

**AN INVESTIGATION OF THE IMMUNOMODULATORY PROPERTIES  
OF *SUTHERLANDIA FRUTESCENS* AND *HYPOXIS  
HEMEROCALLIDEA***

by

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## DECLARATION

I declare that this dissertation is my own work. I have discussed the paper with others and used advice and suggestions from others in writing it, but the paper is neither copied from another source without proper acknowledgement, nor written for me by another person, in whole or in part. It is being submitted for the degree of MSc Pharmacology at the University of Pretoria, Pretoria and has not been submitted before for any degree or examination at this or any other University.

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(Signature of Candidate)

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## ABSTRACT

Human immunodeficiency virus (HIV) is currently the most significant infectious pathogen and the causative agent of acquired immune deficiency syndrome (AIDS). Unfortunately, due to lack of resources, delivery of antiretroviral therapy (ART) to countries where they are most needed, such as South Africa, Botswana, Lesotho, Malawi and Swaziland, is limited and inefficient. Moreover, the short supply and high cost of antiretroviral drugs have caused researchers to turn to plants as prospective therapies in the search for alternative anti-HIV or immunomodulatory compounds. In an African context, traditional medicines are of great importance, not so much as an alternative to treatment, but in many cases as the only source of treatment. There are various South African plants used medicinally which possess phytochemical constituents that target certain mediators of inflammation and the immune system. In African regions where patients do not have access or financial capability to obtain conventional antiretroviral treatment, traditional herbal medicines are used as primary treatment of HIV/AIDS, regardless of the fact that the safety, toxicity and efficacy of these products are not yet fully understood and that a risk for adverse effects exists. *Hypoxis hemerocallidea* Fisch & C. A. Mey. (Hypoxidaceae) as well as *Sutherlandia frutescens* L. R. Br. (Leguminosae) have various effects on the immune system and due to claims about their immune boosting properties, they are two of the most common African herbal compounds being used for HIV management in South Africa.

In this study, the immune modulating properties of *H. hemerocallidea* and *S. frutescens* were investigated in order to determine whether anecdotal claims made about these plants could be supported. Differentiated THP-1 and U937 macrophages were treated with aqueous extracts of *H. hemerocallidea* and *S. frutescens* as well as with solutions of compound standards reputedly isolated from these plants such as beta-sitosterol, found in *H. hemerocallidea*, canavanine, pinitol and gamma-aminobutyric acid (GABA) which are present in *S. frutescens*.

Cytotoxicity of the test compounds was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyl tetrazolium bromide (MTT) assay.

Antioxidant capacity was assessed using the Trolox equivalence antioxidant capacity (TEAC) and Oxygen radical antioxidant capacity (ORAC) assays. Determination of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) concentration in treated THP-1 and U937 cell culture supernatants was performed by ELISA. Concentrations of cytokines (IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, TNF- $\alpha$  and IFN- $\gamma$ ) in treated THP-1 and U937 cell culture supernatants were determined by flow cytometry. Curcumin, a well-known immunomodulatory compound, was used as a positive control.

Results of cytotoxicity assessments showed that *H. hemerocallidea* (0.1 – 1.9 mg/ml), *S. frutescens* (0.1 – 1.6 mg/ml), beta-sitosterol (0.2 – 25  $\mu$ M), canavanine, pinitol and GABA (1.5 – 200  $\mu$ M) were not cytotoxic to THP-1 and U937 macrophages and had cytotoxicity profiles comparable to that of the positive control, curcumin (0.8 - 25  $\mu$ M). The TEAC and ORAC assays showed different results in the antioxidant capacities of the test compounds. The purported antioxidant activity of *H. hemerocallidea* was confirmed by the TEAC assay with antioxidant effects equivalent to 0.2 mg/ml Trolox. Canavanine showed antioxidant activity equivalent to approximately 0.17 mg/ml Trolox and comparable to that of curcumin in the ORAC assay, suggesting its involvement in the inhibition of peroxy radical-induced oxidation. Flow cytometry results showed that curcumin (20  $\mu$ g/ml and 10  $\mu$ g/ml) and beta-sitosterol (25  $\mu$ g/ml and 12.5  $\mu$ g/ml) reduced IL-1 $\beta$  and IL-8 production and significantly ( $p < 0.05$ ) decreased the production of TNF- $\alpha$ . This suggests that beta-sitosterol could indeed possess anti-inflammatory properties, with effects comparable to the known anti-inflammatory effect of curcumin in terms of cytokine profiles.

Beta-sitosterol (25 µg/ml) and pinitol (50 µg/ml) significantly ( $p < 0.001$ ) decreased extracellular PGE<sub>2</sub> levels in U937 macrophages by 233.4 pg/ml and 281.7 pg/ml, respectively and were the only two compounds showing greater reductions in PGE<sub>2</sub> than curcumin.

In conclusion, results of this study do not provide enough evidence to support all anecdotal claims about the 'immune boosting' properties of *S. frutescens* and *H. hemerocallidea*, but the compounds canavanine, beta-sitosterol and pinitol were found to have modulatory effects on certain aspects of the immune system.

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## LIST OF ABBREVIATIONS

AAPH	2,2-azobis(2-amidinopropane) dihydrochloride
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
AOC	Antioxidant capacity
ART	Antiretroviral therapy
ATCC	American Tissue Culture Collection
AUC	Area under curve
BAL	Bronchoalveolar fluid
BSS/BSSG	Beta-sitosterol/beta-sitosterolglucoside
CBA	Cytometric bead array
COX	Cyclooxygenase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2, 2-diphenylpicrylhydrazyl
ELISA	Enzyme-linked immunosorbent assay
FMLP	<i>L</i> -formyl- <i>L</i> -methionyl- <i>L</i> -leucyl- <i>L</i> -phenylalanine
GABA	Gamma-aminobutyric acid
<i>H. hemerocallidea</i>	<i>Hypoxis hemerocallidea</i>
HAT	Hydrogen atom transfer
HIV	Human immunodeficiency virus
IC <sub>50</sub>	50% Inhibitory concentration
iCAM	Intercellular adhesion molecule
IFN	Interferon

IL	Interleukin
LD <sub>50</sub>	50% Lethal dose
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MAPKs	Mitogen-activated protein kinases
MCP	Monocyte chemoattractant protein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NF	Nuclear Factor
NO	Nitric oxide
NOS	Nitric oxide synthase
ORAC	Oxygen radical antioxidant capacity
PBS	Phosphate buffered saline
PE	Phycoerythrin
PG	Prostaglandin
PHA	Phytohaemagglutinin
PMA	Phorbol-12-myristate-13-acetate
pNpp	p-nitrophenyl phosphate
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Reverse transcriptase
<i>S. frutescens</i>	<i>Sutherlandia frutescens</i>
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SET	Single-electron transfer
TBARS	Thiobarbituric acid reactive substances

TEAC	Trolox equivalence antioxidant capacity
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TPA	12-O-tetradecanoylphorbol-13-acetate
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

## **CHAPTER 1: LITERATURE REVIEW**

### **1.1 Human immunodeficiency virus and the immune system**

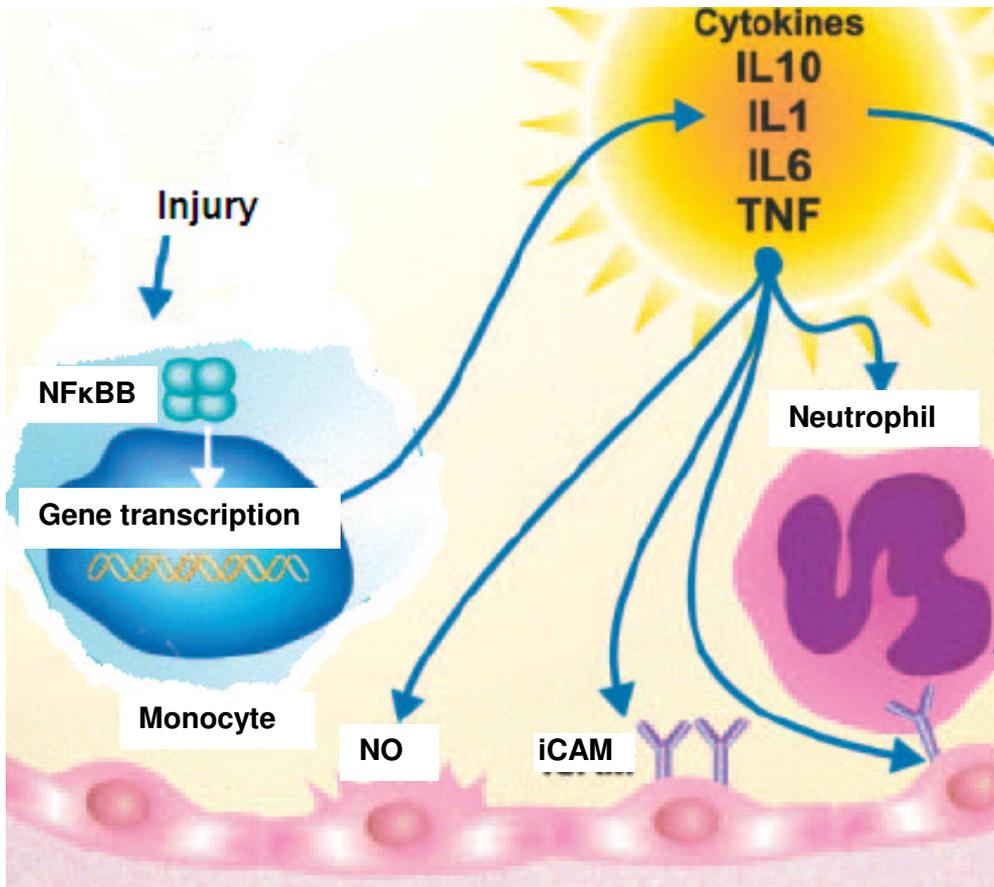
Human immunodeficiency virus (HIV) is currently one of the most significant infectious pathogens and the causative agent of acquired immune deficiency syndrome (AIDS) (Bessong and Obi 2006). Sub-Saharan Africa is affected mostly by the HIV/AIDS pandemic, with 68% of global HIV prevalence, 76% of global AIDS mortality and 90% of global HIV-infected children (UNAIDS and WHO 2009). Unfortunately, due to lack of resources, delivery of antiretroviral therapy (ART) to countries where they are most needed, such as South Africa, Botswana, Lesotho, Malawi and Swaziland, is limited and inefficient (UNAIDS 2005; Bessong and Obi 2006). Despite attempts to improve ART delivery and availability in rural areas, 7 out of 10 people who require treatment, still do not receive treatment and for every person placed on ART, approximately 4 to 6 others acquire AIDS (UNAIDS and WHO 2009; Zachariah et al. 2010). Due to the short supply and high cost of antiretroviral drugs, plants have received much attention as potential therapies in the search for alternative anti-HIV or immunomodulatory compounds.

In order to obtain a better understanding of the immunomodulatory effects of medicinal plants on HIV/AIDS patients, one must consider the immune cells, the cytokines involved in the pathogenesis of HIV as well as general aspects of the immune system. Macrophages and monocytes represent a suitable model for investigating immunomodulating activity of potential immunomodulators. These cells are involved in several immune functions which include removal of cell debris, killing pathogenic microorganisms as well as antigen presentation to lymphocytes (Kinne et al. 2000).

When the body is subjected to certain noxious stimuli such as pathogens (including viruses) or injury, macrophages are activated due to the interaction between cell surface receptors (e.g. Toll-like receptors, TLR) and their stimulation as well as other intracellular signaling mechanisms involving enzymes such as protein tyrosine kinases, phosphatidylinositol 3-kinase, mitogen-activated protein kinases (MAPKs) and various transcription factors, e.g. nuclear factor NF- $\kappa$ B (Sekine et al. 2006).

Following a sequence of events, macrophages then release various pro-inflammatory cytokines (e.g. tumor necrosis factor [TNF]- $\alpha$  and interleukin [IL]-1), chemokines and chemoattractants (e.g. IL-8 and monocyte chemoattractant protein MCP-1), and cytotoxic and inflammatory mediators (e.g. nitric oxide [NO], reactive oxygen species [ROS] and prostaglandin E<sub>2</sub> [PGE<sub>2</sub>]) (Burmester et al., 1997; Bresnihan 1999; Gracie et al. 1999). *In vitro* and *in vivo* investigations have demonstrated the increased production of PGE<sub>2</sub> by monocytes and macrophages of HIV-infected patients (Fernandez-Cruz et al. 1989; Longo et al. 1993). The functions of macrophages as well their release of inflammatory mediators can lead to severe collateral damage linked to various diseases such as rheumatoid arthritis and arteriosclerosis (Michaelsson et al. 1995; Gracie et al. 1999). Figure 1.1 shows a simplified diagram of the immune response involving some of these inflammatory mediators.

In terms of HIV infected /AIDS patients, inflammatory processes mediated by various immune cells, including macrophages, lead to destruction of infected CD4<sup>+</sup> lymphocytes and result in a gradual decrease in the function of the immune system. Estcourt et al. (1997) reported that circulating monocytes from HIV-infected individuals constitutively produce IL-1, tumor necrosis factor (TNF)- $\alpha$ , IL-8 and IL-6.



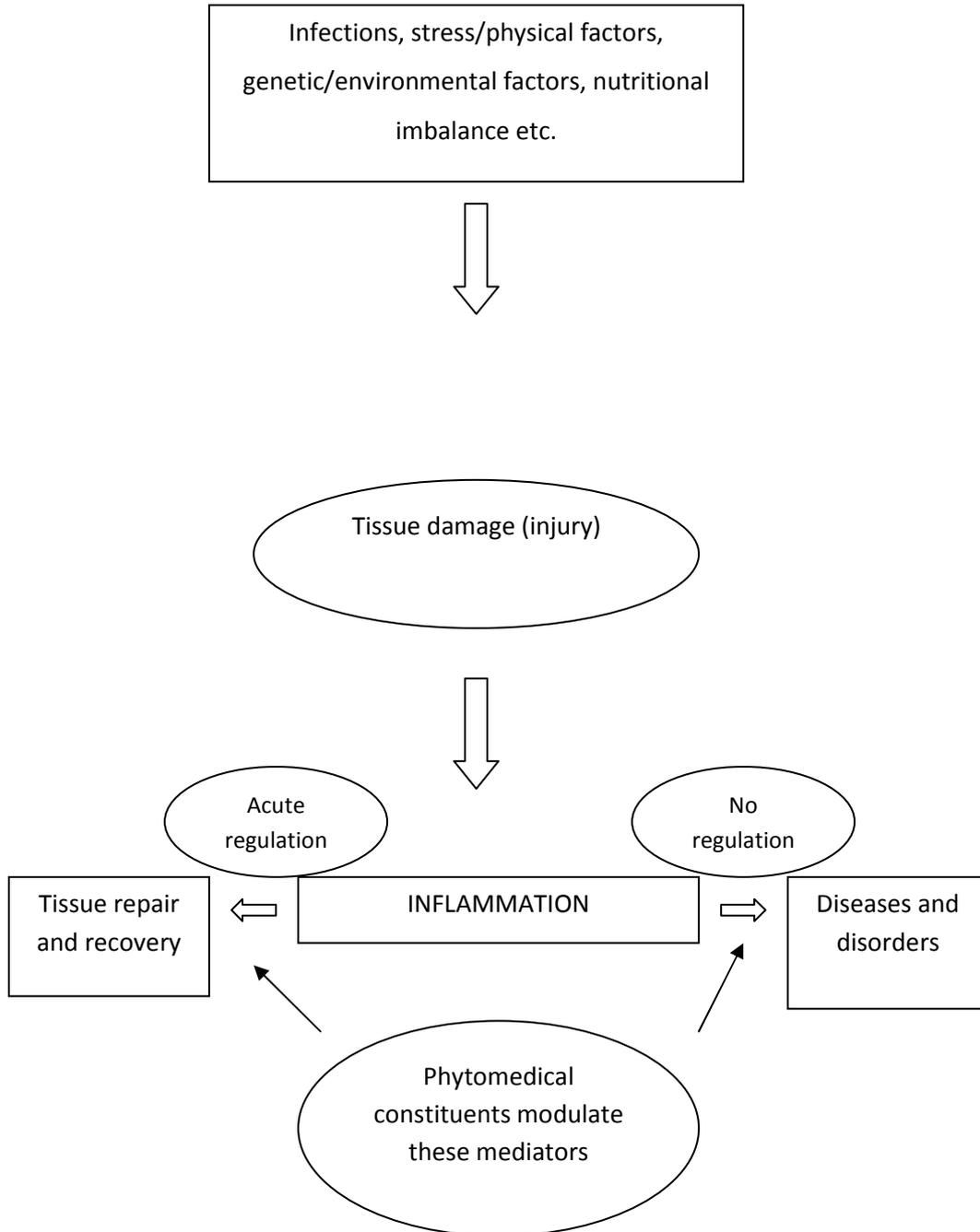
**Figure 1.1** A simplified diagram of the immune response to tissue injury or infection involving inflammatory cytokines (Holmes et al. 2003). Injury or infection stimulates the release of NF- $\kappa$ B from its inactive I- $\kappa$ B complex. NF- $\kappa$ B then leads to the transcription of pro-inflammatory (and anti-inflammatory) cytokines that activate endothelial cells causing increased production of NO via NO synthase. Cytokines also stimulate neutrophils to increase production of ROS, causing further tissue damage.

Other cytokines such as IL-2, IL-12, interferon gamma (IFN- $\gamma$ ) as well as IL-4, 5, 6 and 10 are also implicated in HIV infections (Babakhanian 1995). More recently, Alfano and Poli (2005) stated that IL-1 $\beta$ , IL-6, IL-12, IL-15, IFN- $\gamma$  and TNF- $\alpha$  are key pro-inflammatory cytokines involved in HIV infections. Abovementioned immunological factors need to be taken into consideration in the search and development of novel immune modulating drugs or plant substances.

## 1.2 Traditional medicines

In an African context, traditional medicines are of great importance, not so much as an alternative to treatment, but in many cases as the only source of treatment (Harnett et al. 2005). Epidemiological data suggest that the incidence of chronic inflammatory diseases such as atherosclerosis, diabetes, acquired immune deficiency syndrome (AIDS), asthma etc., is lower in patients who frequently consume fruits and vegetables (Ames et al. 1993; Choi et al. 2002; Chu et al. 2002). Some of the natural occurring substances from foods, herbs and other natural sources include plant phenolics, vitamins, carotenoids, phytoestrogens and terpenoids.

The latter have been shown to possess anti-inflammatory properties, which play an important role in disease prevention and immune promotion (Weisburger 2002). Various South African plants used medicinally possess phytochemical constituents that target certain mediators of inflammation and the immune system. Figure 1.2 represents the pathways of tissue damage and the involvement of phytomedicines in immune modulation (Iwalewa et al. 2007).



**Figure 1.2.** Pathways of tissue damage and the involvement of phytomedicines in immune modulation (Iwalewa et al. 2007).

Traditional herbal medicine is commonly used by HIV-infected patients (Fairfield et al. 1998). In African regions where patients do not have access or financial capability to obtain conventional antiretroviral treatment, traditional herbal medicines are used as primary treatment of HIV/AIDS and for other symptomatic HIV-related problems such as weakness, nausea, depression and insomnia (Babb et al. 2004; Peters et al. 2004). This is regardless of the fact that the safety, toxicity and efficacy of these products are not fully understood and that a risk for adverse effects exists. Nevertheless, due to the perceived beneficial effects of these plants, the Ministry of Health in South Africa promotes the use of these traditional medicines in combination with antiretroviral treatments (Mills et al. 2005). HIV/AIDS patients seek treatment from traditional healers who administer concoctions prepared from various medicinal plants (Bessong and Obi 2006). Because HIV weakens the immune system, making it susceptible to opportunistic infections, the beneficial effect of these plant-derived therapies could be due to their direct inhibitory effect on HIV, a boosting of the immune system, or inhibition of the opportunistic infection (Bessong and Obi 2006). South Africa has a great variety of medicinal plants and studies performed on water and organic extracts of South African medicinal plants, have demonstrated their inhibitory effects on HIV. *Terminalia sericea* Burch. Ex DC (Combretaceae), *Lebostemon trigonum* Buek. (Boraginaceae) and *Peltophorum africanum* Sond. (Fabaceae) showed inhibitory effects on the activity of the reverse transcriptase (RT) enzyme, reducing the ability of HIV to form complementary DNA (Bessong et al. 2004; Bessong et al. 2005; Harnett et al. 2005). African food plants which exert beneficial effects on immunity by modulating immunological factors include *Amaranthus spp* (Amaranthaceae), *Bidens pilosa* L. (Asteraceae), *Brassica spp.* (Brassicaceae), *Centella asiatica* (L.) Urb. (Apiaceae), *Cleome gynandra* L. (Cleomaceae), *Cucumis africanus* L. f. (Cucurbitaceae), *Pelargonium spp* (Geraniaceae), *Solanum nigrum* L. (Solanaceae) and *Urtica dioica* L. (Urticaceae) (Watt and Breyer-Brandwijk 1962; Kayser et al. 2001; Akbay et al. 2003; Lin et al. 2005; Lee and Kim 2006; Chang et al. 2007; Iwalewa et al. 2007; Jagetia and Aggarwal 2007; Narendhirakannan et al. 2007; Thejass and Kuttan 2007).

*Hypoxis hemerocallidea* Fisch & Mey. (Hypoxidaceae) as well as *Sutherlandia frutescens* L. R. Br. (Leguminosae) also have various effects on the immune system and due to claims about their immune boosting properties, are two of the most common African herbal compounds being used for HIV management in South Africa (SADC 2002; Mills et al. 2005).

### 1.2.1 *Hypoxis hemerocallidea*

*H. hemerocallidea* (Hypoxidaceae) is commonly known as the African potato or 'inkomfe' in Zulu (van Wyk et al. 1997; Mills et al. 2005) and is widely distributed in the grassland areas of South Africa especially the Eastern Cape, KwaZulu-Natal, Mpumalanga and Limpopo (van Wyk et al. 1997). As shown in Figure 1.3, it is easily recognizable by its bright yellow, star-shaped flowers and strap-like leaves (Mills et al. 2005; van Wyk 2008). The tuberous rootstock (corm) of the plant is dark brown on the outside, but yellow on the inside when freshly cut (Figure 1.4; van Wyk et al. 1997). It is used to prepare herbal teas or tinctures and is reported to be therapeutically effective at a daily dose of 2 400 mg raw plant (Albrecht et al. 1995). The traditional way to prepare *H. hemerocallidea* involves chopping the plant's corms and boiling for 20 min, corresponding to a daily dose of approximately 20 g in 250 ml of water (Nair and Kanfer 2006). Infusions of the corms are used by traditional healers to treat dizziness, bladder disorders and insanity (Pujol 1990; Hutchings 1996) as well as for strengthening tonics in children with wasting diseases (Watt and Breyer-Brandwijk, 1962). Decoctions are made with leaves to treat prostate problems (Pujol 1990). Traditionally, *H. hemerocallidea* has also been used to treat prostate and urinary tract disorders (Albrecht 1996). The South African press is currently advertising preparations containing *H. hemerocallidea* as an immune stimulant and the corms are also used to treat other immune-related diseases including arthritis, cancer, flu and the common cold (Mills et al. 2005). Indirect evidence has indicated that the sterols and sterolins in the corms of *H. hemerocallidea* have potential immunostimulatory activity (Bouic et al. 1996; 1999; Bouic 2001; Muwanga 2006).



**Figure 1.3.** *Hypoxis hemerocallidea* (van Wyk 2008)

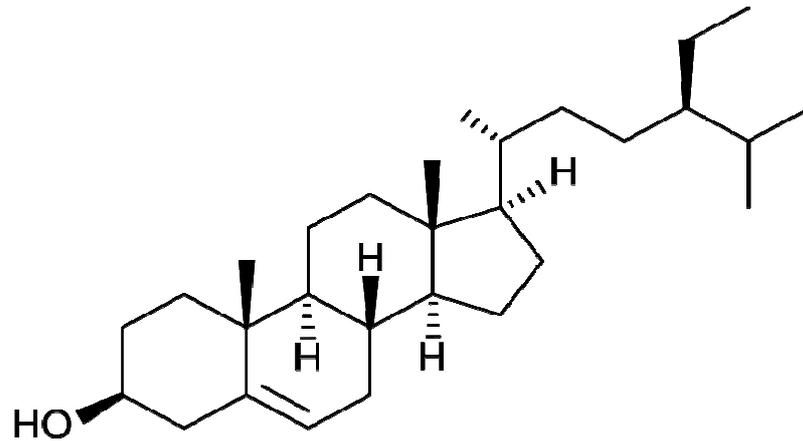


**Figure 1.4.** Corms of *Hypoxis hemerocallidea* (van Wyk et al. 1997)

The sterol found in *H. hemerocallidea*, beta-sitosterol (Figure 1.5), has been found to enhance human T cell proliferation (Bouic et al. 1996; 1999; Bouic 2001). Bouic et al. (1999) treated enriched phytohaemagglutinin (PHA) T-cells with various concentrations of beta-sitosterol and found a several-fold increase in the proliferative response. *In vivo* investigations on mononuclear cells obtained from volunteers who ingested tablets containing 20 mg of beta-sitosterol or placebo, 3 times daily for 4 weeks showed a 20 – 920% enhancement in the proliferation of PHA-stimulated T-cells from treated volunteers compared to baseline of placebo and treatment patients (Bouic et al. 1999).

Much attention has been given to the immune modulating properties of beta-sitosterol (Bouic 2002) and the use of a beta-sitosterol/beta-sitosterolglucoside (BSS/BSSG) mixture (ratio 100:1) has been found to balance the Th1-Th2 cell function which is affected during HIV infection. An open-labeled study in HIV-infected patients which were treated with only the sterol mixture and no anti-retroviral treatment revealed that patients who entered the trial with CD4<sup>+</sup> cell counts >500 cells/ $\mu$ l blood, showed a significant ( $p < 0.05$ ) decrease in plasma viral loads and stable CD4<sup>+</sup> cell counts after the 40 month treatment period (Bouic et al. 2001).

Aqueous extracts of *H. hemerocallidea* (50-800 mg/kg p.o.) have been shown to significantly ( $p < 0.05-0.001$ ) inhibit fresh egg albumin-induced acute inflammation in rats (Ojewole 2006). Furthermore, Gaidamashvili and van Staden (2006) reported that lectin-like proteins isolated from the aqueous extracts of *H. hemerocallidea* can inhibit cyclooxygenase (COX) enzyme which regulates prostaglandin synthesis *in vitro*, lending further support to the anti-inflammatory effects of this plant.



**Figure 1.5.** Chemical structure of beta-sitosterol

### 1.2.2 *Sutherlandia frutescens*

*S. frutescens* (cancer bush) is a shrub belonging to the pea family (Fabaceae) which has attractive red flowers and leaves which are slightly hairy, sometimes giving the plant a silvery appearance (van Wyk et al. 1997). The flowers of *S. frutescens*, which are followed by swan shaped balloon-like, papery pods, has led to the Afrikaans name, *gansies* (Figure 1.6; van Wyk et al. 1997; van Wyk and Gericke 2000).

*S. frutescens* is widely distributed in South Africa, Botswana and Namibia and has even become a popular garden plant (Schrire and Andrews 1992; van Wyk et al. 1997). The leaves are commonly used, but other aerial parts of the plant can also be included to make strong decoctions or alcoholic tinctures (van Wyk et al. 1997). Its medicinal use originated with the Khoi and Nama people who used it to treat a variety of ailments which include cancer, tuberculosis, diabetes, fatigue, influenza, rheumatoid arthritis, osteoarthritis, peptic ulcers, gastritis, reflux oesophagitis, menopausal symptoms, anxiety and clinical depression as well as piles and inflammation (van Wyk et al. 1997; Gericke et al. 2001; Dalvi 2003; Tai et al. 2004).

This plant contains, amongst others, *L*-canavanine, gamma-aminobutyric acid (GABA), which is an amino acid, and *D*-pinitol (Mills et al. 2005). *L*-canavanine (Figure 1.7) is an arginine metabolite and has been reported to have anti-viral activity against influenza and retroviruses, including HIV (Green 1988). Together with its metabolite, canaline, canavanine has been reported to exert antitumor properties, making it one of the likely factors contributing to the anticancer effect of *S. frutescens* (Stander et al. 2007). GABA (Figure 1.8) is an inhibitory neurotransmitter and could therefore be a contributing factor to the use of *S. frutescens* for anxiety and stress (van Wyk and Gericke 2000). *D*-pinitol (Figure 1.9) is a unique sugar; suggested for treating wasting in cancer and AIDS patients (Oslund and Sherman 1996).



**Figure 1.6.** Flowers and fruits of *Sutherlandia frutescens* (van Wyk et al. 1997)

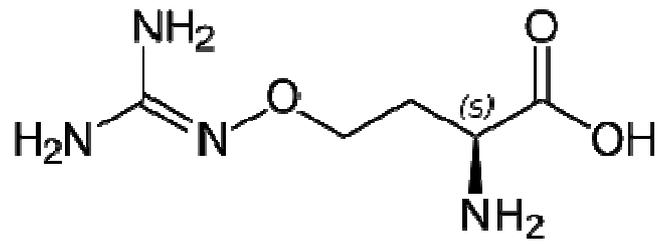


Figure 1.7. Chemical structure of canavanine

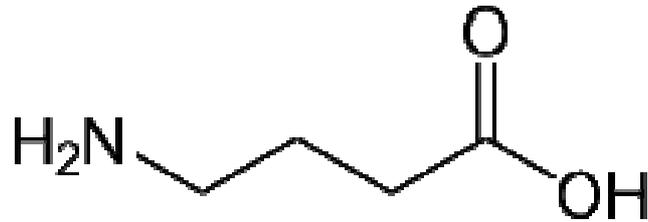


Figure 1.8. Chemical structure of GABA

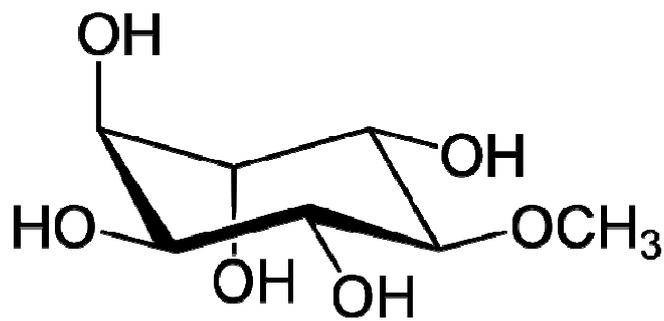


Figure 1.9. Chemical structure of pinitol

The recommended therapeutic dose of dried *S. frutescens* leaves is 9 mg/kg/day (Seier et al. 2002). *S. frutescens* has been reported to increase CD4<sup>+</sup> counts, decrease viral loads, increase appetite and weight gain as well as to generally improve the mood of these patients (Chaffy and Stokes 2002). Ojewole (2004) reported the analgesic, anti-inflammatory and hypoglycemic effects of aqueous *S. frutescens* extracts in mice, lending some scientific support to the folklore use of this plant in the management of painful or inflammatory as well as diabetic conditions.

Fernandes et al. (2004) investigated the antioxidant potential of hot water extracts of *S. frutescens* and suggested that the superoxide and hydrogen peroxide scavenging activity of these extracts could contribute to anti-inflammatory properties of this plant, but the exact mechanism by which *S. frutescens* exerts its effects on the immune system is unknown. Harnett et al. (2005) reported that the anti-HIV activity of *S. frutescens* could be partly attributed to its inhibitory effect against HIV target enzymes. A South African company investigated the effects of this herbal remedy on the health of HIV/AIDS patients (Gericke et al. 2001), however results are inconclusive. Anecdotal reports made by clinicians, state that treatment of HIV/AIDS patients with *S. frutescens*, resulted in weight gain, better appetites and even improvements in CD4<sup>+</sup> counts and decreased viral loads (Chaffy and Stokes 2002).

### 1.3 Study Aim

To investigate the immunomodulatory properties of *Sutherlandia frutescens* and *Hypoxis hemerocallidea*.

### 1.4 Study Objectives

#### Using U937 macrophages:

1. Determine the *in vitro* cytotoxicity of *Sutherlandia frutescens* and *Hypoxis hemerocallidea* (crude extracts as well as standards of compounds reputedly isolated from these plants) using the MTT assay.
2. Determine antioxidant capacity of the various extracts and compound standards using ORAC and TEAC antioxidant assays.
3. Determine cytokine expression in treated U937 macrophages using Cytometric Bead Array (CBA) kits.
4. Determine PGE<sub>2</sub> (pro-inflammatory prostaglandin) production of U937 cells *in vitro* using ELISA.

#### Using the THP-1 macrophage cell line:

1. Determine the *in vitro* cytotoxicity of *Sutherlandia frutescens* and *Hypoxis hemerocallidea* (crude extracts as well as standards of compounds reputedly isolated from these plants) using the MTT assay.
2. Determine antioxidant capacity of the various extracts and compound standards using ORAC and TEAC antioxidant assays.
3. Determine cytokine expression in treated THP-1 cells using Cytometric Bead Array (CBA) kits.
4. Determine PGE<sub>2</sub> (pro-inflammatory prostaglandin) production of THP-1 cells *in vitro* using ELISA.

## CHAPTER 2: CYTOTOXICITY ASSESSMENT

### 2.1 Introduction

In South Africa alone, approximately 27 million people rely on traditional herbal medicine for their primary health care needs (Mander 1998). Due to greater accessibility and affordability of herbal medicines, they have become an important part of African culture and there is an increasing trend internationally, to integrate traditional herbal medicine with primary, pharmacological healthcare (Mander 1998; Fennell et al. 2004). Information and knowledge obtained from traditional healers helps scientists identify and target plants with the potential of being medicinally useful and various *in vitro* and *in vivo* assays are then performed to determine biological activity and toxicity (Cox and Balick 1994). As the prescription of traditional medicine in South Africa is not well-regulated, there are chances of misadministration and toxicity (Fennell et al. 2004). Factors such as correct identification, methods of collection, processing and storage, undesirable contaminants or adulterations as well as contamination of water sources and natural habitats, make the safety of plant medicines questionable (Tadmor et al. 2002; Street et al. 2008). Despite a paucity of evidence on the efficacy and safety of *Hypoxis hemerocallidea* and *Sutherlandia frutescens*, the South African Ministry of Health recommends the use of these herbal remedies for the management of HIV and associated symptoms (SADC 2002; Mills et al. 2005).

Among the various cytotoxicity assays used in *in vitro* toxicological studies, the lactate dehydrogenase (LDH) leakage assay and methyl tetrazolium (MTT) assay are the two most commonly used to determine cell viability after exposure to test substances (Fotakis and Timbrell 2006).

A study comparing the sensitivity of LDH, neutral red, MTT and protein assay in hepatoma cells after exposure to cadmium chloride showed that the neutral red and MTT assays were the most sensitive in detecting cytotoxic events (Fotakis and Timbrell 2006).

The MTT assay was selected for determination of cytotoxicity of test compounds due to easy accessibility and availability in our laboratories as well as familiarity with the standard tetrazole assay procedures. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyl tetrazolium bromide] is a pale yellow, water-soluble salt which is converted to a water-insoluble highly coloured purple formazan in the mitochondria of living cells. The formazan crystals cannot permeate the cell membrane and accumulate inside healthy cells (Mossman 1983). The accumulation of formazan is directly correlated to the activity of the mitochondria, giving a linear response between cell number or activity to the colour intensity.

Macrophages and monocytes represent a suitable model for investigating immunomodulating activity of potential immunomodulators, because they are involved in several critical functions and processes of the immune system (Gingras and Margolin 2000; Kinne et al. 2000). The human myelomonocytic leukemia cell lines, U937 and THP-1 differentiate to form mature monocyte/macrophage cells upon exposure to different substances (Gingras and Margolin 2000) and were thus selected for *in vitro* cytotoxicity investigations. Testing the cytotoxicity of herbal extracts *in vitro* gives a better understanding of their toxicity profile and may be helpful to enhance their effectiveness and/or predict any undesirable effects.

## 2.2 Materials and Methods

### 2.2.1 Plant material

*Sutherlandia frutescens* leaves were a gift from Prof. Carl Albrecht, CANSA (Cape Town). Corms of *Hypoxis hemerocallidea* were collected from the gardens of the Research Centre for Plant Growth and Development, University of KwaZulu-Natal and a voucher specimen is deposited at this institution.

### 2.2.2 Preparation of extracts

One kilogram of the leaves of *S. frutescens* and corms of *H. hemerocallidea* were air-dried and coarsely ground with a Wiley laboratory mill (Arthur Thomas Co. Philadelphia, USA). The dried plant material was ground to a finer powder using a Yellowline laboratory mill (Merck, USA). Water extracts were prepared by adding 25 g of powdered plant material to 250 ml of distilled water. Cold water was used in order to avoid degradation of any potential antioxidants present in the plants.

The mixture was sonicated in a water bath for 30 min and allowed to stand overnight at 4°C, after which it was centrifuged (400 x g, 15 min), filtered (0.45 µm, Millipore) and filter-sterilized (0.22 µm, Millipore). The yields were determined gravimetrically and were 16 mg/ml and 19 mg/ml for *S. frutescens* and *H. hemerocallidea*, respectively. Extracts were diluted with dH<sub>2</sub>O to the desired concentrations of 0.1 – 1.6 mg/ml for *S. frutescens* and 0.1 – 1.9 mg/ml for *H. hemerocallidea* for use in cytotoxicity determination.

### 2.2.3 Compound standards

Gamma-aminobutyric acid (GABA), *D*-pinitol, *L*-canavanine, present in *S. frutescens* were purchased from Sigma Aldrich and beta-sitosterol found in *H. hemerocallidea* was purchased from Merck.

The compounds were dissolved in distilled water or ethanol (beta-sitosterol) and filter sterilized before use in experiments. Test concentrations for GABA, pinitol and canavanine were 1.5 – 200  $\mu$ M and 0.2 – 25  $\mu$ M for beta-sitosterol. The final concentration of ethanol was 0.5%. The known antioxidant and immunomodulator, curcumin, was purchased from Merck and was used as positive control. The curcumin stock solution was prepared in ethanol (100 mM) and diluted with distilled water to the test concentrations of 0.8 to 25  $\mu$ M.

### 2.2.4 Cells and cell culture

THP-1 (ATCC TIB 2.2) and U-937 (ATCC CRL 1593) are macrophage cell lines used to study monocyte and macrophage activity. Fetal calf serum (FCS), RPMI culture medium and phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma Aldrich. THP-1 and U937 cells were cultured in RPMI+ medium, at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. Cells were maintained in 50 ml, 25 cm<sup>2</sup> culture flasks and RPMI+ medium was replaced every 3 days. Before the experiment, cells were washed in serum-free RPMI-1640 medium and resuspended in RPMI-1640 supplemented with 10% heat-inactivated FCS and antibiotics (1% penicillin/streptomycin). Cells were added to each well of a microtitre plate at a concentration of 1 x 10<sup>5</sup> cells/well. The cells were stimulated to differentiate into adherent cells by incubating with 0.1  $\mu$ g/mL PMA at 37 °C for 48 h.

### 2.2.5 Cytotoxicity

One hundred microlitres of the cell suspension ( $1 \times 10^5$  cells/well) was added to 60  $\mu$ l of complete RPMI 1640 medium in the wells of 96 well microtiter culture plates. Twenty microlitres of PMA (0.1  $\mu$ g/ml final concentration) was added to the wells and the plate incubated for 48 h.

Twenty microlitres of the various concentrations of curcumin, *S. frutescens* or *H. hemerocallidea* solutions as well as the compound standards was added to the wells. The plates were incubated for 72h at 37°C in a 5% CO<sub>2</sub> atmosphere.

Cell viability was assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyl tetrazolium bromide (MTT) assay as described by Mossman (1983). After addition of MTT, the plates were incubated for 4 h, centrifuged (800 x g, 10 min) and the supernatant removed by aspiration. Plates were washed with phosphate buffered saline (PBS) and again centrifuged. The supernatant was discarded and the plates allowed to dry.

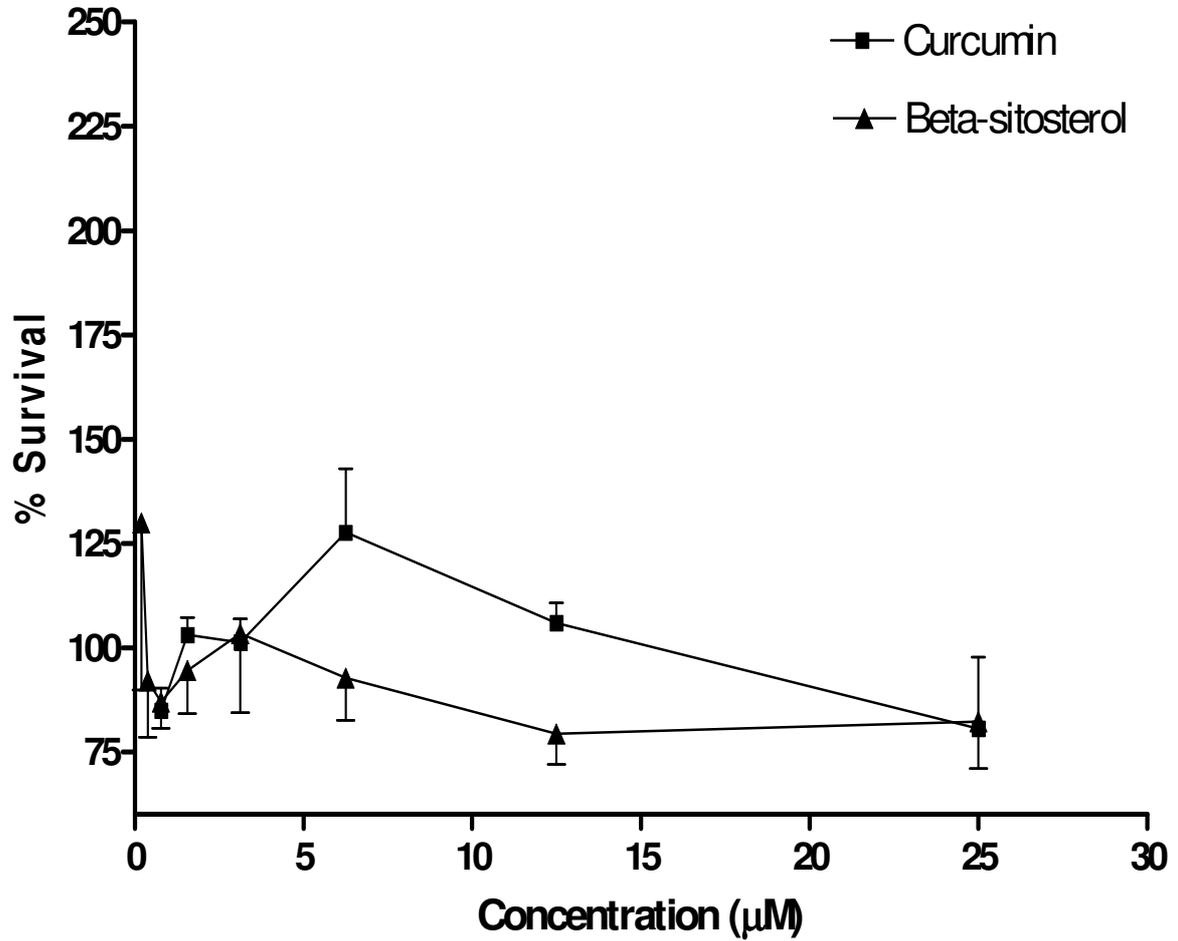
The formazan was solubilised in 100  $\mu$ l of dimethyl sulfoxide (DMSO) and the plates then read on a microplate reader (Bio-Tek Instruments, Inc.) using a test wavelength of 540 nm and a reference wavelength of 620 nm. Three independent experiments were performed in triplicate wells. Survival curves were generated using the Prism GraphPad 4 statistics computer software program.

## 2.3 Results

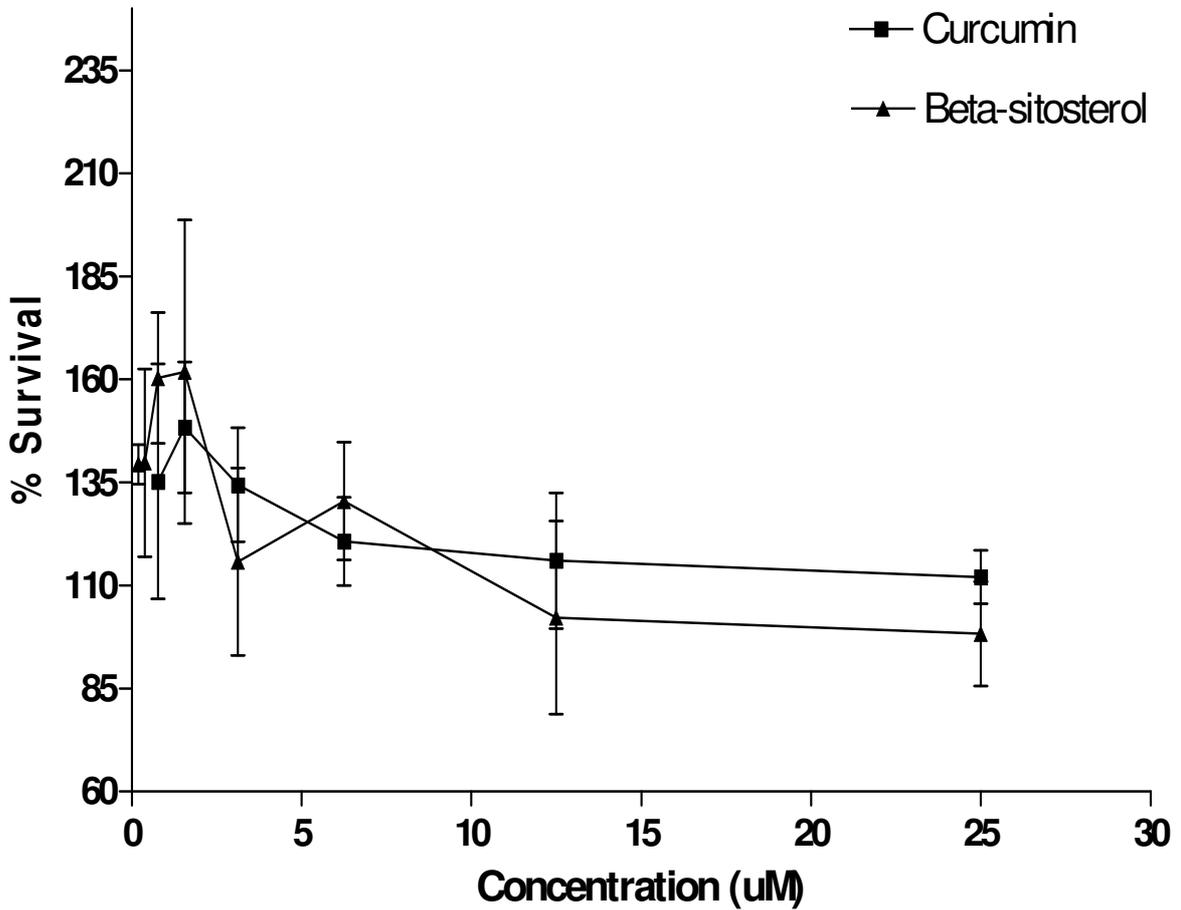
Both curcumin (positive control) and beta-sitosterol showed a slight inhibition (maximum 20%) of THP-1 cell growth at concentrations of 0.25  $\mu$ M (Figure 2.1). No cytotoxicity could be detected in U-937 cells, but stimulation of cell growth  $\leq$ 60% could be seen at most concentrations of curcumin and beta-sitosterol tested (Figure 2.2). *H. hemerocallidea* aqueous extract showed no cytotoxicity in THP-1 cells, but rather stimulated macrophage proliferation by 20-70% (Figure 2.3).

*S. frutescens* aqueous extract caused growth inhibition in THP-1 cells at concentrations <0.5 mg/ml, but stimulated cell growth by approximately 20% at concentrations >0.75 mg/ml (Figure 2.3).

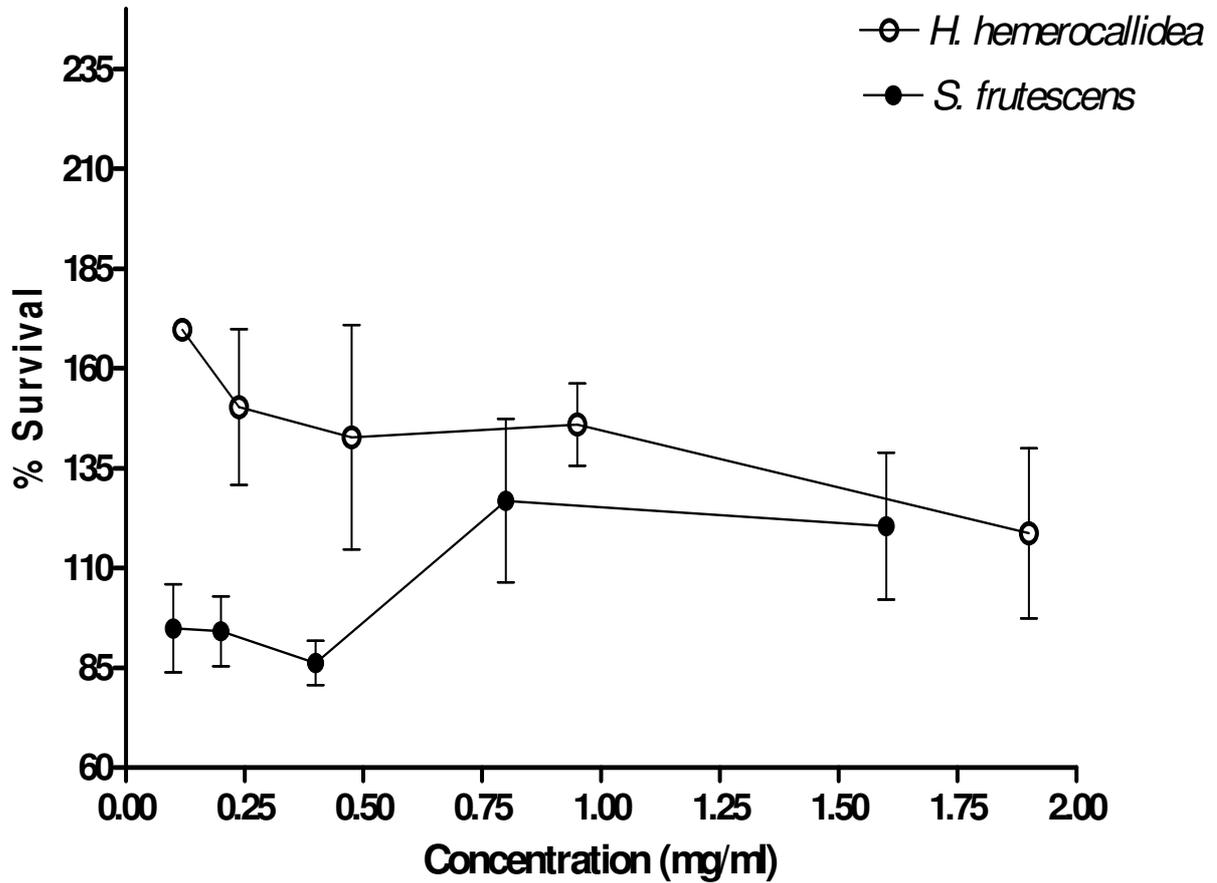
*H. hemerocallidea* caused no inhibition of U937 cell growth, but rather stimulation of up to 75% (Figure 2.4). *S. frutescens* was also not cytotoxic in U937 cells (Figure 2.4). Canavanine, pinitol and GABA showed no cytotoxicity in THP-1 cells (Figure 2.5), nor in U937 cells (Figure 2.6).



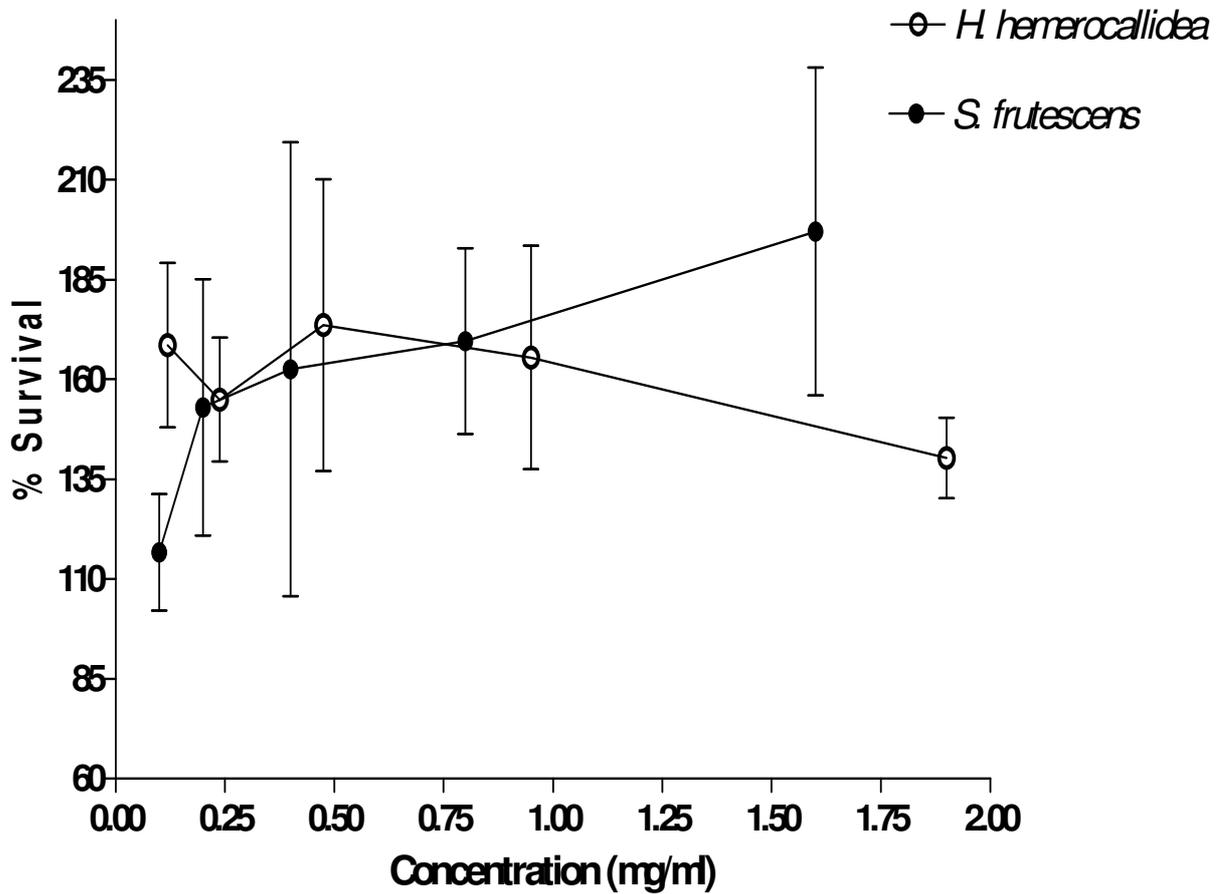
**Figure 2.1.** Survival curve of THP-1 cells ( $1 \times 10^5$ ) treated with varying concentrations of curcumin or beta-sitosterol compared to untreated control cells that had also undergone differentiation treatment with PMA for 48 h. The cells were incubated in the presence of the various compounds for 72 h.



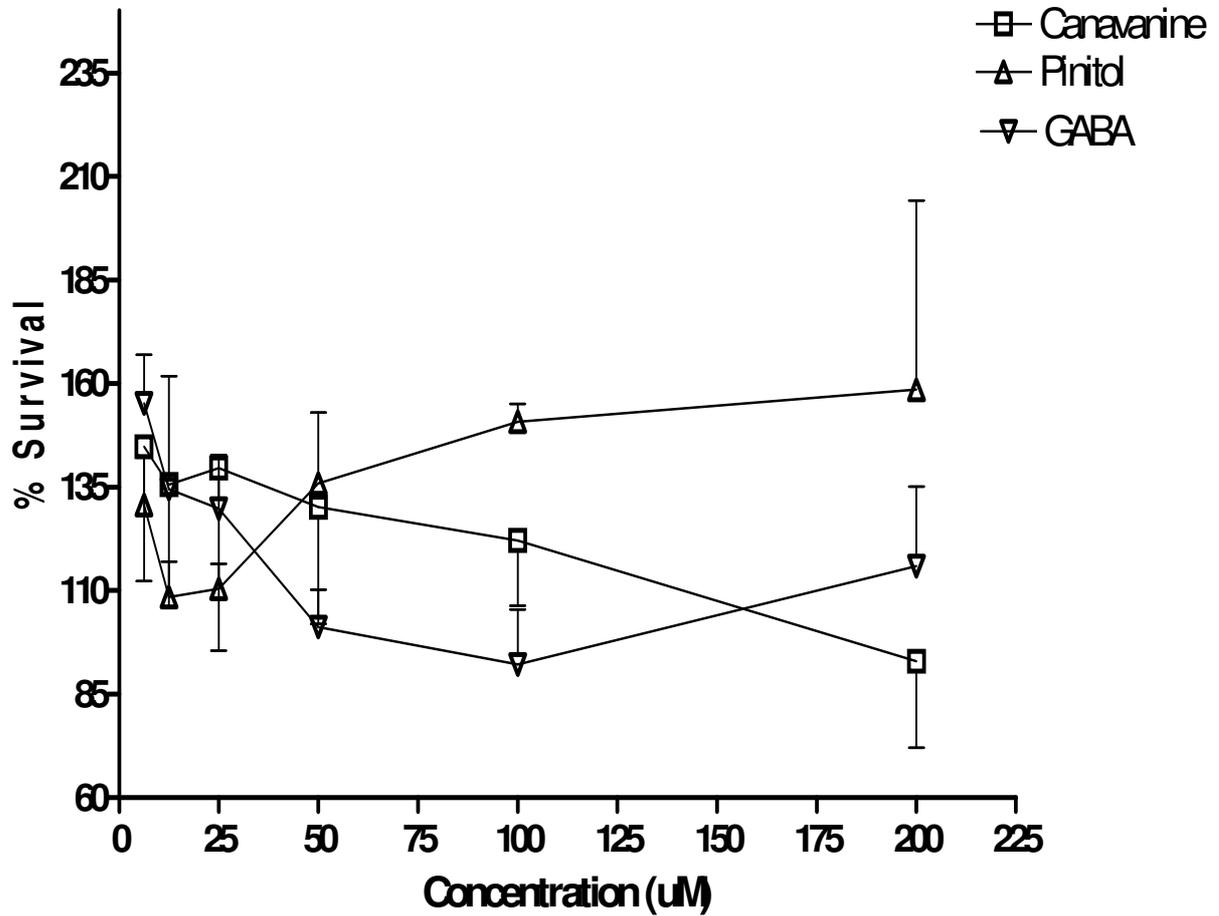
**Figure 2.2.** Survival curve of U-937 cells ( $1 \times 10^5$ ) treated with varying concentrations of curcumin or beta-sitosterol compared to untreated control cells that had also undergone differentiation treatment with PMA for 48 h. The cells were incubated in the presence of the various compounds for 72 h.



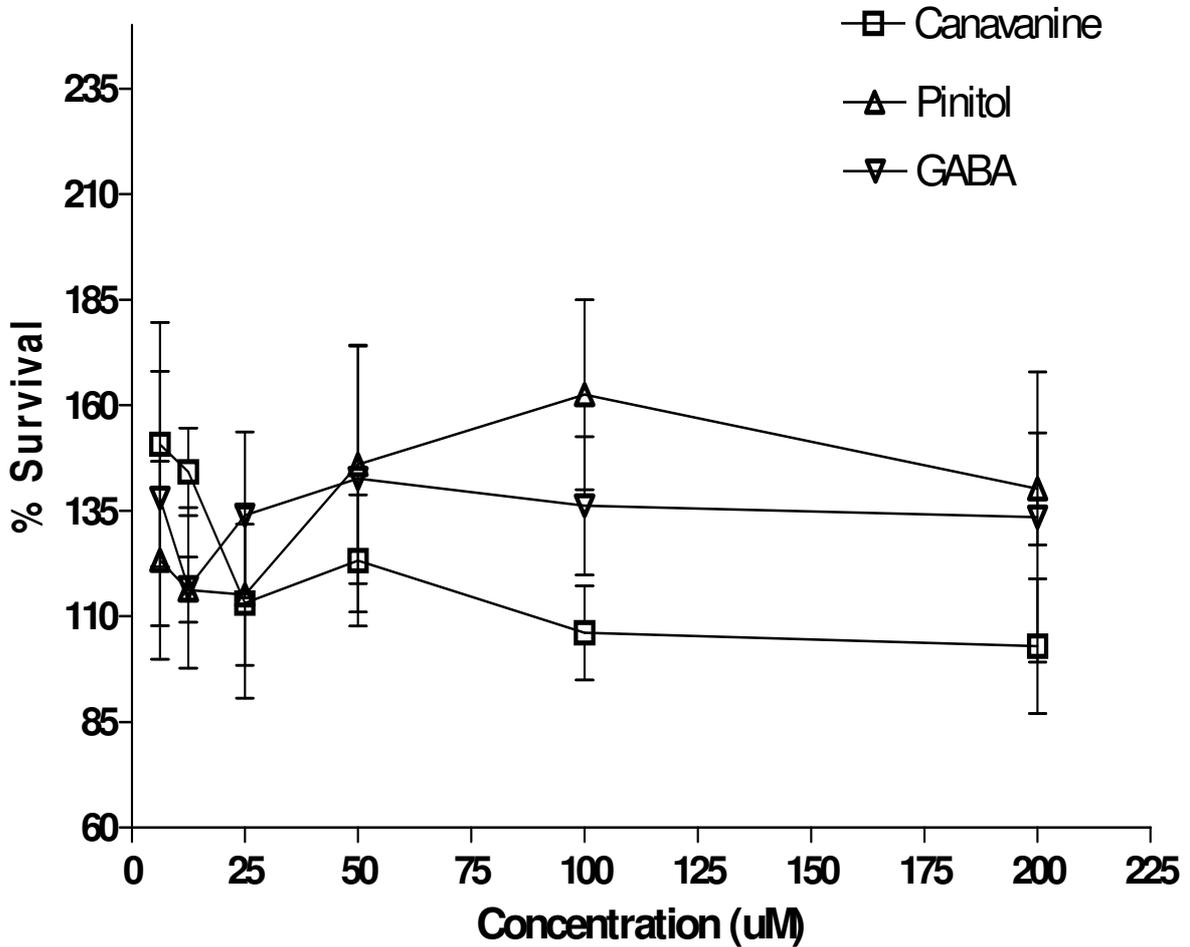
**Figure 2.3.** Survival curve of THP-1 cells ( $1 \times 10^5$ ) treated with varying concentrations of *S. frutescens* or *H. hemerocallidea* compared to untreated control cells that had also undergone differentiation treatment with PMA for 48 h. The cells were incubated in the presence of the various compounds for 72 h.



**Figure 2.4.** Survival curve of U-937 cells ( $1 \times 10^5$ ) treated with varying concentrations of *S. frutescens* or *H. hemerocallidea* compared to untreated control cells that had also undergone differentiation treatment with PMA for 48 h. The cells were incubated in the presence of the various compounds for 72 h.



**Figure 2.5.** Survival curve of THP-1 cells ( $1 \times 10^5$ ) treated with varying concentrations of Canavanine, Pinitol or GABA compared to untreated control cells that had also undergone differentiation treatment with PMA for 48 h. The cells were incubated in the presence of the various compounds for 72 h.



**Figure 2.6.** Survival curve of U-937 cells ( $1 \times 10^5$ ) treated with varying concentrations of Canavanine, Pinitol or GABA compared to untreated control cells that had also undergone differentiation treatment with PMA for 48 h. The cells were incubated in the presence of the various compounds for 72 h.

## 2.4 Discussion

*S. frutescens* has a long history of use in Africa (Mills et al. 2005). The effective dose (2.5 – 5 g of dried leaves) is considered to be safe and use of 5 g daily, for 6 years produced no side effects (van Wyk and Albrecht 2008). A study using fasted BALB/c mice found the lethal dose (LD<sub>50</sub>) of *S. frutescens* to be 1280±71 mg of extract per kg (Ojewole 2004). Suppliers of commercial preparations of *S. frutescens* recommend a 300 mg tablet twice daily (van Wyk and Albrecht 2008). Seier et al. (2002) investigated the effects of consumption of a dose 1, 3 and 9 times higher than the recommended dose of 9 mg/kg/day on vervet monkeys. They concluded that at the highest dose (9 times the recommended dose), no clinical, haematological or physiological toxicities were evident.

A phase I clinical study which involved the treatment of 25 healthy adults with 800 mg of *S. frutescens* leaf powder per day showed no side effects or toxicities during or after the 3 month trial period (Johnson et al. 2007). Steenkamp and Gouws (2006) tested aqueous extracts of *S. frutescens* (at a final concentration of 50 µg/ml) on various cancer cell lines and found that it stimulated the growth of the normal transformed MCF-12A and the metastatic cancer MDA-MB-231 breast cell lines. Canavanine has been linked to systemic lupus erythematosus syndrome as a potential causative agent (Capasso et al. 2000). As canavanine is an arginine analogue, it may be incorporated into proteins after long term usage, resulting in autoimmunity (Prete 1985). Canavanine has been shown to inhibit growth of L1210 murine leukemic cells as well as rat colon tumours (Green et al. 1980; Thomas et al. 1986).

From the *in vitro* cytotoxicity investigations it is evident that neither of the test substances were cytotoxic at the concentrations tested, but rather that stimulation of cell growth took place. Testing the substances at higher concentrations would not be clinically applicable, because administration of recommended daily dosages of plant substances cannot produce higher concentrations in the blood.

Commercially available tablets of *S. frutescens* contain 3 mg/g of canavanine and 0.4 mg/g of GABA (Tai et al. 2004). Based on physiological blood volumes, this would translate to a blood concentration of approximately 0.3 µg/ml and 0.04 µg/ml, respectively, assuming the compounds are instantly absorbed. No cytotoxicity was observed after testing *S. frutescens* extract at a dose of 1.6 mg/ml in an *in vitro* setting which is 20 times higher than the maximum concentration that can be absorbed.

Results of the present study are confirmed by the study of Steenkamp and Gouws (2006) which also reported stimulation of cell growth by *S. frutescens*. Canavanine, pinitol and GABA were tested at concentrations much higher than physiologically achievable (with recommended daily intake) but still showed no toxicity. Although canavanine has been shown to have cytotoxic activity against various tumour cell lines (Green et al. 1980; Thomas et al. 1986), results from the present study did not confirm those findings and showed that canavanine did not inhibit, but in fact stimulated the growth of THP-1 as well as U937 cells.

Regarding the cytotoxicity of *H. hemerocallidea*, Steenkamp and Gouws (2006) reported that the aqueous extracts of this plant inhibited growth of MCF-12A and MCF-7 cell lines, but stimulated growth of DU-145 cells *in vitro*. A case report of an ischaemic heart disease patient who presented with ventricular tachycardia after chronic infusion of *H. hemerocallidea*, suggests cardiotoxic effects of certain components in *H. hemerocallidea* (Ker 2005). Beta-sitosterol has been shown to be effective in the treatment of ailments such as rheumatoid arthritis, allergic rhinitis and sinusitis without any concerns about safety or toxicity (Myers 1998; Louw 2002; Nair 2006). The safety of plant sterols is further confirmed in that phytosterols are present in many fruits and vegetables consumed daily (Nair 2006). In a clinical study involving 2400 patients treated with 25 g/day of phytosterols and phytostanols, no adverse effects were observed and a series of studies conducted over 4 years showed complete safety of phytosterol consumption in animal and human models (Allayee et al. 2000; Berge et al. 2000).

*H. hemerocallidea* is reported to be effective at a daily intake of 2400 mg of raw plant material (Albrecht et al. 1995). This plant has been found to possess a maximum of 9 mg beta-sitosterol per 100g of *H. hemerocallidea* (Nair and Kanfer 2008). If 2400 mg of *H. hemerocallidea* is consumed daily, it would result in an intake of only 0.2 mg of beta-sitosterol. Based on physiological blood volumes, only 0.03 µg of beta-sitosterol per ml of blood could be attained.

In this study, the highest test concentration of *H. hemerocallidea* (1.9 mg/ml) and beta-sitosterol (25 µM) did not show any cytotoxicity, suggesting that taking 2400 mg of *H. hemerocallidea* daily should not produce adverse effects in humans. *H. hemerocallidea* caused stimulation of THP-1 and U937 cell growth, similar to the findings of Steenkamp and Gouws (2006) where stimulation of DU-145 cells was reported. Beta-sitosterol, when consumed as *H. hemerocallidea*, produces such low concentrations in the blood, that the effect of this compound would be negligible and no toxicity should be expected from the beta-sitosterol. The finding that beta-sitosterol caused a maximum of 20% inhibition in THP-1 cell growth and no inhibition in U937 cells, supports previous studies concerning the safety of phytosterols in immune cells.

Curcumin (or diferuloylmethane) is a compound found in *Curcuma longa* (turmeric) and has reputed anti-inflammatory, anti-oxidant as well as immunomodulatory properties (Jagetia and Aggarwal 2007; Itokawa et al. 2008). Present findings show that curcumin caused a maximum of 20% inhibition of cell growth and was not considered to be cytotoxic even at concentrations above physiological levels (Figures 2.1 and 2.2). It can be seen that the effects of the extracts of *S. frutescens* and *H. hemerocallidea* and their compound standards tested in the present study are comparable to curcumin (positive control).

## 2.5 Conclusion

*In vitro* investigations showed that water extracts from *H. hemerocallidea* and *S. frutescens* as well as compound standards isolated from these plants show no cytotoxicity, even at test concentrations much higher than recommended for daily intake.

## CHAPTER 3: DETERMINATION OF ANTIOXIDANT CAPACITY

### 3.1 Introduction

There has been much research investigating antioxidant properties of natural compounds such as fruits, vegetables, herbs, spices and teas. Studies suggest that the natural antioxidants found in fruits and vegetables lower oxidative stress and decrease free radical production, reducing the risk for degenerative diseases (Chen 2007).

A free radical is an atom or molecule with an unpaired electron and tends to be unstable and highly reactive (Yoshikawa et al. 2007). Free radicals or reactive oxygen species (ROS) are involved in various biological reactions, starting with the abstraction of an electron from a stable molecule which creates a new free radical and continues as a chain reaction (Chen 2007). The most-studied of the free radical chain reactions is that of lipid peroxidation, which compromises cell membranes and causes irreversible damage to cells (Yoshikawa et al. 1997). Other free radicals such as the superoxide anion and the hydroxyl radical, react with biological macromolecules and also induce tissue damage (Chen 2007). Alternative mechanisms by which free radicals and ROS are involved in tissue damage and pathogenesis of diseases include DNA damage, inactivation of nitric oxide (NO) and oxidation of LDL (low density lipoprotein) (Chen 2007). Free radicals do not always have negative effects, in fact they are produced within the human body and do have beneficial effects, as long as they do not exceed the capacity of the antioxidant defence systems (Chen 2007). When free radicals or ROS levels increase to a level intolerable to the body's defence system, oxidative stress increases, contributing to the pathogenesis of many human diseases (Chen 2007).

During infection or inflammation, there is an increase in the levels of the nitric-oxide synthase (NOS) and cyclooxygenase (COX) enzymes which lead to increased production of pro-inflammatory NO and prostaglandins (PGs) (Iwalewa et al. 2007). Oxygen free radicals and non-radical ROS are released by neutrophils and other phagocytes during phagocytosis of bacteria or foreign particles, resulting in the simultaneous destruction of these immune cells (Iwalewa et al. 2007). It is therefore critical to assess the role of antioxidant activity when investigating immunomodulatory properties of plant extracts.

Since a standardized method for the determination of antioxidant capacity does not exist, the use of more than one assay at a time is recommended in order to obtain a comprehensive assessment of antioxidant potency (Prior et al. 2005). Antioxidant assays differ in terms of assay principles and experimental conditions, causing specific antioxidants to have varying contributions to the total measured antioxidant capacity (AOC) of a test sample (Cao and Prior 1998). In this study, the Trolox equivalence antioxidant capacity (TEAC) and oxygen radical antioxidant capacity (ORAC) assays were selected for assessment of antioxidant capacity. The TEAC assay, as described by Re et al. (1999), is based on the comparison of the capacity of a sample to reduce the highly coloured ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] radical (ABTS•+) relative to a water soluble reference antioxidant standard, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Trolox is a potent antioxidant which promotes the reduction of the ABTS radical. When Trolox is added to a solution of preformed ABTS radical, there is a reduction in the colour and therefore absorbance of the solution. There is thus a linear quantitative relationship between the reduction in absorbance and the apparent concentration of antioxidant present in the sample.

The ORAC assay, as described by Ou et al. (2001), uses fluorescein as a fluorescent probe and AAPH [2,2'-azobis (2-amidinopropane) dihydrochloride] as the free radical. When AAPH is added to fluorescein, it leads to oxidative degeneration of the fluorescent molecule, which leads to a decrease in fluorescence.

Addition of an antioxidant delays the degeneration of fluorescein. Trolox is used as a standard and antioxidant activities of test samples are compared to a Trolox standard curve.

Quantification of the antioxidant properties of a herbal extract and remedies compared to the extraction method or mass of extract could provide a better insight to its therapeutic mechanism.

## 3.2 Materials and Methods

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2,2-azobis(2- methyl propanimidamide) dihydrochloride (AAPH), potassium persulfate ( $K_2S_2O_8$ ) and fluorescein sodium salt were purchased from Sigma Aldrich.

### 3.2.1 TEAC assay

The ABTS radical was generated by chemical reaction with potassium persulfate ( $K_2S_2O_8$ ). Twenty five millilitres of ABTS (175  $\mu$ moles) was spiked with 440 $\mu$ l of  $K_2S_2O_8$  solution (45  $\mu$ moles) and allowed to stand in the dark overnight (12 - 16 h), giving it enough time for formation of the ABTS radical. The working solution was prepared by diluting the stock solution with dH<sub>2</sub>O to an absorbance at  $\lambda = 734$  nm of  $0.70 \pm 0.02$  (Perkin-Elmer UV/Vis Lambda 2 spectrophotometer). Test solutions of *S. frutescens*, *H. hemerocallidea*, canavanine, pinitol and GABA were prepared in distilled water (1 mg/ml). Curcumin and beta-sitosterol were made up to 1 mg/ml in ethanol, as they were not water-soluble at the desired concentrations. Two millilitres of the ABTS radical solution was added to the measuring cuvette and the absorbance ( $A_0$ ) measured. Thereafter, 20  $\mu$ l sample or standard was added, solutions mixed and the absorbance measured again. Any antioxidants present in the sample would reduce the radical, causing a reduction in the measured absorbance.

A linear Trolox standard curve was created for a concentration range of 0.065 – 1 mg/ml (0.025 - 0.4 mM). The percentage radical reduction obtained for the sample was compared to this calibration curve to calculate the antioxidant concentration in terms of Trolox concentrations ( $\mu\text{M}$  or mg/ml TE). Three independent experiments were performed and each sample was analysed in triplicate. Mean and SEM (standard error of the mean) values were determined using Prism GraphPad 4 statistics software.

### 3.2.2 ORAC assay

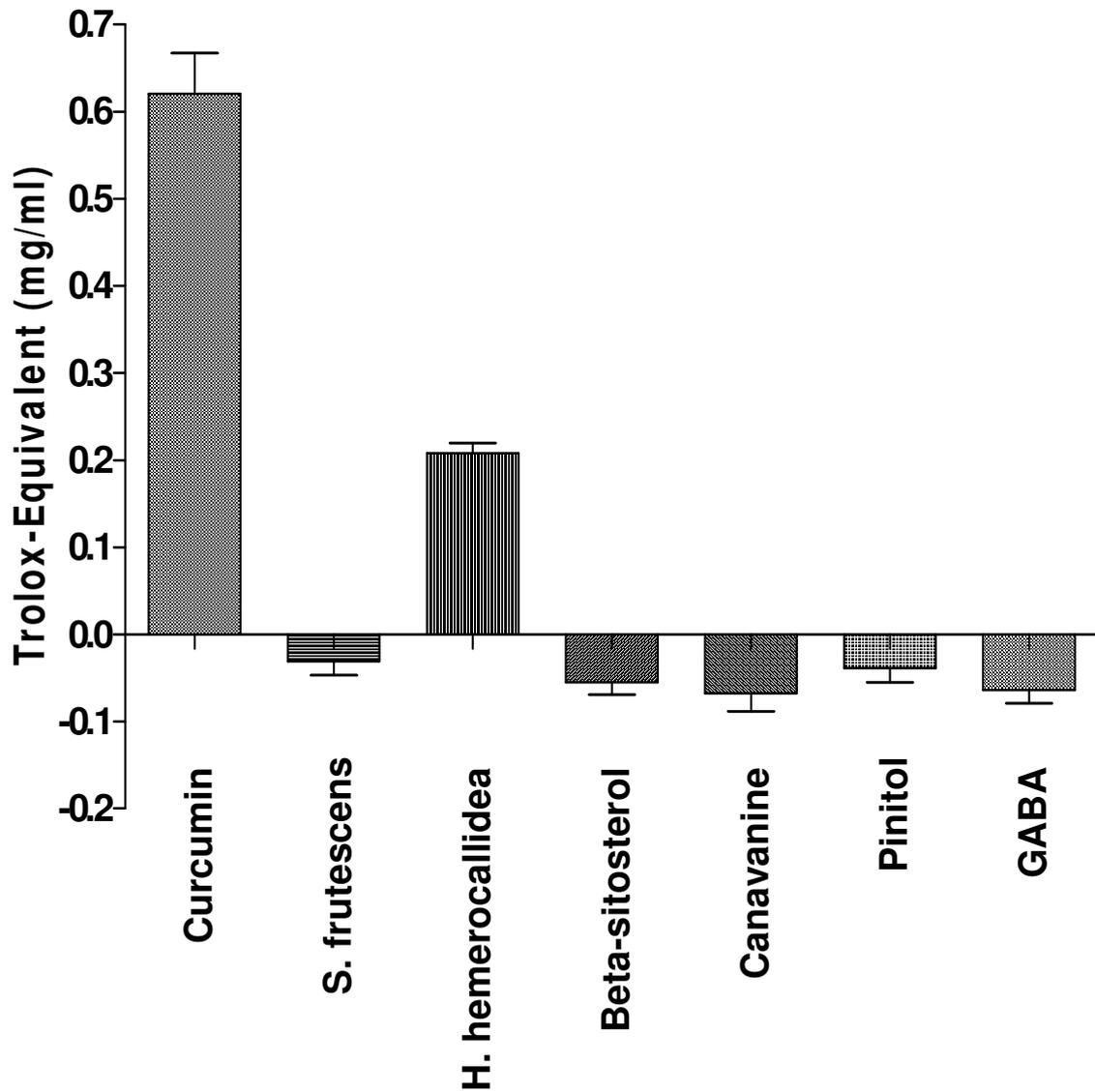
A stock solution of fluorescein sodium salt was made by dissolving 16.7 mg in 10 ml phosphate buffer solution (PBS) (75 mM, pH 7.0). The working solution (60 nM) was obtained by subsequent dilution in PBS. A 10 ml aliquot of AAPH was freshly prepared at a concentration of 153 mM and maintained on ice before addition to the reaction mixture. Trolox solutions were used to establish a calibration curve (50, 100, 200 and 400  $\mu\text{M}$ ) and were prepared by dilution of the 1 mM stock solution with PBS. The analysis was performed using microplates (96-well, opaque white) and a plate reading fluorimeter (Fluostar Optima). Sample (7  $\mu\text{l}$ ) was mixed with 140  $\mu\text{l}$  of fluorescein (60 nM) in the microplate. AAPH (40  $\mu\text{l}$ ) solution was immediately added and the microplate shaken to ensure a homogenous solution. The fluorescence ( $\lambda_{\text{excitation}} = 485 \text{ nm}$ ,  $\lambda_{\text{emission}} = 520 \text{ nm}$ ) was recorded every 5 min over 4 h. The quantification of the antioxidant activity was based on the calculation of the area under the curve (AUC). Three independent experiments were performed.

## 3.3 Results

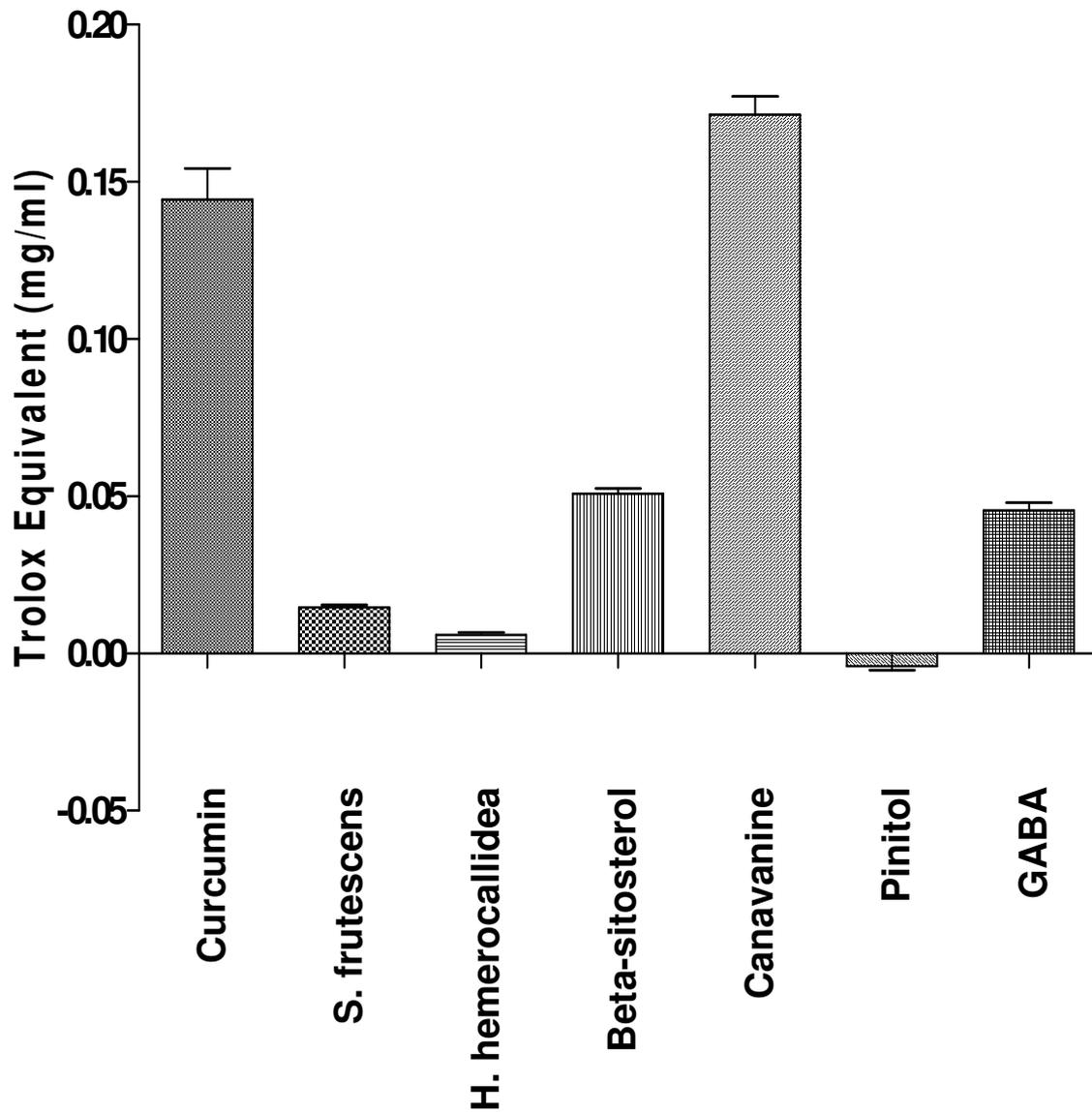
Results of the TEAC assay revealed that of all the test samples, only curcumin possesses antioxidant activity at 1 mg/ml. Figure 3.1 represents the activity of all the test compounds and extracts as compared to each other in Trolox equivalents. The negative values obtained, imply a slight pro-oxidative effect.

Curcumin, which has been reported to be a strong antioxidant, was used as the positive control and exhibited the highest antioxidant activity of all compounds tested. *H. hemerocallidea* aqueous extract (1 mg/ml) showed an antioxidant capacity of 0.208 mg/ml Trolox, whereas *S. frutescens*, beta-sitosterol, canavanine, pinitol and GABA had no measurable antioxidant activity when using the TEAC assay.

With the exception of pinitol, all test samples showed antioxidant activity when determined using the ORAC assay (Figure 3.2). Canvanine showed the highest antioxidant activity, comparable to that of curcumin.



**Figure 3.1.** Antioxidant capacity of test compounds as determined by the TEAC assay. Curcumin, *S. frutescens* aqueous extract, *H. hemerocallidea* aqueous extract, beta-sitosterol, canavanine, pinitol and GABA were all tested at a concentration of 1 mg/ml.



**Figure 3.2.** Antioxidant capacity of test compounds as determined by the ORAC assay. Curcumin, *S. frutescens* aqueous extract, *H. hemerocallidea* aqueous extract, beta-sitosterol, canavanine, pinitol and GABA were all tested at a concentration of 1 mg/ml.

### 3.4 Discussion

Fernandes et al. (2004) investigated the potential antioxidant activity of hot water extracts of *S. frutescens* (10-40 µg/ml) on luminol and lucigenin enhanced chemiluminescence by *L*-formyl-*L*-methionyl-*L*-leucyl-*L*-phenylalanine (FMLP)-stimulated neutrophils as well as its superoxide and hydrogen peroxide scavenging properties. Authors reported that hot water extracts of *S. frutescens* significantly ( $p < 0.05$ ) decreased both the luminol and lucigenin enhanced chemiluminescence responses of neutrophils stimulated by FMLP in a dose related manner and that this inhibition was detectable at concentrations as low as 10 µg/ml. Furthermore, the hot water extract inhibited superoxide as well as horseradish peroxidase/hydrogen peroxide induced chemiluminescence at concentrations as low as 10 µg/ml in the case of superoxide and 0.62 µg/ml in the case of the horseradish peroxidase/hydrogen peroxide system.

A study where the antioxidant activity of *S. frutescens*, using a cell free ABTS radical scavenging assay (TEAC assay), and inhibition of LPS-activated NO production by RAW 264.7 cells revealed that 0.5 µl ethanolic extract of *S. frutescens* had hydroxyl radical scavenging activity equivalent to 10 µM of Trolox (Tai et al. 2004). Furthermore, LPS-stimulated nitric oxide production in RAW 264.7 was found to be reduced in a concentration-dependant manner by *S. frutescens* extract. Lower concentrations of *S. frutescens*, that were non-toxic to the cells, could not reduce LPS-activated nitric oxide production.

Canavanine and pinitol (components of *S. frutescens*) were also tested using RAW 264.7 cells and it was shown that both canavanine (at 0.5 mM and 2 mM) and pinitol (at 10 mM), significantly inhibited LPS-induced nitric oxide production.

GABA has been shown not to possess any antioxidant activity in AAPH-induced lipid oxidation systems (Kohen et al. 1988). However, the 2, 2-diphenylpicrylhydrazyl (DPPH) radical scavenging assay showed that *S. frutescens* possesses concentration-dependant DPPH radical scavenging activity at both 0.1 and 0.5 mg/ml (Chen 2007). The author attributed this antioxidant activity to the phenolic compounds present in *S. frutescens*.

In the present study, no antioxidant activity was found for *S. frutescens* extracts using the TEAC assay (Figure 3.1) and only slight activity was seen in the ORAC assay (Figure 3.2), being contradictory to those of other researchers who claimed high activity (Fernandes et al. 2004; Chen 2007). It should be noted that the test concentration of *S. frutescens* in the present study was 1 mg/ml and that it is possible that higher concentrations of the extract could produce higher antioxidant effects, however the clinical relevance of such a high concentration is questionable. The findings of the present study are supported by the findings of Tai et al. (2004) who found that aqueous extracts of *S. frutescens* have no antioxidant effects at low, non-toxic concentrations.

Canavanine was shown to possess the highest antioxidant activity of all the test samples in the ORAC assay, which is supported by the findings of Tai et al. (2004). Contrary to Tai et al. (2004) who reported antioxidant activity for pinitol, no activity for this compound was detected in either of the antioxidant assays. The antioxidant effects seen for GABA and canavanine in the ORAC assay (Figure 3.2), suggest that both compounds may contribute to the observed antioxidative effect of *S. frutescens*.

Using the DPPH assay, acetone extracts of leaves and corms of *H. hemerocallidea* have been shown to possess high anti-oxidant activity (Katerere and Eloff 2008). Antioxidant activity of *H. hemerocallidea* extracts has been reported when using the thiobarbituric acid reactive substances (TBARS) and TEAC assays (Laporta et al. 2007) as well as Electron Spin Resonance Spectrometry (Steenkamp et al. 2006).

Beta-sitosterol showed an antioxidant effect in the ORAC assay (Figure 3.2), which is supported by the findings of Vivancos and Moreno (2005). Good antioxidant activity for curcumin has previously been reported (Khanna 1999), which supports the present findings of the TEAC and ORAC assays.

Antioxidant assays are used regularly, especially in the field of plant and phytochemical research, but determining the antioxidant capacity of a plant extract or individual compound via one or more antioxidant assays remains difficult (Phipps et al. 2007). Differences between *in vitro* assays as well as the complexity of biological systems, make finding a single assay to measure total antioxidant capacity (AOC), nearly impossible (Phipps et al. 2007). The ORAC assay measures AOC using an hydrogen atom transfer (HAT) mechanism, whereas the TEAC assay employs a single-electron transfer (SET) reaction to make its measurements (Huang et al. 2005). HAT-based procedures measure the ability of an antioxidant to quench free radicals by hydrogen donation (Phipps et al. 2007):



SET-based assays, on the other hand, measure the ability of an antioxidant to transfer an electron to another molecule, reducing it as follows (Phipps et al. 2007):



The ORAC assay measures AOC by detecting the inhibition of peroxy radical-induced oxidation of a fluorescent probe (Caldwell 2001). Preservation of the fluorescence signal is directly proportional to the amount of protection by the antioxidant, and can be quantitated by the determination of the AUC from the addition of a known oxidant of the fluorescent probe.

The fact that this assay uses AUC calculations is an advantage, because it can be used in cases where the test antioxidant might exhibit a lag phase as well as in cases where the antioxidant has an immediate effect, making the assay particularly useful to test food samples, plants, as well as fruit and vegetable extracts which have multiple ingredients and complex reaction kinetics (Phipps et al. 2007). One disadvantage of the ORAC assay is that it only measures antioxidant ability against peroxy radicals, not measuring other important lipophilic antioxidants which can work against free radicals such as  $\text{HO}^\cdot$ ,  $\text{ONOO}^\cdot$  and  $\text{O}_2^{\cdot-}$  (Phipps et al. 2007). The issue of temperature control whilst performing the assay could also lead to intra-assay variability and decreased reproducibility of results (Phipps et al. 2007).

In the TEAC assay, AOC is measured by the ability of a test antioxidant to reduce the colour of an ABTS radical solution; this AOC is then compared to and expressed relative to Trolox (Re et al. 1999; Phipps et al. 2007). The TEAC assay is widely applied and has the advantage of being a simple procedure to perform (Prior et al. 2005). Furthermore, it can be applied over a wide pH range and can be used to test a variety of samples because it accommodates lipophilic as well as hydrophilic compounds (Phipps et al. 2007). A disadvantage of this procedure is that it has a short endpoint time (4-6 min) and TEAC values may differ when slow reactions are involved or reactions that have long lag phases, which make it difficult to assess the correct antioxidant potential of the test sample (Phipps et al. 2007).

As the ORAC and TEAC assays rely on very different reaction mechanisms when measuring the AOC of test samples, it could be one of the reasons for the discrepancy between the results obtained from the two assays in the present study. Furthermore, seeing that plant extracts with multiple compounds were tested, it could be that the different assays reported AOC of different constituents of the extracts, making it difficult to compare one result to the other.

### 3.5 Conclusion

The TEAC assay showed that none of the test samples possess stronger antioxidant activity than curcumin, whereas the ORAC assay showed that only canavanine produced an antioxidant effect comparable to that of curcumin. This suggests that canavanine, present in *S. frutescens*, may be involved in the inhibition of peroxy radical-induced oxidation.

## CHAPTER 4: DETERMINATION OF CYTOKINE PROFILES

### 4.1 Introduction

In response to certain stimuli such as HIV infection, macrophages release various pro-inflammatory cytokines which in turn activate other immune functions and lead to the activation of select immune cells with a subsequent elevation in immune system functions. Circulating monocytes from HIV-infected individuals are known to constitutively produce high concentrations of TNF- $\alpha$ , IL-1, IL-6 and IL-8. Other cytokines such as IL-2, IL-12, IFN- $\gamma$  as well as IL-4, 5, and 10 are also implicated in HIV (Babakhanian 1995; Estcourt et al. 1997). More recently, Alfano and Poli (2005) stated that IL-1 $\beta$ , IL-6, IL-12, IL-15, IFN- $\gamma$  and TNF- $\alpha$  are key pro-inflammatory cytokines involved in HIV. Although studies confirm increased expression of cytokines by HIV-infected monocytes/macrophages, there are inconsistencies as to exactly which cytokines are increased or decreased, especially in *in vitro* studies (Bornemann et al. 1997). These inconsistencies have been linked to technological variabilities, different types of cell cultures as well as technical error (Bornemann et al. 1997). HIV infection and replication causes macrophages to be in a pro-inflammatory 'status', making inflammatory cytokines important in HIV investigations (Porcheray et al. 2006).

The aim of this phase of the study was to investigate the potential effects of aqueous extracts from *S. frutescens* and *H. hemerocallidea* as well as active compounds from these plants on the cytokine profiles of LPS-stimulated THP-1 and U937 macrophage cell lines after forcing differentiation with PMA. ELISA is one of the most common methods used for the detection of cytokines secreted from cells because of its cost-effectiveness (Chen et al. 1999). The main disadvantage of using this method is its restriction to measuring one cytokine at a time. This means that multiple sample aliquots are required and that the procedure must be repeated for each cytokine of interest (Chen et al. 1999).

Flow cytometry is an efficient, high-throughput method of detecting fluorescently labelled particles (Prussin 1996). This technique of cytokine detection has advantages such as quantitative detection of up to six cytokines simultaneously from a single sample and can be used for intra- or extracellular cytokines. Chen et al. (1999) compared the quantification of six cytokines using microparticle-based flow cytometric technology and the conventional ELISA method. In the flow cytometric method, one 50  $\mu$ l sample could provide results for all six cytokines, whereas the ELISA method required 600  $\mu$ l. The authors concluded that the microparticle-based flow cytometric technology has 'comparable analytical sensitivity', but a 'wider dynamic range than conventional ELISA'. Flow cytometric bead array kits were used for cytokine quantitation in this study. These kits enable multiple soluble analytes to be captured onto antibodies bound to select beads with different size and with fluorescent intensity variation. A second quantitating antibody with a specific fluorescent emission wavelength is then bound to the captured analyte and the fluorescence intensities of each bead then gives relative concentration information. The CBA kits used in this study were designed to detect the concentration of various cytokines in cell culture supernatants or total concentration of lysed cells. The capture beads have green fluorescence while the detection antibodies used to form sandwich antibody complexes are phycoerythrin (PE)-conjugated. Standards or the test samples are incubated with the beads in a similar way to ELISA kits. The advantage of the CBA kit, is that it can give counts of thousands of beads equivalent to an ELISA plate well each.

## **4.2 Materials and Methods**

### **4.2.1 Treatment of cells**

Culturing and preparation of THP-1 and U937 cells were performed as described in Section 2.2.4 (p.17). Cells were added to each well of a microtitre plate (1 x 10<sup>5</sup> cells/well) and stimulated to differentiate into adherent cells by incubating with 0.1  $\mu$ g/mL PMA at 37 °C for 48 h.

Twenty microlitres of the respective test compounds were then added at the following concentrations; extracts of *S. frutescens* and *H. hemerocallidea* at 100 µg/ml, curcumin at 10 µg/ml and 20 µg/ml, beta-sitosterol at 12.5 µg/ml and 25 µg/ml and canavanine, pinitol and GABA at 50 µg/ml.

Abovementioned test concentrations were selected to ensure detectable biological activity and are at least ten times the physiological concentrations. Due to their low solubility, two concentrations of beta-sitosterol and curcumin were tested, as it was not sure if the higher concentration would be stable during the long incubation times. Cells were incubated with the respective test compounds for 72h, after which they were stimulated with 1 µg/ml of LPS for 24h (to promote cytokine production). After incubation, plates were centrifuged (2000 x *g*, 10 min). Supernatants were aspirated from each well and stored at -70 °C for determination of extracellular cytokine concentrations. Cells were lysed with 0.01% w/v (or 100 µg/ml) SDS for 30 min. After centrifugation (2000 x *g*, 10 min), supernatants were aspirated from each well and stored at -70 °C for determination of intracellular cytokine concentration.

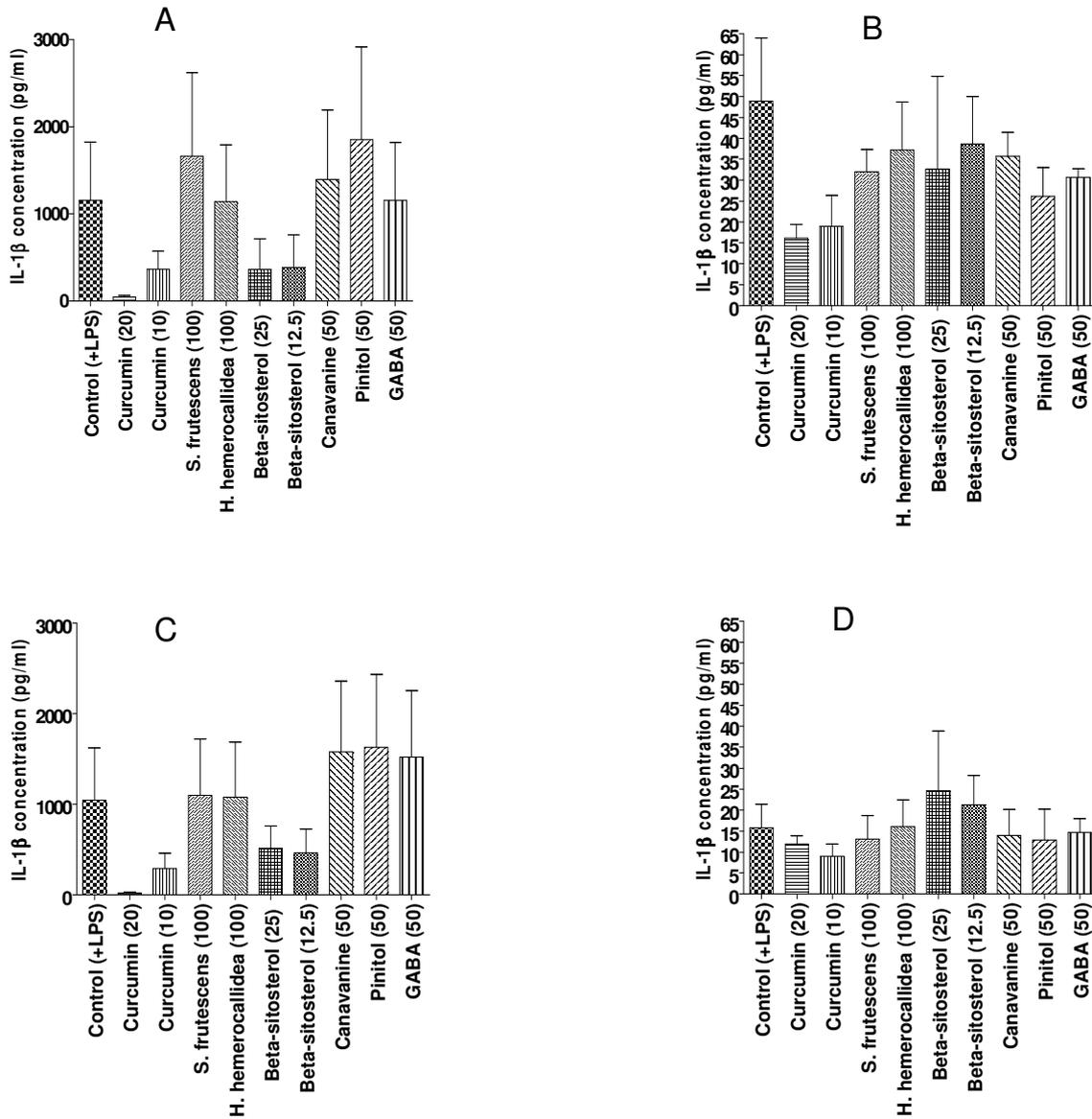
#### **4.2.2 Cytokine determination**

Flow cytometric BD™ Cytometric Bead Array (CBA) kits for determination of cytokine production were purchased from Becton, Dickinson and Company, SA. The Human Inflammatory Kit contained antibodies directed against IL-1β, IL-6, IL-8, IL-10, TNF-α and IL-12p70. The Human Th1/Th2 Cytokine Kit contained antibodies directed against IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ.

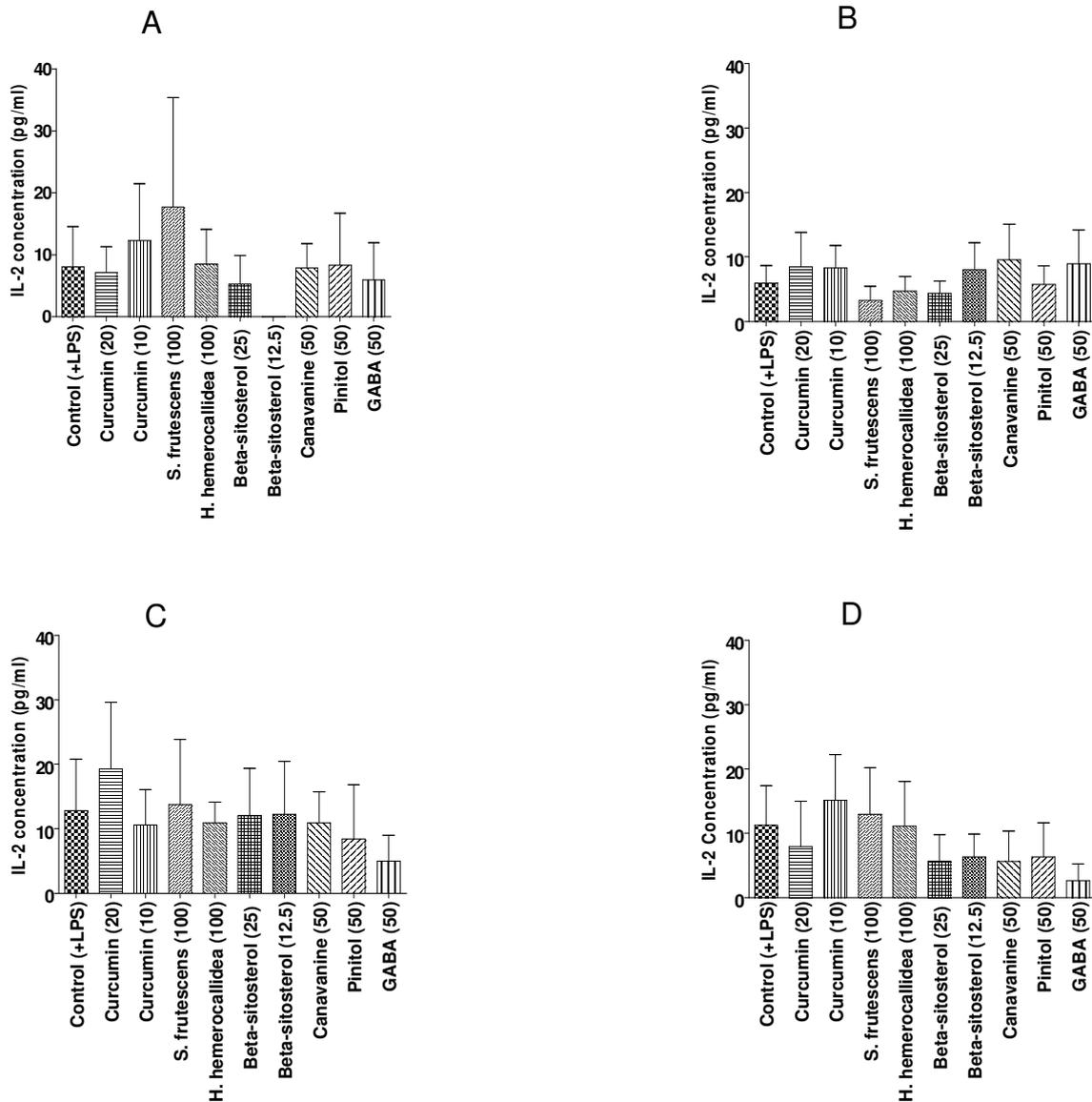
The procedure was performed according to the *BD CBA Cytokine Kit Assay Procedures*. A series of standards (0 - 5000 pg/ml) were prepared by serial dilution of the Top Standard Cytokine for each of the analysed cytokines in order to generate a series of standard curves. Fifty microlitres of mixed capture beads were added to assay tubes. Thereafter, 50 µl Cytokine Standard dilutions or the various test samples were added and the assay tubes incubated for 3h at room temperature and protected from direct exposure to light to allow capture of the soluble cytokines onto the beads. After incubation the beads were washed free of any unbound protein using one millilitre wash buffer that was added to each assay tube, then the tubes were centrifuged (200 x *g*, 5 min) and the supernatants discarded. After washing, 50 µl PE detection reagent was added and the suspension again incubated for 30 min. The bead pellet was resuspended in 300 µl wash buffer, vortexed and 150 µl of each control or test sample transferred to a well of a 96-well plate for analysis on the flow cytometer. Two independent experiments were performed in duplicate wells. Data was acquired using a BD FACS Array flow cytometer and BD CBA Analysis Software or FCAP Array™ Software.

### 4.3 Results

Figures 4.1A-D show the concentration of IL-1β present in intracellular and extracellular supernatants collected from differentiated U937 and THP-1 macrophages after 72 h incubation with the various test compounds or plant extracts. Neither of the test compounds caused statistically significant changes in either intra- or extracellular IL-1β production of the LPS-stimulated macrophages (Figures 4.1A-D). Curcumin (10 µg/ml and 20 µg/ml) and beta-sitosterol (12.5 µg/ml and 25 µg/ml) reduced intracellular IL-1β production in both U937 (Figure 4.1A) and THP-1 macrophages (Figure 4.1C). *S. frutescens* and pinitol increased intracellular IL-1β production in U937 cells (Figure 4.1A), whereas canavanine, pinitol and GABA increased intracellular IL-1β production in THP-1 cells (Figure 4.1C). None of the test compounds produced any significant effect on IL-2 production in either U937 or THP-1 macrophages (Figures 4.2A-D).



**Figure 4.1.** Intracellular IL-1 $\beta$  concentration in differentiated U937 macrophages (A), extracellular IL-1 $\beta$  concentration in differentiated U937 macrophages (B), intracellular IL-1 $\beta$  concentration in differentiated THP-1 macrophages (C), extracellular IL-1 $\beta$  concentration in differentiated THP-1 macrophages (D). Cells were treated with various test compounds (concentration in  $\mu\text{g/ml}$ ), stimulated with LPS (for extracellular cytokine determination) and lysed with SDS (for intracellular cytokine determination). IL-1 $\beta$  concentration in cell culture supernatants was then determined using Flow Cytometric CBA kits. (One-way ANOVA, n = 4)

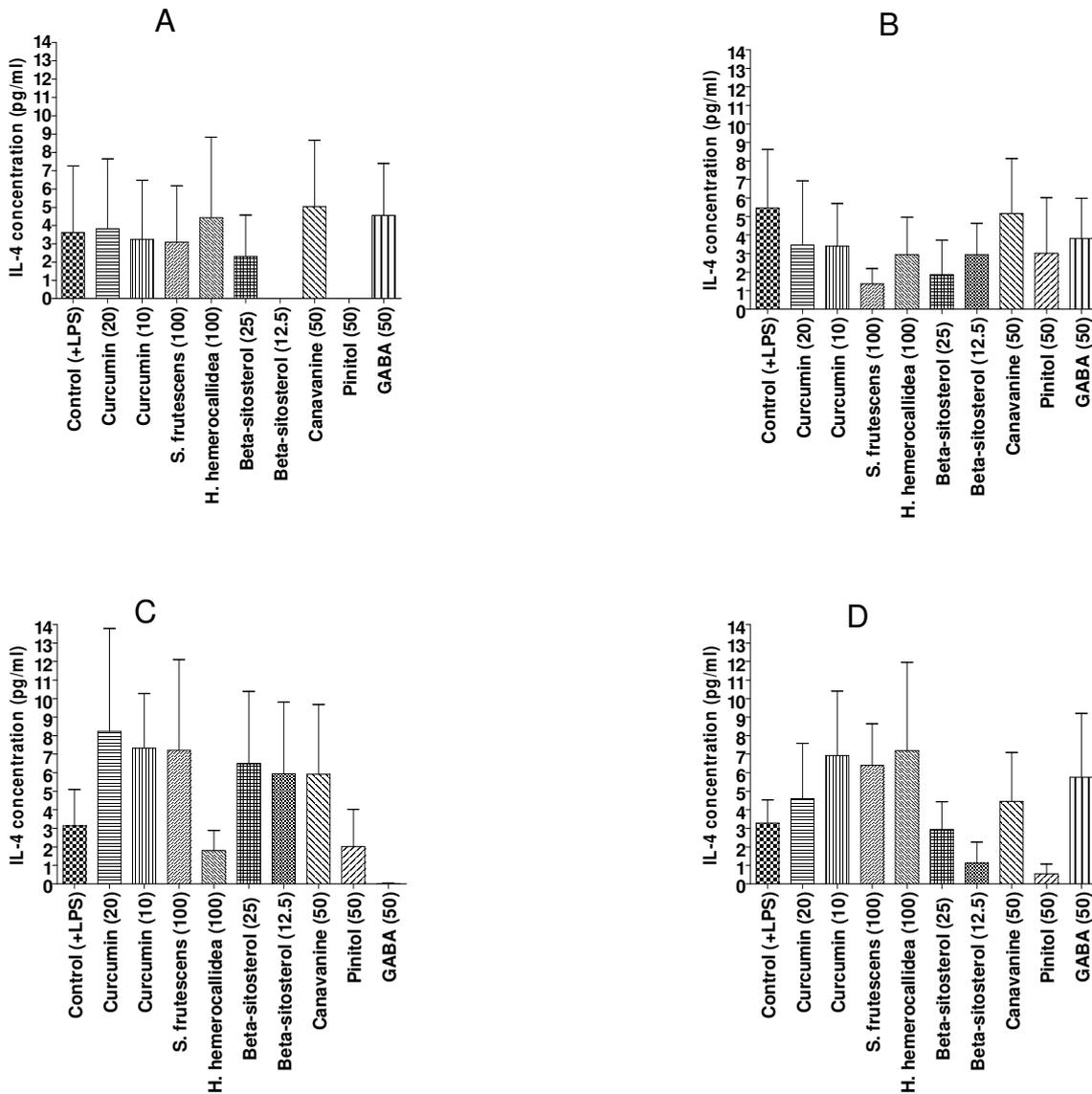


**Figure 4.2.** Intracellular IL-2 concentration in differentiated U937 macrophages (A), extracellular IL-2 concentration in differentiated U937 macrophages (B), intracellular IL-2 concentration in differentiated THP-1 macrophages (C), extracellular IL-2 concentration in differentiated THP-1 macrophages (D). Cells were treated with various test compounds (concentration in  $\mu\text{g/ml}$ ), stimulated with LPS (for extracellular cytokine determination) and lysed with SDS (for intracellular cytokine determination). IL-2 concentration in cell culture supernatants was then determined using Flow Cytometric CBA kits. (One-way ANOVA,  $n = 4$ )

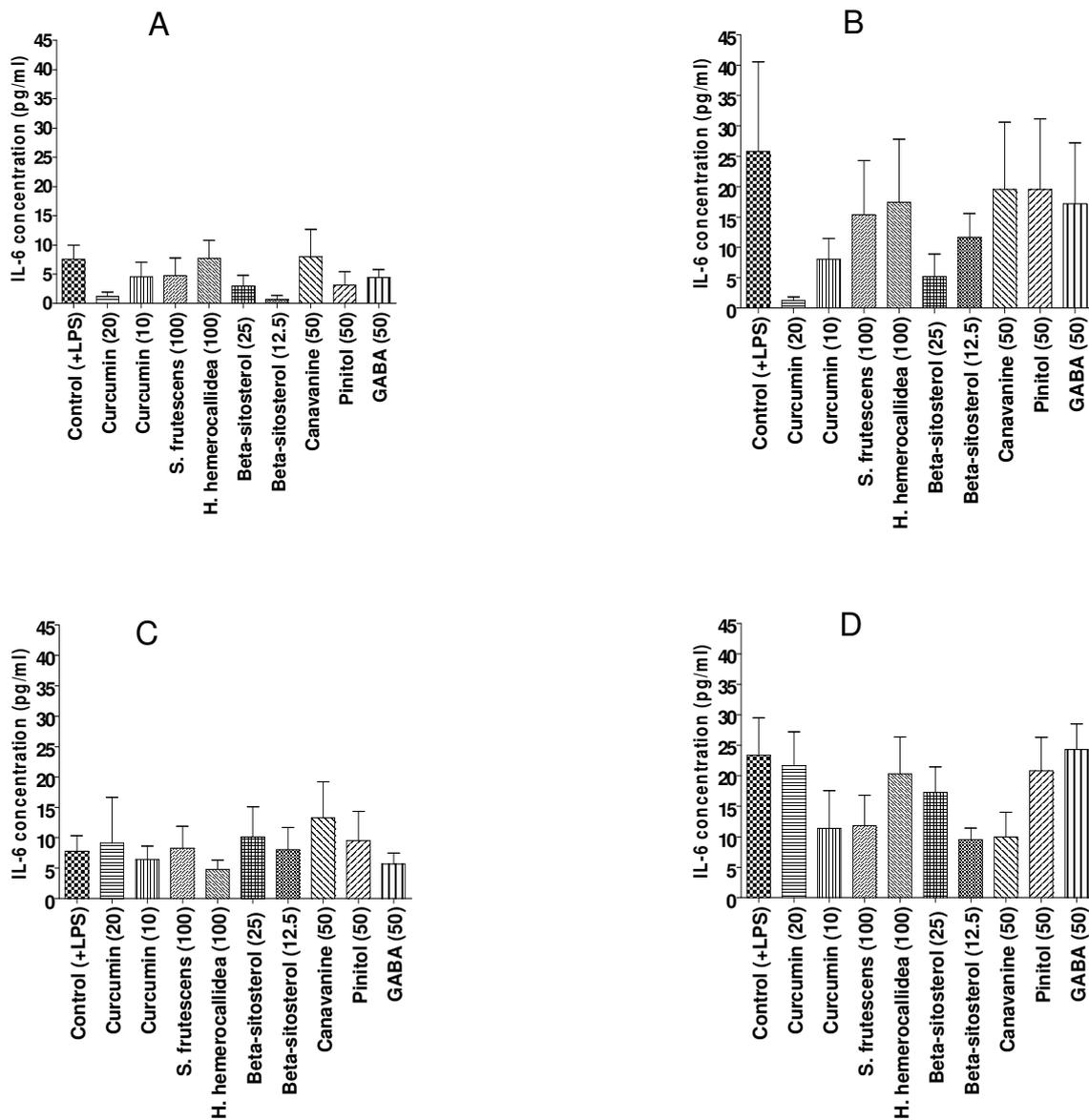
Beta-sitosterol (12.5 µg/ml) and pinitol reduced intracellular IL-4 in U937 cells from approximately 4 pg/ml to 0 pg/ml (Figure 4.3A) and GABA reduced intracellular IL-4 production in THP-1 cells from 3 pg/ml to 0 pg/ml, however these reductions were not significant (Figure 4.3C). All test compounds caused slight reductions in extracellular IL-4 in U937 cells compared to untreated controls (Figure 4.3B). Curcumin, *S. frutescens*, beta-sitosterol, and canavanine slightly increased intracellular IL-4 production in THP-1 cells (Figures 4.3C). Extracellular IL-4 increased upon exposure to curcumin, *S. frutescens*, *H. hemerocallidea* and GABA, but was decreased by beta-sitosterol (12.5 µg/ml) and pinitol in THP-1 cells (Figure 4.3D). These changes were also not significant.

Figure 4.4A depicts how curcumin (20 µg/ml) reduced the intracellular concentration of IL-6 in U937 cells. Although not statistically significant, beta-sitosterol (12.5 µg/ml) caused the same reduction in IL-6 production, having effects comparable to that of 20 µg/ml curcumin. Curcumin (10 µg/ml and 20 µg/ml) and beta-sitosterol (12.5 µg/ml and 25 µg/ml) caused the concentration of extracellular IL-6 in U947 cells to decrease slightly (Figure 4.4B). These changes were not statistically significant. The intracellular IL-6 concentration in THP-1 cells (Figure 4.4C) remained relatively unaffected by the test compounds. Curcumin (10 µg/ml), *S. frutescens*, beta-sitosterol (12.5 µg/ml) and canavanine had comparable effects on extracellular IL-6 in THP-1 cells (Figure 4.4D), decreasing the concentration by 12 -14 pg/ml.

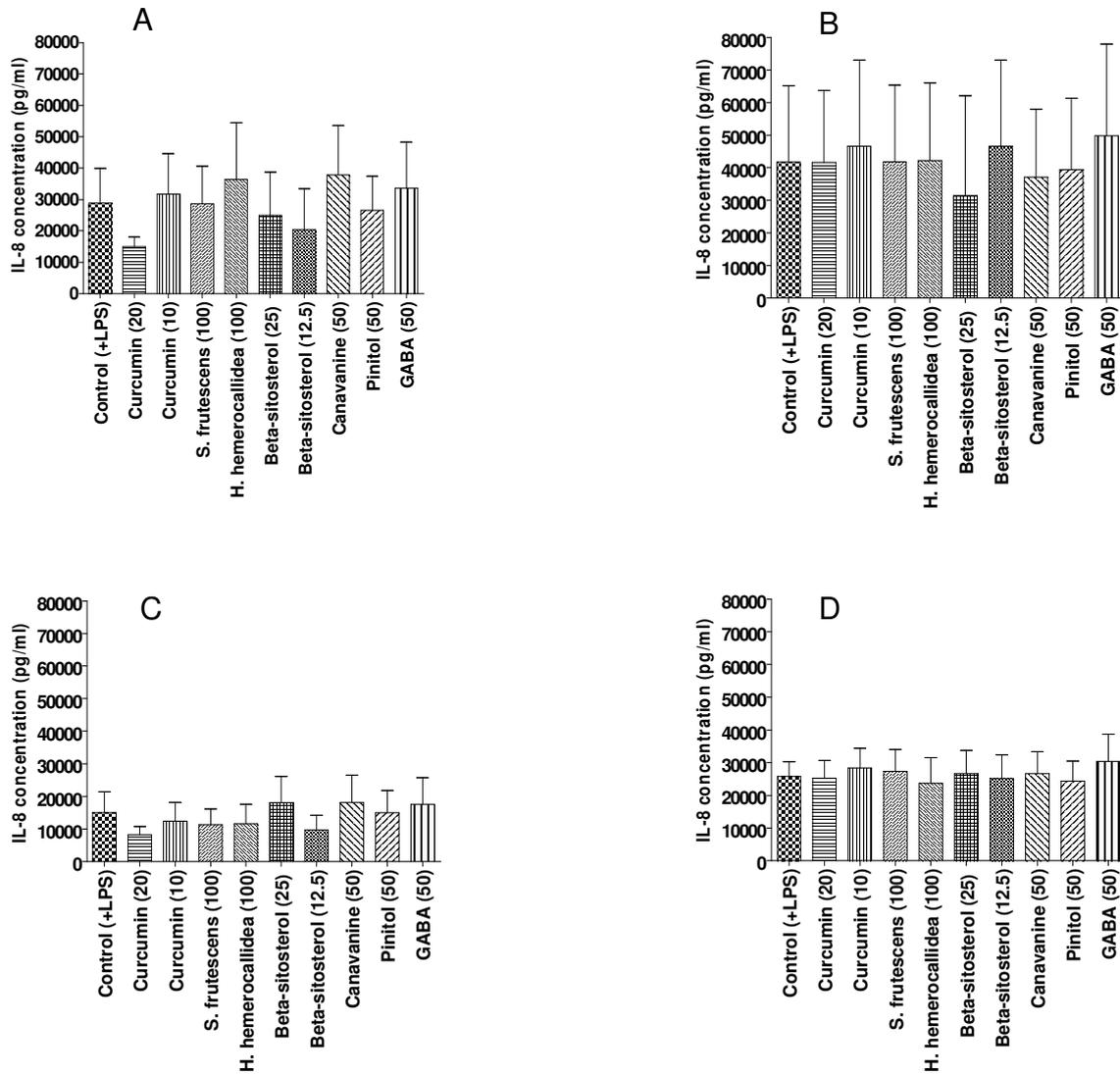
Both curcumin (20 µg/ml) and beta-sitosterol (12.5 µg/ml) affected IL-8 production, although non-significantly. In U937 cells, intracellular IL-8 concentration was reduced by 14 000 pg/ml and 8 000 pg/ml for curcumin and beta-sitosterol, respectively, which translates to decreases of 51% and 28% (Figure 4.5A). Extracellular IL-8 in U937 cells was reduced by 10 000 pg/ml (or 24%) under the influence of beta-sitosterol (12.5 µg/ml) (Figure 4.5B). The intra- and extracellular concentrations of IL-8 remained relatively unchanged in the THP-1 cells (Figures 4.5C and 4.5D).



**Figure 4.3.** Intracellular IL-4 concentration in differentiated U937 macrophages (A), extracellular IL-4 concentration in differentiated U937 macrophages (B), intracellular IL-4 concentration in differentiated THP-1 macrophages (C), extracellular IL-4 concentration in differentiated THP-1 macrophages (D). Cells were treated with various test compounds (concentration in  $\mu\text{g/ml}$ ), stimulated with LPS (for extracellular cytokine determination) and lysed with SDS (for intracellular cytokine determination). IL-4 concentration in cell culture supernatants was then determined using Flow Cytometric CBA kits. (One-way ANOVA,  $n = 4$ )



**Figure 4.4.** Intracellular IL-6 concentration in differentiated U937 macrophages (A), extracellular IL-6 concentration in differentiated U937 macrophages (B), intracellular IL-6 concentration in differentiated THP-1 macrophages (C), extracellular IL-6 concentration in differentiated THP-1 macrophages (D). Cells were treated with various test compounds (concentration in  $\mu\text{g/ml}$ ), stimulated with LPS (for extracellular cytokine determination) and lysed with SDS (for intracellular cytokine determination). IL-6 concentration in cell culture supernatants was then determined using Flow Cytometric CBA kits. (One-way ANOVA,  $n = 4$ )

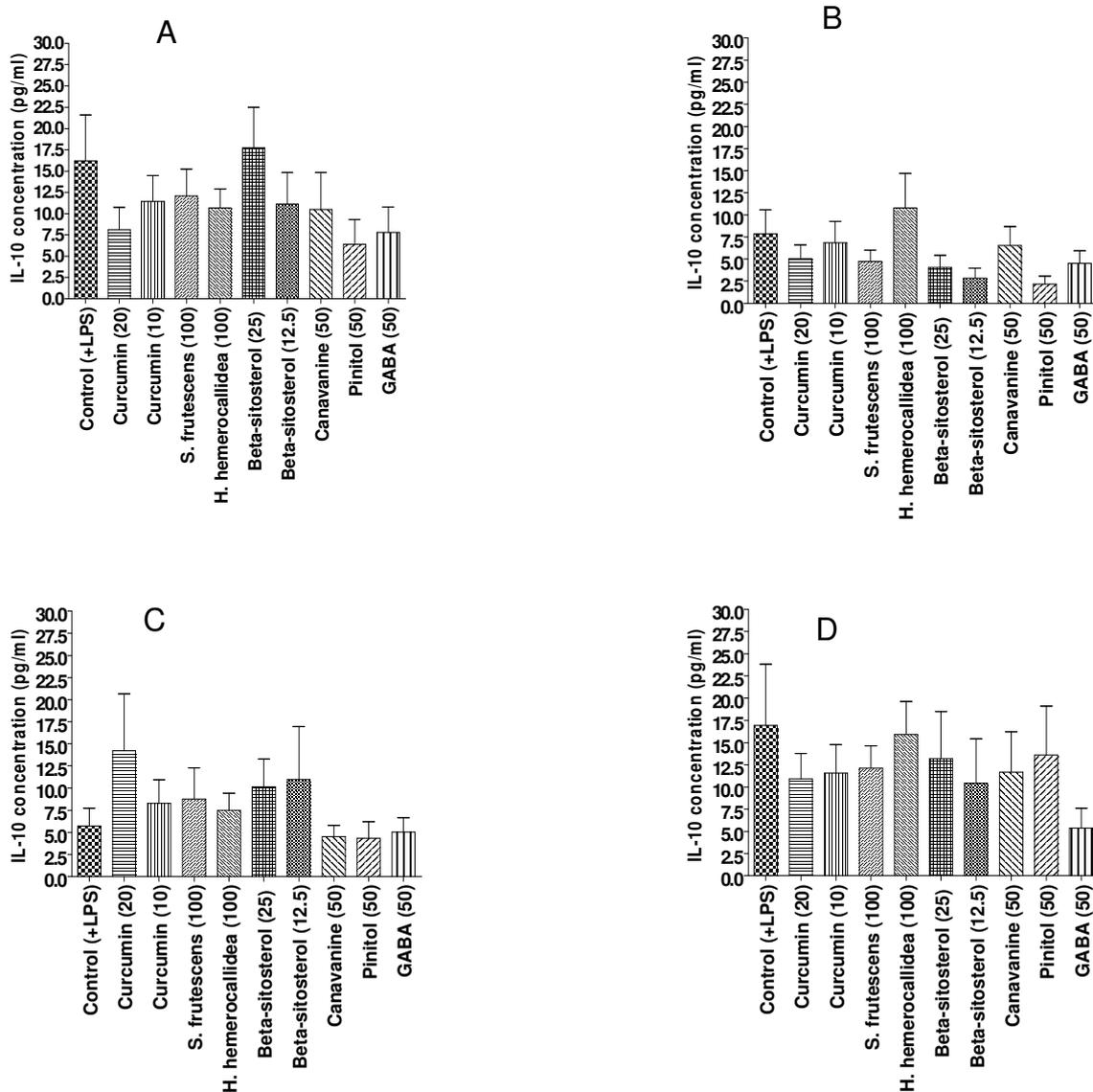


**Figure 4.5.** Intracellular IL-8 concentration in differentiated U937 macrophages (A), extracellular IL-8 concentration in differentiated U937 macrophages (B), intracellular IL-8 concentration in differentiated THP-1 macrophages (C), extracellular IL-8 concentration in differentiated THP-1 macrophages (D). Cells were treated with various test compounds (concentration in  $\mu\text{g/ml}$ ), stimulated with LPS (for extracellular cytokine determination) and lysed with SDS (for intracellular cytokine determination). IL-8 concentration in cell culture supernatants was then determined using Flow Cytometric CBA kits. (One-way ANOVA,  $n = 4$ )

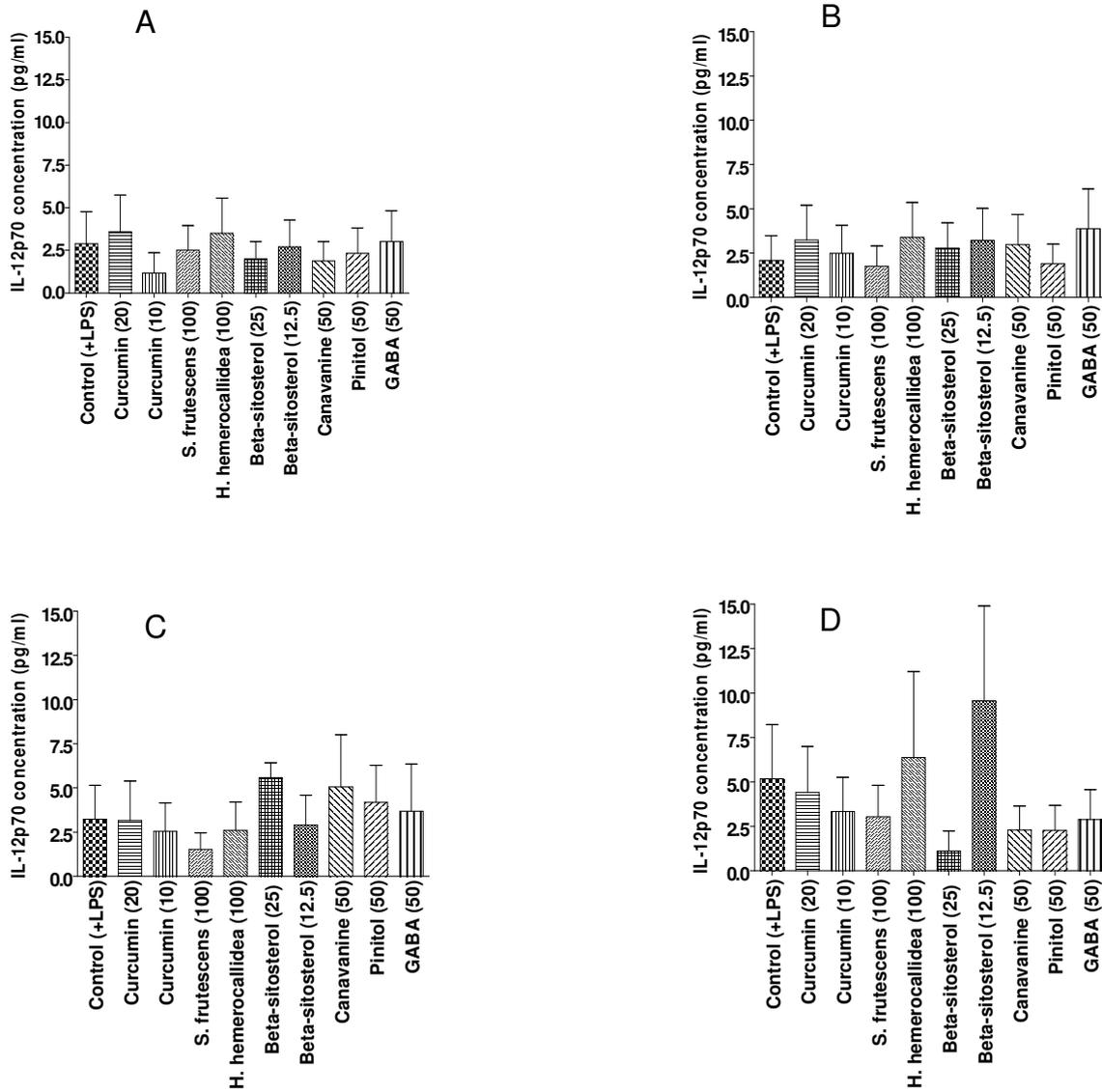
In terms of IL-10, curcumin (20 µg/ml), pinitol and GABA reduced intracellular production non-significantly (Figure 4.6A). Likewise, the effect of beta-sitosterol (12.5 µg/ml) and pinitol on extracellular IL-10 reduction was minimal (Figure 4.6B). In THP-1 cells, curcumin (20 µg/ml) increased intracellular IL-10 (Figure 4.6C) and extracellular IL-10 was slightly reduced by all test compounds (Figure 4.6D).

All the test compounds had a weak effect on IL-12p70 production (Figure 4.7A-D). Curcumin (20 µg/ml) decreased the intracellular production of TNF-α in U937 cells (Figure 4.8A), the extracellular production of TNF-α in U937 cells (Figure 4.8B) and significantly ( $p < 0.01$ ) reduced extracellular TNF-α in THP-1 cells by 43 pg/ml (Figure 4.8D). Similarly, curcumin (10 µg/ml) reduced extracellular TNF-α in U937 cells (Figure 4.8B) and significantly ( $p < 0.05$ ) reduced extracellular TNF-α in THP-1 cells (Figure 4.8D). Curcumin (10 µg/ml) caused a slight reduction in intracellular TNF-α in THP-1 cells (Figure 4.8C). Beta-sitosterol (12.5 µg/ml and 25 µg/ml) reduced intra- and extracellular TNF-α production in U937 cells (Figure 4.8A and 4.8B) and significantly ( $p < 0.05$ ) decreased the extracellular concentration of TNF-α in THP-1 cells (Figure 4.8D).

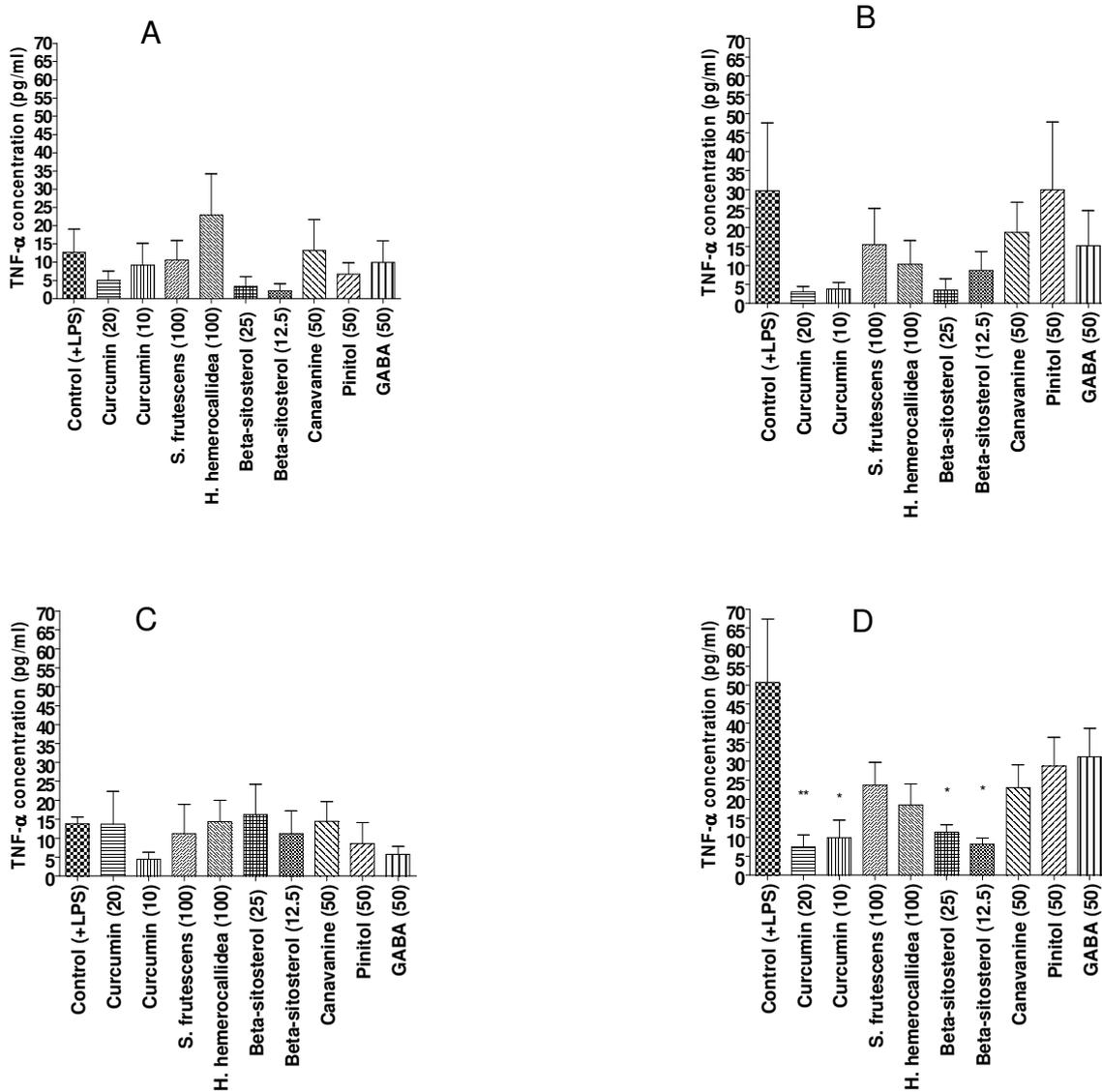
Beta-sitosterol (12.5 µg/ml) and pinitol reduced the intracellular concentration of IFN-γ in U937 cells (Figure 4.9A). *S. frutescens* reduced the extracellular concentration of IFN-γ in U937 cells (Figure 4.9B) and pinitol reduced the intracellular concentration in THP-1 cells from 4.8 pg/ml to 0 pg/ml (Figure 4.9C). *H. hemerocallidea* increased the extracellular concentration of IFN-γ in THP-1 cells non-significantly (Figure 4.9D).



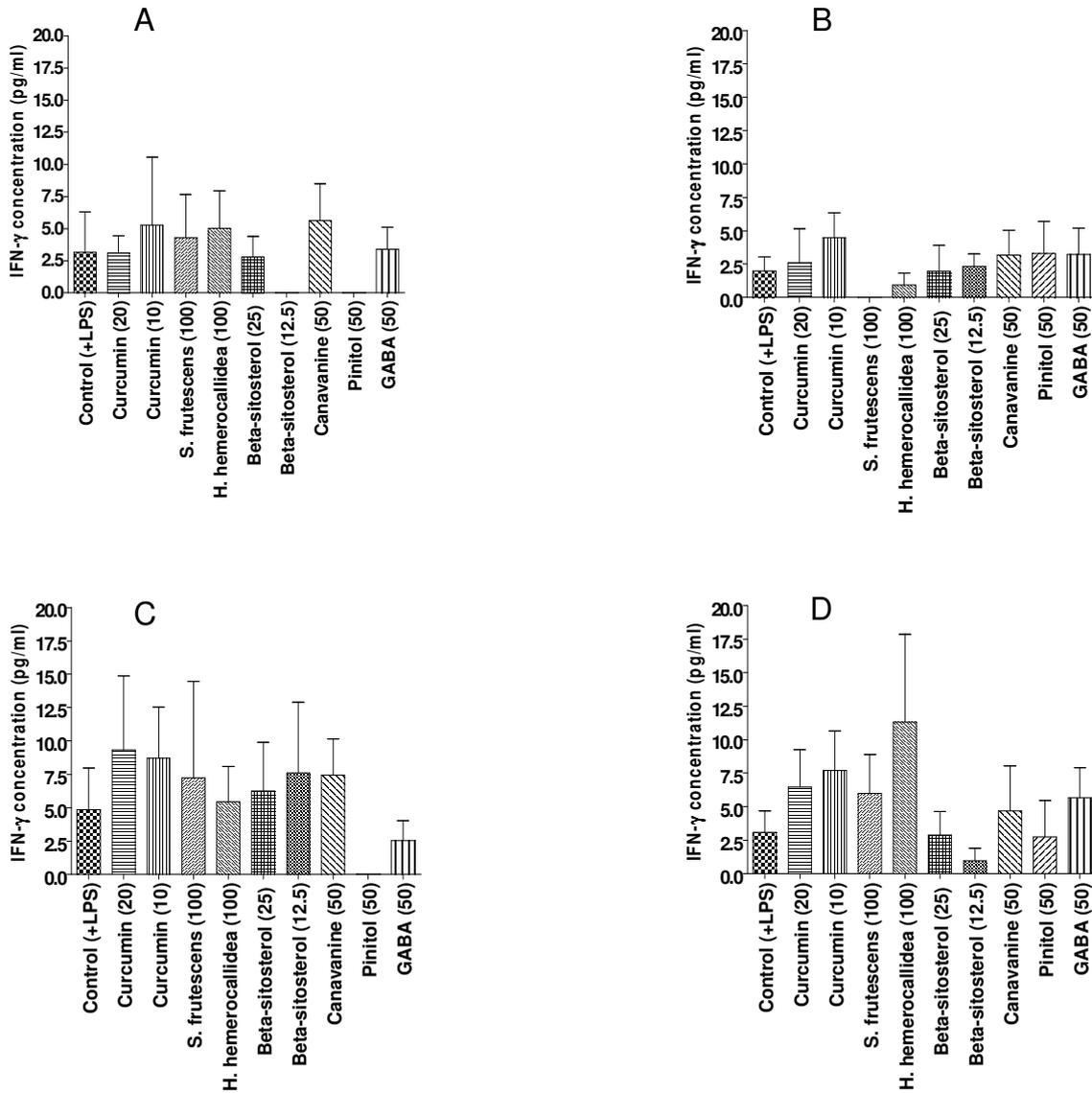
**Figure 4.6.** Intracellular IL-10 concentration in differentiated U937 macrophages (A), extracellular IL-10 concentration in differentiated U937 macrophages (B), intracellular IL-10 concentration in differentiated THP-1 macrophages (C), extracellular IL-10 concentration in differentiated THP-1 macrophages (D). Cells were treated with various test compounds (concentration in  $\mu\text{g/ml}$ ), stimulated with LPS (for extracellular cytokine determination) and lysed with SDS (for intracellular cytokine determination). IL-10 concentration in cell culture supernatants was then determined using Flow Cytometric CBA kits. (One-way ANOVA,  $n = 4$ )



**Figure 4.7.** Intracellular IL-12p70 concentration in differentiated U937 macrophages (A), extracellular IL-10 concentration in differentiated U937 macrophages (B), intracellular IL-12p70 concentration in differentiated THP-1 macrophages (C), extracellular IL-12p70 concentration in differentiated THP-1 macrophages (D). Cells were treated with various test compounds (concentration in  $\mu\text{g/ml}$ ), stimulated with LPS (for extracellular cytokine determination) and lysed with SDS (for intracellular cytokine determination). IL-12p70 concentration in cell culture supernatants was then determined using Flow Cytometric CBA kits. (One-way ANOVA,  $n = 4$ )



**Figure 4.8.** Intracellular TNF- $\alpha$  concentration in differentiated U937 macrophages (A), extracellular TNF- $\alpha$  concentration in differentiated U937 macrophages (B), intracellular TNF- $\alpha$  concentration in differentiated THP-1 macrophages (C), extracellular TNF- $\alpha$  concentration in differentiated THP-1 macrophages (D). Cells were treated with various test compounds (concentration in  $\mu\text{g/ml}$ ), stimulated with LPS (for extracellular cytokine determination) and lysed with SDS (for intracellular cytokine determination). TNF- $\alpha$  concentration in cell culture supernatants was then determined using Flow Cytometric CBA kits. (One-way ANOVA,  $n = 4$ , \*  $p < 0.05$ , \*\*  $p < 0.01$ )



**Figure 4.9.** Intracellular IFN- $\gamma$  concentration in differentiated U937 macrophages (A), extracellular IFN- $\gamma$  concentration in differentiated U937 macrophages (B), intracellular IFN- $\gamma$  concentration in differentiated THP-1 macrophages (C), extracellular IFN- $\gamma$  concentration in differentiated THP-1 macrophages (D). Cells were treated with various test compounds (concentration in  $\mu\text{g/ml}$ ), stimulated with LPS (for extracellular cytokine determination) and lysed with SDS (for intracellular cytokine determination). IFN- $\gamma$  concentration in cell culture supernatants was then determined using Flow Cytometric CBA kits. (One-way ANOVA,  $n = 4$ )

## 4.4 Discussion

Beta-sitosterol has a multitude of important uses, amongst which, is to regulate the function and proliferation of lymphocytes and NK cells as well as many other immunomodulatory properties involving cytokine processes (Bouic et al. 1996; Bouic and Lamprecht 1999). Yuk et al. (2007) investigated the effect of beta-sitosterol on ovalbumin-induced lung inflammation in mice. Beta-sitosterol (1 mg/kg) was administered intraperitoneally, and the bronchoalveolar (BAL) fluid collected by lavage, where it was found that beta-sitosterol inhibited the increased mRNA and protein expression of IL-4 and IL-5 (Yuk et al. 2007). Furthermore, after the induction of inflammation in mice, phytosterols were administered and shown to alter the cytokine profile of immune responses by significantly ( $p < 0.05$ ) increasing the production of IL-2 and IFN- $\gamma$  (Calpe-Berdiel et al. 2007). Investigation into the immune modulating properties of *H. hemerocallidea* (ethanolic extract) and beta-sitosterol indicated moderate immunostimulatory activity by both compounds, which was evident from the increased cellular expression of IFN- $\gamma$  (Muwanga 2006).

*S. frutescens* is used to treat a variety of ailments such as rheumatoid arthritis, osteoarthritis and inflammation, but the exact mechanism by which it exerts its anti-inflammatory effects has yet to be described (Gericke et al. 2001; Dalvi 2003). In an attempt to explain why *S. frutescens* is useful in so many immune diseases, much research has been focused on the effects of its chemical constituents on immunological processes. GABA (5 ng/ml) has been shown to significantly ( $p < 0.05$ ) reduce IL-6 and IL-12 production after being added to the culture medium of LPS-stimulated macrophages (Reyes-Garcia et al. 2007).

Pinitol has been reported to have various roles in immune modulation. Flow cytometric and ELISA analysis showed that pinitol impairs the ability of dendritic cells to produce IL-12p70 and other pro-inflammatory cytokines (Lee et al. 2007a). Furthermore, *in vivo* data indicated that pinitol inhibits IFN- $\gamma$  production in the CD4+ T-cells of LPS-treated mice (Lee et al. 2007a).

In ovalbumin-induced asthmatic mice, pinitol reduced the normal increased production of IL-4 and IL-5 in the bronchoalveolar fluid of the challenged mice (Lee et al. 2007b).

Results of the present study did not reveal significant changes in many of the cytokine profiles of differentiated U937 and THP-1 macrophages. IL-1 $\beta$  is a pro-inflammatory cytokine which is involved in the immune response against infections (Dinarello 1994). Although no statistical significance was obtained, the 80-100% reduction in IL-1 $\beta$  caused by curcumin and beta-sitosterol suggests potential anti-inflammatory activity, while *S. frutescens* and its constituents, canavanine, pinitol and GABA increased IL-1 $\beta$  production (by approximately 50%) and seem to have a pro-inflammatory effect (Figure 4.1). Beta-sitosterol, pinitol and GABA reduced IL-4 concentrations (Figure 4.3). In Figure 4.4 the levels of IL-6 were very low, making the observed changes in concentration insignificant. IL-8 is a chemotactic and inflammatory cytokine which attracts neutrophils to inflammation sites or in response to foreign antigens (Baggiolini and Clarke-Lewis 1992). Again, although non-significant, the 51% and 28% reductions in IL-8 production caused by curcumin and beta-sitosterol, respectively (Figure 4.5), indicate the potential anti-inflammatory activity of these two compounds.

Curcumin and beta-sitosterol significantly reduced extracellular TNF- $\alpha$  production in THP-1 cells (Figure 4.8), confirming the purported anti-inflammatory activity of curcumin and suggesting comparable anti-inflammatory activity by beta-sitosterol. *S. frutescens*, beta-sitosterol and pinitol reduced IFN- $\gamma$  concentrations, but these changes are most likely due to technical problems such as capture beads being lost during the experiment, causing the levels of IFN- $\gamma$  to be undetected (Figure 4.9).



**Table 4.1.** Summary of the most noteworthy observations made in cytokine investigations.

	<b>IL-1<math>\beta</math></b>	<b>IL-8</b>	<b>TNF-<math>\alpha</math></b>	<b>Potential Effect</b>
<b>Curcumin</b> (10 $\mu$ g/ml and 20 $\mu$ g/ml)	Decreased by 80-100%	Decreased by $\pm$ 51%	Decreased by $\pm$ 80% ( $p < 0.05$ )	Anti-inflammatory
<b>Beta-sitosterol</b> (12.5 $\mu$ g/ml and 25 $\mu$ g/ml)	Decreased by $\pm$ 80%	Decreased by 24-28%	Decreased by $\pm$ 80% ( $p < 0.05$ )	Anti-inflammatory
<b><i>S. frutescens</i></b> (100 $\mu$ g/ml)	Increased by $\pm$ 50%	-	-	Pro-inflammatory
<b>Canavanine</b> (50 $\mu$ g/ml)	Increased by $\pm$ 50%	-	-	Pro-inflammatory
<b>Pinitol</b> (50 $\mu$ g/ml)	Increased by $\pm$ 50%	-	-	Pro-inflammatory
<b>GABA</b> (50 $\mu$ g/ml)	Increased by $\pm$ 50%	-	-	Pro-inflammatory

Curcumin has anti-inflammatory and immunomodulatory properties and was shown to downregulate expression of various cytokines involved in the immune response such as TNF- $\alpha$ , IL-1, IL-2, IL-6, IL-8, and IL-12, most likely through the inactivation of the NF- $\kappa$ B transcription factor (Jagetia and Aggarwal 2007; Itokawa et al. 2008). Table 4.1 shows that present findings confirm the anti-inflammatory effects of curcumin by the reductions in IL-1 $\beta$ , IL-8 and TNF- $\alpha$  concentrations. Muwanga (2006), Calpe-Berdiel et al. (2007) and Yuk et al. (2007) reported that *H. hemerocallidea* and beta-sitosterol caused changes in IL-2, IL-4 and IFN- $\gamma$  levels. Results of the present study obtained for *H. hemerocallidea* and beta-sitosterol are thus not supported, since *H. hemerocallidea* caused no significant effect on the cytokine profiles of IL-2, IL-4 or IFN- $\gamma$ . Beta-sitosterol did however, produce noteworthy changes in the pro-inflammatory cytokines IL-1 $\beta$ , IL-8 and TNF- $\alpha$  concentrations (Table 4.1), confirming claims made about its immune modulating activity (Bouic et al., 1996; Bouic and Lamprecht, 1999). In the present study, *S. frutescens* showed a pro-inflammatory effect by increasing the concentration of IL-1 $\beta$  and although this observation was not significant, it failed to lend support to many claims made about the anti-inflammatory effects and uses of this plant. Although Lee et al. (2007a; 2007b) reported that pinitol was able to alter the concentrations of IL-4, IL-5, IL-12p70 and IFN- $\gamma$ , similar results were not found in this study. Furthermore, results obtained for GABA are not supported by the findings of Reyes-Garcia et al. (2007) who reported that GABA caused a reduced production of IL-6 and IL-12 in LPS-stimulated macrophages. On the contrary, GABA caused an increase in IL-1 $\beta$  production which suggests pro-inflammatory activity in this instance.

Inconsistencies and conflicting results are commonly found with *in vitro* cytokine investigations. The complex nature of cytokine functions within and between macrophages as well as the large number of different cytokines, make the clinical significance of observed changes in cytokine profiles questionable. Variables which affect cytokine concentration and detection include macrophage type, cell culture purity, cytokine assays, time at which cytokine levels are measured and of course, technical and methodological inconsistencies (Bornemann et al. 1997).

The use of two different cell lines in the present study, under very similar experimental conditions, highlights the variability of the assay techniques commonly used to assess activity of plant extracts. This is a probable reason for the different measured effects observed in different laboratories.

It is difficult to compare the biological activity observed in this study to that of previously reported literature. Environmental factors such as season and location could have caused variations in the observed activity of the plant extracts. Post-harvesting variables were kept as constant as possible through careful storage and experimental conditions during the course of the present study, but one must consider the changes in activity of the chemical constituents of plants which may occur due to preparatory treatments such as drying, re-dissolving, sonication, etc. (Houghton and Raman 1998). Factors such as temperature, light and pH could also cause degradation of plant material.

#### **4.5 Conclusion**

It is important to remember that recorded cytokine concentrations are very close to the detection limit, making the data variable. Due to lack of statistical significance, definite conclusions cannot be drawn from the present data.

Furthermore, due to large variability in the cytokine concentrations of the two cell lines tested, it would be difficult to confirm the results reported in previous studies. It was however observed that curcumin and beta-sitosterol reduced IL-1 $\beta$  and IL-8 production and significantly ( $p < 0.05$ ) decreased the production of TNF- $\alpha$ . This suggests that beta-sitosterol could indeed possess anti-inflammatory properties, with effects comparable to the known anti-inflammatory effect of curcumin.

## CHAPTER 5. DETERMINATION OF PGE<sub>2</sub> PRODUCTION

### 5.1 Introduction

When the body is subjected to certain stimuli such as pathogens (including viruses) or injury, macrophages are activated and, following a sequence of events, these cells release various pro-inflammatory cytokines, chemokines and chemoattractants, cytotoxic and inflammatory mediators as well as prostaglandins (PG's) (Bresnihan 1999; Gracie et al. 1999). The chronic release of inflammatory mediators, leads to collateral damage linked to various diseases such as rheumatoid arthritis and arteriosclerosis, but in terms of HIV/AIDS-infected patients, these inflammatory processes lead to destruction of infected CD4<sup>+</sup> lymphocytes, resulting in a decreased functionality of the cell mediated immune system (Michaelsson et al. 1995; Gracie et al. 1999).

The role of PG's in the immune system is well known, as is the relationship between PG's and immunodeficiency. It has been proposed that PGE<sub>2</sub> plays a key role in downregulating the Th1 response and inhibiting IL-12 production, which in turn causes a dominance of Th0/Th2 immune responses (van der Pouw Kraan et al. 1995). In HIV-infected patients, an overproduction of PGE<sub>2</sub>, corresponds with decreased levels of IL-12, which could in turn, exacerbate the disease by enhancing the Th2 immune response which leads to the destruction of immune cells (Mastino et al. 1993; Chehimi et al. 1994). PGE<sub>2</sub> is said to affect both Th1 and Th2 cytokine production, possibly resulting in increased HIV replication (Miao et al. 1996). Monocytes isolated from HIV/AIDS subjects showed *ex vivo* increased PGE<sub>2</sub> production and high levels of circulating immunoreactive PGE<sub>2</sub> have been reported in HIV-infected patients (Tarter et al. 1997; Barrios-Rodiles et al. 1999). This information makes PGE<sub>2</sub> a key marker when investigating immune function or modulation of the immune system by external substances.

Due to the relationship between PG levels and the immune function of HIV/AIDS patients, *H. hemerocallidea* and *S. frutescens* as well as standards of compounds reputedly isolated from these plants were tested for their effect on PGE<sub>2</sub> production.

## 5.2 Materials and Methods

### 5.2.1 Treatment of cells

Culturing and preparation of THP-1 and U937 cells was performed as described in section 2.2.4 (p.17). Cells were treated with test samples as described in Section 4.2.1 (p.41, 42).

### 5.2.2 PGE<sub>2</sub> determination

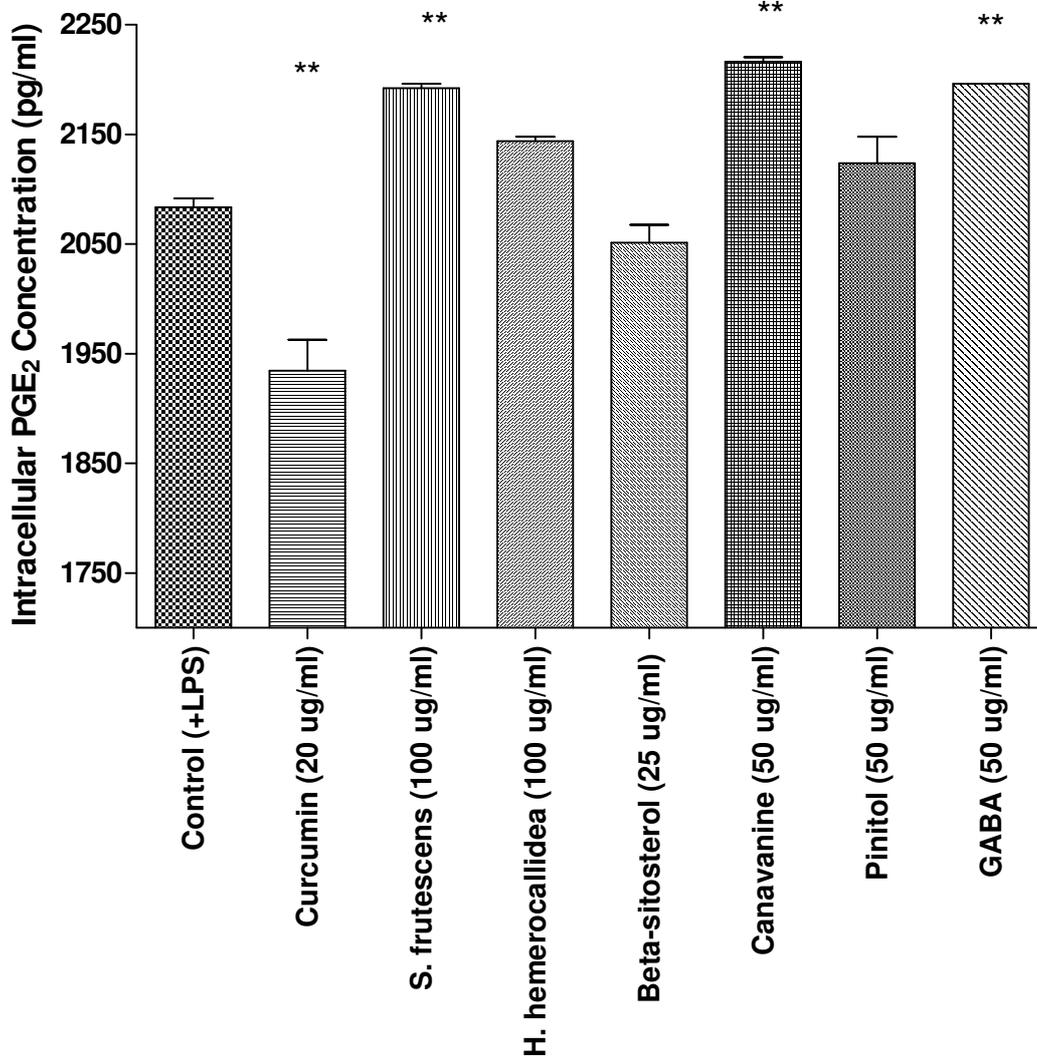
PGE<sub>2</sub> concentration was determined using an Assay Designs Correlate-EIA PGE<sub>2</sub> ELISA kit according to the procedure described in the package insert. This kit was purchased from Biocom Biotech. Supernatants were thawed, acidified by addition of 2M HCl to a pH of 3.5 and centrifuged (500 x *g*, 2 min) to remove any precipitate. C<sub>18</sub> reverse phase SPE cartridges from the ELISA kits were then prepared by washing with 10 ml ethanol followed by 10 ml deionized water. Each sample was then applied to the column under a slight positive pressure and the samples allowed to penetrate the stationary phase slowly, making sure that they did not run dry. Columns were washed with 10 ml water, followed by 10 ml 15% ethanol and finally, 10 ml hexane. The sample was eluted from the column with 10 ml ethyl acetate. Collected samples were dried under a stream of nitrogen and 250 µl of the Assay Buffer (Tris buffered saline containing proteins and sodium azide) was added to resuspend the dried sample. PGE<sub>2</sub> standards were prepared by serial dilution of the supplied standard with tissue culture medium (RPMI+) to obtain a concentration range of 39 – 2500 pg/ml.

One hundred microlitres of standard or sample was pipetted into the appropriate wells of a 96-well plate. Fifty microlitre conjugated PGE<sub>2</sub> (alkaline phosphatase conjugated with PGE<sub>2</sub>) and 50 µl antibody was added. The plate was incubated at room temperature while gently shaking for 2h. The contents of the wells were washed twice with 400 µl wash buffer (Tris buffered saline containing detergents) and the supernatant discarded. Five microlitres of conjugate was added to the appropriate wells to serve as the total activity wells.

At this point, blank wells contained only culture medium and total activity wells (positive control) contained only conjugate. Two hundred microlitres of pNpp substrate solution (p-nitrophenyl phosphate in buffer) was added to each of the washed wells and the plates incubated (room temperature, 45 min) after which 50 µl of stop solution (trisodium phosphate in water) was added to each well. Plates were read for optical density at 405 nm using a reference wavelength of 570 nm. The mean optical density of the Blank wells was manually subtracted from all the readings. Net optical density was then used to calculate the binding of standards and samples as a percentage of the maximum binding wells. “Percent Bound” versus “Concentration of PGE<sub>2</sub> standards” was used to generate a standard curve. A linear calibration curve was approximated through the transformed data points and the concentration of PGE<sub>2</sub> in the samples was determined by interpolation. This experiment was performed once, in duplicate wells.

### 5.3 Results

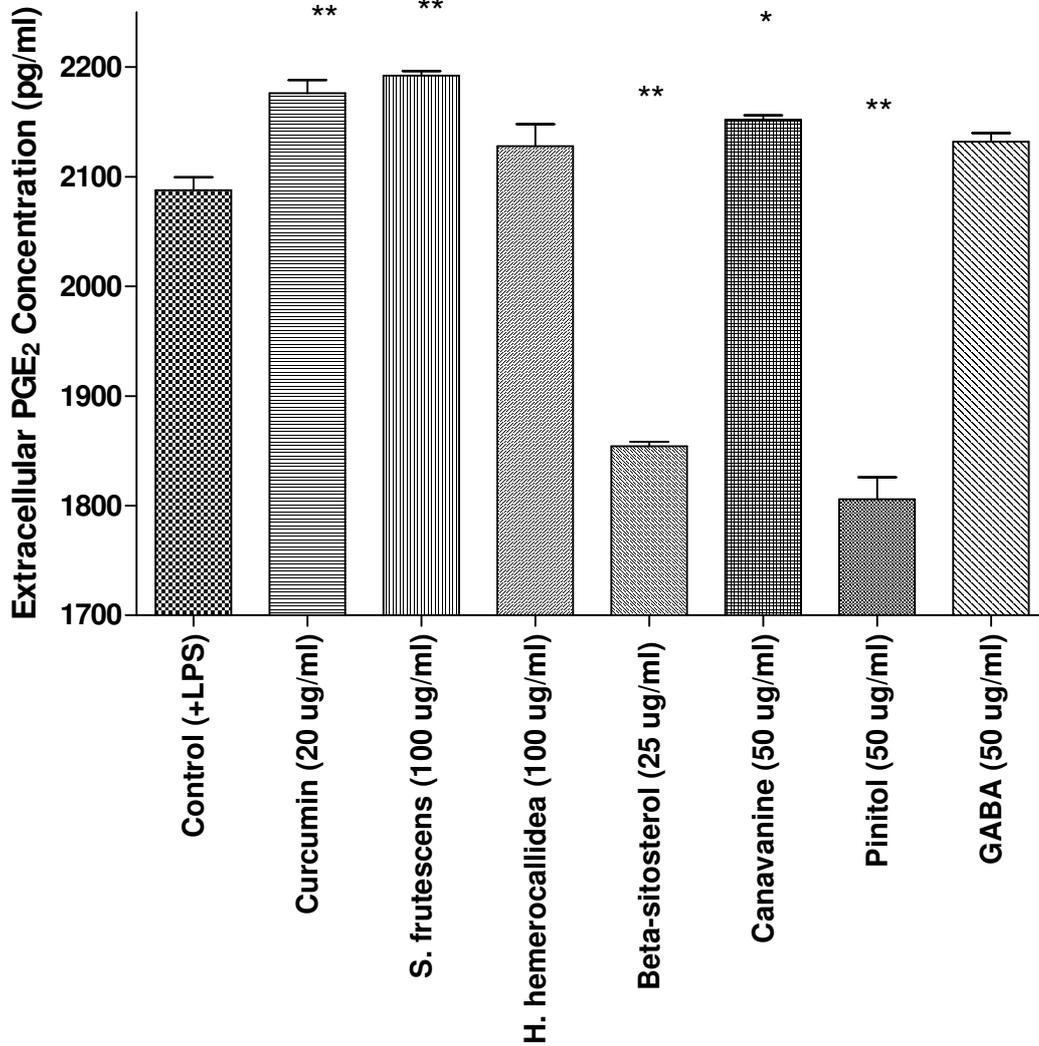
Curcumin caused a significant reduction in the intracellular PGE<sub>2</sub> production of U937 macrophages, whereas *S. frutescens* significantly ( $p < 0.01$ ) stimulated the production of intracellular PGE<sub>2</sub> in these cells (Figure 5.1). Canavanine and GABA also caused significant ( $p < 0.01$ ) increases in PGE<sub>2</sub> concentrations (Figure 5.1), suggesting their contribution to the pro-inflammatory effect of *S. frutescens* in this instance.



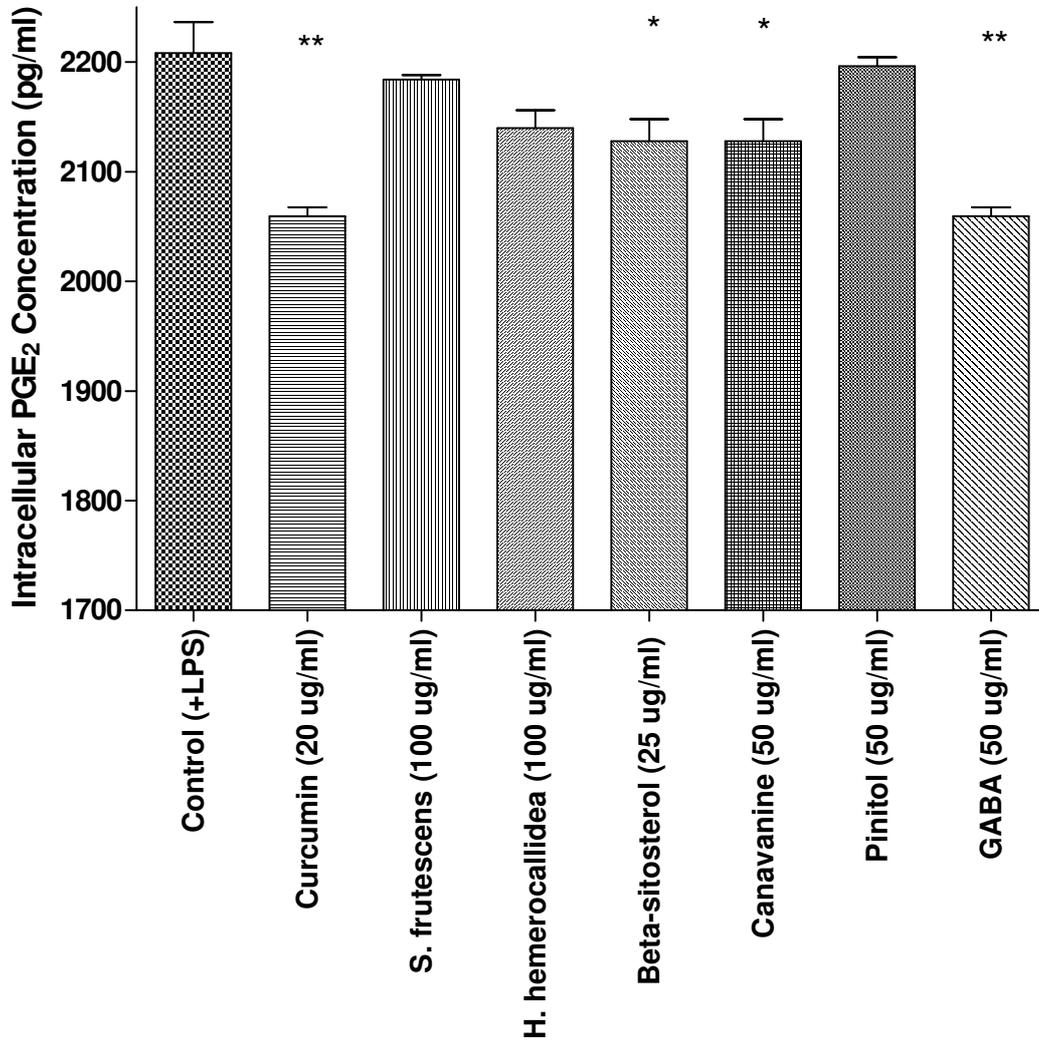
**Figure 5.1.** Intracellular PGE<sub>2</sub> concentration in differentiated U937 macrophages. Cells were treated with various test compounds, stimulated with LPS and lysed with SDS. PGE<sub>2</sub> concentration in cell culture supernatants was then determined using ELISA kits. (One-way ANOVA, n = 2, \*\* p<0.01 relative to the control)

Curcumin produced a significant ( $p < 0.01$ ) increase in the extracellular PGE<sub>2</sub> production of U937 macrophages, suggesting a pro-inflammatory effect, comparable to that of *S. frutescens*, when compared to untreated controls (Figure 5.2). Canavanine also stimulated PGE<sub>2</sub> production, however, pinitol had a significant ( $p < 0.01$ ) anti-inflammatory effect (Figure 5.2). Although *H. hemerocallidea* extract produced no significant effect on extracellular PGE<sub>2</sub> in U937 cells, beta-sitosterol (one of its constituents) caused a significant ( $p < 0.01$ ) reduction in PGE<sub>2</sub> production (Figure 5.2).

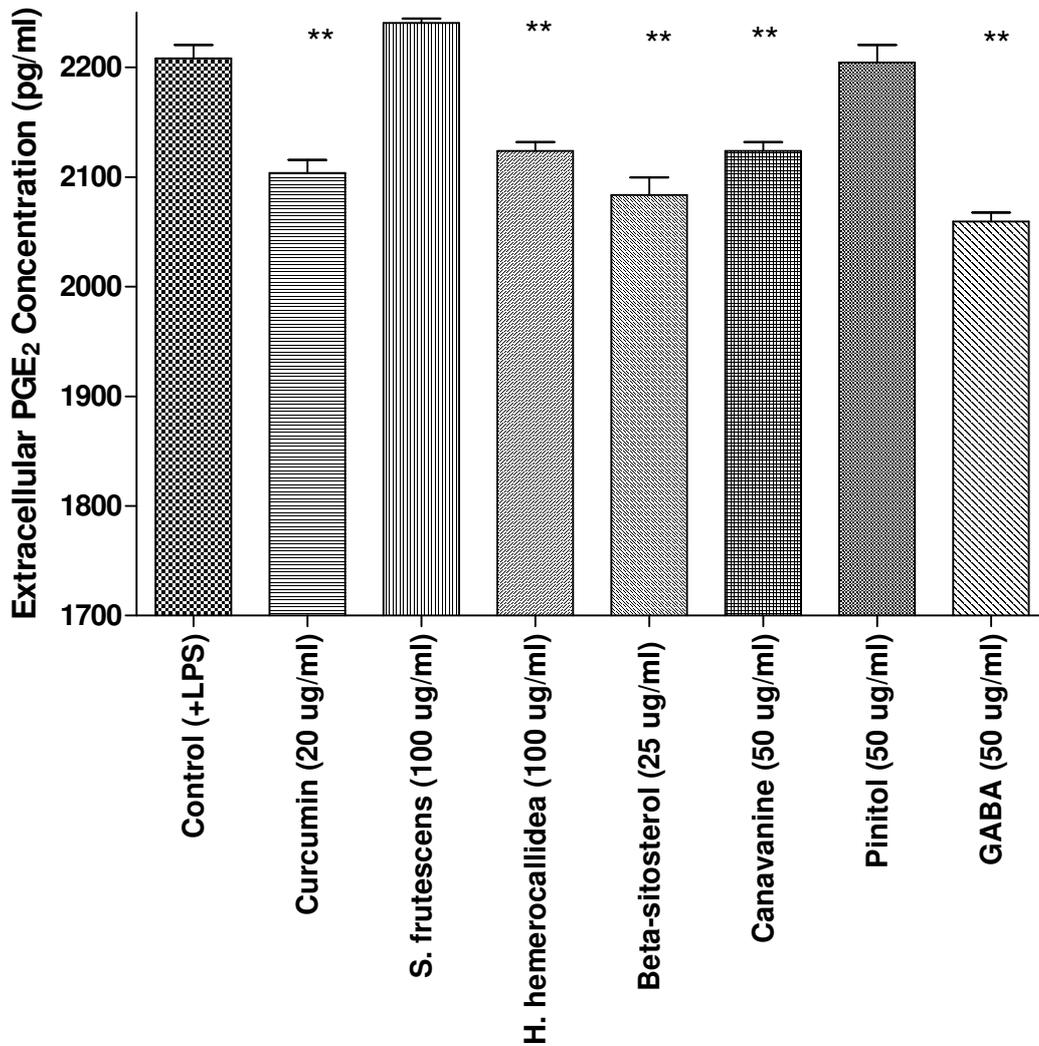
Curcumin caused significant ( $p < 0.01$ ) anti-inflammatory activity in THP-1 cells both intra- and extracellularly (Figures 5.3 and 5.4). Beta-sitosterol, canavanine and GABA reduced intracellular as well as extracellular PGE<sub>2</sub> concentrations significantly ( $p < 0.05$ ), comparable to that of curcumin. *S. frutescens* extract had no significant effect on either intra- or extracellular PGE<sub>2</sub> production in THP-1 macrophages (Figures 5.3 and 5.4) but *H. hemerocallidea* extract caused a significant decrease in the concentration of extracellular PGE<sub>2</sub> (Figure 5.4).



**Figure 5.2.** Extracellular PGE<sub>2</sub> concentration in differentiated U937 macrophages. Cells were treated with various test compounds and stimulated with LPS. PGE<sub>2</sub> concentration in cell culture supernatants was then determined using ELISA kits. (One-way ANOVA, n = 2, \* p<0.05, \*\* p<0.01 relative to the control)



**Figure 5.3.** Intracellular PGE<sub>2</sub> concentration in differentiated THP-1 macrophages. Cells were treated with various test compounds, stimulated with LPS and lysed with SDS. PGE<sub>2</sub> concentration in cell culture supernatants was then determined using ELISA kits. (One-way ANOVA, n = 2, \* p<0.05, \*\* p<0.01 relative to the control)



**Figure 5.4.** Extracellular PGE<sub>2</sub> concentration in differentiated THP-1 macrophages. Cells were treated with various test compounds and stimulated with LPS. PGE<sub>2</sub> concentration in cell culture supernatants was then determined using ELISA kits. (One-way ANOVA, n = 2, \*\* p<0.01 relative to the control)

## 5.4 Discussion

According to Plummer et al. (1999), curcumin inhibits COX-2 expression in colon cells, resulting in decreased PGE<sub>2</sub> production. Sharma et al. (2004) determined the levels of PGE<sub>2</sub> in blood samples taken from 15 colorectal cancer patients who had received 3.6 g curcumin daily. A significant ( $p < 0.05$ ) decrease in plasma PGE<sub>2</sub> concentration of 57% after 29 days of treatment was noted. Figures 5.1, 5.3 and 5.4 confirm claims made in terms of the anti-inflammatory properties of curcumin. Beta-sitosterol, canavanine and GABA caused significant reductions in intracellular as well as extracellular PGE<sub>2</sub> concentrations comparable to that of curcumin.

No significant prostaglandin-synthesis inhibitory activity was reported for *H. hemerocallidea* (Jager et al. 1996). Gaidamashvili & van Staden (2006) confirmed this when they used the cyclooxygenase assay to investigate the inhibition of PG production by lectin-like proteins isolated from *H. hemerocallidea* and found that *H. hemerocallidea* had a relatively weak effect, inhibiting only 29% of cyclooxygenase activity compared to 59% inhibition by the indomethacin control. Furthermore, Ojewole (2002) reported that the methanolic extract of *H. hemerocallidea* produced a more pronounced anti-inflammatory effect than its aqueous extract. The latter is not promising in terms of applicability, because the principle ways of administration of these traditional remedies, results in the uptake of mainly aqueous extracts (Gaidamashvili and van Staden 2006). Topical application of a methanolic extract of *S. frutescens* was reported to significantly inhibit 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced COX-2 production in mouse skin (Kundu et al. 2005). Results of the present study suggest that *S. frutescens* and canavanine stimulated PGE<sub>2</sub> production and that beta-sitosterol and pinitol inhibited PGE<sub>2</sub> production in U937 macrophages (Figures 5.1 and 5.2). Furthermore, it appears as though *H. hemerocallidea*, beta-sitosterol, canavanine and GABA inhibit PGE<sub>2</sub> production in differentiated THP-1 macrophages (Figures 5.3 and 5.4).

The results of this study are contradictory to those of Kundu et al. (2005) who reported the anti-inflammatory activity of *S. frutescens*, but this discrepancy could be attributed to the fact that an aqueous extract of *S. frutescens* was used as opposed to the methanolic extract used by Kundu et al. (2005).

Results of Gaidamashvilli and van Staden (2006) support the present findings where weak anti-inflammatory activity of aqueous extracts of *H. hemerocallidea* in terms of PGE<sub>2</sub> production were found. It should also be noted that the significant effects caused by most of the test samples in this experiment were comparable to that of curcumin and that only beta-sitosterol and pinitol (Figure 5.2) showed greater reductions in PGE<sub>2</sub> production than the curcumin positive control. Although the beta-sitosterol component of *H. hemerocallidea*, showed significant reductions in PGE<sub>2</sub> production (Figures 5.2–5.4), it appears as though this effect is not seen when beta-sitosterol is administered as part of the crude *H. hemerocallidea* extract (Figures 5.1-5.3). *S. frutescens* had no significant effect on either intra- or extracellular PGE<sub>2</sub> production in differentiated THP-1 macrophages (Figures 5.3 and 5.4). The fact that the individual components of this plant produced anti-inflammatory effects when used as standard solutions, but the crude extract did not, suggests that this anti-inflammatory activity could not be achieved by administration of the plant extract alone, and that components of this plant would have to be isolated and concentrated in order to produce a significant effect.

It is however important to remember that the statistically significant differences seen in these results were obtained by only two observations of each event. Furthermore, the differences in PGE<sub>2</sub> concentrations are all between 5% and 10%, which is physiologically debatable. Perhaps the most noteworthy observations, are the reductions in extracellular PGE<sub>2</sub> concentrations caused by beta-sitosterol and pinitol in U937 macrophages.

## 5.5 Conclusion

*S. frutescens*, *H. hemerocallidea* and compounds isolated from these plants do seem to have significant effects on PGE<sub>2</sub> production *in vitro* with beta-sitosterol and pinitol the two most promising candidates for anti-inflammatory activity in terms of reducing PGE<sub>2</sub> production. They are the only two compounds showing greater reductions in PGE<sub>2</sub> than curcumin which has previously been shown to inhibit PG production.

## CHAPTER 6: SUMMARY

Assessment of the cytotoxicity of the aqueous extracts of *S. frutescens* and *H. hemerocallidea* as well as compound standards from these plants revealed that neither of the test samples was cytotoxic, even at test concentrations much higher than recommended for daily intake. *S. frutescens* was tested at a concentration range of 0.1 - 1.6 mg/ml, *H. hemerocallidea* at 0.1 - 1.9 mg/ml, beta-sitosterol at 0.2 – 25  $\mu$ M, canavanine, pinitol and GABA at 1.5 – 200  $\mu$ M. Test samples showed cytotoxicity profiles comparable to that of the positive control (curcumin), which is a well known immune modulator.

TEAC and ORAC antioxidant assays were employed to assess the antioxidant capacity of the test samples. The poor correlation between data obtained for the two assays was most likely due to the different free radical sources used in the two procedures. Previously reported antioxidant activity of the curcumin positive control was confirmed in both assays. In the TEAC assay, only *H. hemerocallidea* showed an antioxidant effect, with 1 mg/ml of its aqueous extract being equivalent to approximately 0.2 mg/ml Trolox. *S. frutescens*, beta-sitosterol, canavanine, pinitol and GABA produced a slight pro-oxidative effect. Results of the ORAC showed canavanine (1 mg/ml) to be the strongest antioxidant, producing an effect equivalent to that of curcumin. *S. frutescens*, *H. hemerocallidea*, beta-sitosterol, pinitol and GABA produced only slight antioxidant activity which was not as potent as that of curcumin.

There was great variation in the data obtained from flow cytometric analysis of cytokine profiles in treated THP-1 and U937 macrophages, probably due to the fact that recorded concentrations were very close to the detection limit. It was however, observed that curcumin and beta-sitosterol reduced IL-1 $\beta$  and IL-8 production and significantly ( $p < 0.05$ ) decreased the production of TNF- $\alpha$ .

ELISA results revealed that *S. frutescens*, *H. hemerocallidea* and compound standards from these plants did seem to have significant effects on PGE<sub>2</sub> production *in vitro*. Beta-sitosterol and pinitol were the only two compounds that showed greater reductions in PGE<sub>2</sub> than curcumin, significantly ( $p < 0.01$ ) decreasing extracellular PGE<sub>2</sub> levels in U937 macrophages.

The results of this study do not provide enough evidence to support all claims made about the 'immune boosting' activity of *S. frutescens* and *H. hemerocallidea*, but canavanine, beta-sitosterol and pinitol were found to have potential immune modulating effects comparable to those of curcumin.

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