03	8964.05	2655.95
3.00	10522.76	11265.76	6042.73	8497.35	11350.24	9857.77	9589.44	10190.26	2031.03
5.00	10461.37	10035.30	5862.79	8725.29	11193.41	2403.07	8113.54	9380.30	3368.70

(**Key**: STDev – Standard Deviation)

Table 5: IL-6 modulation by hot *S. personii* of 6 volunteers under therapeutic conditions

	DONORS								
mg/ml	**1**	**2**	**3**	**4**	**5**	**6**	**Average**	**Median**	**STDev**
endotoxin	3132.06	5720.38	2899.82	5907.92	6446.52	7905.45	5335.36	5814.15	1954.61
0.30	5912.73	6545.69	4183.39	3753.95	5071.55	3915.12	4897.07	4627.47	1145.45
0.50	1607.96	6693.52	4416.82	5792.52	6251.03	7307.53	5344.90	6021.77	2074.52
1	5757.81	6945.00	4616.06	6087.26	6956.90	6255.45	6103.08	6171.36	870.83
2	5680.95	6454.45	4829.18	6896.01	6860.04	7096.28	6302.82	6657.24	880.15

Table 6: IL-6 modulation by cold *S. personii* of 6 volunteers under therapeutic Conditions

	DONORS								
mg/ml	**1**	**2**	**3**	**4**	**5**	**6**	**Average**	**Median**	**STDev**
endotoxin	3132.06	5720.38	2899.82	5907.92	6446.52	7905.45	5335.36	5814.15	1954.61
0.30	5566.40	6286.56	4488.24	4586.42	6518.78	6259.88	5617.71	5913.14	895.94
0.50	6056.15	6901.93	4856.11	6399.97	6837.06	8015.97	6511.20	6618.52	1047.09
1	7326.90	7526.94	5285.44	6715.82	7839.87	6514.27	6868.21	7021.36	921.10
2	7726.94	6751.98	6244.62	6923.62	7031.09	7147.67	6970.99	6977.35	486.64

Table 7: IL-6 modulation by hot *C. sativa* of 6 volunteers under therapeutic conditions

	DONORS								
mg/ml	**1**	**2**	**3**	**4**	**5**	**6**	**Average**	**Median**	**STDev**
endotoxin	3132.06	5720.38	2899.82	5907.92	6446.52	7905.45	5335.36	5814.15	1954.61
0.10	3005.62	6299.62	4963.22	5916.99	6541.30	7350.10	5679.47	6108.31	1525.60
1.10	5248.61	8060.02	7012.36	5803.45	7981.60	7035.74	6856.96	7024.05	1137.09
3.00	7505.13	10147.48	6676.34	6491.70	7411.77	6956.90	7531.55	7184.34	1341.72
5.00	9696.87	9320.64	6274.42	6533.94	6568.37	7487.97	7647.04	7028.17	1504.30

Table 8: IL-6 modulation by cold *C. sativa* of 6 volunteers under therapeutic conditions

	DONORS								
mg/ml	**1**	**2**	**3**	**4**	**5**	**6**	**Average**	**Median**	**STDev**
endotoxin	3132.06	5720.38	2899.82	5907.92	6446.52	7905.45	5335.36	5814.15	1954.61
0.10	7267.47	7643.63	6624.92	5543.55	8403.28	7708.91	7198.63	7455.55	998.28
1.10	8966.88	9735.10	6798.55	186.35	6929.17	7402.26	6669.72	7165.72	3386.09
3.00	10784.03	10555.20	7765.97	6935.46	8178.87	7957.08	8696.10	8067.97	1587.10
5.00	11143.20	9980.47	7941.97	6951.28	8278.28	7708.91	8667.35	8110.13	1574.49

Table 9: IL-6 modulation by hot *S. personiii* of 6 volunteers under prophylactic conditions

	DONORS								
mg/ml	1	2	3	4	5	6	Average	Median	STDev
endotoxin	4929.56	795.66	1916.89	3640.28	3561.75	7700.56	3757.45	3601.02	2413.87
0.30	2036.10	636.27	1297.87	2294.48	802.15	5428.30	2082.53	1666.98	1765.67
0.50	2439.27	498.20	1084.34	4463.70	1797.11	6718.73	2833.56	2118.19	2345.01
1	3025.38	738.35	2034.25	1254.27	2855.64	7354.20	2877.01	2444.94	2365.80
2	3926.56	1768.39	1881.41	3925.59	3286.24	5173.18	3187.14	2903.50	1654.03

Table 10: IL-6 modulation by cold *S. personiii* of 6 volunteers under prophylactic conditions

	DONORS								
mg/ml	1	2	3	4	5	6	Average	Median	STDev
endotoxin	4929.56	795.66	1916.89	3640.28	3561.75	7700.56	3757.45	3601.02	2413.87
0.30	3793.70	4358.00	3887.16	5242.67	4132.76	7509.23	4820.59	4245.38	1415.62
0.50	5206.84	5072.62	3879.97	5378.44	6772.50	8084.85	5732.54	5292.64	1475.41
1	4625.65	4980.48	4063.48	5925.43	6652.79	7933.72	5696.93	5452.96	1435.15
2	3964.85	4504.62	2456.83	4438.64	2897.62	5638.20	3987.18	4438.64	1296.78

Table 11: IL-6 modulation by hot *C. sativa* of 6 volunteers under prophylactic conditions

	DONORS								
mg/ml	1	2	3	4	5	6	Average	Median	STDev
endotoxin	4929.56	795.66	1916.89	3640.28	3561.75	7700.56	3757.45	3601.02	2413.87
0.10	3159.32	606.06	627.98	1298.16	782.93	2336.19	1468.44	1040.55	1054.61
1.10	5249.78	869.70	1238.02	3617.11	396.15	4686.81	2676.26	2427.57	2101.51
3.00	7080.11	2147.13	3209.38	5358.29	2233.61	8594.55	4770.51	4283.84	2685.45
5.00	7004.84	4823.73	3942.68	6453.97	4515.42	10349.96	6181.77	5638.85	2356.26

Table 12: IL-6 modulation by cold *C. sativa* of 6 volunteers under prophylactic conditions

	DONORS								
mg/ml	1	2	3	4	5	6	Average	Median	STDev
endotoxin	4929.56	795.66	1916.89	3640.28	3561.75	7700.56	3757.45	3601.02	2413.87
0.10	8356.02	5028.87	4994.75	2744.64	8251.96	7807.54	6197.30	6418.20	2289.40
1.10	9466.21	6926.30	4929.51	7839.02	8756.73	10416.23	8055.67	8297.87	1956.97
3.00	9337.94	8037.01	5455.46	8276.23	10158.21	11918.95	8863.97	8807.09	2185.40
5.00	8093.62	7659.81	6057.01	8773.36	10634.33	10634.33	8642.08	8433.49	1783.45

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