Immunomodulatory activities of non-commercialized leafy vegetables in Kwa-Zulu Natal, South Africa

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Submitted in complete fulfillment of the requirements for the Degree of

Master of Technology (Biotechnology)

Department of Biotechnology and Food Technology

Faculty of Applied Sciences

Durban University of Technology

South Africa

2012

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Submitted for examination	**:** January 2012

REFERENCE DECLARATION IN RESPECT OF A MASTER'S DISSERTATION

I, **Berushka Padayachee – Student Number: (20823441)** and **Prof Bharti Odhav** (full name of supervisor) do hereby declare that in respect of the following dissertation:

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AUTHOR'S DECLARATION

This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Prof Bharti Odhav** and **Dr. Lalini Reddy**

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Student's signature

DEDICATION

I dedicate this work to my late grandparents and my parents who have always taught me to perservere and strive for success no matter what the odds.

ACKNOWLEDGEMENTS

I would like to extend my sincere gratitude and appreciation to the following people who have made this thesis possible:

- My supervisor and mentor Prof B. Odhav for her incredible support, encouragement, guidance and insight throughout the course of this study.
- My co-supervisor Dr. L. Reddy and Prof H. Baijnath for their support.
- I am extremely grateful to Mr. Vivek Naranbhai for his time and effort in assisting me with the flow cytometry tests and cell culture techniques.
- The National Research Foundation (NRF) for funding this project.
- The South African Blood Bank (East Coast region, Pinetown) for the donation of buffy coats to run my experiments.
- My friends Alveera, Depika, Vashka, Naazlene, Diantha, Upasana and Kyle for their constant support and motivation at all times.
- The Staff at the Department of Biotechnology and Food Technology, Durban University of Technology especially Dr. Raju, Dr. Venu, John Mellem and Viresh Mohanlall who helped me with the editing of my thesis.
- I am indepted to my family for their love and prayers and emotional support.

ABSTRACT

Immunomodulation using plants is of primary interest in scientific communities because it provides an alternative to conventional chemotherapy for a wide range of diseases. It is based on the ability of the plants to effectively modulate immune functions, thus being able to promote positive health and maintain the body's resistance to infection. This research is aimed to evaluate the immunomodulatory potential of fourteen traditional leafy vegetables from Kwa-Zulu Natal, South Africa on human peripheral blood mononuclear cells (PBMC). In this study the methanolic and aqueous extracts were screened for lymphocyte proliferation using the MTT assay. The cytokine response was evaluated by measuring the secretion of interleukin 10 (IL-10) and interferon-gamma (IFN-γ) using the ELISA assay. The subpopulation of T cells viz., $CD4^+$, $CD8^+$, NK and B cells were measured by flow cytometry.

Most of the methanolic extracts stimulated PBMC's whilst a few suppressed lymphocyte proliferation. Most of the aqueous extracts were inactive. The methanolic extracts of *Amaranthus hybridus* and *Centella asiatica* stimulated PBMC's and showed an increase in IFN-γ secretion and the $CD8^+$ cytotoxic T cells and B cells. Thus, they induced the Tc-1 immune response and stimulated cell mediated immunity. The methanolic extracts of *Asystasia gangetica, Bidens pilosa, Emex australis, Justicia flava Momordica balsamina, Oxygonum sinuatum, Senna occidentalis* and *Sonchus oleraceous* and the aqueous extracts of *Amaranthus spinosus* and *Asystasia gangetica, Ceratotheca triloba, Oxygonum sinuatum, Physalis viscosa and Sonchus oleaceous* stimulated PBMC's and showed an increase in IL-10 secretion and the $CD8^+$ cytotoxic T cells and B cells. Thus, they induced the Tc-2 immune response and stimulated humoral immunity. Also, the methanolic extracts of *Amaranthus spinosus* and *Ceratotheca triloba* and the aqueous extracts of *Bidens pilosa* and *Justicia flava* increased both IL-10 and IFN-γ secretion and the $CD8^+$

cytotoxic T cells indicating the stimulation of both the Tc1 and Tc2 cytokine profiles. The elevated secretion of IFN-γ and IL-10 caused by the extracts can be attributed to the $CD8^+$ cytotoxic T cells and B cells.

The findings of this study show that leafy vegetables hold promise as immunomodulatory candidates. They may enhance cell-mediated immune functions by a pro-inflammatory response whilst some can promote humoral immune functions by means of an anti-inflammatory response. Further investigation should be considered on the effect of the extracts on other immune parameters.

TABLE OF CONTENTS

ABBREVIATIONS

%	percentage
℃	degree celsius
µl	microlitre
AIM	Acquired immune model
APC	Antigen presenting cells
B-TGCF	B-cell derived growth factor
BRM	Biological response modulators
CCM	Complete culture media
CO_2	Carbon dioxide
CON A	Concanavallin A
CRM	Cytokine response model
CSA	Cyclosporin A
CSIF	Cytokine synthesis inhibitory factor
CTL	Cytotoxic T lymphocyte
DMSO	Dimethyl sulphoxide
DP	double-positive
DPBS	Dulbecco's phosphate buffered saline solution
ELISA	Enzyme-Linked Immunosorbent Assay
FCS	Fetal Calf Serum
FITC	fluorescein isothiocyanate
g	gram
h	hour
HUVECS	Human umbilical vein endothelial cells

IFN-γ	Interferon-gamma
IL-10	Interleukin 10
LPS	Lipo-polysaccharide
mABs	monoclonal antibodies
ml	millilitre
MTT	3-(4,5-dimethylthiazolyl-2)-2,5 diphenyltetrazolium bromide
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate buffered saline
PE	Phycoerythrin
PerCP	peridinin chlorphyll protein
PHA	Phytohaemagglutinin
RPMI	Roswell Park Memorial Institute
SP	single-positive
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
TGIF	T-cell growth inhibitory factor
TMB	Tetramethylbenzidine
Th cell	T helper cell
Tc cell	T cytotoxic cell
TNF-α	Tumor necrosis factor- alpha
WHO	World Health Organization

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE: INTRODUCTION

1.1. Study background

South Africa is home to an exceptionally rich cultural diversity as well as a large floral biodiversity. Of the 30 000 higher plant species found, 3 000 species are used for medicinal purposes (Van Wyk *et al.,* 1997). It is estimated that 27 million South Africans utilize traditional herbal medicines (Stafford *et al.,* 2004). Moreover, the World Health Organization estimates that approximately 80% of the world's inhabitants rely on traditional medicine for their primary health care (WHO, 2002). These statistics emphasize the importance of traditional herbal medicine, more especially in a developing country such as South Africa where it forms the backbone of rural healthcare and is particularly prevalent in areas where western medicine is inaccessible.

A large number of plants used in traditional medicine are used to treat diseases, including infections, immunological disorders and cancer (Ganju *et al.,* 2003). The basis of this appears to be the ability of the plants to effectively modulate immune functions, thus being able to promote positive health and maintain the body's resistance to infection (Jayathirtha and Mishra, 2004). Traditionally many plant preparations are used to treat inflammation, which act by affecting the immune system, whose main function is to protect against disease by identifying and killing pathogens and tumour cells. This system is made up of two arms: the innate and adaptive systems. The innate immune system comprises cells that defend the host from infection by other organisms in a non-specific and generic manner by molecular interaction and by the expression of inducible cytokines and chemokines. The adaptive immune system is composed of specialized effector cells (T and B cells) which recognize antigens processed and presented by macrophages and dendritic cells, leading to the activation of cytotoxic T cells and

generation of antibodies that are involved in the elimination or prevention of pathogenic challenges.

The immune system is a carefully controlled system that under normal conditions balances the pro- and anti-inflammatory principles. However, malfunctions of the immune system often occur due to some loss of internal control (Littman and Rudensky, 2010). An overactive immune system is seen in numerous pathologies including autoimmunity (Kamradt and Mitchison, 2001), chronic inflammatory diseases, systemic vasodilation, carcinogenesis (Aggarwal *et al.,* 2006; Galgani *et al.,* 2009), sepsis and anaphylactic shock. On the other hand immunosuppression leads to greater susceptibility to infection and has also been implicated to play a role in tumour development (De Souza and Bonorino, 2009; Sethi *et al.,* 2009).

Different immune cells like regulatory T cells have been suggested to play a key function for the balance between autoimmunity and the anti-tumour response of the immune system (Yamaguchi and Sakaguchi, 2006; Littman and Rudensky, 2010). Inflammation is, in general terms, beneficial when acute and tightly regulated, but detrimental when chronic. Thus, an altered level of activity of the immune system may be either beneficial or harmful to the organism, depending on the overall degree of modulation and the pathophysiological context. Pharmacological manipulation (i.e. exogenous modulation) of the immune system is desirable and indicated in certain pathologies. To achieve this, both anti-inflammatory (immunosuppressive) and immunostimulatory drugs (e.g. vaccine adjuvants, interferon-α and recombinant cytokines) are currently available to the physician.

From an ethnopharmacological point of view, the concept of inflammation and associated symptoms (pain and fever) has evolved independently in all ethnic groups and human civilizations. As a consequence, traditional phytotherapeutic intervention for inflammatory

disorders has been reported widely in the ethnopharmacological literature (Abad *et al.,* 1996; Rios, 2009; Zhang *et al.,* 2009). In contrast, the concept of immunostimulation is not empirical (as in treating inflammation), but largely based on theoretical considerations of the immune system. Thus, the notion of immunostimulation lacks a solid ethnopharmacological background, despite the fact that in ancient Chinese medicine the zheng qi (healthy energy) was a kind of conception of immune defense which could be positively influenced by means of phytotherapy (Tan and Vanitha, 2004). The Chinese version of Taoist and Buddist philosophies strives for a balance between active and passive or any kind of natural duality (yin yang), thus also looking for homoestasis in health.

The utilization of traditional medicinal practices has greatly receded due to the advent of modern treatment modalities. As a consequence, relatively little attention has been devoted to studies on the immunomodulatory effects of medicinal plants to treat diseases. Hence, there is a substantial need to develop new immunomodulatory agents with novel mechanisms of action due to the alarming increase in the incidence of these immune related diseases. The present study is therefore aimed to evaluate the immunomodulatory potential of selected plants in vitro.

Fourteen non-cultivated leafy vegetables indigenous to Kwa-Zulu Natal, South Africa were selected in this study. They are: *Amaranthus dubius, Amaranthus hybridus, Amaranthus spinosus, Asystasia gangetica, Bidens pilosa, Centella asiatica, Ceratotheca triloba, Emex australis, Justicia flava, Momordica balsamina, Physalis viscosa, Oxygonum sinuatum, Senna occidentalis,* and *Sonchus oleraceous*. The selection of these plants is based on the fact that they supplement commercial vegetables and are thus safe for consumption; they form an important aspect of subsistence farming, and are often used for medicinal purposes (Odhav *et al.,* 2007). They are also readily available, do not require any form of cultivation and have great cultural significance.

1.2. Objectives of the study

The objectives of this study were to:

1. Collect and prepare aqueous and methanolic extracts of fourteen (14) traditional leafy vegetables from Kwa-Zulu Natal, South Africa.
2. Isolate lymphocytes from buffy coats of whole human blood.
3. Screen all 14 plants for lymphocyte proliferation activity using the (MTT) assay.
4. Determine the levels of Interleukin 10 and Interferon-gamma released by the extract-stimulated lymphocytes using the ELISA assay.
5. Determine the effect of the plant extracts on lymphocytes by evaluating the cell sub-population responses, namely; natural killer (NK) cells, $CD4^+$ helper T cells, $CD8^+$ cytotoxic T cells and B cells using flow cytometry.
6. Correlate the T cell sub population responses with the cytokine responses, to indicate a possible immunomodulatory pathway i.e. Th1/Tc1 and Th2/Tc2 response.

CHAPTER TWO: LITERATURE REVIEW

2.1. Plants used in this study

Traditional indigenous vegetables have nutritional value and are used as medicine for many rural communities in most parts of Africa. Many people, however, are still unaware of the importance and medicinal value of these plants, possibly because it constitutes an area which is still relatively under-researched in South Africa (Odhav *et al.,* 2007). Interest in recent years has been diverted to the introduced non-native vegetables and to excessive cultivation of field crops, accounting for the under-utilization of traditional leafy vegetables. This has led to poor nutrition, widespread diseases and nutritional disorders among the rural communities.

More consideration should be given to these traditional leafy vegetables since they prove to be beneficial in many aspects. They grow readily in the wild, do not require any formal cultivation, and represent an inexpensive means of high quality sources of nutrition for the poverty-stricken areas of South Africa. Traditional leafy vegetables that can potentially modulate immune functions, in addition to supplementing the nutritional diet of rural communities, will not only strengthen its awareness among local populations, but can also open up the possibility of commercialization of these plants.

It is against this background of information and knowledge of medicinal plants acquired that the present study will be designed to investigate the immunomodulatory potential of traditional leafy vegetables in Kwa-Zulu Natal, South Africa. Table 1 is a summary of the plants, their common names, their traditional names, and the source from which they were collected. This is followed by a detailed description (as found in literature) of the plants habitat and distributions, its medicinal properties and phytochemistry.

Table 1: Summary of leafy vegetables used in this study

Scientific name	Family name	Common name	Zulu name	Active compounds	Source
Amaranthus dubius	Amaranthaceae	Wild spinach	Imbuya	β-carotene Niacin Thiamin Riboflavin Ascorbic acid	Reservoir Hills, Durban
Amaranthus hybridus	Amaranthaceae	Rough pigweed	Imbuya	Flavonoids Terpenoids Steroids Cardiac glucosides	Reservoir Hills, Durban
Amaranthus spinosus	Amaranthaceae	Spiny amaranth	Imbuya	Amaranthoside Amaranthine Amaricin	Reservoir Hills, Durban
Asystasia gangetica	Acanthaceae	Creeping foxglove	Isihobo	Salidroside Apigenin Ajugol Megastigmane Glucoside Benzyl b-D-glucopyranoside	Reservoir Hills, Durban
Bidens pilosa	Asteraceae	Beggar's ticks	Amalenjane	**Flavonoids:** centaurein, centaureidin Polyacetylene2-*O*-b-D-glucosyltrideca-11 *E*-en-3,5,7,9-tetrayn-1,2-diol	Reservoir Hills, Durban
Centella asiatica	Apiaceae	Gotu kola	Icudwane	**Triterpenoids**: Asiatic acid, Asiaticoside, Madecassic acid Madecassoside	Reservoir Hills, Durban
Ceratotheca triloba	Pedaliaceae	Wild foxglove	Udonqabathwa	Anthraquinones Saponins Steroids Terpenoids	Reservoir Hills, Durban
Emex australis	Polygonaceae	Devil's thorn	Inkunzane	Phenols	Reservoir Hills, Durban
Justicia flava	Acanthaceae	Yellow justicea	Ipela	Helioxanthin, Isolariciresinol Justicinol Docosanoic acid	Reservoir Hills, Durban
Momordica	Cucurbitaceae	Balsam	Umkaka	Momordin II	National

balsamina		pear		**Phenols:** Rosmarinic acid **Cucurbitacins:** Karavilagenin Balsaminapentaol Cucurbalsaminol	Botanical Institute, Durban
Oxygonum sinuatum	Polygonaceae	Stars talk	Untabane	Emodin	Reservoir Hills, Durban
Physalis viscosa	Solanaceae	Sticky ground cherry	Uqadolo	Withanolides	Park Rynie
Senna occidentalis	Fabaceae	Coffee senna	Isinyembane	**Anthraquinones:** Dianthrone	Reservoir Hills, Durban
Sonchus oleraceous	Asteraceae	Sow thistle		15-O-β-glucopyranosyl-11β,13-dihydrourospermal Ursolic acid **Monoterpene:** Loliolide	Reservoir Hills, Durban

2.1.1. *Amaranthus dubius*

Figure 1: *Amaranthus dubius*

Amaranthus dubius (A. dubius) belongs to the family Amaranthaceae. It is a flowering plant, also known as wild spinach or spiny amaranth (Odhav *et al*., 2007) and it is widespread throughout the humid lowland tropics. The plant grows up to 150 cm tall, has slender to stout stems and simple, spiral leaves without stipules (Fig. 1). It is a protected weed cooked as a pot herb in many African countries. The leaves of Amaranth are generally recommended as a good food, with medicinal properties for young children, lactating mothers and for people with haemorrhage, anaemia, constipation, stomach pains and kidney complaints (Grubben and Denton, 2004). The leaves contain considerable amounts of β-carotene, niacin, thiamin, riboflavin and ascorbic acid. However, it is not that popular because of the presence of a high content of hydrocyanic acid and oxalic acid, which are considered as anti-nutrients (Grubben and Denton, 2004).

2.1.2. *Amaranthus hybridus*

Figure 2: *Amaranthus hybridus*

Amaranthus hybridus (A. hybridus), a member of the Amaranthaceae family is a popular nutritious leafy vegetable that is cultivated for the leaves which are eaten as spinach. It is a 2.5 m high erect, branched, perennial herb that reaches maturity in six weeks (Fig. 2). It is found on wastelands or near old kraals. The leaves are edible and can be taken as an infusion to treat anaemia, chronic fatigue, heavy menstrual bleeding intestinal bleeding, diarrhoea and coughs (Beekrum 2003). The juice of the whole plant is used to relieve constipation. Externally, the crushed leaves can be used as a poultice for bleeding wounds and infusions can be splashed onto the skin to alleviate burning and itchy skin. Leaf infusions also have cosmetic uses as a cleansing rinse for oily skin since it tightens pores and moistens the skin (Grubben and Denton, 2004). The plant is rich in minerals, proteins and vitamins and is thus consumed widely in Southern Africa (Mellem 2008). Methanolic, hexane, ethyl acetate and dichloromethane extracts of the leaves of *A. hybridus* have pharmacologically active compounds such as flavonoids, steroids, terpenoids and cardiac glucosides (Maiyo *et al.*, 2010).

2.1.3. *Amaranthus spinosus*

Figure 3: *Amaranthus spinosus*

Amaranthus spinosus (A. spinosus) is another member of the Amaranthaceae family and is commonly referred to as spiny pigweed, thorny amaranth or prickly amaranth. It is an annual plant that grows as an erect, branched monoecious herb and it is found mostly on roadsides and wastelands (Fig. 3). Rural communities in Africa consume this plant as a non-conventional leafy vegetable (Globeinmed, 2009). A number of traditional uses have been documented for this plant. It improves appetite, biliousness, blood diseases, burning sensations, bronchitis, piles, leucorrhoea and also acts as a laxative, diuretic and antipyretic. The leaves and roots are used to relieve burns, abscesses, inflammatory swellings and bruises (Kirtikar and Basu, 2001). The chemical constituents of this plant include; amaranthoside, amaricin, stigmasterol glycoside, spinoside, quercetin and betaine (Kirtikar and Basu, 2001; Zeashan *et al.*, 2009).

2.1.4. *Asystasia gangetica*

Figure 4: *Asystasia gangetica*

Asystasia gangetica (A. gangetica), also known as Hunter's spinach and creeping foxglove is an erect, clambering herb that can grow up to 1.25 m (Fig. 4). It has green, oval shaped, smooth leaves and is generally found along roadsides and waterbanks and in semi-water logged areas (Akah *et al.,* 2003; Globeinmed, 2009). The traditional medicinal uses of this plant are based on its antihelmintic and anti-parasitic properties. The plant is used for relieving childbirth pains and for stiff neck, nose bleeding, stomach aches, fever and it is also used to treat asthma (Akah *et al.* 2003; Beekrum 2003). The sap is applied to sores, wounds and piles. A leaf decoction is used as an analgesic and treats epilepsy and urethral discharge (Grubben and Denton, 2004). The leaves are also good sources of proteins, amino acids, sugars, lipids, fibres and minerals, accounting for its potential as a food source. Known compounds of *A. gangetica* include; salidroside, apigenin, ajugol, megastigmane glucoside and benzyl b-D-glucopyranoside (Anderson, 2007).

2.1.5. *Bidens pilosa*

Figure 5: *Bidens pilosa*

Bidens pilosa (B. pilosa) commonly known as beggar's ticks belongs to the Asteraceae family, the largest flowering plant family in the world. This plant inhabits mainly plantations, roadsides and wastelands thriving in moist, nutrient-rich soil. Its characteristic features include the petiolate leaves, erect, ramified stems and the flowers which have white ray florets and yellow disk florets (Bayer Crop Science, 2010) as shown in Fig. 5. The plant is used traditionally in several forms of treatment, namely as anti-inflammatory, anti-diabetic, diuretic, anti-rheumatic and antibiotic agents (Chang *et al.*, 2007). The juices of the leaves are used to dress wounds and ulcers (Beekrum, 2003). Previous studies reported on the bioactivities of *B. pilosa* plant extracts such as, anti-hyperglycemic (Ubillas *et al*., 2000), anti-hypertensive (Dimo *et al*., 2001), anti-leukemic (Chang *et al.*, 2001) and anti-microbial (Khan *et al.*, 2001) activities. Phytochemical studies indicate the presence of several photochemical constituents in the plant namely, chalcones, flavonoids, polyacetylenes, glucosides and diterpenes (Chiang *et al.*, 2007).

2.1.6. *Centella asiatica*

Figure 6: *Centella asiatica*

Centella asiatica (C. asiatica) is a member of the Apiaceae family and is also referred to as Marsh pennywort (Odhav *et al.*, 2007). It is a perennial creeper with long stems and heart shaped bright green leaves (Fig. 6). It grows abundantly in moist areas such as riverbanks and is distributed widely in tropical and subtropical areas. *C. asiatica* has many traditional medicinal uses in various parts of the world. In the Ayurvedic system of medicine, it is used to treat central nervous system disorders such as depression, insomnia, stress and memory loss (Ganachari *et al*., 2004). In China, it is used for the treatment of leprosy, scar formation and for wound healing purposes (Yen *et al.*, 2001). Other medicinal actions of this plant include its ability to treat fevers, diarrhoea, leprosy, stress and cancer (Jayathirtha and Mishra, 2004; Sagrawat and Khan, 2007). The crushed leaves can be mixed with aqueous cream to form a paste that can be applied to the skin for ailments such as eczema, wounds and sores. The plant also has anti-bacterial, anti-fungal, anti-inflammatory and anti-tumor properties (Grubben and Denton, 2004). Known phytochemicals of this plant include triterpenoids; asiatic acid and madecassic acid.

2.1.7. *Ceratotheca triloba*

Figure 7: *Ceratotheca triloba*

C. triloba is a South African annual plant that is found in the summer rainfall areas of South Africa, mainly the grasslands. There are only four known species of *Ceratotheca* that are found in Southern Africa (Smithies, 2000). The genus name *"Ceratotheca"* means a horned capsule (Fig. 7) which is derived from the Greek words kerato (horned) and theke (a case). The species name *triloba* is derived from Latin, meaning three-lobed, alluding to the leaves (Hutchings *et al.,* 1996). According to the current classification system (Table 1) it belongs to the family Pedaliaceae. This family of plants is characterized by having mucilaginous hairs which give the stems and leaves a slimy or clammy feel. *C. triloba* germinates optimally in disturbed areas like roadsides, where they grow, flower and seed before winter. The height of the plant depends on the water uptake during summer. The leaves are soft, green and are about 50 mm long covered with fine white hairs. *C. triloba* is slightly sticky and when it is crushed it produces a strong unpleasant smell (Van der Walt, 2001).

It is used in many traditional cultures to treat painful menstruation, stomach cramps, nausea, fever and diarrhoea (Tredgold, 1986). Other traditional uses include the infusion of the leaves which is administrated as an abortifacient and also its use for the treatment of diarrhoea, gastro-intestinal cramps and dysmenorrhoea (Pooley, 1998; Van Wyk and Gericke, 2000). Studies show that *C. triloba* serves as a good source of energy and magnesium (Odhav *et al.,* 2007). Literature on the biological activities of *C. triloba* shows that the plant has no angiotensin 1-converting enzymes (Ramesar *et al.,* 2008). Extracts of the plant can be used to control diabetes as it inhibits α-amylases and it also has anti-oxidant activity (Odhav *et al.,* 2010). In addition the plant extract inhibits lipo-oxygenase and can be used as an anti-inflammatory agent. Known compounds of *C. triloba* include anthraquinones, saponins, steroids and terpenoids (Mohanlall *et al.,* 2011).

2.1.8. *Emex australis*

Figure 8: *Emex australis*

Emex australis (E. australis) also known as Devil's thorn is a herbaceous plant of the Polygonaceae family. It is an annual herb spreading from a dense rosette with a thick taproot

system. The leaves are pear-shaped, broadly rounded and dull green in colour as shown in Fig. 8 (Factsheet, 2005). It inhabits suptropical and temperate regions, mainly and sandy and loamy soils. When consumed in large quantities, the leaves have a laxative effect and also contain oxalates (Natural medicinal herbs, 2006). The leaves have also been used to relieve biliousness and to stimulate appetite. Decoctions of the roots are used for stomach cramps and are also given to infants suffering from restlessness and constipation. Unspecified parts of the plant are used to treat stomach and intestinal complaints (Dold and Cocks, 2000). *E. australis* also displays antioxidant activity due to the presence of phenolic compounds in the plant (Akula and Odhav, 2008).

2.1.9. *Justicia flava*

Figure 9: *Justicia flava*

Justicia flava (J. flava) is widespread in parts of tropical and Southern Africa and is found in different veld types, more commonly in disturbed habitats, growing in sunny or semi-shady areas. Yellow justicia, as it is commonly known is a perennial herb or shrublet that grows up to 450 mm high. The leaves are lanceolate or broadly ovate, with the leaf stalk growing up to 1-25

mm long (Fig 9). It is able to tolerate dry conditions. In a study conducted to determine the potential antihypertensive properties of plants, *J. flava* demonstrated angiotensin-converting enzyme (ACE) inhibition activity (Science in Africa, 2007). In Tanzania, the leaves are used for their emetic properties. In Ivory Coast, the pulped leaves are rubbed onto the skin to treat convulsions and feverish pains in babies. In Ghana, the plant is used internally and externally against fever, yaws and diarrhoea in children (Grubben and Denton, 2004). The main phytochemicals found in *J. flava* were sterols and salicyclic acid from the leaves, stems and roots. In addition, the leaves contain the three lignins, helioxanthin, isolariciresinol and justicinol as well as docosanoic acid (Grubben and Denton, 2004).

2.1.10. *Momordica balsamina*

Figure 10: *Momordica balsamina*

Momordica balsamina (M. balsamina) is widespread throughout Africa and is found in all the provinces of South Africa except the Western Cape. The balsam apple, as it is known, occurs in sandy and calcareous soil, clay and loam. It thrives in areas such as grasslands, savanna and

river bank vegetations and forest margins. The monoecious, perennial herb has a tuberous rootstock, mostly annual stems and broadly ovate to orbicular waxy leaves as shown in Fig. 10 (Hutchings *et al.,* 1996). The leaves and young fruits of this plant are cooked and eaten as a vegetable in many parts of Africa. The bitter taste of *M. balsamina* is due to the presence of saponins. Its medicinal uses are wide and diverse. The fruits, seeds and leaves are used for its anthelmintic properties and is also used to treat fevers, uterine bleeding, syphilis, rheumatism and skin disorders (Grubben and Denton, 2004). The crude extracts of the leaves and the fruits have shown hypoglycaemic and anti-malarial activity (Ramalhete *et al.,* 2009). Seven compounds were isolated from *M. Balsamina*, five of which had cytotoxic effects on a breast cancer cell line MCF-7; balsaminapentaol, balsaminol A, balsaminol B, cucurbalsaminol B, cucurbita-5,23(E)-diene-3,7,25-triol and karavilagenin E. Other compounds which have been isolated from the plant include a ribosome inactivating protein called momordin II and the caffeic acid ester, rosmarinic acid which has shown anti-inflammatory, antiviral and antioxidant activities (Grubben and Denton, 2004).

2.1.11. *Oxygonum sinuatum*

Figure 11: *Oxygonum sinuatum*

Oxygonum sinuatum (O. sinuatum) also known as Stars talk is an erect, annual herb that has green to red-brown pubescent stems, white or pink flowers and grows up to 1 m tall (Fig. 11). It is found mainly as a weed on fields or on waste grounds and prefers well-drained loamy soils. The plant is distributed in parts of eastern and southern Africa, where the leaves are eaten raw or boiled as a vegetable. The raw leaves of *O. sinuatum* have an acidic taste while in powdered form, the taste is mild. Medicinally, the leaves are used to treat boils and the stems are chewed to treat tonsillitis. The whole plant is used for treating gastric ulcers, malaria and hepatitis while the leaf sap is used for coughs (Grubben and Denton, 2004). The juice of the leaves are used for fungal and eye infections (Maundu *et al.,* 1999). An anthraquinone, emodin, was isolated from *O. sinuatum* (Crawford *et al.,* 2011).

2.1.12. *Physalis viscosa*

Figure 12: *Physalis viscosa*

Physalis viscosa (P. viscosa) also known as sticky gooseberry belongs to the Solanaceae family. It is a perennial plant that grows up to 1.8 m high and inhabits coastal areas and

mountains (Michail, 2005). It is an erect, sprawling plant with bell-shaped greenish-yellow flowers and yellow berries (Fig. 12). The plant is used as a tonic, sedative, laxative, and diuretic. The juice of the berries are used in urinary disorders and inflammatory diseases (Beekrum, 2003). The aerial part of *P. viscosa* contain withanolides such as 4β-hydroxywithanolides and its 5,6-desoxi analogue, withaphysanolides and withanolide related pregnanes such as 4-hydroxy-5β, 6β-epoxypregn-2-ene-1,20-dione. Withanolides are a group of steroidal lactones that are known for their broad spectrum of biological activity (Silva *et al.*, 1993).

2.1.13. *Senna occidentalis*

Figure 13: *Senna occidentalis*

Senna occidentalis (S. occidentalis) belongs to the Fabaceae family and is also known as coffee senna. The species varies from a semi-woody annual herb in warm areas to a short-lived perennial shrub in frost-free areas. The crushed foliage of the plant has an unpleasant odor. The compound, alternate leaves have four to six pairs of leaflets, which are ovate to ovate-lanceolate and pointed at the tip as shown in Fig. 13 (Demel and Deketay, 1996). Many phytoactive

chemicals present in the tissues of the plant support its numerous applications in folk medicine. The leaves are used as an analgesic, anti-inflammatory, antibacterial, antifungal, antiviral, febrifuge, purgative and as an immunostimulant. The foliage of the plant is poisonous and generally avoided by livestock (Natural Resources Conservation Service, 2002 and Rain-tree, 2002). Anthraquinone compounds are common in this species and contribute to its widespread use as a purgative and laxative.

2.1.14. *Sonchus oleraceous*

Figure 14: *Sonchus oleraceous*

Sonchus oleraceous (S. oleraceous) also known as sow thistle, is an erect annual herb that grows up to 30-110 cm in height and inhabits fields, roadsides, pastures and waste areas. The leaves are thin, soft and dark green in colour while the flowers blossom yellow and are 5-6 mm in diameter (Fig. 14). The distinguishing feature of the plant is its hollow stem which exude latex (a whitish milk) if damaged (Auld and Meld, 1992). *S. oleraceous* is reported to have an emmenagogue effect and it also aids in liver functionality and is used in the treatment of diarrhoea (Natural medicinal herbs, 2009). The latex in the sap is used to treat warts while the

leaves are applied as a poultice to inflammatory swellings. An infusion of the leaves and the roots are used as a febrifuge and tonic (Natural medicinal herbs, 2009). A phytochemical study of the roots of *S. oleraceous* revealed that two compounds; loliolide and 15-O-β-glucopyranosyl-11β,13-dihydrourospermal displayed antibacterial activity against *Staphylococcus aureus, Bacillus subtilis* and *Escherichia coli* as well as *in vitro* cytotoxic activity against mouse lymphoma cells (L5178Y) (Elkhayat, 2009). Loliolide exerts immunosuppressive activity against B and T lymphocytes (Okada *et al.,* 1994). There have also been reports investigating the free radical scavenging activity of *S.oleraceous*, which may be due to the phenolic and flavonoid compounds present in the plant (Yin *et al.,* 2007). These authors also show that the ethanolic extracts has activity against the proliferation of stomach cancer cells.

2.2. Immunomodulation

Immunomodulation is the process of modifying an immune response in a positive or negative manner by the administration of a drug or compound. Immunomodulation using medicinal plants is of primary interest in scientific communities as it could provide an alternative to conventional chemotherapy for a wide range of diseases (Ganju *et al.,* 2003). An "immunomodulator" which functions as the agent, is any substance, synthetic or biological in origin which can modulate the components of the immune system by a specific stimulatory or suppressive effect (Juyal and Singla, 2001). Natural adjuvants, synthetic agents and biological compounds such as antibodies and cytokines are used as immunosuppressive and immunostimulatory agents (Sagrawat and Khan, 2007).

Why do we need immunomodulators? The human body is continuously exposed to a series of stress factors, which weaken the function of the immune response, generating immunosuppression. In addition, no effective vaccines against some severe infections and

diseases exist e.g. HIV, malaria and meningitis (European Commision, 2008). Therefore, a non-specific stimulation of the immune system is thought to be very useful when the immune system of the host is impaired. Immunostimulants are drugs or compounds that predominantly lead to a non-specific activation of immunological defence mechanisms (Wagner *et al.,* 1999). These mechanisms relate to the function and efficiency of macrophages, complement, granulocytes, natural killer cells, lymphocytes and also to the production of effector molecules such as cytokines generated by activated cells (Jayathirtha and Mishra, 2004). These non-specific effects are expected to provide protection against pathogens including bacteria, fungi, viruses etc.

Immunosuppressants, on the other hand, are used in situations where the immune response is detrimental, such as autoimmune diseases, transplant rejection, allergies and immediate and delayed type hypersensitivity immune reactions (Pereira *et al.,* 1999; Juyal and Singla, 2001). They reduce resistance to infections and stress which may be due to environmental or chemotherapeutic factors. Both aspects of immunomodulation i.e. immunostimulation and immunosuppression are equally important to regulate the normal immunological functioning.

2.3. Plants and the immune response

It is generally assumed that a "healthy immune system" is able to combat infections and inhibit tumour development. Because the immune system is so dynamic and complex it is difficult to define what a “good immune system” means. The concept of plant immunostimulants has almost exclusively originated from in vitro cell culture work, *ex vivo* preparations (Vomel, 1985), or from studies using direct systemic injections mainly based on activation of the innate immune system.

From a historical perspective, the first reports on plant immunostimulants were described by Wagner *et al*., (1999), Thereafter, there are several studies that report of research conducted *in vivo* with plant lectins (Bloksma *et al*., 1979; Ganguly and Das, 1994), plant polysaccharides (Luettig *et al.,* 1989; Laskova and Uteshev, 1992) and saponins (Song and Hu, 2009) reporting immunostimulation by plant constituents injected systemically (intraperitoneally). Other studies of *in vitro*, high molecular weight polysaccharide fractions of numerous medicinal plants have been shown to stimulate cells of the innate immune system (Stimpel *et al*., 1984; Luettig *et al.,* 1989; Guo *et al.,* 2009).

Several bioavailable small organic compounds have been reported to stimulate the immune system in vivo, including the plant natural products phytol, aristolochic acid, and plumbagin as well as the synthetic antibiotic levamisole (Wagner, 1990; Wagner *et al*., 1999; Aachoui and Gosh, 2009). Although the mechanisms of immunostimulation is not known, other cytotoxic plant compounds viz., colchicine, vincristine, isopteropodin, cytotoxic compounds bryostatin-1 and phorbol esters (Wagner, 1990; Wagner *et al.,* 1999) as well as the anti-cancer agent taxol (paclitaxel) show immunostimulating properties.

The clinical concept of immunostimulation primarily focuses on the administration of endogenous immune system enhancing factors that primarily act via T-lymphocytes. These include recombinant interferon alpha (IFN-α) and interleukins 2 and 12 which are used in anti-viral and anti-leukemia therapies respectively, and also involves the use of adjuvants and other emerging novel immune enhancing materials (Hubbell *et al.,* 2009). There is no reason to eliminate plant compounds as potential non-specific immunostimulants, the immune system has evolved to defend the body against invading pathogens, not against the bioavailable plant constituents which make up a major portion of our diet. At the present time, there are very few actual immunostimulants in worldwide clinical use and none of those are of plant origin. A

notable exception is the saponin QS-21 obtained from the bark of *Quillaja saponaria* which is under development as experimental adjuvant to improve the T-cell response for vaccines (Sun *et al*., 2009).

2.3.1. Examples of plant immunomodulators

In Africa traditional healers have been treating immune-related conditions for decades, and many plants have been used successfully (Richter, 2003; Peters *et al.,* 2004). Numerous medicinal plants used in Indian traditional medicine have attracted the attention of many researchers due to their actions on the immune system. Systems of medicines such as Siddha and Ayurveda have suggested ways to increase the body's natural resistance to infection (Sagrawat and Khan, 2007). Examples of plant immunomodulators are; Aloe *vera, Asparagus racemose, Curcuma longa, Eclipta alba, Emblica officinalis* and *Tinospora cordifolia* (Juyal and Singla, 2001; Sagrawat and Khan, 2007). A few will be discussed.

Aloe vera (Aloeaceae) also known as *Curacae aloe* or the silent healer is used widely in folk medicine for its burn healing properties and immunomodulatory effects. Due to these therapeutic properties, it is used in a variety of commercial products (Choi and Chung, 2003). Isolated components of the plant have been shown to exert pharmaceutical activity. It was found that Acemannan, a saccharide from this plant activated macrophages, increased cytokine release and enhanced the generation of cytotoxic T-lymphocytes (Choi and Chung, 2003). Two dihyrdocoumarin derivatives exhibited immunomodulatory activity by increasing the phagocytic activity and stimulated the production of superoxide anions in the oxygen respiratory burst of rat peritoneal macrophages (Zhang *et al.,* 2009).

Curcuma longa known as turmeric belongs to the family Zingiberaceae. Several reports have documented the ability of curcumin, a chemical component of *C. longa*, to modulate the growth

and cellular responses of various cell types of the immune system (Gupta and Ghosh, 1999). Curcumin inhibits the PHA-induced proliferation of peripheral blood mononuclear cells (PBMC) and also suppresses interleukin-2 (IL-2) expression and NF-Kb. Evidence also suggests that curcumin modulates both the proliferation and activation of T cells (Yadav *et al.*, 2005). The most active component of *C. longa*, diferuloylmethane, has been shown to inhibit the TNF-α expression of adhesion molecules on human umbilical vein endothelial cells (HUVECs) (Gupta and Ghosh, 1999).

Eclipta alba (Asteraceae), also called "Bhringraj" in India is a dried herb powder which has a wide range of uses in traditional medicine. A study conducted by Jayathirtha and Mishra (2004), has reported the ability of *E. alba* to induce immunomodulatory activity in albino mice by significantly increasing the phagocytic index, antibody titer and the white blood cell count. Other immunomodulators of plant origin are outlined in Table 2 by Sagrawat and Khan, (2007).

Table 2: Examples of other plant immunomodulators (Adapted from Sagrawat and Khan, 2007).

Name	Family	Chemical moiety	Pharmacological activity
Actinidia macrosperma	Actinidiaceae	Alkaloids	Immunostimulatory
Boswellia carteri	Burseraceae	Triterpenoids	Immunostimulant action on T-lymphocytes
Euphorbia tirucalli	Euphorbiaceae	Biopolymeric	Suppression of $CD4^+$ and $CD8^+$ T cells, inhibition of intracellular Interleukin-2 and Interferon-gamma change
Scutellaria baicalensis	Lamiaceae	Wogonin	Stimulates TNF-α, activates iNOS

Zingiber officinale	Zingiberaceae	Gingerols	Potent stimulator of B cells

2.4. Types of immune modulatory reponses

Advances have been made towards understanding host immune responses to infectious diseases. Due to these developments, it has now become possible to strategically manipulate the signalling pathways involved in effector functions in order to influence host responses (Tzianabos, 2000). The modulation of the immune system is an advanced and sophisticated process that is essential in many ways to maintain disease prevention. However, it is a process that still requires thorough scientific investigation due to the vast complexities of the interactions within the immune system (Juyal and Singla, 2001).

There are three types of immunomodulatory responses after treatment with phytochemicals. The type 1 response results in enhanced lymphocyte activation and IFN-γ secretion. The type 2 response results in augmented lymphocyte activation and suppressed IFN-γ secretion. Lastly, the type 3 response causes an increased IFN-γ secretion and suppressed lymphocyte proliferarion (Cherng *et al* 2007; Cherng *et al*., 2008).

To better understand immunomodulation, one requires an understanding of the intricate interactions and networking within the immune system, on which it is dependent. Hence, a summarised overview of the immune system is given below.

2.5. Overview of the immune system

The immune system is a complex network of cells and processes that has evolved to defend the human body against attacks by "foreign invaders". An impaired immune system can manifest in a range of ailments such as infections, aging, allergies, disorders of various organs as well as a

torrent of diseases ranging from cancer to AIDS (Cherng *et al.*, 2008). The role of the immune system has become increasingly important in understanding the mechanisms of disease prevention (Volman *et al*., 2008). This system is also under constant threat by the invasions of pathogens, such as bacteria, fungi, viruses, parasites and toxins (Figure 15), which also target and compromise the immune system. The resulting immune dysfunction is classified as either: an underactive, weakened immune function (immunodeficiency) or an overactive immune function (autoimmunity or autoimmune disease) (Figure 16). A weak immune system can lead to various infections by pathogens and, in some cases, cancer. An overactive immune system could result in allergic reactions such as asthma and sinusitis or autoimmune disorders such as lupus and multiple sclerosis.

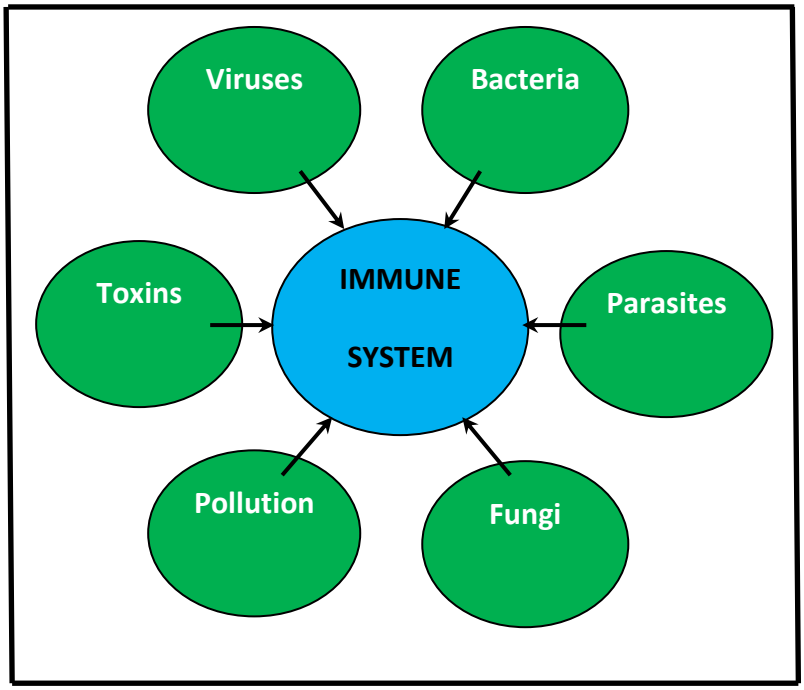


Figure 15: Threats to the immune system

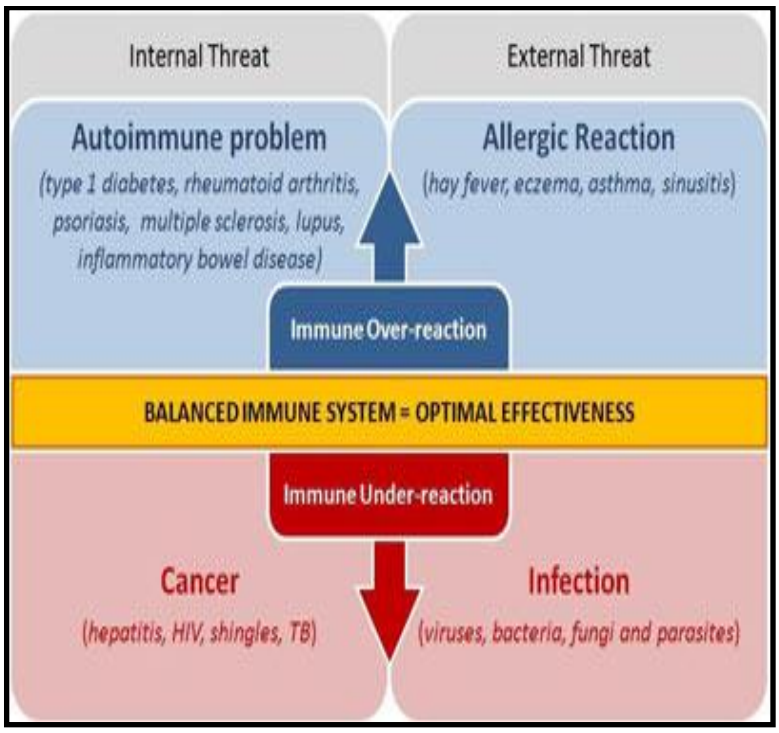
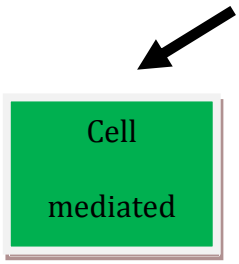
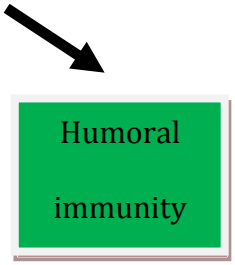


Figure 16: A balanced immune system

The most effective function of the immune system is to mount a response that eradicates the infectious agent from the body. This is accomplished by the actions of mutually interactive systems, the innate (rapid but non-specific) and adaptive (highly specific) immune systems as shown in table 3 (Volman *et al.*, 2008).

Table 3: Cells and processes involved in the innate and adaptive immune systems (Adapted from Volman *et al.*, 2008).

	INNATE/NATURAL	ADAPTIVE/SPECIFIC	
Cells/Systems	Natural killer (NK) cells Complement-system Neutrophils Monocytes/macrophages	 Cytotoxic T cells Helper T cells	 B cells
Processes:	Phagocytosis Antigen presentation Oxidative burst Cytokine production	Cytokine production (Th1+Th2)	Antibody production
		Macrophage activation (Th1)	
		Lysis of infected cells (Tc)	
		B-cell activation (Th2)	

2.5.1. Innate and adaptive immunity

The innate immune system is the defense system that an individual is born with and includes epithelial barriers such as the skin, gastrointestinal tract, lungs and the urinary tract, all of which constitute the first line of defense. The epithelium acts as a physical barrier to the entry of microbes and also produces a wide range of antimicrobial factors. Since the innate immune response is generally non-specific, it does not respond vigorously to second encounters with particular microbes hence it does not exhibit a memory response (Pruett, 2003). Cells which mediate these responses include: neutrophils, eosinophils, basophils, macrophages, natural killer (NK) cells and the complement system (Table 3).

Since viruses lack the apparatus to renew and replicate themselves, it is necessary for them to penetrate the cells of the infected host to dominate its replicative machinery. Therefore, it is essential for the host to find a way to kill the infected cells before the virus can reproduce. Natural killer cells mediate this function by facilitating the lysis of virally infected or tumor cells (Roitt *et al.*, 2005). Macrophages aid in eliminating intracellular microbes and infectious agents via phagocytosis. Certain mediators of T cells can enhance the ability of macrophages to destroy such microorganisms. This is achieved by many mechanisms including; the development of nitric oxide, development of reactive forms of oxygen, activation of proteolytic enzymes and the production of cytokines (Paul, 2003). In addition, macrophages can act as antigen presenting cells (APCs) and therefore, recruit the "help" of activated cytokine producing $CD4^+$ T cells in regulating their function. These cells play a central role in phagocytosis of pathogens, free radical production (oxidative burst), cytokine production and the presentation of antigens to lymphocytes as seen in Table 3 (Paul, 2003).

In contrast to rapid and non-specific innate immunity, acquired or adaptive immunity refers to the type of specific immunity a host develops after exposure to a suitable antigen (Prescott *et al.,* 1996). It is made up of two components; humoral immunity and cell mediated immunity. (Table 3). The major effector cells of the humoral response are the B lymphocytes which are stimulated to develop into antibody-secreting plasma cells. The major effector cells of the cell-mediated response include the T helper cells (Th), which produce cytokines that activate macrophages for increased microbicidal activity, and cytotoxic T cells (Tc), which directly eradicate invading organisms (Pruett, 2003). After leukocytes of the innate immune system are activated, they produce cytokines and present antigens to T- and B-lymphocytes, which in turn activates the adaptive immune system. This represents the interaction between both systems, which ultimately generates an immune response (Volman *et al.,* 2008).

2.5.2. Lymphocytes

The major classes of lymphocytes are the T lymphocytes (which mature in the thymus) and the B lymphocytes (which mature in the bone marrow). Both are morphologically and functionally different. They are distinguished by antigen-specific surface receptors and molecules called clusters of differentiation (CD), which can differentiate between the various subsets of cells that behave differently. For example, a mature T cell carries a marker known as T3 (or $CD3^+$). Most T helper cells carry a T4 ($CD4^+$) marker, a molecule that recognizes MHC class II antigens, whereas, cytotoxic T cells carry a T8 marker ($CD8^+$), which recognizes MHC class I antigens. The $CD3^-$ cells are the B lymphocytes and natural killer cells, also known as CD56 (Understanding the immune system, 2002).

2.5.2.1. Development of thymocytes and the T cell subsets

During development of T cells in the thymus, genes of the T cell receptor (TCR) are transcribed and translated; their products are assembled and then expressed on the cell surface. However, gene expression of each TCR subunit is not synchronous in thymocyte development. Genes encoding the CD3 complex and the C chain are transcribed in the most immature thymocytes. It is believed that the low surface expression of TCR on double-positive (DP) thymocytes which are immediate precursors of mature single-positive (SP) thymocytes, is due to the low levels of transcription of the TCR a-chain gene. This is due to the fact that cell surface expressions of TCRs are dependent on expression and assembly of all six TCR proteins (Kosugi *et al.*, 1992).

The key proteins that are involved in the development of thymocytes are the CD3/TCR complex and the antigen co-receptors (CD4 and CD8). As T cells mature in the thymus, they lose their expression of CD4 and CD8 co-receptors. The expression of TCR-beta (pre-TCR complex) is critical for cell development of $CD4^-CD8^-$ to $CD4^+CD8^+$. Expression of TCR-alpha-beta is critical for further development into either $CD4^+$CD8- helper T cells or CD4-$CD8^+$

cytotoxic T cells. The maturation of $CD4^+CD8^+$ immature T cell into $CD4^+CD8^-$ helper T cell or $CD4^-CD8^+$ cytotoxic T cell involves the upregulation of the TCR/CD3 complex and CD5 proteins as well as the down modulation of CD4 and CD8 molecules (Reese *et al*., 2001).

2.5.2.2. T-lymphocytes

Peripheral blood T lymphocytes are non-antibody producing lymphocytes which are produced in the bone marrow but sensitized in the thymus and constitute the basis of cell-mediated immunity (Immune system, 2001). T-cells function as mediators of many immunologic functions since they secrete cytokines (e.g., IFN-γ, IL-4 etc), mediate other immune cells (macrophages, B cells, leukocytes etc) and maintain immunity against pathogens (Chang *et al.*, 2007). Among the T cells are two important sublineages: those that express the co-receptor molecule CD4 ($CD4^+$T cells) and those that express CD8 ($CD8^+$ T cells). T-cells can act as both regulators (T-suppressor cells and T-helper cells) and effectors (cytotoxic Tc cells). T suppressor cells inhibit the production of cytotoxic T cells to prevent more damage than is necessary. Tc ($CD8^+$) cells kill virus infected cells and cells undergoing malignant transformation. T helper cells are special subpopulations of $CD4^+$ T cells that assist other immune competent cells in mounting an immune response by causing cell activation or the secretion of cytokines. They are divided into three subsets: Th-1, Th-2, and Th-3 cells.

- Th-1 cells participate in cellular immunity. They are needed for controlling intracellular pathogens such as viruses and bacteria and also provide cytokine mediated help to cytotoxic T lymphocytes.
- Th-2 cells participate in humoral immunity. Their activity results in the stimulation of antibody production so that the antigens outside the cells are eliminated. These antibodies are needed to control extracellular pathogens.

- Th-3 cells are inflammation regulating cells. The main inflammation regulating mediator is TGF-β (Transforming growth factor-beta). Th-3 cells inhibit both the Th-1 and Th-2 pathway and therefore down regulate their activity.

2.5.2.3. Helper T lymphocytes ($CD4^+$)

$CD4^+$ T cells play a central role in the immune system as it mediates both innate and adaptive immune responses. They secrete lymphokines that stimulate the growth and division of cytotoxic T cells and B cells and also enhance the ability of macrophages to destroy the microbes. $CD4^+$ T cells are an essential component of effective $CD8^+$ T-cell responses and are particularly crucial for the establishment of functionally optimal CD8 memory populations. As mentioned earlier, they commit to either a Th-1 or Th-2 subtype, and are distinguished by the cytokines they secrete. Th-1 lymphocytes predominately secrete inflammatory effector cytokines such as interferon–gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) which are involved in macrophage activation, protection against intracellular pathogens, clearance of viruses and perpetuating autoimmune responses. IFN-γ is the main Th1 cytokine. Thus, Th-1 cells are effective inducers of cell-mediated immune responses. In contrast, Th-2 cells secrete a number of interleukins (IL) such as IL3, IL-4, IL-5, IL-10 and IL-13 which are essential in promoting high affinity antibody responses and protection against extracellular infections (Paul, 2003; Stockinger *et al.,* 2004). This is depicted in Figure 17. Th-1 activity will inhibit Th-2 activity and vice versa. In healthy individuals, there is a harmonious balance between Th-1 and Th-2 activity.

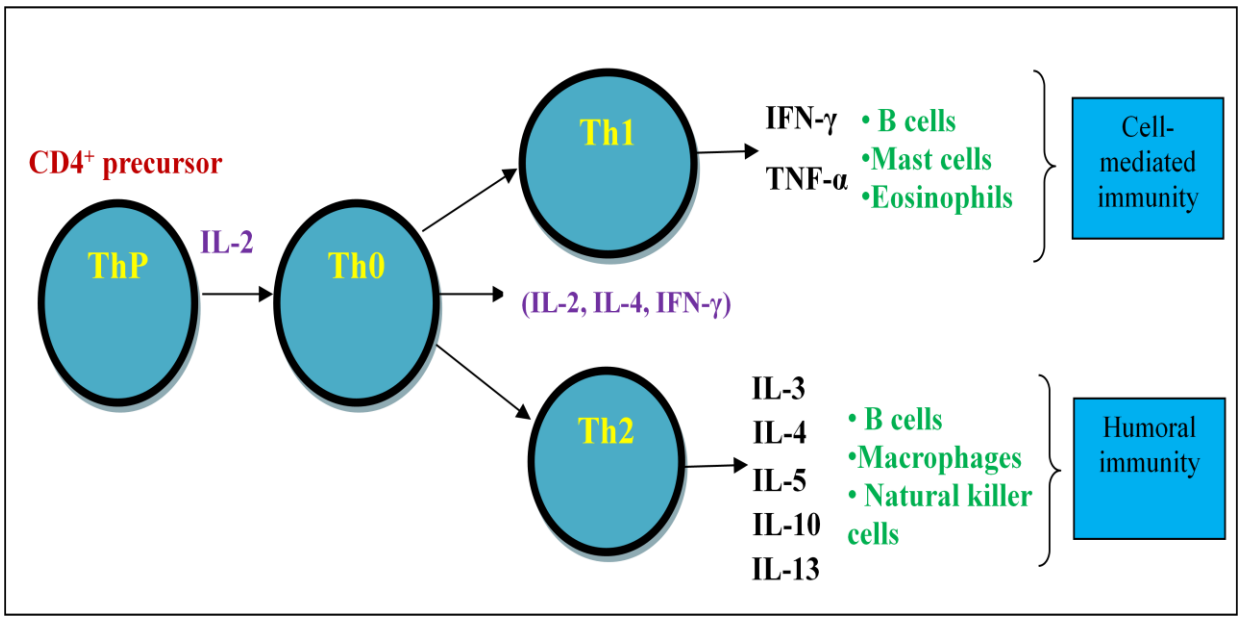


Figure 17: Differentiation patterns of $CD4^+$ T cells (Adapted from Mosmann and Sad 1996)

$CD4^+$ T cells differentiate from naïve precursors (ThP), which do not secrete either IL-4 or IFN-γ into Th0 cells, since they have both Th1 and Th2 characteristics. With further stimulation, Th0 cells deviate towards Th1 or Th2 cells, distinguished by the pattern of cytokines they secrete. Th1 cells secrete mainly TNF-α and IFN-γ, promoting cell-mediated immunity whereas the Th2 cells secrete IL-3, IL-5, IL-4, IL-10 and IL-13, promoting humoral immunity (Mosman and Sad, 1996) as shown in Figure 17. The differences between the Th1 and Th2 responses are summarized in table 4.

Table 4: Comparison between Th 1 and Th 2 immune responses

Type of response	**Type 1**	**Type 2**
Main cell type	Macrophage	B cell
Cytokines produced	IFN-γ, TNF-α, IL-1	IL-4, IL-5, IL-6, IL-10, IL-13
Immune response stimulated	Cellular immune system - Maximizes the killing efficacy of the macrophages and the proliferation of CD8+ T cells - Promotes pro-inflammatory action	Humoral immune system - Stimulates B cells into proliferation to increase neutralizing antibody production - Promotes anti-inflammatory action
Other functions	-Involved in the development of organ-specific autoimmune diseases - IFN-γ inhibits production of Th2 cytokine IL-4, thus down-regulates Th2 response thereby preserving its own response	-Helps eradicate extracellular pathogens - Induces a pronounced allergic response - IL-10 inhibits IL-2 and IFN-γ in helper T cells

2.5.2.4. Cytotoxic T lymphocytes ($CD8^+$)

$CD8^+$ T cells are historically regarded as a homogenous population of cytotoxic cells that are responsible for cytokine production and cytotoxic lysis of infected or tumour cells. Distinct subsets of $CD8^+$ T cells that are similar to their $CD4^+$ counterparts have been established in the

mouse, rat and human models (Salgame *et al.*, 1991; Sad *et al.*, 1995; MacAry *et al.*, 1998). These subsets were termed Tc1 and Tc2 (Mosmann and Sad, 1996; Vukmanovic-Stejic *et al.*, 2000). Figure 18 demonstrates the Tc1 and Tc2 immune responses of the $CD8^+$ T cells.

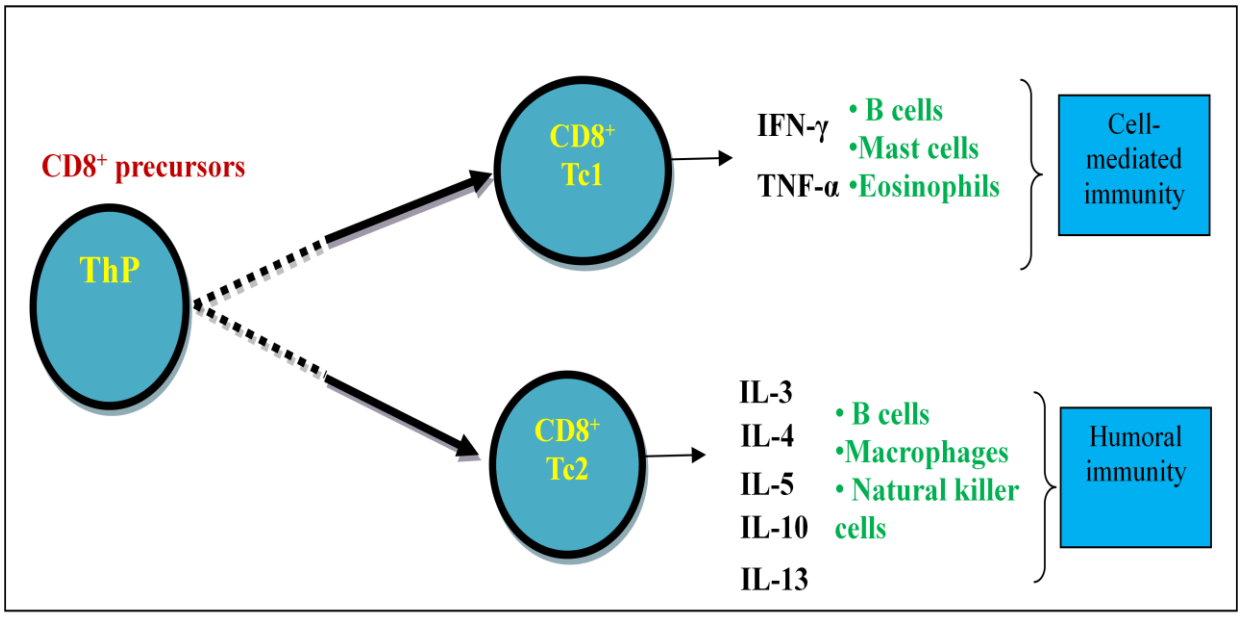


Figure 18: Differentiation patterns of $CD8^+$ T cells (Adapted from Mosmann and Sad 1996)

In addition to their role in killing infected cells, $CD8^+$ T cells also play a role in the activation and differentiation of $CD4^+$ T cells. This regulation could be mediated through secreted products (cytokines and chemokines) or by cell-cell interaction. Other functions of $CD8^+$T cells include influencing other components of the immune response, such as recruitment of eosinophils into the lungs during virus infection or allergic asthma, activation of macrophages and regulation of antibody production by B cells (Vukmanovic-Stejic *et al.*, 2000).

2.5.2.5. Natural killer cells (CD56)

Natural killer cells provide a first line of defence against infectious agents and hence, form a very important component of the innate immune system. They are distinguished from the rest of the lymphocytes due to their lack of expression of T cell receptor genes and surface

immunoglobulins (Middleton *et al*., 2002). Their characteristic morphology is unique in that they are large, cytotoxic, granular lymphocytes which possess activating receptors which recognize and bind to glycoproteins on the surface of virally infected cells or tumour cells, thus bringing the killer and target cells into close proximity. The binding process triggers the release of perforin and cytolysin molecules from its granule contents. These molecules then polymerize to form transmembrane channels, which facilitate lysis of the target cell. The granules of NK cells also contain lymphotoxin α and serine proteases such as granzyme B, which can induce apoptosis (programmed cell death) of the target cell (Roitt *et al*., 2005). Apart from their ability to effectively kill virally infected cells, NK cells also produce a wide range of cytokines such as IL-1 and TNF, which play an important role in inflammation and IFN-γ and transforming growth factor-β (TGF-β), which modulate the cell mediated immune response (Roitt *et al*., 2005).

2.5.2.6. B lymphocytes (CD19)

About 5-15 % of lymphocytes in the bloodstream are composed of B cells. The major function of the B lymphocytes is to develop into plasma cells, which produce and secrete antibodies in response to pathogens such as bacteria, viruses, and tumour cells which circulate in the bloodstream. The production and binding of antibodies to a foreign antigen is a critical means of signalling to other immune cells to engulf or remove that antigen. There are five classes of antibodies (also called immunoglobulins): IgA, IgD, IgE, IgG and IgM. Each antibody is specific for a certain antigen (Immune system, 2001).

2.5.3. Cytokines

Cytokines are the hormonal messengers responsible for most of the biological effects in the immune system. They attach to receptors on the outside portion of cell membranes, causing the target cell to produce a certain reaction, depending on the cell and cytokine. These proteins are

divided into several families and they include the type I cytokines that encompass many of the interleukins (IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-12, IL-13, IL-15, IL-21, and IL-23) and the type II cytokines, which include several hematopoietic growth factors such as the interferons, Tumour necrosis factor (TNF), lymphotoxin, fas ligand, chemokines and IL-1, IL-8 and IL-10 (Paul, 2003).

Helper T-cells i.e. $CD4^+$ T cells are prolific cytokine producers and they produce enormous amounts of two types of cytokines: Th1 and Th2. Also, both cytokine patterns can be expressed by an individual T cell, called the Th_o cells (Mosmann and Sad 1996). The type of cytokine produced determines whether a naïve helper T-lymphocyte (Th0) develops into a type 1 helper T-lymphocyte (Th1) or a type 2 helper T-lymphocyte (Th2) (Volman *et al*., 2008). Th1-type cytokines such as IFN-γ tend to produce proinflammatory responses responsible for killing intracellular parasites and for perpetuating autoimmune responses. Excessive proinflammatory responses can lead to uncontrolled tissue damage, so there needs to be a mechanism to counteract this.

The Th2-type cytokines include interleukins 4, 5, and 13, which are associated with antibody production and the promotion of IgE and eosinophilic responses in atopy, and also interleukin-10, which has more of an anti-inflammatory response. In excess, Th2 responses will counteract the Th1 mediated microbicidal action. Thus, IFN-γ selectively inhibits the proliferation of Th2 cells and IL-10 inhibits the cytokine synthesis of Th1 cells. Therefore, it is essential that the Th1/Th2 response in humans is well balanced and suited to the immune challenge.

For instance, interleukin-12, produced by activated macrophages, stimulates Th1 cell development. This stimulates the production of Th1 cytokines such as IL-1, IFN-γ, IL-2, and TNF-α, which play an important role in cell-mediated immunity. In contrast, IL-4, results in the

development of Th2 cells, producing IL-5, IL-6, IL-10, and IL-13, which are involved in the humoral immune response (Volman *et al*., 2008). Cytokines such as IL-1 and TNF upregulate the expression of adhesion molecules for neutrophils on the surface of endothelial cells, increase capillary permeability and promote the chemotaxis and activation of the polymorphonuclear neutrophils themselves.

The cytokine network plays a key role in facilitating communication within the immune system and orchestrating a suitable reaction to various threats such as bacteria, fungi, viruses and in some cases, tumours. The main cytokines in this study are IL-10 and IFN-γ which are discussed.

2.5.3.1. Interleukin-10 (IL-10)

Interleukin-10 is a homodimeric, multifunctional cytokine of 35 kD that is produced by the Th2 cells, B cells and macrophages. It is also known as B-cell derived growth factor (B-TGCF), cytokine synthesis inhibitory factor (CSIF) and T-cell growth inhibitory factor (TGIF). Human IL-10 was found to be produced by all T helper cell subsets, activated $CD8^+$ peripheral blood cells, B-cell lymphomas and by mast cells (Aman *et al*., 1996; Haddad *et al*., 2003).

IL-10 is primarily responsible for down-regulating the expression of the proinflammatory Th1 cytokines such as IFN-γ, IL-2 and TNF-β (Roitt *et al.*, 2005). In human monocytes, IL-10 and IFN-γ antagonize each other's production and function. IL-10 acts as a stimulator of the proliferation of mast cells and peripheral lymphocytes. It also stimulates and promotes the growth and differentiation of B cells. Among these activities, IL-10 also plays a key role in regulating the growth and differentiation of NK cells, cytotoxic and helper T cells, mast cells, granulocytes, dendritic cells and keratinocytes (Haddad *et al*., 2003). The pleiotropic actions of IL-10 on macrophages and T cells include its ability to inhibit T cell proliferation and to block

accessory cell functions such as antigen presentation and major histocompatibility class II expression. Because of these activities, IL-10 has been regarded widely as an immunosuppressive cytokine (Aman *et al*., 1996).

2.5.3.2. Interferon-γ (IFN-γ)

IFN-γ is a multipotent cytokine with both biological and pathological functions. It is secreted by both cells of the innate immune system (Natural killer cells) and the adaptive immune system ($CD8^+$T cells and $CD4^+$ T cells). IFN-γ plays a key role in the immune response against infection. Apart from controlling the activation of the host immune system to combat microbial infections, it is also involved in the repression of autoimmune disorders, by turning on T-regulatory cells and enhancing apoptosis of T effector cells (Chen and Liu, 2009).

Th1 cells and the pathway they dominate are heavily reliant on IFN-γ. It enhances the Major histocompatibility (MHC) class I and class II molecules and inhibits the proliferation of the Th2 response (Roitt *et al*., 2005). IFN-γ also modulates a variety of immune functions such as pathogen clearance, tumor eradication, T-cell activation and inflammatory responses (Chang *et al.*, 2007). Its broad biological functions include: facilitating antigen presentation through the MHC-1 and MHC-II restricted pathway; influencing the cell cycle; growth and apoptosis and immune regulating functions such as inhibiting Th2 $CD4^+$ T cell development.

The deficiency of IFN-γ and IFN-γ receptor genes have been related to the pathogenesis of many diseases such as, Type I diabetes, rheumatoid arthritis and multiple sclerosis. This is due to the fact that IFN-γ is a multi-copy gene and its expression is widely regulated at multiple levels by the host. Also, almost every type of cell is changed upon interaction with IFN-γ due to the vast range of biological effects that it exerts, therefore, any defects that occur in these processes has implications for disease (Chen and Liu, 2009).

CHAPTER THREE: MATERIALS AND METHODS

3.1. Overview of the methodology

A schematic representation of the methods used in the study is presented in Figure 19.

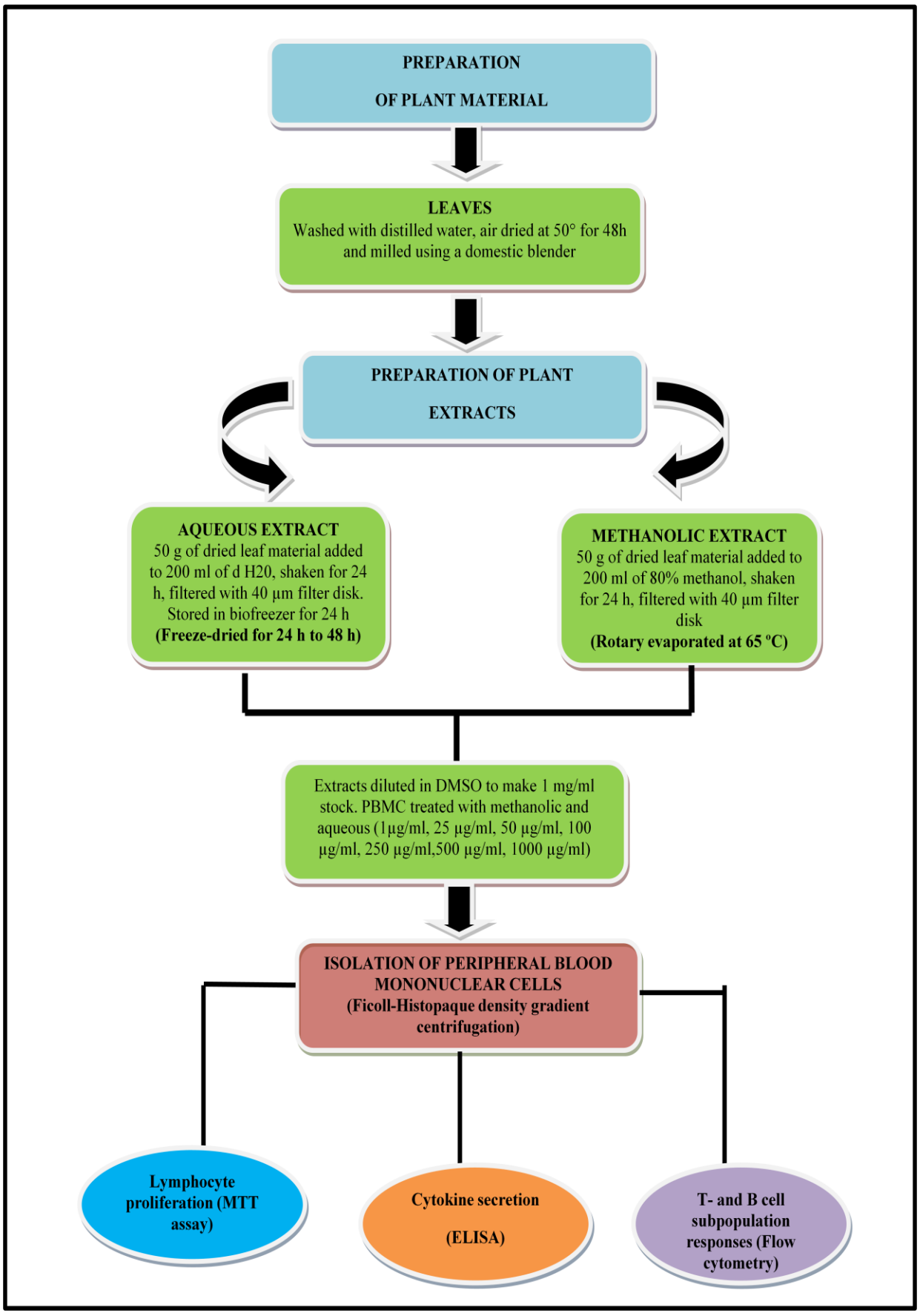


Figure 19: Overview of the methodology

3.2. Collection and processing of plant material

The fourteen leafy vegetables: *A. dubius A. hybridus, A. spinosus, A. gangetica, B. pilosa, C. asiatica, C. triloba, E. australis, J. flava, M. balsamina, P. viscosa, O. sinuatum, S. occidentalis, and S. oleraceous* were collected from Reservoir Hills, Durban. They were identified by a botanist and voucher specimens are stored in the Ward Herbarium (UKZN). The scientific name, family name, common name, and the location from which they were collected are listed in Table 1. The plants were collected, de-leafed, washed thoroughly with distilled water and dried at 50°C in an air dryer for 48 h. The dried leaf material was ground to a fine powder using a domestic blender (Waring Commercial) and then stored at room temperature in amber bottles until required for extraction.

3.3. Preparation of plant extracts

3.3.1. Aqueous extracts

Fifty grams of the leaf material of each plant were used for the aqueous extraction. Ground leaf material was added to 200 ml of distilled water in a 250 ml flask and thereafter shaken at 156 rpm for 24 h at 37ºC in a shaking incubator (New Brunswick Scientific). After agitation, the contents of the flask were filtered through with a 0.22 µm filter disk (Whatman No. 1, UK).The filtrate was stored in the biofreezer at -70ºC for 24 h and then freeze dried for 24 h to 48 h. The freeze dried aqueous extracts (1 mg) were dissolved in 1 ml DMSO yielding a concentration of 1000 µg/ml. Dilutions of the plant stock solution was made to yield test concentrations of 1000 µg/ml - 1µg/ml.

3.3.2. Methanolic extracts

The methanolic extracts were prepared as the aqueous extracts except that 50 g of dried leaf material was added to 200 ml of 80% methanol in a 250 ml flask and after filtration the methanol was removed by evaporation using a rotary evaporator (Heidolph Laborata 4000) at a

temperature of 65ºC. The remaining extract was freeze-dried using a freeze-dryer (Virtis Benchtop) forming a powdery residue.

3.4. Isolation of lymphocytes

For optimal tissue culture conditions, certain protocols were adhered to so that contamination was avoided and proper laboratory procedure was followed. All work surfaces and equipment were disinfected with 70% ethanol and 2% Virkon. The isolation of the peripheral blood mononuclear cell layer (PBMC) was conducted according to the standard operating procedures from the HIV pathogenesis programme at the HPP laboratories in Durban. Buffy coats were obtained from the South African Blood Bank (East Coast region, Pinetown). Density gradient centrifugation on Ficoll-Histopaque 1077 (according to the SOP/HPP/01 method) was used to isolate the PBMC layer.

The buffy coat was carefully layered onto an equal volume of pre-warmed Phosphate buffered saline (pH 7.2) with 1% Pen/Strep and Fungizone (Sigma) and Ficoll Histopaque 1077 in Greiner 15 ml centrifuge tubes. The blood, PBS and Ficoll (1: 1: 1) was added to each tube. The mixture was centrifuged at 1500 rpm for 30 min at room temperature. The upper plasma layer was aspirated and discarded. The mononuclear cell layer (Figure 20), which contained the PBMC was carefully removed using a 3 ml sterile Pasteur pipette and transferred into a new sterile tube. Care was taken to avoid taking up red blood cells. PBS (pH 7.2) (45 ml) was added immediately to prevent the cells from lysing. The cells were then washed 3 times with PBS (pH 7.2) by centrifugation at 1700 rpm for 10 min at room temperature. After the third wash, the PBS was removed and the pellet was re-suspended in RPMI-1640 media (Sigma). Cells were enumerated using a haemocytometer. An equal volume of the cell suspension and 0.2% Trypan Blue dye were mixed in an eppendorf tube and vortexed. Equal aliquots (20 µl) of the suspension were added into both chambers of the haemocytometer and observed under the

microscope (10X objective). The mixture was drawn across the grid by capillary action. The cells were counted within an area of 16 squares which is equivalent to the number of cells x 10^4 /ml. Only the viable translucent cells were counted within the squares. The number of viable cells per ml was calculated as follows:

Cells/ml = average number of cells per primary square x 10^4 x dilution factor.

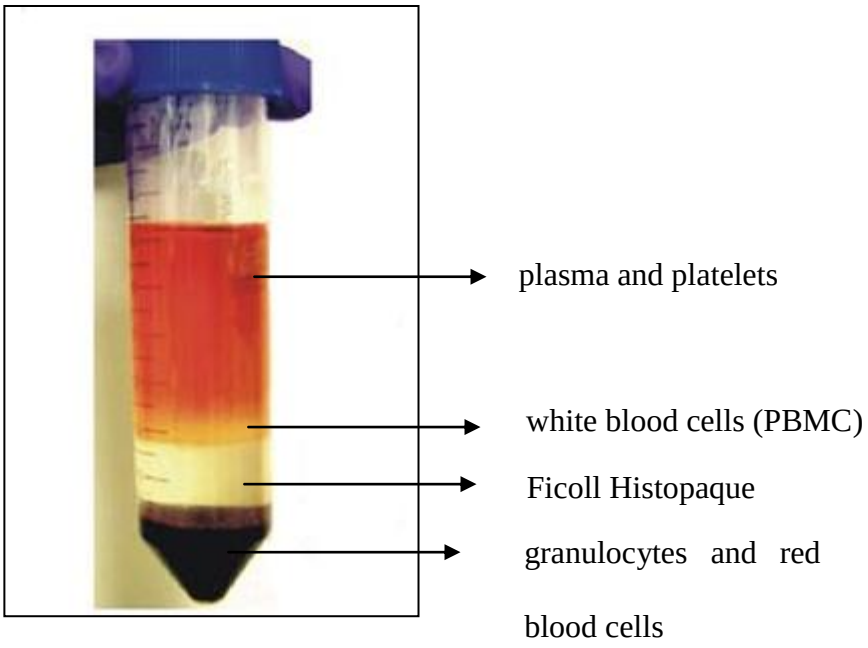


Figure 20: Isolation of human peripheral blood mononuclear cells (PBMC) showing separation of white blood cells and granulocytes

3.4.1. Lymphocyte culture and maintenance

The PBMC ($3x10^5$ cell/ml) were cultured in 75 cm^2 tissue culture flasks (Greiner) with complete culture medium (RPMI-1640, 10% fetal calf serum (FCS), and 1% Penicillin / Streptomycin) in a 5% CO_2 and 95% humidified incubator at 37°C for 24 h. The cultures were examined daily for changes in colour, turbidity, density and growth patterns using an inverted light microscope (Nikon) shown in Figure 21.

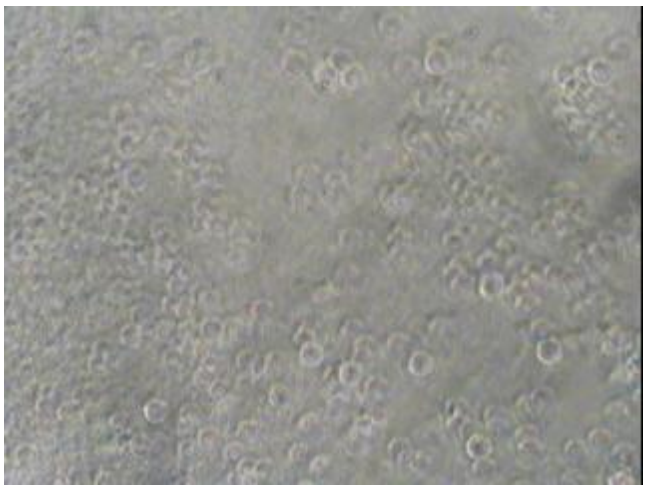


Figure 21: PBMC cultures observed after 24 hours (x20).

3.4.2. Preservation and storage of cells

The PBMC pellet (from cultured cells) was washed 3 times with pre-warmed PBS (pH 7.2) and suspended in 0.5 ml FCS and cooled on ice. To this an equal aliquot of a cooled solution of 20% dimethyl sulphoxide (DMSO) in RPMI-1640 media (V\V 1:4) was added as a cyroprotectant in cyrovials (Nunc) and refrigerated overnight at -20°C for 24 h. The PBMC was subsequently transferred to a -70°C bio-freezer and stored until required.

3.4.3. Regeneration of cells

When needed the PBMC were removed from the –70°C biofreezer, disinfected with 70% ethanol and rapidly thawed in a 37ºC water bath with moderate shaking. The cells were transferred to 20 ml of pre-warmed complete culture medium (CCM) in 75 cm^2 tissue culture flasks and incubated in a 5% CO_2 incubator at 37°C. The cell viability was determined by the trypan blue assay as described above.

3.5. Immunomodulatory studies

3.5.1. Lymphocyte proliferation assay

Principle

The MTT assay is a standard assay that is used to measure cell viability. It is a colorimetric assay that measures cell proliferation. It is based on the reduction of the yellow tetrazolium compound, 3-(4,5-dimethythiazol-2)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. MTT enters the cells and passes into the mitochondria where it is reduced to insoluble, coloured (dark purple) formazan crystals. The cells are then solubilised with an organic solvent and the released, solubilised formazan reagent is measured using a spectrophotometer (Patel *et al.*, 2009). The optimal wavelength for absorbance is 570 nm, but any filter that absorbs between 550 nm and 600 nm may be used. The data is analyzed by plotting cell number versus absorbance, allowing quantitation of changes in cell proliferation. The rate of tetrazolium reduction is proportional to the rate of cell proliferation.

Procedure

3.5.1.1. Optimization of controls

The proliferation of the PBMC was determined by the methyl-thiazolyl tetrazolium (MTT) method described by Mosman, (1983). Initially assay parameters were optimised by determining the effect of the mitogens PHA, Con A, LPS and immunosuppressant CSA and comparing them to untreated cells. Hundred µl of $6x10^5$ cells/ml were incubated in 96 well, flat-bottomed microtiter plates (Cellstar, Greiner Germany) with 20 µl of PHA (250 µg/ml, 125 µg/ml), 20 µl of Con A (20 µg/ml, 10 µg/ml and 5 µg/ml), 20 µl of LPS (20 µg/ml, 10 µg/ml and 5 µg/ml), 20 µl of CSA (20 µg/ml, 10 µg/ml and 5 µg/ml), 10 µl and 5 µl of DMSO and 20 µl of RPMI-1640 media in a 5% CO_2 and 95% humidified incubator at 37°C for 24 h.

Following incubation, the absorbance was read at 570 nm with a reference wavelength of 650 nm on an ELISA microplate reader. The percentage cell proliferation was calculated as follows:

$$\% \text{ cell proliferation} = \frac{\text{average absorbance of treated cells}}{\text{average absorbance of untreated cell}} \times 100$$

3.5.1.2. Effect of the plant extracts on PBMC

Two µl (1000 µg/ml - 1 µg/ml of plant extracts in DMSO) of the aqueous or methanolic extracts was added to 100 µl of PBMC ($6x10^5$ cells/ml) in a 96 well flat-bottomed microtiter plate (Cellstar, Greiner, Germany) for 24 h at 37ºC in a 5% CO_2 humidified incubator. Twenty µl of PHA (250 µg/ml), or 20 µl of Con A (20 µg/ml) were used as positive controls and 20 µl of CSA and RPMI-1640 were used as the negative controls and the latter was regarded as 100% (untreated cells). After 24 h, 10 µl of MTT salt (5mg/ml) was added to each well and incubated for 4 h in a 37ºC humidified incubator. The purple formazan precipitate formed was dissolved in DMSO (100 µl) and incubated for a further 30 min. Following incubation, the absorbance was read at 570 nm with a reference wavelength of 650 nm in an ELISA microplate reader. The percentage cell viability was calculated as follows:

$$\% \text{ cell proliferation} = \frac{\text{average absorbance of treated cells}}{\text{average absorbance of untreated cell}} \times 100$$

The average viability and their standard deviation were then used to determine the % change in the cell concentration and compared to untreated cells. All the experiments were carried out in triplicate and the results were expressed as the mean ±SD.

3.5.2. Determination of IFN-γ and IL-10 cytokine response using ELISA

Principle

The secretion of cytokines by PBMC in response to plant extracts was tested using the enzyme-linked immunosorbent assay (ELISA) BD OptEIA ELISA kit II (Becton, Dickinson, San Jose, CA, USA). The ELISA immunoassay can detect and quantitate the concentration of soluble cytokine proteins (range) by the use of highly-purified anti-cytokine antibodies which are non-covalently adsorbed onto microtiter plates (Staley *et al*., 2007). Standards and samples are added to the wells, and any IL-10 or IFN-γ that is present binds to the immobilized antibody. The wells are washed and, Streptavidin-horseradish peroxidase conjugate mixed with biotinylated anti-human IFN-γ antibody is added, producing an antibody-antigen-antibody "sandwich". The wells are again washed and the TMB substrate solution is added, which produces a blue color in direct proportion to the amount of IFN-γ present in the initial sample. The stop solution changes the color from blue to yellow, and the microwell absorbances are measured spectrophotometrically using an ELISA plate reader at an appropriate optical density (OD) of 450 nm. The mean absorbance for each set of duplicate standards and samples are calculated and the standard curve is plotted with IFN-γ concentration on the x-axis and absorbance on the y-axis to determine the IFN-γ concentration of the samples.

Procedure

The cultivation and treatment of human PBMC was performed as previously described in the lymphocyte proliferation assay. The sample diluent (100 µl) was added to each well and then 100 µl/well of IFN-γ standard or supernatant sample was added to the appropriate wells. The plate was covered with an adhesive strip and incubatedfor 2 h at room temperature. Each well was aspirated and washed with wash buffer, and the process was repeated four times. The IFN-γ and IL-10 cytokine conjugates (100 µl/well respectively) were added, covered with anew

adhesive strip, and incubated for 1 h at room temperature. The washing process was repeated seven times. The TMB substrate solution (200 µl/well) was added and incubated for 30 min at room temperature. Finally, the stop solution (50 µl/well) was added and the optical density of each well was determined within 30 min, using an ELISA plate reader at a wavelength of 450 nm. A standard curve was plotted, displaying the relationship between the cytokine concentration and the corresponding OD (absorbance) values of the replicates.

3.5.3. Effect of plant extracts on lymphocyte subset populations using flow cytometry

Principle

The effects of plant extracts on the T-cell sub population (activated T cells, $CD4^+$, $CD8^+$, total B cells and NK cells) was determined by flow cytometry, according to Cherng *et al.* (2008). All fluorescein isothiocyanate (FITC), Phycoerythrin (PE), Allophycocyanin (APC), Peridinin chlorophyll protein (PerCP), PerCP-Cy5.5–conjugated monoclonal antibodies (mAbs) were purchased from Becton Dickinson (San Jose, CA, USA). Optimal concentrations of mAbs were determined for each mAb by titration. Flow cytometry simultaneously measures and analyzes the physical properties of particles such as cells as they flow through a fluid stream through a beam of light. The light scatter properties of cells can be used to analyse changes in size, granularity, internal complexity and relative fluorescence intensity (Sgonc and Gruber 1998). Flow cytometric analyses are performed to determine which lymphocytes direct the patterns of immunomodulation, by using conjugated monoclonal antibodies (mAbs).

Procedure

The cultivation and treatment of human PBMC was completed as previously described in the lymphoproliferation assay. After treatment with the plant extracts, the cultured cells (0.25- 1

million) were aliquoted into a FACS tube then washed with 4 ml Dulbecco's phosphate buffered saline solution (DPBS), centrifuged for 5 min at 2000 rpm at RT and the supernatant was decanted. The pellet was suspended in DPBS and washed again by centrifugation at 1800 rpm for 8 min. Cells were stained with a yellow viability dye (1 µl dye/ 1000 µl DPBS), vortexed and incubated at 4 °C for 15 min. Thereafter, cells were washed with 4 ml of DPBS with 1% FCS, centrifuged at 1800 rpm for 8 min and the supernatant was decanted. The cells were then stained with the exact volume of required mAbs, as shown in table 5 below, vortexed and incubated in the refrigerator for 20 min. After washing with cold DPBS and 1% FCS, cells were centrifuged for 8 min at 1800 rpm and the supernatant was decanted. Therafter, cells were vortexed again and 100 µl of a fixative (Reagent A) was added to samples and refrigerated for 10 min. Finally, 50 ul fixative (Reagent A) was added to each sample, covered with foil and store in the fridge until time of acquisition on LSR2 flow cytometer. The stained PBMC were analyzed by a flow cytometric analyzer (LSR 11 Sorvall RT7 Plus, Becton Dickinson, USA) with Cell Quest software (Becton Dickinson, USA). Results were analysed using Flow Jo 7.0 (USA) software. The numbers of NK cells ($CD3^{-}$, $CD16^{+,}$ $CD56^{+}$), activated PBMC ($CD25^{+}$), T cell subsets ($CD3^{+}$, CD $4^{+,}$ $CD8^{+}$), total T cells (CD $3^{+,}$), total B cells (CD 3^{-}, HLA-DR^{+}) and active T cells (CD 3^{+}, HLA-DR^{+}) were determined by standard FACScan procedures with mAbs according to the manufacturers protocol.

Table 5: List of fluorochromes used for each monoclonal antibody

Conjugated monoclonal antibodies (mAbs)	**Volume (µl)**
Alexa fluor AF 700 (Anti-CD3)	3
Phycoerythrin PE-Cy (Anti-CD4)	20
Q655 (Anti-CD8)	2
Peridinin chlorphyll protein PerCP-Cy55 (Anti-CD4)	20
PB (Anti-CD19)	12

CHAPTER FOUR: RESULTS

4.1. Immunomodulatory activities of plants evaluated by lymphocyte proliferation

4.1.1. Effect of the plant extracts on PBMC

The optimal proliferation of PBMC's required to evaluate the effect of the plant extracts was determined by optimising the dose and time response of PBMC's exposed to the mitogens PHA, LPS, Con A and the immunosuppressant CSA. It was found that PHA, Con A and LPS increased the number of PBMC's in a dose-dependent manner while CSA suppressed the number of PBMC's (Figure 22). PHA caused a two and a half fold increase in proliferation and Con A and LPS caused a two-fold increase in proliferation when compared to the untreated cells. The IC50 values were 250 µg/ml for PHA and 20 µg/ml for LPS and Con A and CSA (Figure 22). The optimum time for exposure was after 24 h. These values were subsequently used as the positive and negative controls when testing the plant extracts on PBMC's.

The effects of the methanolic and aqueous extracts of the 14 plants (listed in Table 1) and 250 µg/ml PHA, 20 µg/ml Con A, LPS and CSA were tested on PBMC's. The results are displayed in Table 6. The optimum concentration of the plant extracts was 1000 µg/ml as it was most effective for the proliferation of PBMC's and was used for subsequent experiments. The methanolic extracts of *A. hybridus, B. pilosa, C. asiatica, C. triloba, J. flava, M. balsmina* and *S. occidentalis* and the aqueous extracts of *A. gangetica, E. australis, J. flava* and, the positive controls; PHA, LPS and Con A increased the number of PBMC's whilst all the other extracts and CSA decreased the number of PBMC's when compared to the untreated cells (Table 6).

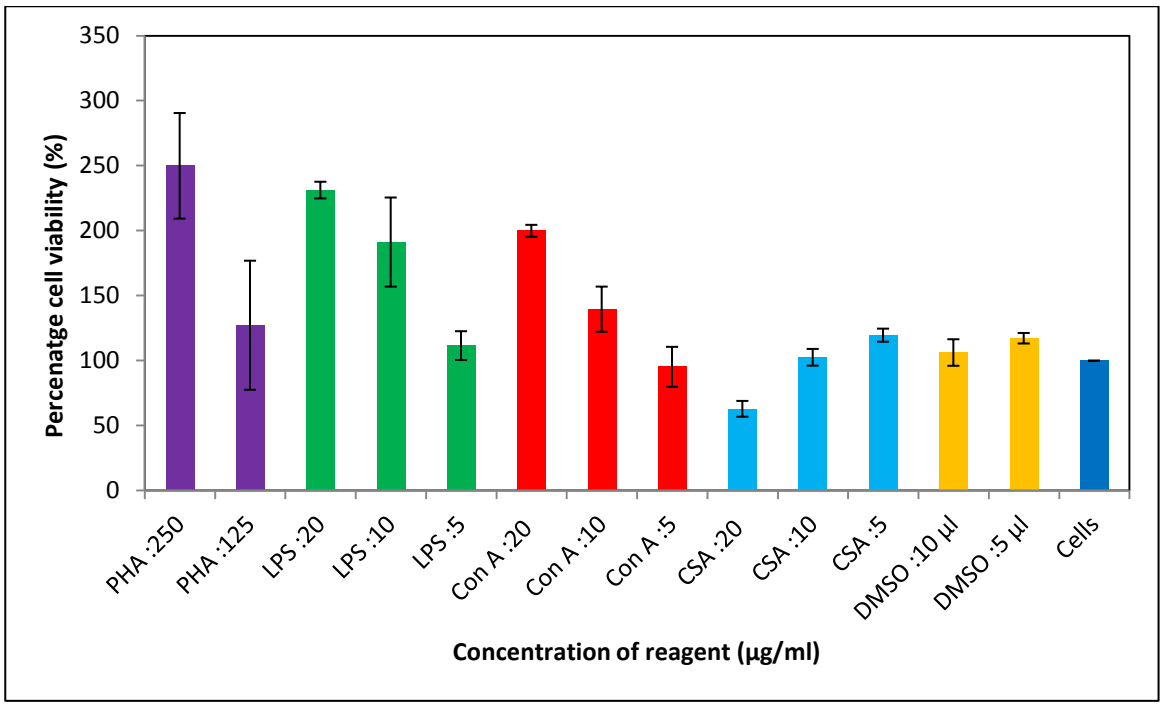


Figure 22: PMBC's exposed to concentrations of the positive controls: PHA, LPS and Con A and the negative controls: CSA and untreated cells

Table 6: Effect of the plant extracts on the proliferation of PBMC'S

Plant extract and Controls	**Effect of plant extracts (1000 µg/ml) on % proliferation/inhibition of PBMC**	
	Methanolic	**Aqueous**
A. dubius	94	71
A. hybridus	232	51
A. spinosus	43	106
A. gangetica	62	121
B. pilosa	275	48
C. asiatica	363	57
C. triloba	348	32
E.australis	51	152
J.flava	221	198
M.balsamina	184	38
O.sinuatum	64	66
P.viscosa	n/t	53
S.occidentalis	189	47
S.oleraceous	60	47
-ve controls:		
Untreated cells	100	100
CSA(20 µg/ml)	70	70
+ve controls:		
PHA (250 µg/ml)	250	250
ConA (20 µg/ml)	193	193
LPS (20 µg/ml)	232	232

[a] = IC_{50} values represent the mean ±S.D. of three determinations

* n/t = Methanolic extract of *P. viscosa* not tested

4.2. Immunomodulatory activity of plants evaluated by the lymphocyte secretion of cytokines IFN-γ and IL-10

The secretion of IFN-γ and IL-10 in extract-treated and untreated PBMC cultures after 24 h was measured using an ELISA procedure. The viability of PBMC after thawing was greater than 80%. The results are displayed in Table 7. PBMC's that were not exposed to the plant extracts released very low levels of IFN-γ (24 pg/ml) and IL-10 (6 pg/ml). This is in accordance with the results of Andersson *et al.,* (1988) and Kabilan *et al.,* (1990). Their studies showed that intracellular IFN-γ is not present in unstimulated PBMC's from healthy adults, and that the activation of T lymphocytes with specific mitogens or non-specific stimuli such as PHA is required for the detection of intracellular IFN-γ. There was also no detectable IFN-γ (3 pg/ml) and IL-10 (7 pg/ml) when PBMC's were exposed to CSA. This is expected since CSA is known to be a potent inhibitor of IFN-γ. PBMC's that were exposed to PHA and Con A showed high levels of IFN-γ (519 pg/ml and 72 pg/ml respectively) and IL-10 (352 pg/ml and 235 pg/ml respectively). These mitogens are well known to stimulate the release of cytokines.

The methanolic extracts of *A. hybridus* and *C. asiatica* and the aqueous extracts of *A. dubius* and *B. pilosa* caused an increase in the secretion of IFN-γ and a decrease in the secretion of IL-10 when compared to the untreated cells (Table 7). The methanolic extracts of *A. gangetica, E. australis, J. flava, M. balsamina, O. sinuatum, S. occidentalis and S. oleraceous* and the aqueous extracts of *A. gangetica, A. spinosus, E. australis, M. balsamina, O.sinuatum, P. viscosa, S. occidentalis and S. oleraceous* caused high levels of IL-10 secretion but low levels of IFN-γ secretion. Furthermore, the methanolic extracts of *A. spinosus* and *C. triloba* and the aqueous extracts of *B. pilosa* and *J. flava* caused an increase in both IL-10 and IFN-γ secretion (Table 7).

Table 7: Effect of the plant extracts on the levels of cytokines (IFN-γ and IL-10) released by PBMC's

Plant extract and controls	Secretion of IFN-γ (pg/ml)		Secretion of IL-10 (pg/ml)	
	Methanolic extracts (1000 µg/ml)	Aqueous extracts (1000 µg/ml)	Methanolic extracts (1000 µg/ml)	Aqueous extracts (1000 µg/ml)
A. dubius	-12 ± 0.001[a]	489 ± 0.005	8 ± 0.000	9 ± 0.001
A. hybridus	89 ± 0.023	39 ± 0.085	12 ± 0.002	8 ± 0.001
A. spinosus	35 ± 0.050	-16 ± 0.00	19 ± 0.008	71 ± 0.026
A. gangetica	-3 ± 0.006	-4 ± 0.023	94 ± 0.006	57 ± 0.011
B. pilosa	16 ± 0.005	567 ± 0.006	14 ± 0.003	16 ± 0.006
C. asiatica	63 ± 0.025	41 ± 0.036	11 ± 0.006	12 ± 0.004
C. triloba	69 ± 0.047	-14 ± 0.006	22 ± 0.029	45 ± 0.002
E. australis	9 ± 0.028	5 ± 0.023	61 ± 0.000	323 ± 0.014
J. flava	-5 ± 0.005	74 ± 0.035	40 ± 0.014	129 ± 0.018
M. balsamina	0 ± 0.010	1 ± 0.020	32 ± 0.045	52 ± 0 .013
O. sinuatum	8 ± 0.013	15 ± 0.021	93 ± 0.081	74 ± 0 .005
P. viscosa	n/t	12 ± 0.029	n/t	72 ± 0 .026
S. occidentalis	10 ± 0.001	0 ± 0.006	92 ± 0.028	104 ± 0.013
S. oleraceous	5 ± 0.011	3 ± 0.007	91 ± 0.006	105 ± 0.025
-ve controls				
Untreated cells	24 ± 0.030	24 ± 0.030	6 ± 0.001	6 ± 0.001
CSA (20 µg/ml)	3 ± 0.035	3 ± 0035	7 ± 0.006	7 ± 0.006
+ve controls				
PHA (250 µg/ml)	519± 0.006	519± 0.006	72 ± 0.008	72 ± 0.008
Con A (20 µg/ml)	352± 0.008	352± 0.008	235 ± 0.009	235± 0.009

[a] = Values represent the mean ±S.D. of two determinations

* n/t = Methanolic extract of *P. viscosa* not tested

4.3. Immunomodulatory activity of plants evaluated by the lymphocyte subset responses

The effect of the plant extracts on the lymphocyte subset populations was determined after 24 h using flow cytometry. The results were obtained on frozen PBMC and viability after thawing was greater than 80%. The results are displayed in Table 8. According to Cherng *et al.,* (2008) the lymphocyte sub-populations are made up of T-cells: $CD4^+$ T ($CD3^+$, $CD4^+$), $CD8^+$ T ($CD3^+$, $CD8^+$), naive $CD4^+CD8^+$ T cells, naive $CD4^-CD8^-$ T cells, B cells (CD3−, HLA-DR^+) and NK cells (CD3−, $CD16^+$, $CD56^+$).

PBMC's that were not exposed to the plant extracts showed high populations of $CD4^+CD8^-$ helper T cells (42%) and naive $CD4^-CD8^-$ T cells (43%) and low populations of the $CD4^-CD8^+$ cytotoxic T cells (1%), naive $CD4^+CD8^+$ T cells (0%), B cells (1%) and NK cells (1%). PBMC's that were exposed to the immunosuppressant CSA displayed similar results to the untreated cells as they showed high populations of $CD4^+CD8^-$ helper T cells (41%) and naive $CD4^-CD8^-$ T cells (41%) and low populations of $CD4^-CD8^+$ cytotoxic T cells (3%), naive $CD4^+CD8^+$ T cells (1%), B cells (1%) and NK cells (2%). The addition of the mitogens PHA and Con A to PBMC's showed an increase in the $CD4^-CD8^+$ cytotoxic T cells (25% and 31% respectively), naive $CD4^+CD8^+$ T cells (14% and 2%), B cells (5% and 1%) and NK cells (8% and 3%) and a decrease in the naive $CD4^-CD8^-$ T cells (2% and 4%) and $CD4^+CD8^-$ helper T cells (24% and 38% respectively) when compared to the untreated cells.

Different plant extracts had varied responses (increased, decreased or no response) on the different subpopulations. Overall, NK cells, B cells and $CD4^-CD8^-$ T cell subpopulations were decreased or increased slightly by both methanolic and aqueous plant extracts except for the aqueous extract of *A. dubius* which had an increase on B cells and the aqueous extract of *A.*

spinosus which had an increase on $CD4^-CD8^-$ T cells. The majority of plant extracts whether methanolic or aqueous had an effect on increasing $CD8^+$ T cells, $CD4^+$ T cells and $CD4^+CD8^+$ T cells and decreasing $CD4^-CD8^-$ T cells. The plant extracts showed varying subpopulation responses. Some of the results were similar to the positive controls. The methanolic and aqueous extracts of *A. hybridus*, *A. gangetica*, *A. spinosus*, *J. flava*, *O. sinuatum* and *S. oleraceous* showed an increase in the numbers of $CD4^-CD8^+$ cytotoxic T cells, naive $CD4^+CD8^+$ T cells and B cells and a decrease in the $CD4^+CD8^-$ helper T cells and naive $CD4^-CD8^-$ T cells, similar to the activity of PHA and Con A. The aqueous extracts of *E. australis* and *M. balsamina* caused an increase in both the $CD4^+CD8^-$ helper T cells and $CD4^-CD8^+$ cytotoxic T cells. The lymphocyte proliferation activity, cytokine responses and effects on the lymphocyte subsets for each plant is described in the sections (4.3.1– 4.3.14).

Table 8: Effect of the plant extracts on the lymphocyte subset populations

Plant extract and Controls	**Percentage cell subset populations (%)**					
	Cytotoxic T cells $CD4^-CD8^+$	**Naïve DP T cells $CD4^+CD8^+$**	**HelperT cells $CD4^+CD8^-$**	**Naive DN T cells $CD4^-CD8^-$**	**B cells CD 19**	**NK cells CD 56**
A. dubius	33	22	31	2	2	1
	22	4	26	3	21	1
A. hybridus	31	5	42	5	3	1
	30	55	0	0	0	0
A. spinosus	35	5	40	3	2	1
	12	2	38	23	5	2
A. gangetica	35	8	39	2	2	1
	35	15	32	1	3	2
B. pilosa	8	18	0	0	2	1
	34	3	42	2	2	1
C.asiatica	37	43	5	0	2	1

	35	13	37	2	3	2
C.triloba	36	5	39	2	2	1
	34	7	42	2	3	1
E. australis	26	3	41	5	3	2
	32	2	43	4	3	2
J. flava	35	6	41	2	3	1
	36	12	30	3	4	2
M. balsamina	22	5	34	5	6	1
	30	4	43	5	3	1
O. sinuatum	32	13	39	1	3	1
	16	11	9	0	5	0
P. viscosa **	n/t	n/t	n/t	n/t	n/t	n/t
	34	3	38	5	2	1
S. occidentalis	35	15	31	1	3	1
	0	0	12	1	2	1
S. oleraceous	14	6	33	8	5	1
	33	17	34	3	2	2
-ve controls						
Untreated cells	1	0	42	43	1	1
CSA	3	1	41	41	1	2
+ve controls						
PHA	25	14	24	2	5	8
Con A	31	2	38	4	1	3

Cell sub-population: $CD4^+$ T cell ($CD3^+$, $CD4^+$), $CD8^+$ T cell ($CD3^+$, $CD8^+$), naive $CD4^+CD8^+$ T cell, naive $CD4^-CD8^-$ T cell, B cell (CD3−, HLA-DR^+) and NK cell (CD3−, $CD16^+$, $CD56^+$), according to Cherng *et al.*,(2008). Values highlighted in orange indicate methanolic extracts. Values highlighted in blue indicate aqueous extracts

4.3.1. *A. dubius*

The proliferation of lymphocytes exposed to the methanolic and to the aqueous extracts of *A. dubius* was compared to the untreated cells at 100% (Figure 23). Both extracts showed a decrease in the number of viable PBMC's when compared to the untreated cells. The aqueous extract showed a greater decrease than the methanolic extracts.

The aqueous extract also showed a significant increase in the levels of IFN-γ (489 pg/ml) and no change in the levels of IL-10 (9 pg/ml) when compared to the untreated cells (24 pg/ml and 6 pg/ml respectively) as shown in Table 7. The methanolic extract showed no change in the levels of both IFN-γ (-12 pg/ml) and IL-10 (8 pg/ml).

A comparison of the subsets of PBMC's that were not treated with the plant extract; (Fig. 24C) to PBMC's that were treated with the methanolic extract (Fig. 24A) and with the aqueous extract (Fig. 24B) showed that in the untreated cells, there is a predominance of naive $CD4^-$ $CD8^-$ cells (43%) and $CD4^+CD8^-$ helper T cells (42%) and low numbers of the $CD4^-CD8^+$ cytotoxic T cells (1%), naive $CD4^+CD8^+$ T cells (0%), B cells (1%) and NK cells (1%). The methanolic extract (Fig. 24A) showed an increase in the $CD4^-CD8^+$ cytotoxic T cells (33%), naive $CD4^+CD8^+$ T cells (22%), a slight increase in the B cells (2%) and no change in the NK cells (1%). The aqueous extract (Fig. 24B) showed an increase in the $CD4^-CD8^+$ cytotoxic T cells (22%) and B cells (21%) and a decrease in the $CD4^+CD8^-$ helper T cells (26%) and naive $CD4^-CD8^-$ T cells (3%). There was no change in the naive $CD4^+CD8^+$ T cells (4%) and NK cells (1%).

a) Dose effect of the plant extracts on PBMC

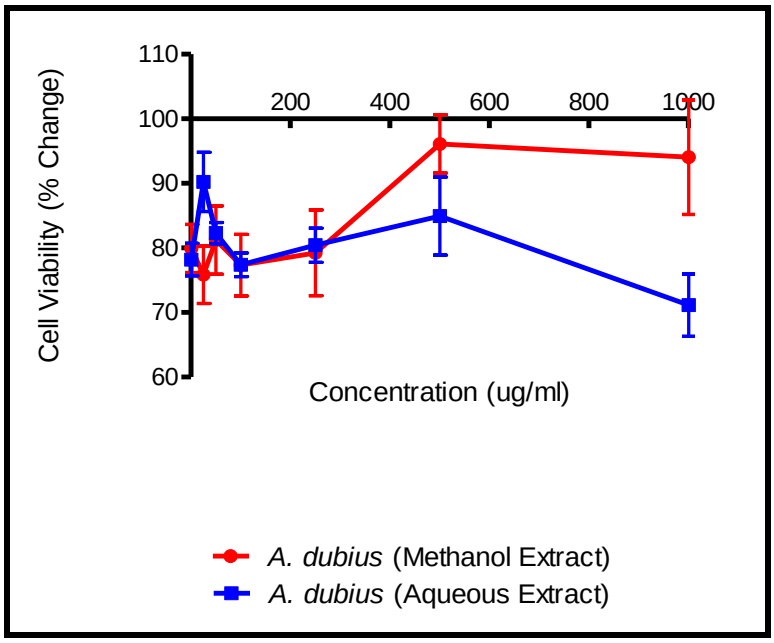
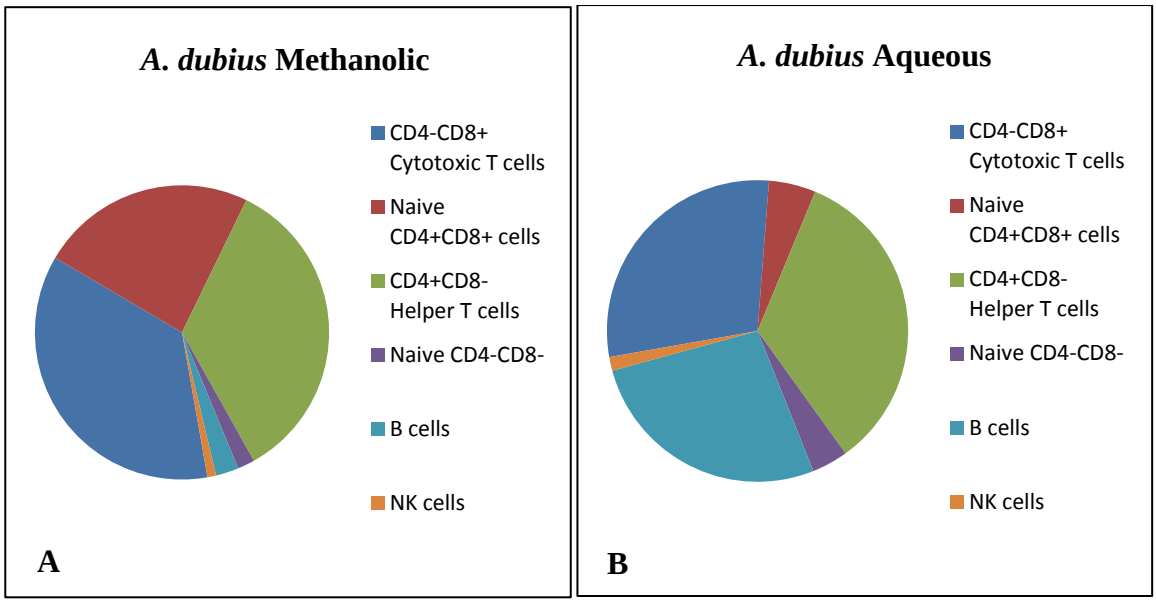


Figure 23**:** Effect of methanolic and aqueous leaf extracts of *A. dubius* (1 µg/ml – 1000 µg/ml) on PBMC over 24 h

b) Effect of the plant extracts on lymphocyte subset populations (as per Table 8)



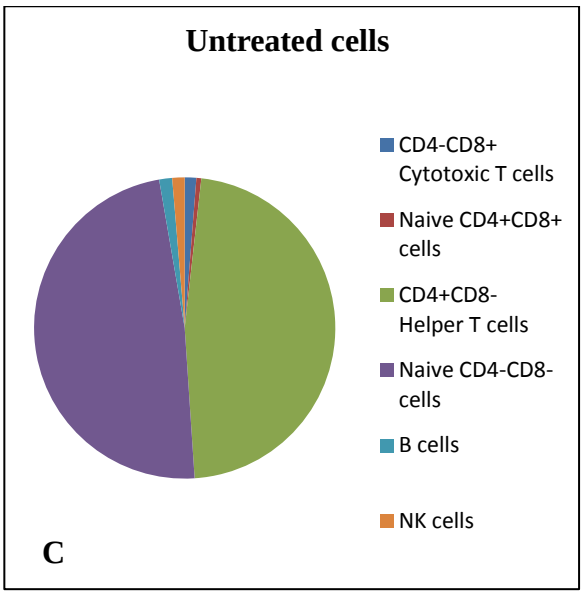


Figure 24: Effect of leaf extracts of *A. dubius* on cell subset populations evaluated by flow cytometry. A) *A. dubius* methanolic extract, B) *A. dubius* aqueous extract and C) Untreated cells.

4.3.2. *A. hybridus*

The methanolic extract of *A. hybridus* stimulated the number of PBMC's whilst the aqueous extract suppressed the number of PBMC's when compared to the untreated cells (Fig. 25).

Both the methanolic and the aqueous extracts showed an increase in the secretion of IFN-γ (89 pg/ml and 39 pg/ml respectively) and no significant change in the secretion of IL-10 (12 pg/ml and 8 pg/ml) when compared to the untreated cells (24 pg/ml and 6 pg/ml) as shown in Table 7.

The predominant subsets of cells that resulted with the methanolic extract (Fig. 26A) were the $CD4^{-}CD8^{+}$ cytotoxic T cells (31%), naive $CD4^{+}CD8^{+}$ T cells (5%) and B cells (3%). The naive $CD4^{-}CD8^{-}$ T cells (5%) were decreased and there no changes in the $CD4^{-}CD8^{+}$ helper T cells (42%) and NK cells (1%) when compared to the untreated cells (Fig. 26C). The aqueous extract (Fig. 26B) showed an increase in the $CD4^{-}CD8^{+}$ cytotoxic T cells (30%) and naive $CD4^{+}CD8^{+}$ T cells (55%) and a decrease in the $CD4^{+}CD8^{-}$ helper T cells (0%) and naive $CD4^{-}CD8^{-}$ T cells

(0%) when compared to the untreated cells (Fig. 26C). There were also no changes in the B cells (0%) and NK cells (0%)

a) Dose effect of the plant extracts on PBMC

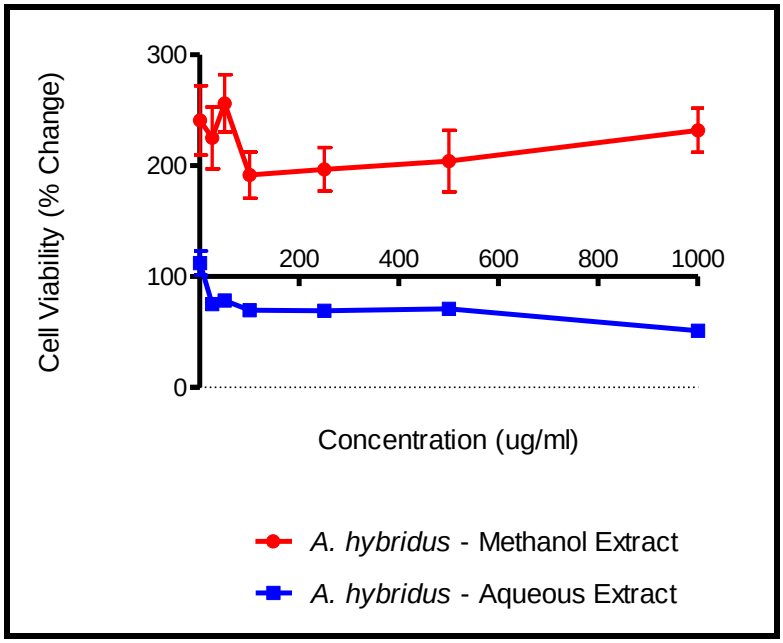
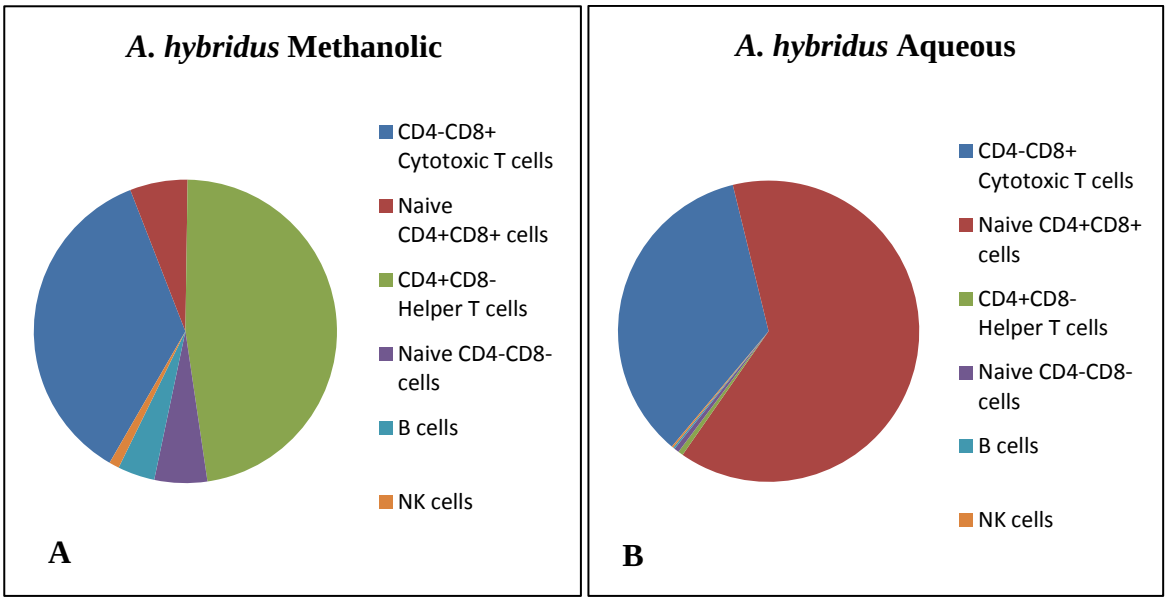


Figure 25: Effect of methanolic and aqueous leaf extracts of *A. hybridus* (1 µg/ml – 1000 µg/ml) on PBMC over 24 h

b) Effect of the plant extracts on lymphocyte subset populations (as per Table 8)



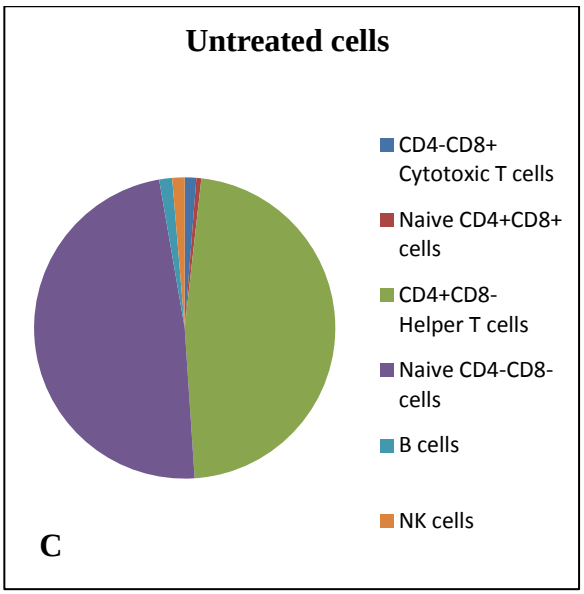


Figure 26: Effect of leaf extracts of *A. hybridus* on T cell subset populations evaluated by flow cytometry. A) *A. hybridus* methanolic extract, B) *A. hybridus* aqueous extract and C) Untreated cells.

4.3.3. *A. spinosus*

The methanolic extract of *A. spinosus* showed a decrease in the number of PBMC's whilst the aqueous extract showed a slight increase in the number of PBMC's when compared to the untreated cells (Fig. 27).

Both the methanolic and the aqueous extracts showed an increase in the secretion of IL-10 (19 pg/ml and 71 pg/ml respectively), while only the methanolic extracts showed an increase in the secretion of IFN-γ (35 pg/ml) when compared to the untreated cells (6 pg/ml and 24 pg/ml) as shown in Table 7.

The cell sub population responses for the methanolic extract (Fig. 28A) showed an increase in the $CD4^{-}CD8^{+}$ cytotoxic T cells (35%), naive $CD4^{+}CD8^{+}$ T cells (5%) and B cells (2%) and a decrease in the naive $CD4^{-}CD8^{-}$ T cells (3%) when compared to the untreated cells (Fig. 28C). No changes were observed with the $CD4^{+}CD8^{-}$ helper T cells (40%) and NK cells (1%). The aqueous extract (Fig. 28B) showed an increase in the $CD4^{-}CD8^{+}$ cytotoxic T cells (12%) and B

cells (5%), a slight increase in the NK cells (2%) and a decrease in the naive $CD4^{-}CD8^{-}$ T cells (23%) when compared to the untreated cells (Fig. 28C). No changes were observed with the $CD4^{+}CD8^{-}$ helper T cells (38%) and naive $CD4^{+}CD8^{+}$ T cells (2%).

a) Dose effect of the plant extracts on PBMC

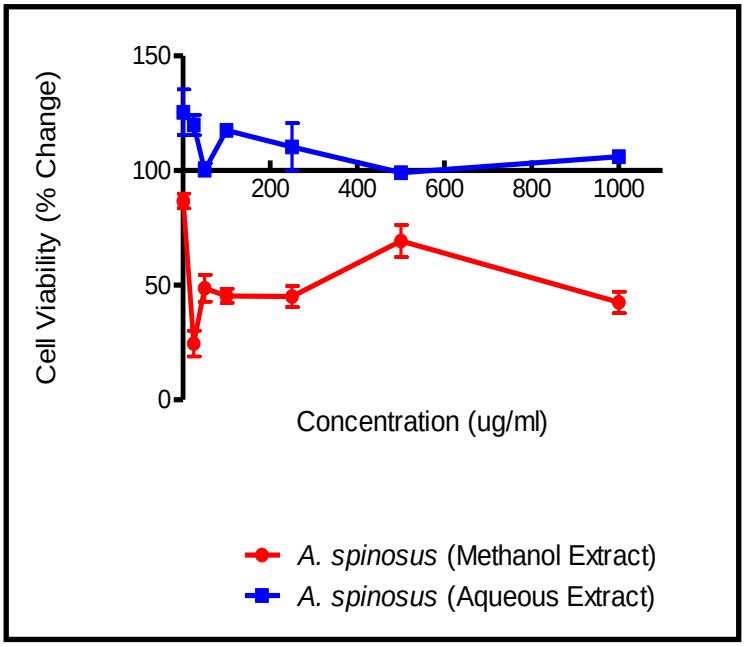


Figure 27**:** Effect of methanolic and aqueous leaf extracts of *A. spinosus* (1 µg/ml – 1000 µg/ml) on PBMC over 24 h

b) Effect of the plant extracts on lymphocyte subset populations (as per Table 8)

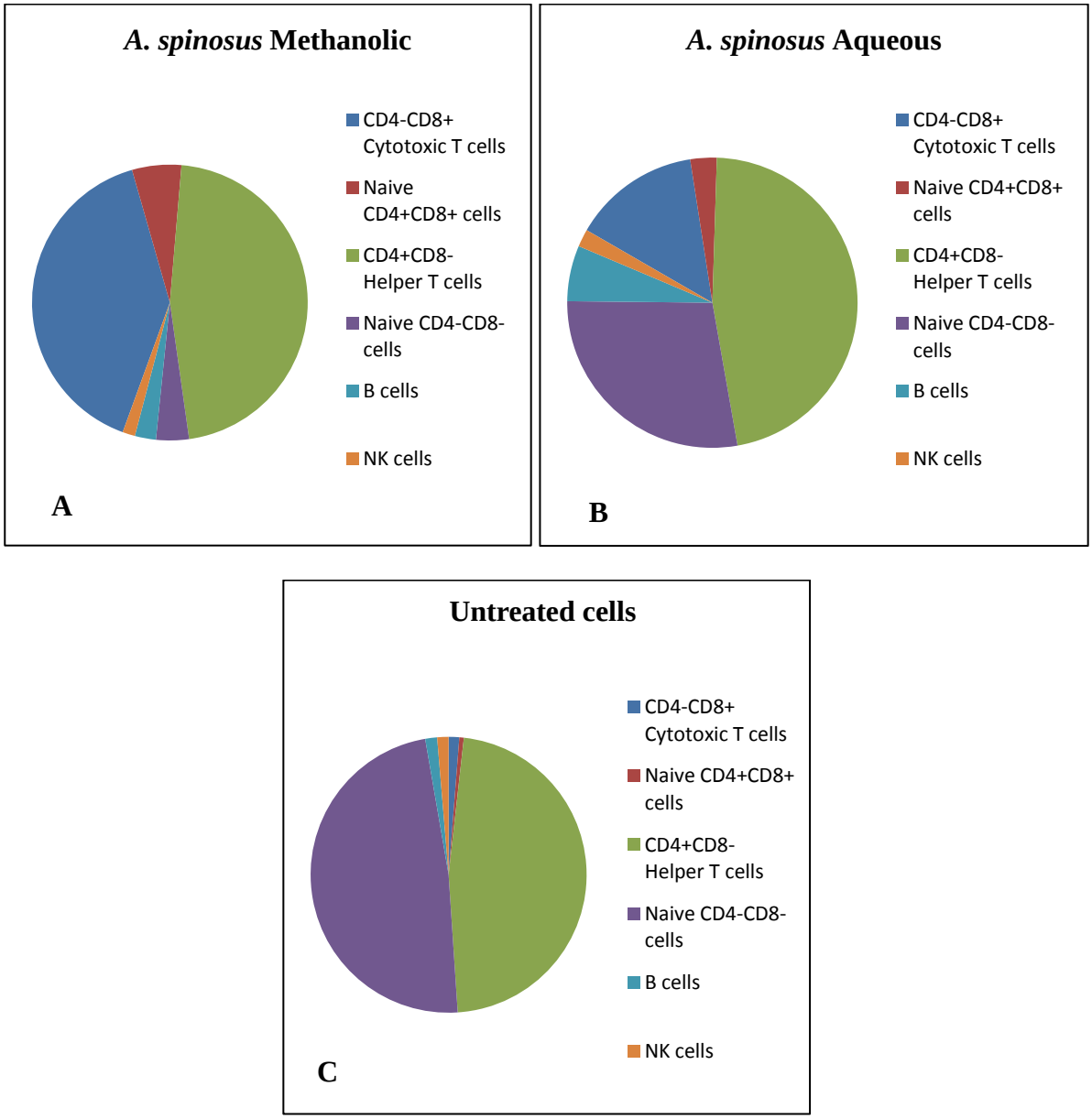


Figure 28: Effect of leaf extracts of *A. spinosus* on T cell subset populations evaluated by flow cytometry. A) *A. spinosus* methanolic extract, B) *A.spinosus* aqueous extract and C) Untreated cells.

4.3.4. *A. gangetica*

The methanolic extract of *A. gangetica* showed a decrease in the number of PBMC's whilst the aqueous extract showed an increase in the number of PBMC's (500 µg/ml - 1000 µg/ml) when compared to the untreated cells (Fig. 29).

Both the methanolic and the aqueous extracts caused an increase in the levels of IL-10 (94 pg/ml and 57 pg/ml respectively) and a decrease in the levels of IFN-γ (-3 pg/ml and -4 pg/ml) when compared to the untreated cells (6 pg/ml and 24 pg/ml) as shown in Table 7.

The methanolic extract (Fig. 30A) and the aqueous extract (Fig.30 B) caused an increase in the numbers of $CD4^{-}CD8^{+}$ cytotoxic T cells (35% and 35% respectively), naive $CD4^{+}CD8^{+}$ T cells (8% and 15%) and a slight increase in the B cells (2% and 3%) when compared to the untreated cells (Fig. 30C). There was a decrease in the numbers of naive $CD4^{-}CD8^{-}$ T cells (2% and 1%) and no change in the numbers of $CD4^{+}CD8^{-}$ helper T cells (39% and 32%) and NK cells (1% and 2%).

a) Dose effect of the plant extracts on PBMC

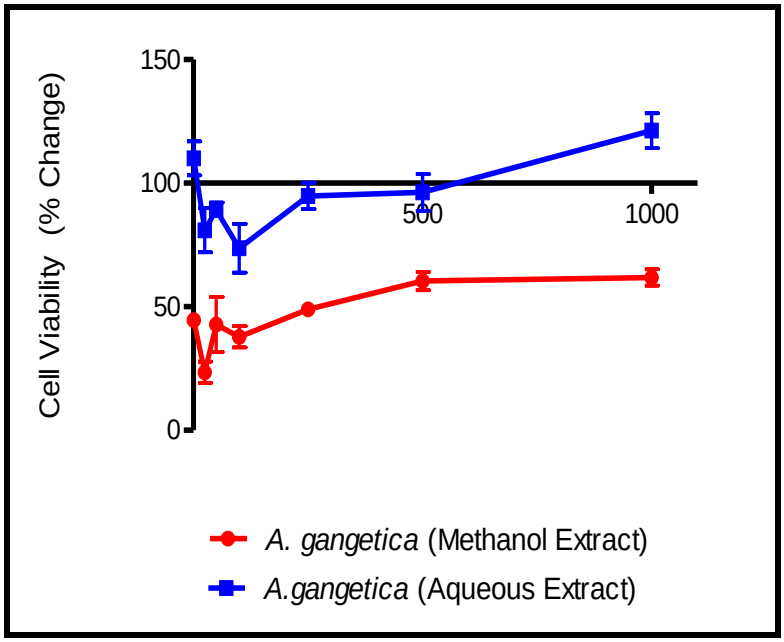


Figure 29: Effect of methanolic and aqueous leaf extracts of *A. gangetica* (1 µg/ml – 1000 µg/ml) on PBMC over 24 h

b) Effect of the plant extracts on lymphocyte subset populations (as per Table 8)

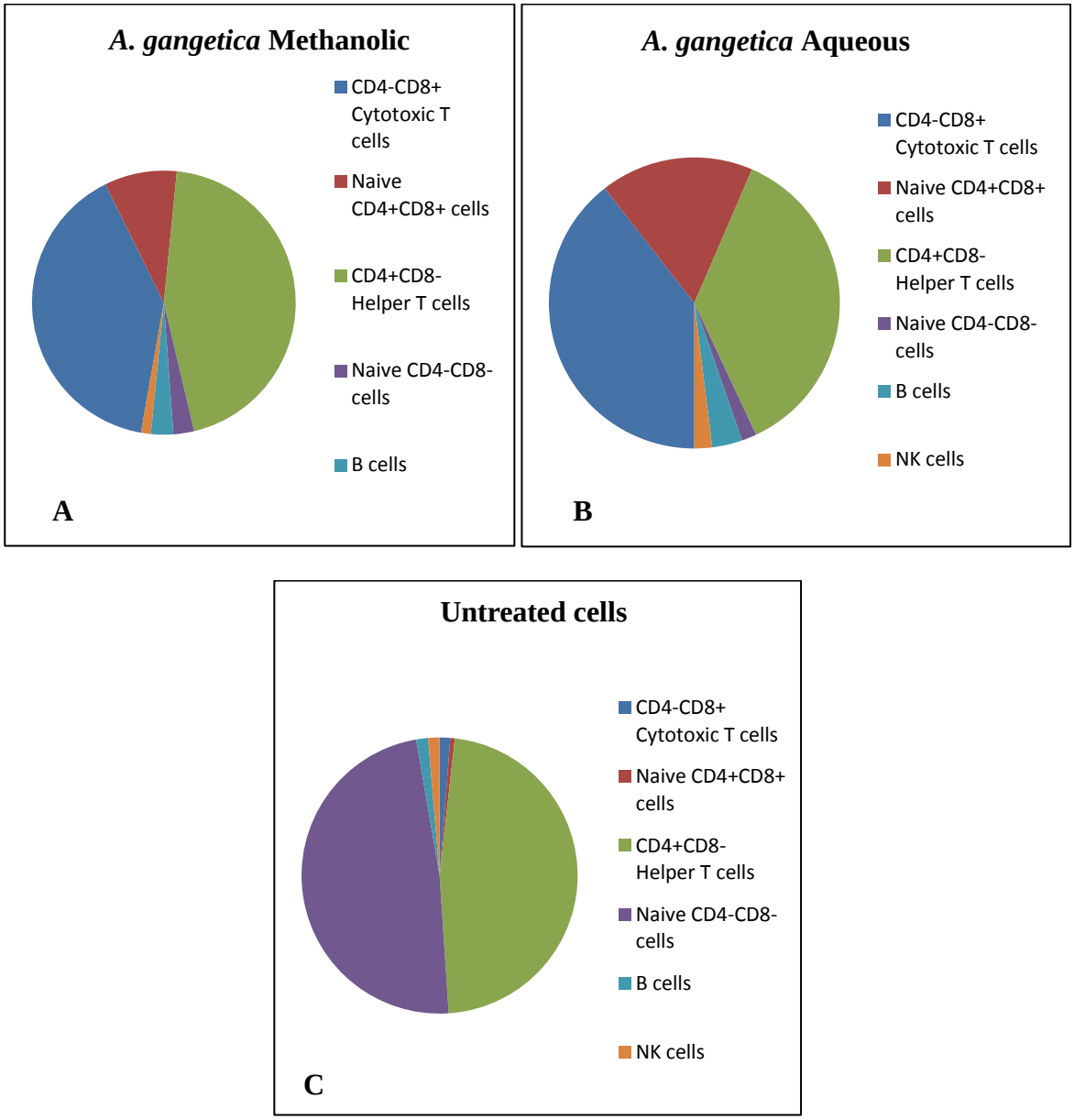


Figure 30: Effect of leaf extracts of *A. gangetica* on T cell subset populations evaluated by flow cytometry. A) *A. gangetica* methanolic extract, B) *A. gangetica* aqueous extract and C) Untreated cells.

4.3.5. *B. pilosa*

The methanolic extract of *B. pilosa* stimulated the number of PBMC's in a dose-dependent manner and showed a three-fold increase in proliferation in comparison to the untreated cells

(Fig. 31). The aqueous extract did not show proliferation of PBMC's when compared to the untreated cells and to the methanolic extract (Fig. 31).

The methanolic extract showed an increase in the release of IL-10 only (14 pg/ml) whilst the aqueous extract showed an increase in the release of both IFN-γ (567 pg/ml) and IL-10 (16 pg/ml) when compared to the untreated cells (24 pg/ml and 6 pg/ml respectively) as shown in Table 7.

The methanolic extract (Fig. 32A) showed an increase in the $CD4^{-}CD8^{+}$ cytotoxic T cells (8%) and naive $CD4^{+}CD8^{+}$ T cells (18%), a slight increase in the B cells (2%) and a decrease in the $CD4^{+}CD8^{-}$ helper T cells (0%) and naive $CD4^{-}CD8^{-}$ T cells (0%) when compared to the untreated cells (Fig. 32C). There were no changes in the NK cells (1%). The aqueous extract (Fig. 32B) showed an increase in the $CD4^{-}CD8^{+}$ cytotoxic T cells (34%) and B cells (2%) and a decrease in the naive $CD4^{-}CD8^{-}$ T cells (2%) when compared to the untreated cells (Fig. 32C). No changes were observed with the $CD4^{+}CD8^{-}$ helper T cells (42%), naive $CD4^{+}CD8^{+}$ T cells (3%) and NK cells (1%).

a) Dose effect of the plant extracts on PBMC

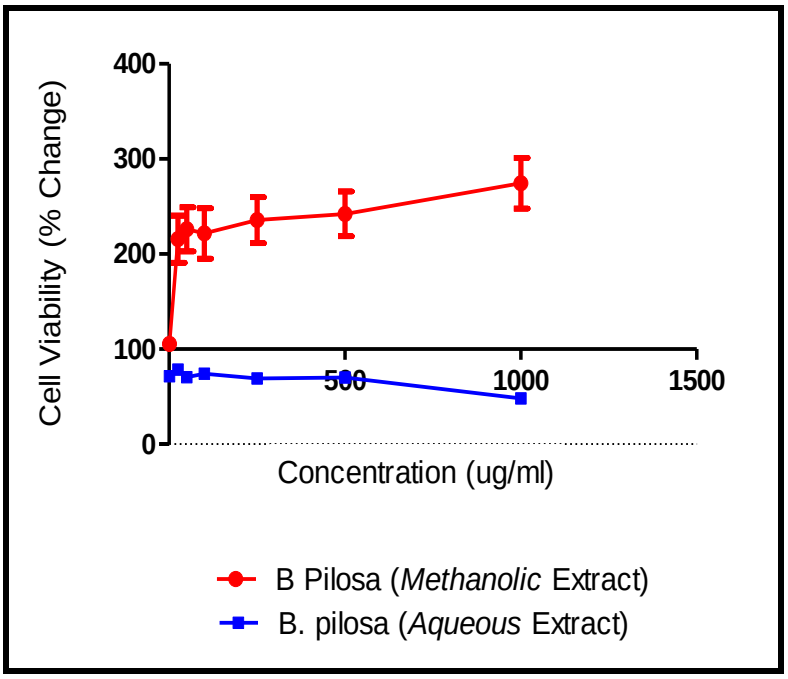
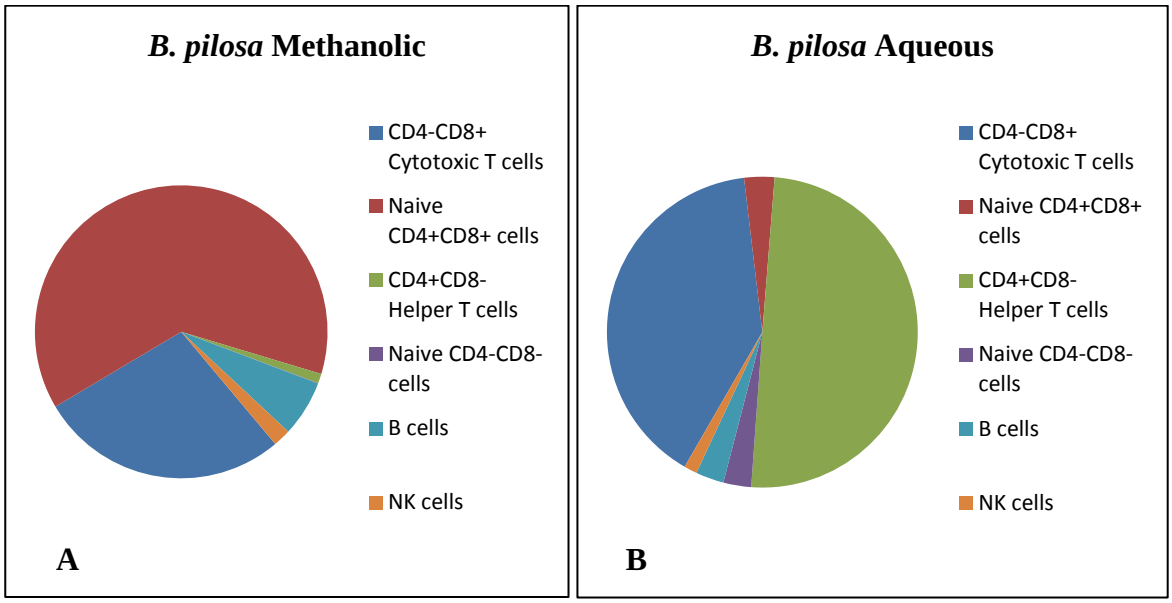


Figure 31: Effect of methanolic and aqueous leaf extracts of *B. pilosa* (1 µg/ml – 1000 µg/ml) on PBMC over 24 h.

b) Effect of the plant extracts on lymphocyte subset populations (as per Table 8)



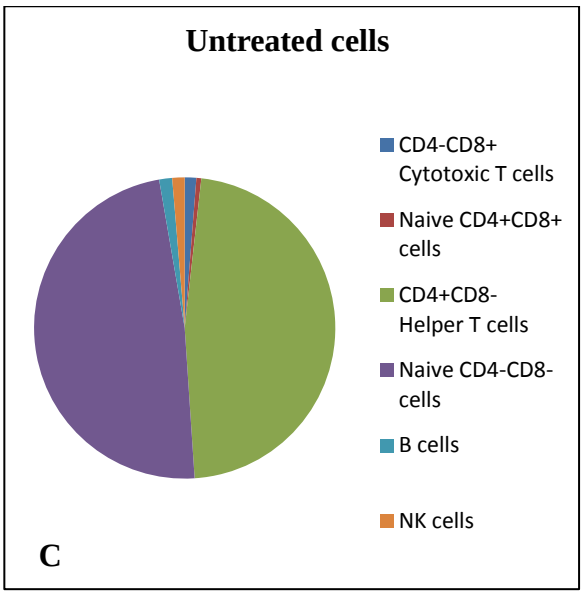


Figure 32: Effect of leaf extracts of *B. pilosa* on T cell subset populations evaluated by flow cytometry. A) *B. pilosa* methanolic extract, B) *B. pilosa* aqueous extract and C) Untreated cells.

4.3.6. *C. asiatica*

The methanolic extract of *C. asiatica* stimulated PBMC's in a dose-dependent relationship and showed a three and a half fold increase in proliferation when compared to the untreated cells (Fig. 33). The aqueous extract suppressed PBMC's when compared to the untreated cells.

Both the methanolic and the aqueous extracts caused an increase in the secretion of IFN-γ (63 pg/ml and 41 pg/ml respectively) and no significant change in the secretion of IL-10 (11 pg/ml and 12 pg/ml) when compared to the untreated cells (24 pg/ml and 6 pg/ml) as shown in Table 7.

The subset of cells that resulted with the methanolic extract (Fig. 34A) showed an increase in the $CD4^{-}CD8^{+}$ cytotoxic T cells (37%), naive $CD4^{+}CD8^{+}$ T cells (43%) and a slight increase in the B cells (2%) when compared to the untreated cells (Fig. 34C). There was a decrease in the $CD4^{+}CD8^{-}$ helper T cells (5%) and naive $CD4^{-}CD8^{-}$ T cells (0%). No changes were observed with the NK cells (1%). Similarly, the aqueous extract (Fig. 34B) showed an increase in the

$CD4^-CD8^+$ cytotoxic T cells (35%), naive $CD4^+CD8^+$ T cells (13%) and B cells (3%), a slight increase in the NK cells (2%) and a decrease in the $CD4^+CD8^-$ helper T cells (37%) and naive $CD4^-CD8^-$ T cells (2%) when compared to the untreated cells (Fig. 34C).

a) Dose effect of the plant extracts on PBMC

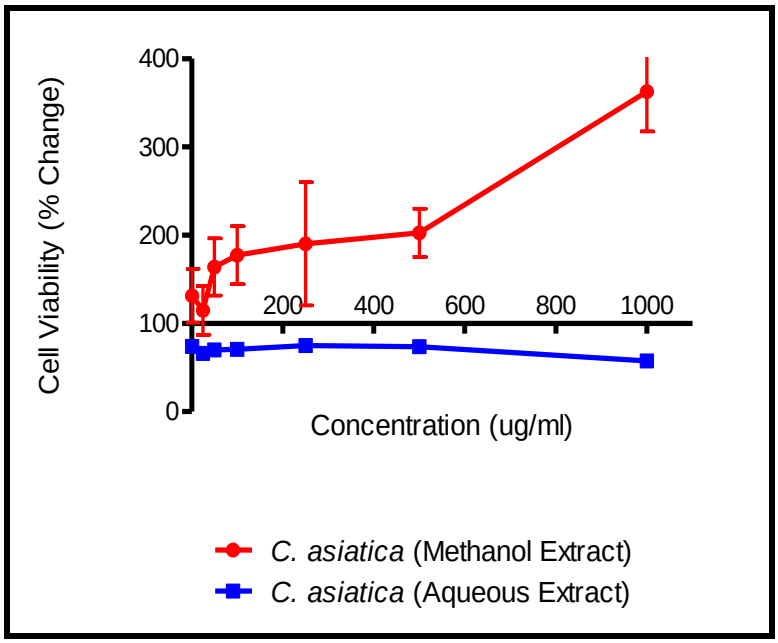
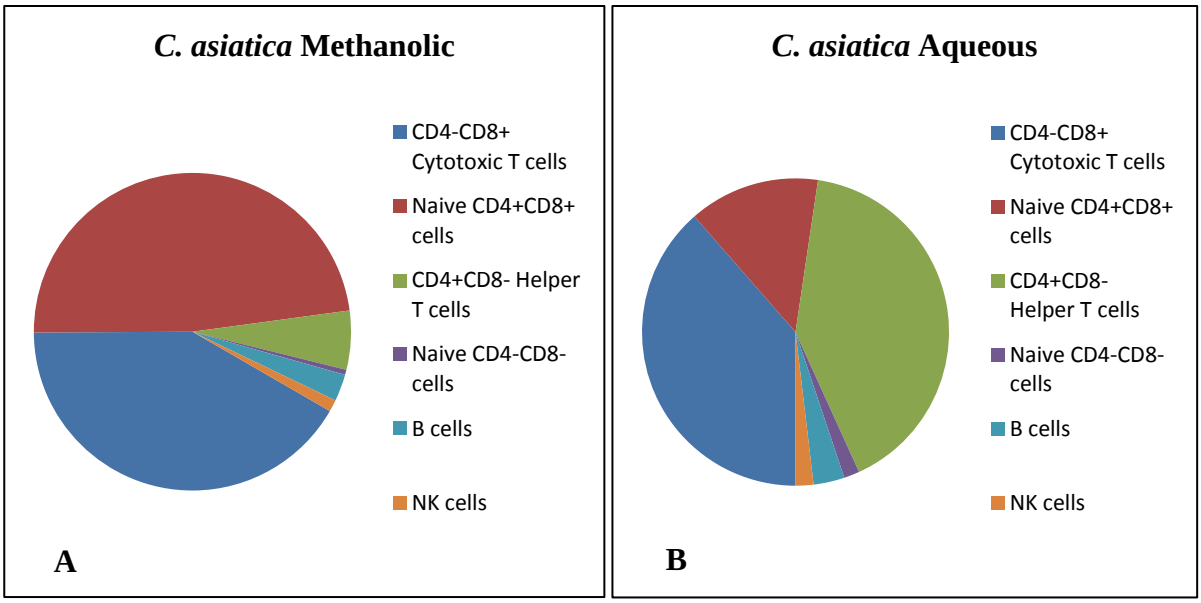


Figure 33**:** Effect of methanolic and aqueous leaf extracts of *C. asiatica* (1 µg/ml – 1000 µg/ml) on PBMC 24 h

b) Effect of the plant extracts on lymphocyte subset populations (as per Table 8)



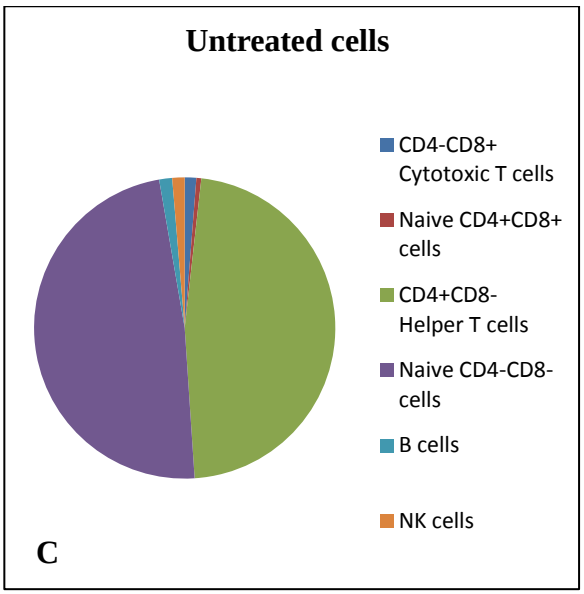


Figure 34: Effect of leaf extracts of *C. asiatica* on T cell subset populations evaluated by flow cytometry. A) *C. asiatica* methanolic extract, B) *C. asiatica* aqueous extract and C) Untreated cells.

4.3.7. *C. triloba*

The methanolic extract of *C. triloba* stimulated the number PBMC's in a dose-dependent manner and showed a three and a half-fold increase in proliferation when compared to the untreated cells (Fig. 35). The aqueous extract suppressed the number of PBMC's (Fig. 35).

The methanolic extract showed an increase in the levels of IFN-γ (69 pg/ml) and IL-10 (22 pg/ml) whilst the aqueous extract showed an increase in the level of IL-10 only (45 pg/ml) when compared to the untreated cells (24 pg/ml and 6 pg/ml respectively) as shown in Table 7.

The methanolic extract (Fig. 36A) and the aqueous extract (Fig. 36B) showed an increase in the $CD4^{-}CD8^{+}$ cytotoxic T cells (36% and 34% respectively) and naive $CD4^{+}CD8^{+}$ T cells (5% and 7%) and a slight increase in the B cells (2% and 3%) when compared to the untreated cells (Fig. 36C). There was also a decrease in the naive $CD4^{-}CD8^{-}$ T cells (2% and 2% respectively) and no changes in the $CD4^{+}CD8^{-}$ helper T cells (39% and 42%) and NK cells (1% and 1%).

a) Dose effect of the plant extracts on PBMC

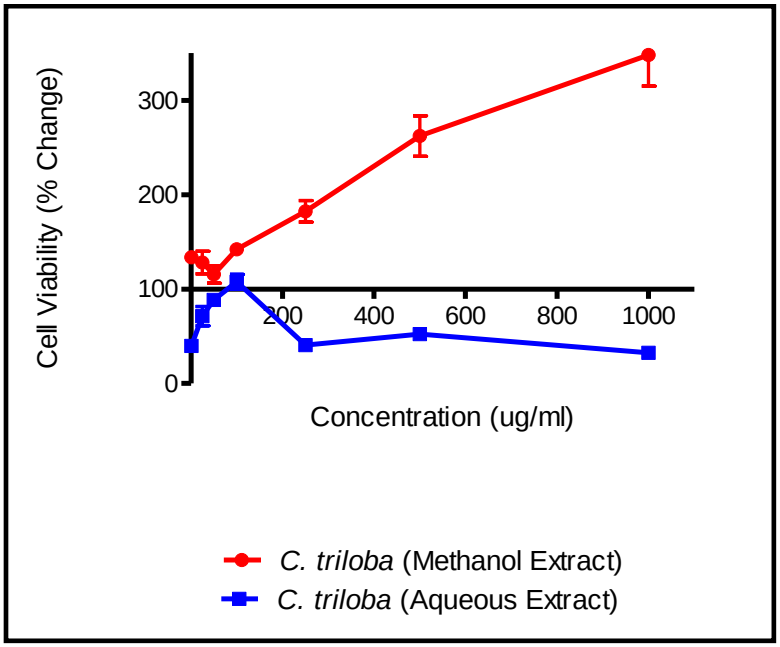
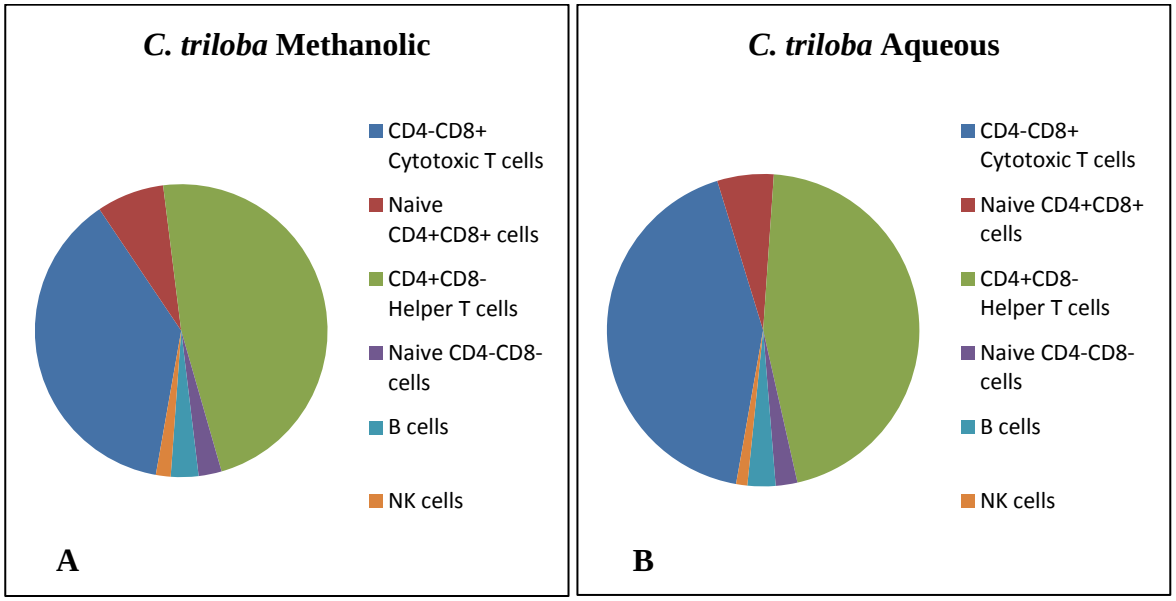


Figure 35: Effect of methanolic and aqueous leaf extracts of *C. triloba* (1 µg/ml – 1000 µg/ml) on PBMC over 24 h

b) Effect of the plant extracts on lymphocyte subset populations (as per Table 8)



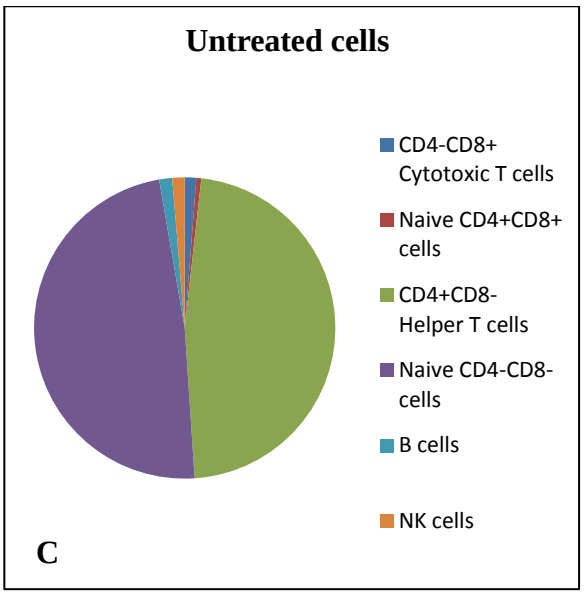


Figure 36: Effect of leaf extracts of *C. triloba* on T cell subset populations evaluated by flow cytometry. A) *C. triloba* methanolic extract, B) *C. triloba* aqueous extract and C) Untreated cells.

4.3.8. *E. australis*

The methanolic extract of *E. australis* did not show proliferation of lymphocytes, however there was a two-fold increase in the number of PBMC's (250 µg/ml – 1000 µg/ml) shown with the aqueous extract when compared to the untreated cells (Fig. 37).

Both the methanolic and aqueous extracts did not affect the release of IFN-γ (9 pg/ml and 5 pg/ml respectively) but did increase the release of IL-10 (61 pg/ml and 323 pg/ml) when compared to the untreated cells (24 pg/ml and 6 pg/ml) as shown in Table 7.

The methanolic extract (Fig. 38A) showed an increase in the $CD4^{-}CD8^{+}$ cytotoxic T cells (26%), B cells (3%) and NK cells (2%) and a decrease in the naive $CD4^{-}CD8^{-}$ T cells (5%) when compared to untreated cells (Fig. 38C). No changes were shown with the naive $CD4^{+}CD8^{+}$ T cells (3%) and $CD4^{+}CD8^{-}$ helper T cells (41%). The aqueous extract (Fig. 38B) showed an increase in the $CD4^{-}CD8^{+}$ cytotoxic T cells (32%) and a slight increase in the $CD4^{+}CD8^{-}$ helper T cells (43%), B cells (3%) and NK cells (2%) when compared to the

untreated cells (Fig. 38C). There was a decrease in the naive $CD4^{-}CD8^{-}$ T cells (4%) and no change in the naive $CD4^{+}CD8^{+}$ T cells (2%).

a) Dose effect of the plant extracts on PBMC

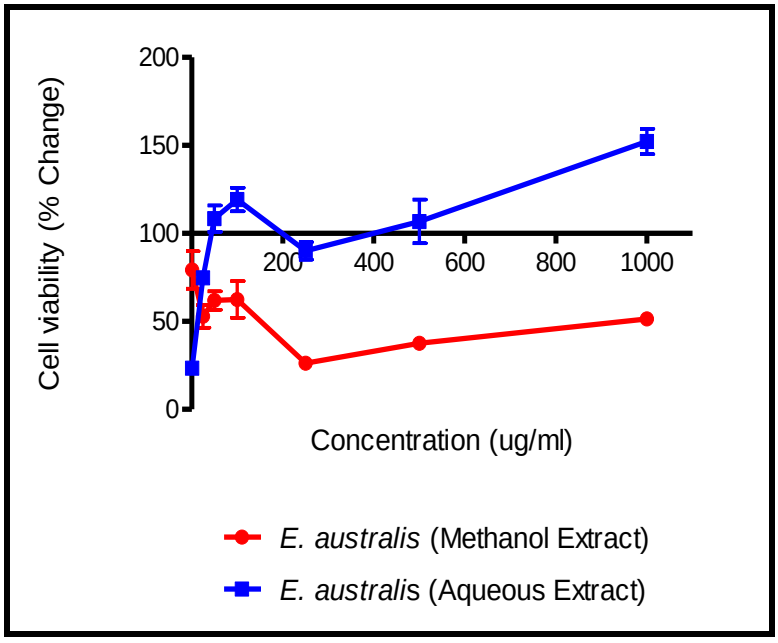
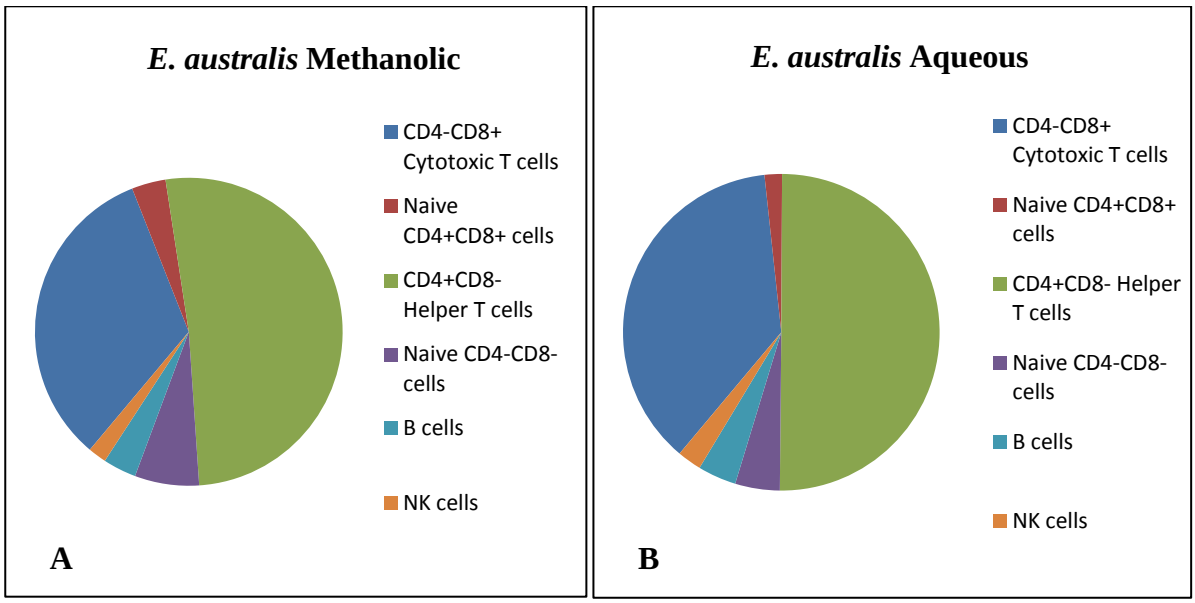


Figure 37: Effect of methanolic and aqueous leaf extracts of *E. australis* (1 µg/ml – 1000 µg/ml) on PBMC over 24 h

b) Effect of the plant extracts on lymphocyte subset populations (as per Table 8)



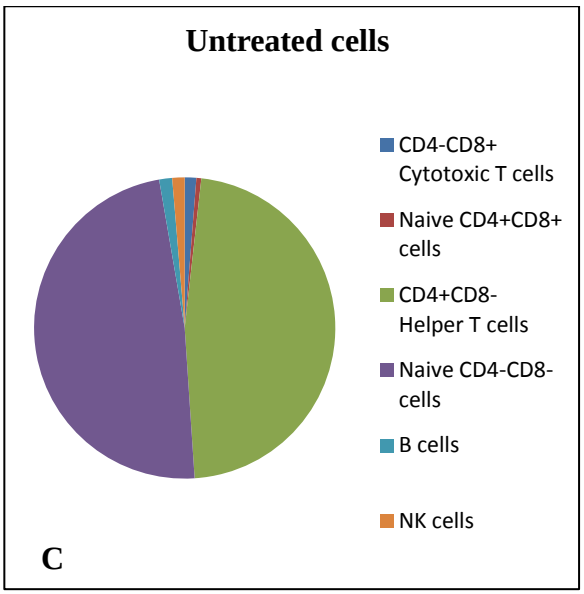


Figure 38: Effect of leaf extracts of *E. australis* on T cell subset populations evaluated by flow cytometry. A) *E. australis* methanolic extract, B) *E. australis* aqueous extract and C) Untreated cells

4.3.9. *J. flava*

Both the methanolic and the aqueous extracts of *J. flava* showed fluctuations in the number of PBMC's (1 µg/ml to 100µg/ml) and thereafter, stimulated PBMC's in a dose-dependent manner (100 µg/ml to 1000 µg/ml). The extracts showed a two-fold increase in the number of PBMC's when compared to the untreated cells (Fig. 39).

The methanolic extract showed an increase in the secretion of IL-10 only (40 pg/ml) whilst the aqueous extract showed an increase in the secretion of both IFN-γ (74 pg/ml) and IL-10 (129 pg/ml) whilst when compared to the untreated cells (24 pg/ml and 6 pg/ml respectively) as shown in Table 7.

The methanolic extract (Fig. 40A) showed an increase in the $CD4^{-}CD8^{+}$ cytotoxic T cells (35%) and naive $CD4^{+}CD8^{+}$ T cells (6%) and a slight increase in the B cells (3%) when compared to the untreated cells (Fig. 40C). There was a decrease in the naive $CD4^{-}CD8^{-}$ T cells (2%) and no change in the $CD4^{+}CD8^{-}$ helper T cells (41%) and NK cells (1%). The aqueous

extract (Fig. 40B) showed an increase in the $CD4^{-}CD8^{+}$ cytotoxic T cells (36%), naive $CD4^{+}CD8^{+}$ T cells (12%) and B cells (4%) and a slight increase in the NK cells (2%) when compared to the untreated cells (Fig. 40C). There was a decrease in the $CD4^{+}CD8^{-}$ helper T cells (30%) and naive $CD4^{-}CD8^{-}$ T cells (3%).

a) Dose effect of the plant extracts on PBMC

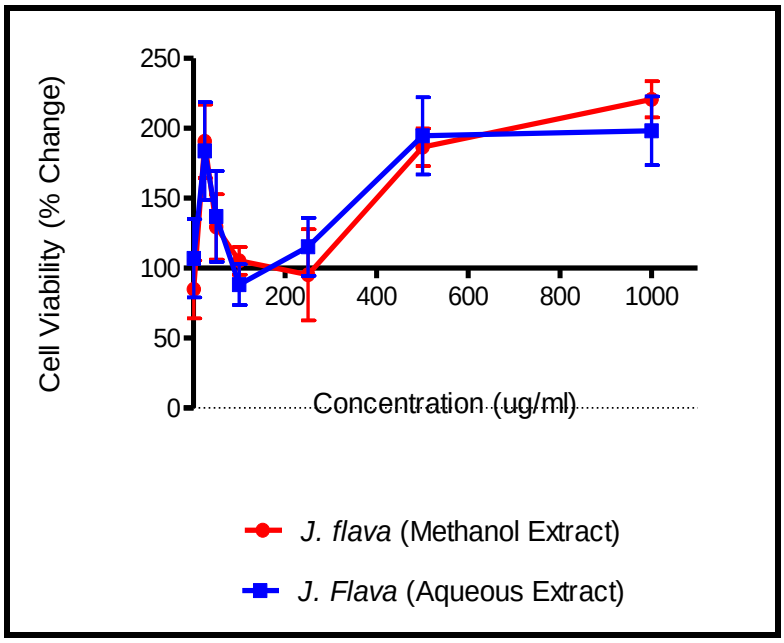
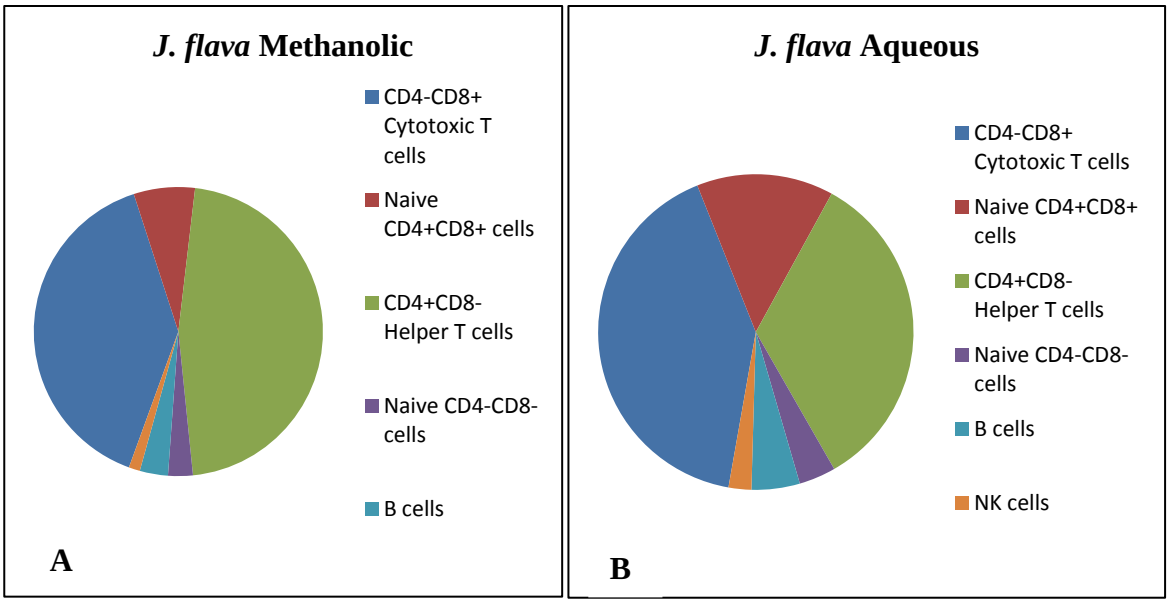


Figure 39**:** Effect of methanolic and aqueous leaf extracts of *J. flava* (1 µg/ml–1000 µg/ml) on PBMC over 24 h

b) Effect of the plant extracts on lymphocyte subset populations (as per Table 8)



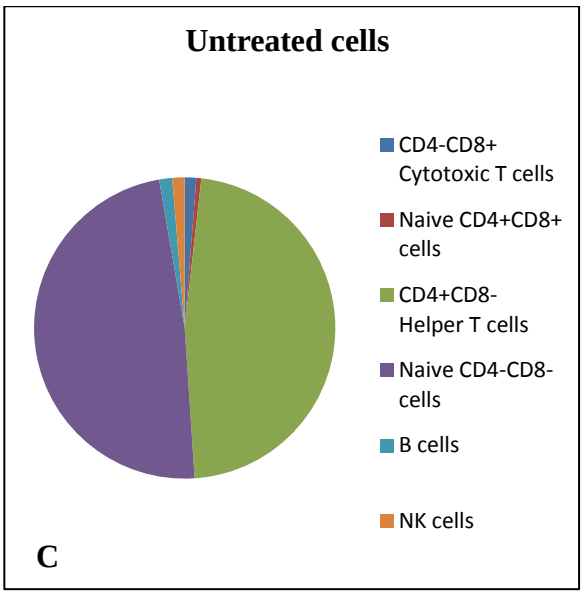


Figure 40: Effect of leaf extracts of *J. flava* on T cell subset populations evaluated by flow cytometry. A) *J. flava* methanolic extract, B) *J. flava* aqueous extract and C) Untreated cells.

4.3.10. *M. balsamina*

The methanolic extract of *M. balsamina* suppressed the number of PBMC's (1 µg/ml - 500 µg/ml) and thereafter stimulated the number of PBMC's (500 µg/ml - 1000 µg/ml) to almost two-fold when compared to the untreated cells (Fig. 41). The aqueous extract showed a different trend as it increased the number of PBMC's (25 µg/ml - 100 µg.ml) to two-fold and thereafter suppressed the number of PBMC's (100 µg/ml- 1000 µg/ml) as shown in Fig. 41.

Both the methanolic and the aqueous extracts did not cause the secretion of IFN-γ (0 pg/ml and 1 pg/ml respectively) but caused an increase the secretion of IL-10 (32 pg/ml and 52 pg/ml) when compared to the untreated cells (24 pg/ml and 6 pg/ml) as shown in Table 7.

The methanolic extract (Fig. 42A) showed an increase in the $CD4^{-}CD8^{+}$ cytotoxic T cells (22%), naive $CD4^{+}CD8^{+}$ T cells (5%) and B cells (6%) and a decrease in the $CD4^{+}CD8^{-}$ helper T cells (34%) and naive $CD4^{-}CD8^{-}$ T cells (5%) when compared to the untreated cells (Fig. 42C). The aqueous extract (Fig. 42B) showed an increase in the $CD4^{-}CD8^{+}$ cytotoxic T cells (30%), naive $CD4^{+}CD8^{+}$ T cells (4%) and B cells (3%) and a slight increase in the $CD4^{+}CD8^{-}$

helper T cells (43%) when compared to the untreated cells (Fig. 42C). No changes were shown in the NK cells for both methanolic and aqueous extracts (1% and 1% respectively).

a) Dose effect of the plants extract on PBMC

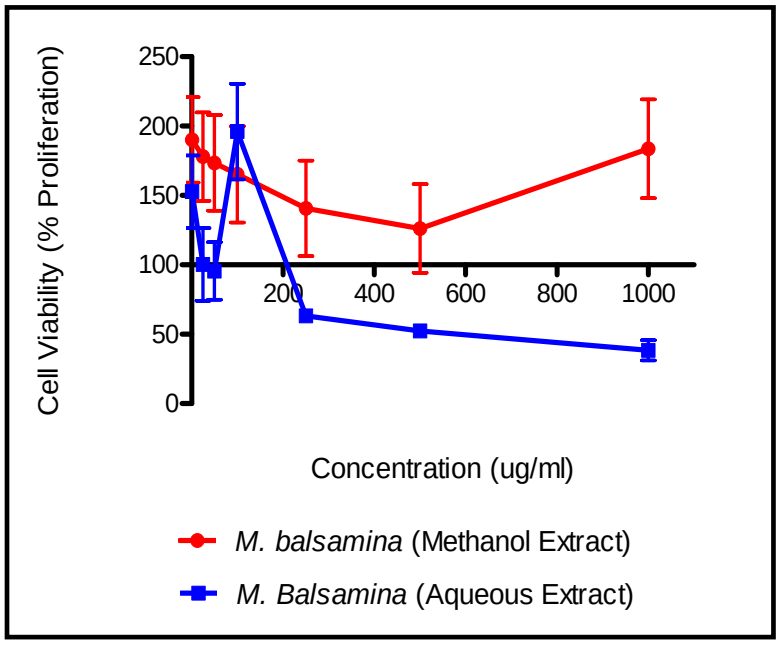
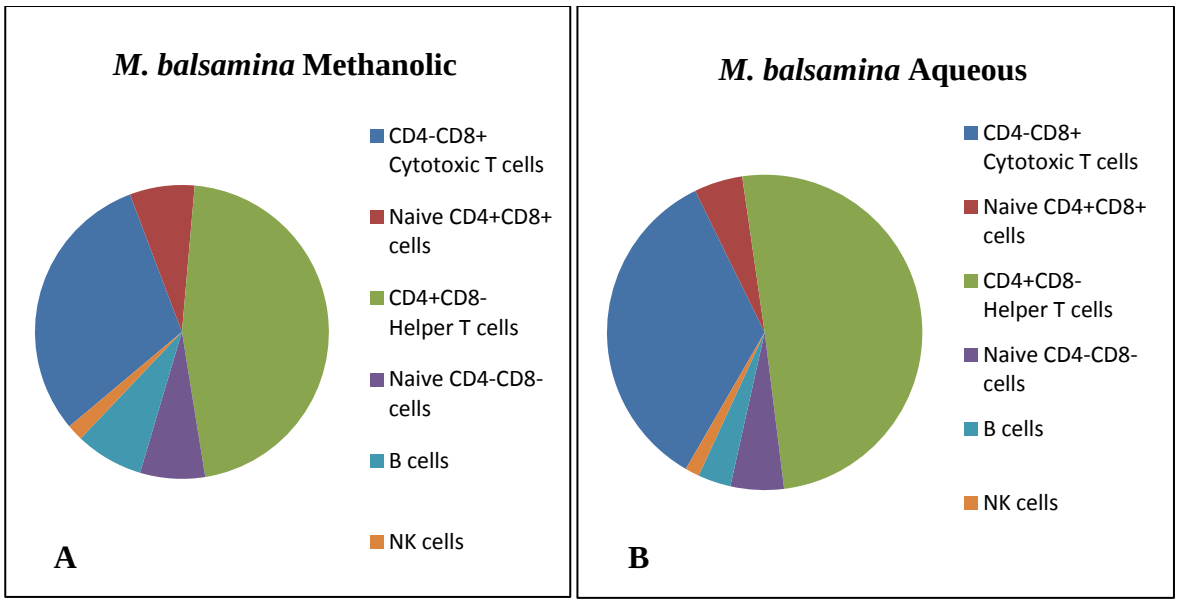


Figure 41: Effect of methanolic and aqueous leaf extracts of *M. balsamina* (1 µg/ml – 1000 µg/ml) on PBMC over 24 h

b) Effect of the plant extracts on lymphocyte subset populations (as per Table 8)



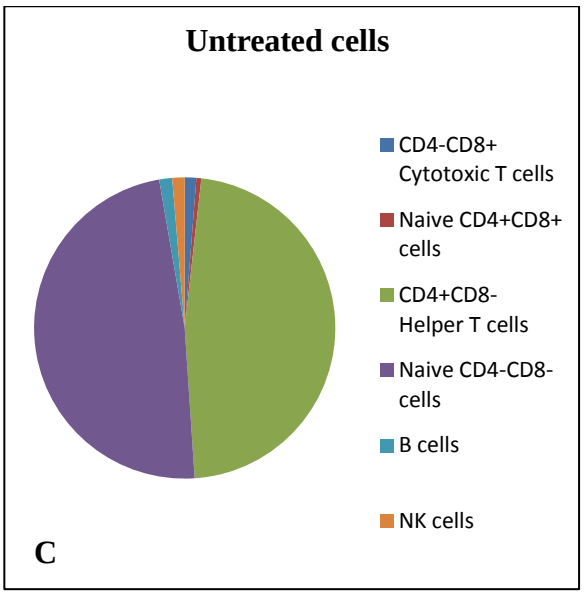


Figure 42: Effect of leaf extracts of *M. balsamina* on T cell subset populations evaluated by flow cytometry. A) *M. balsamina* methanolic extract, B) *M. balsamina* aqueous extract and C) Untreated cells.

4.3.11. *O. sinuatum*

Both the methanolic and the aqueous extracts of *O. sinuatum* suppressed the number of PBMC's when compared to the untreated cells (Fig.43).

Both the methanolic and the aqueous extracts showed a strong secretion of IL-10 (93 pg/ml and 74 pg/ml respectively) and a weak secretion of IFN-γ (8 pg/ml and 15 pg/ml) when compared to the untreated cells (6 pg/ml and 24 pg/ml) as shown in Table 7.

Both the methanolic (Fig. 44A) and the aqueous (Fig. 44B) extracts showed an increase in the $CD4^{-}CD8^{+}$ cytotoxic T cells (32% and 16% respectively), naive $CD4^{+}CD8^{+}$ T cells (13% and 11%) and B cells (3% and 5%) and a decrease in the $CD4^{+}CD8^{-}$ helper T cells (39% and 9%) and naive $CD4^{-}CD8^{-}$ T cells (1% and 0%) when compared to the untreated cells (Fig. 44C). No changes were shown in the NK cells for both methanolic and aqueous extracts (1.% and 0% respectively).

a) Dose effect of the plant extracts on PBMC

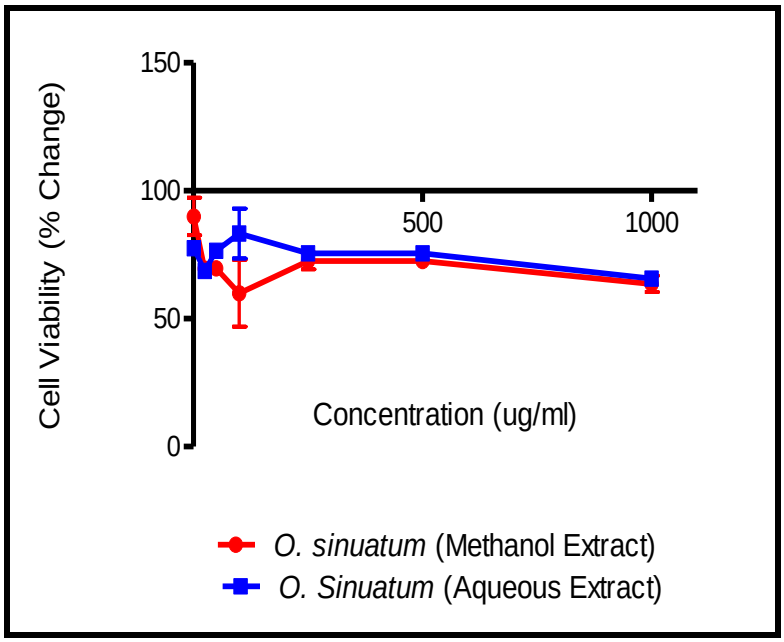
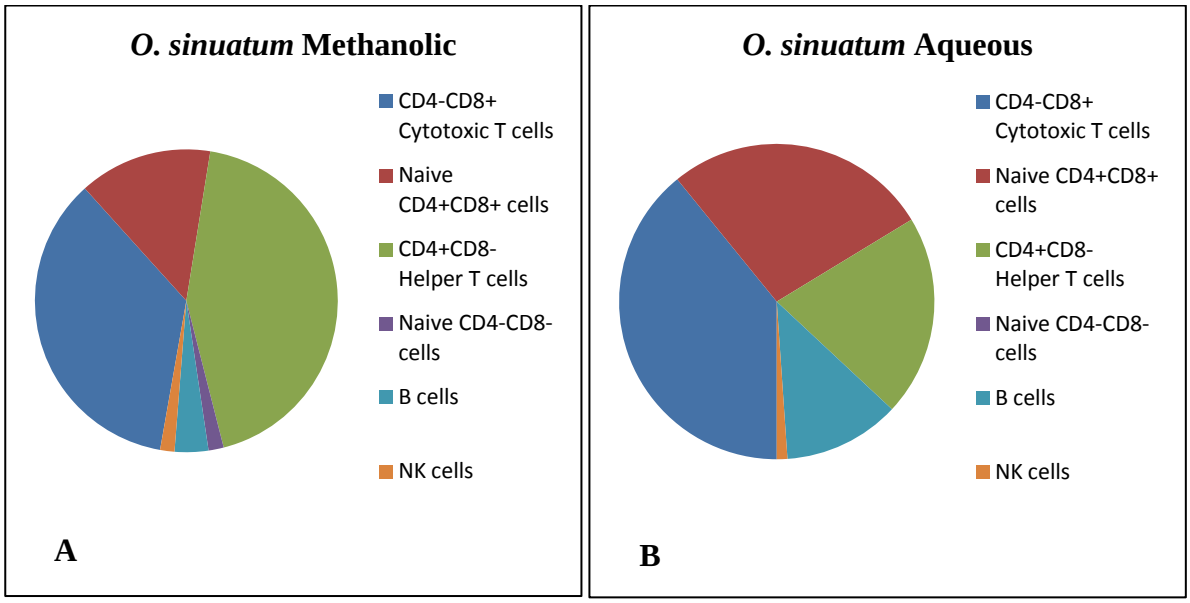


Figure 43: Effect of methanolic and aqueous leaf extracts of *O. sinuatum* (1 µg/ml – 1000 µg/ml) on PBMC over 24 h

b) Effect of the plant extracts on lymphocyte subset populations (as per Table 8)



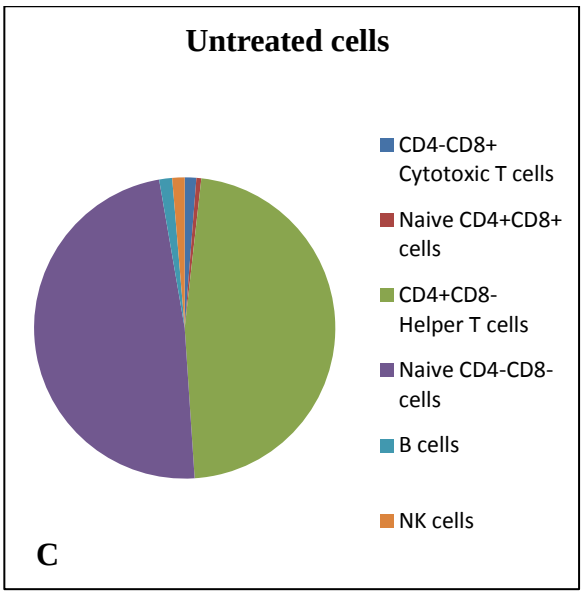


Figure 44: Effect of leaf extracts of *O. sinuatum* on T cell subset populations evaluated by flow cytometry. A) *O. sinuatum* methanolic extract, B) *O. sinuatum* aqueous extract and C) Untreated cells.

4.3.12. *P. viscosa*

The aqueous extract of *P. viscosa* decreased the number of PBMC's when compared to the untreated cells (Fig. 45).

It also stimulated the secretion of IL-10 (72 pg/ml) whilst it inhibited the secretion of IFN-γ (12 pg/ml) when compared to the untreated cells (6 pg/ml and 24 pg/ml respectively) as shown in Table 7.

The aqueous extract (Fig. 46A) showed an increase in the $CD4^{-}CD8^{+}$ cytotoxic T cells (34%), a slight increase in the naive $CD4^{+}CD8^{+}$ T cells (3%) and B cells (2%) and a decrease in the naive $CD4^{-}CD8^{-}$ T cells (5%) when compared to the untreated cells (Fig. 46B). No changes were shown with the $CD4^{+}CD8^{-}$ helper T cells (38%) and NK cells (1%) when compared to the untreated cells.

a) Dose effect of the plant extract on PBMC

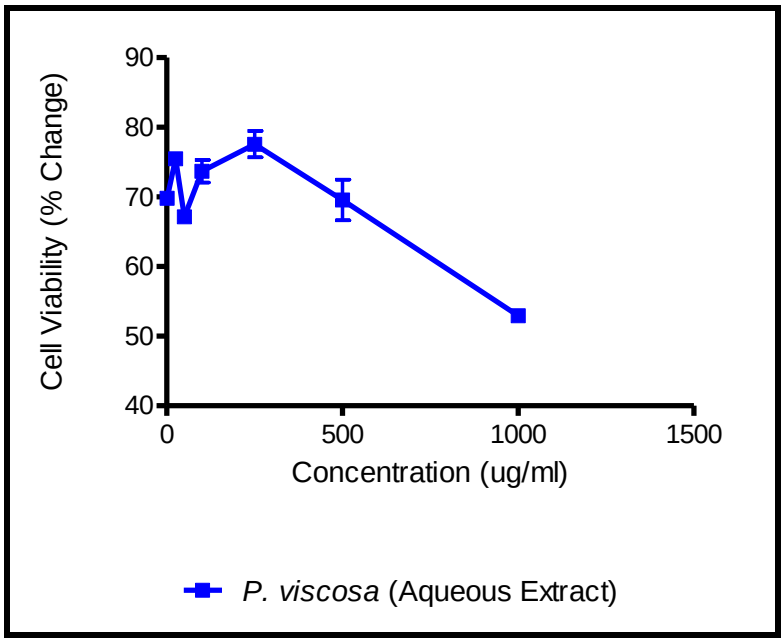


Figure 45: Effect of aqueous leaf extracts of *P. viscosa* (1 µg/ml – 1000 µg/ml) on PBMC over 24 h

b) Effect of the plant extract on lymphocyte subset populations (as per Table 8)

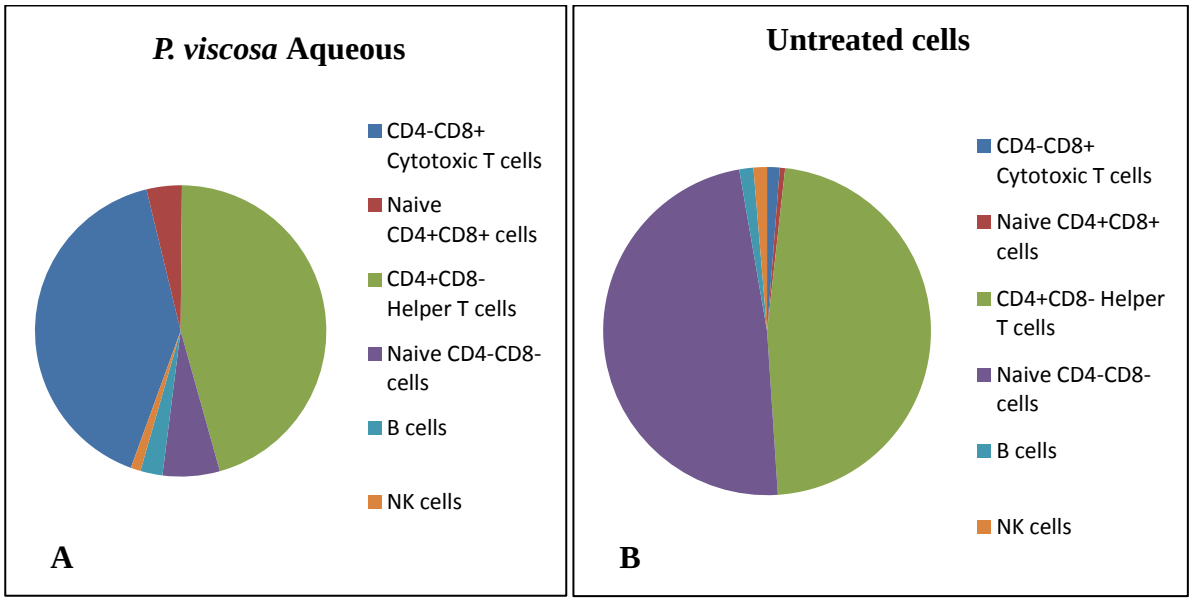


Figure 46: Effect of leaf extracts of *P. viscosa* on T cell subset populations evaluated by flow cytometry. A) *P. viscosa* aqueous extract and B) Untreated cells

4.3.13. *S. occidentalis*

The methanolic extract of *S. occidentalis* showed an increase in proliferation of PBMC's (250 µg/ml-1000 µg/ml) whilst the aqueous extract did not show proliferation of PBMC's when compared to the untreated cells (Fig. 47).

The same pattern of cytokine secretion was observed with the methanolic and the aqueous extracts, as they both showed an increase in the secretion of IL-10 (92 pg/ml and 104 pg/ml respectively) and very little or no secretion of IFN-γ (10 pg/ml and 0 pg/ml respectively) when compared to the untreated cells (6 pg/ml and 24 pg/ml respectively) as shown in Table 7.

The methanolic extract (Fig. 48A) showed an increase in the $CD4^{-}CD8^{+}$ cytotoxic T cells (35%), naive $CD4^{+}CD8^{+}$ T cells (15%) and B cells (3%) and a decrease in the $CD4^{+}CD8^{-}$ helper T cells (31%) and naive $CD4^{-}CD8^{-}$ T cells (1%) when compared to the untreated cells (Fig. 48C). No changes were observed with the NK cells (1%). The aqueous extract (Fig. 48B) showed a decrease in the $CD4^{+}CD8^{-}$ helper T cells (12%) and naive $CD4^{-}CD8^{-}$ T cells (1%) and no changes in all the other cell populations when compared to the untreated cells (Fig. 48C)

a) Dose effect of the plant extracts on PBMC

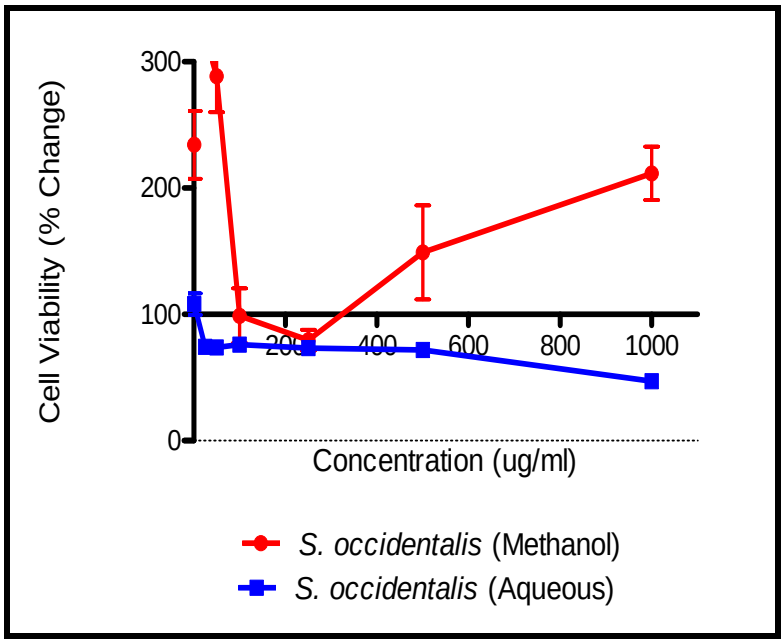
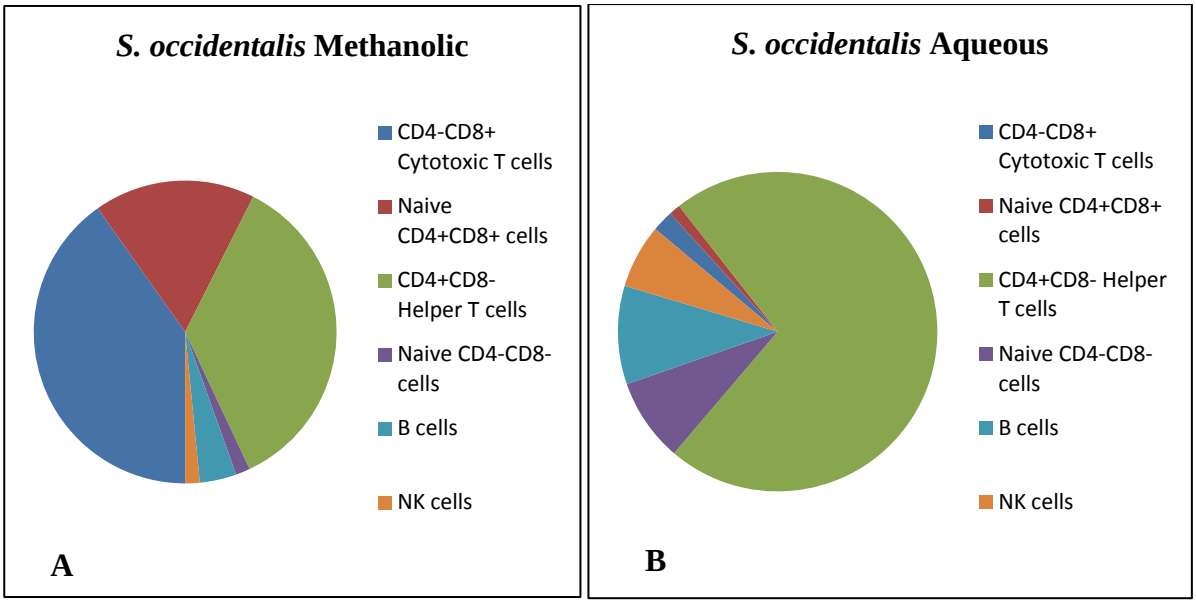


Figure 47: Effect of methanolic and aqueous leaf extracts of *S. occidentalis* (1 µg/ml – 1000 µg/ml) on PBMC over 24 h

b) Effect of the plant extracts on lymphocyte subset populations (as per Table 8)



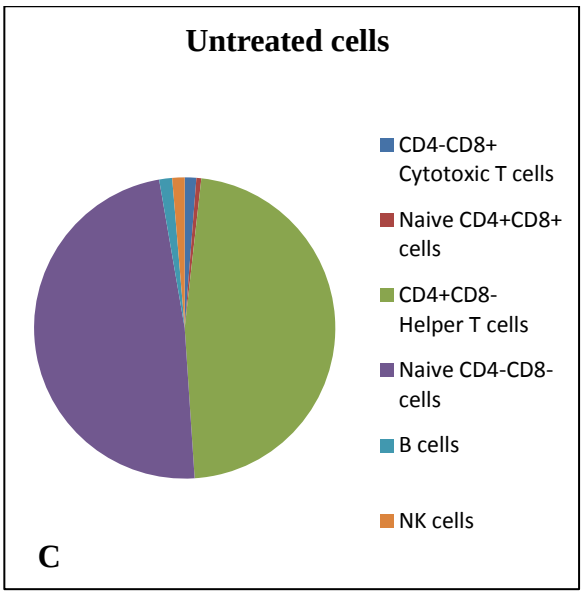


Figure 48: Effect of leaf extracts of *S. occidentalis* on T cell subset populations evaluated by flow cytometry. A) *S. occidentalis* methanolic extract, B) *S. occidentalis* aqueous extract and C) Untreated cells.

4.3.14. *S. oleraceous*

The methanolic and the aqueous extracts of *S. oleraceous* showed a decrease in the number of PBMC's when compared to the untreated cells (Fig. 49).

Both the methanolic and aqueous extracts showed a decrease in the secretion of IFN-γ (5 pg/ml and 3 pg/ml respectively) but an increase in the secretion of IL-10 (91 pg/ml and 105 pg/ml) when compared to the untreated cells (24 pg/ml and 6 pg/ml) as shown in Table 7.

Both the methanolic extract (Fig. 50A) and the aqueous extract (Fig. 50B) showed an increase in the $CD4^{-}CD8^{+}$ cytotoxic T cells (14% and 33% respectively), naive $CD4^{+}CD8^{+}$ T cells (6% and 17%) and B cells (5% and 2%) and a decrease in the $CD4^{-}CD8^{+}$ helper T cells (33% and 34%) and naive $CD4^{-}CD8^{-}$ T cells (8% and 3%) when compared to the untreated cells (Fig. 50C). There were no changes in the NK cells for the methanolic extracts (1%) but a slight increase in the NK cells for the aqueous extracts (2%).

a) Dose effect of the plant extracts on PBMC

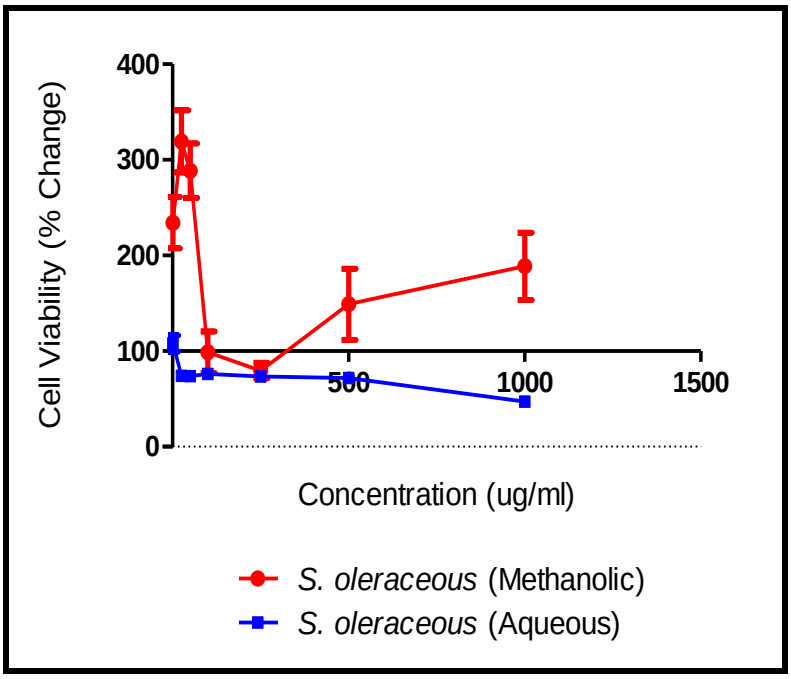
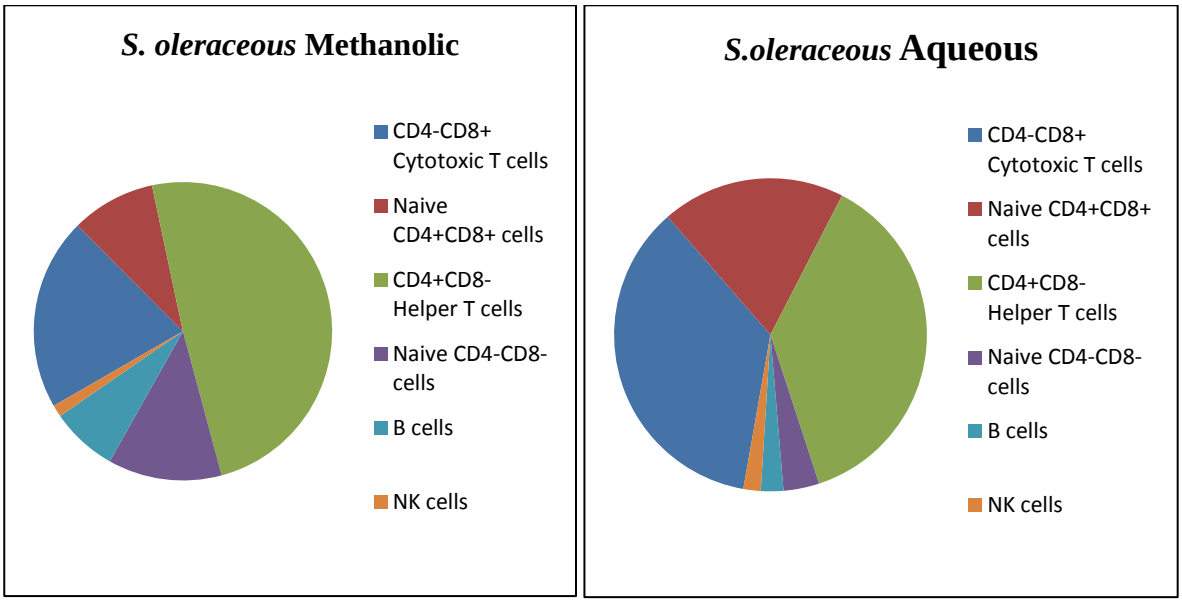


Figure 49: Effect of methanolic and aqueous leaf extracts of *S. oleraceous* (1 µg/ml – 1000 µg/ml) on PBMC over 24 h

b) Effect of the plant extracts on lymphocyte subset populations (as per Table 8)



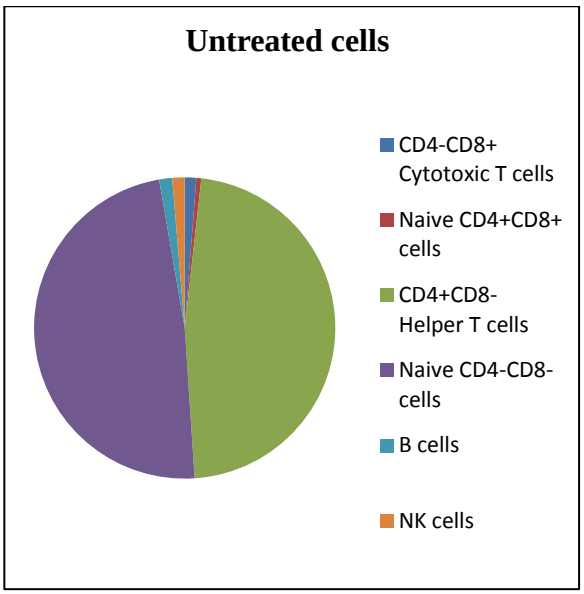


Figure 50: Effect of leaf extracts of *S. oleraceous* on T cell subset populations evaluated by flow cytometry. A) *S. oleraceous* methanolic extract, B) *S. oleraceous* aqueous extract and C) Untreated cells.

CHAPTER FIVE: DISCUSSION

The human immune system has the essential function of protecting the body against the damaging effects of microbial agents that are pathogenic. The system comprises innate (non-specific) and acquired (specific) immunity. Natural killer (NK) cells, complement system, macrophages, antigen presenting cells (APC's) and neutrophils make up innate immunity, and mounts an immediate non-specific response to foreign microbial agents. If microbes by-pass this primary defence, acquired immunity, comprising humoral and cell mediated immune responses, will then act to contain the invaders. The major cells of the humoral response are the B cells which are stimulated to develop into antibody-secreting plasma cells. The major cells of the cell-mediated response are the helper T cells (Th), which produce cytokines that activate macrophages for increased microbicidal activity, and the cytotoxic T cells (Tc), which directly eradicate invading organisms (Pruett, 2003). The T cells commit to either a Th-1/Tc-1 or Th-2/Tc-2 subtype, and are distinguished by the cytokines they secrete.

Th-1/Tc-1 cells secrete inflammatory effector cytokines such IFN-γ and TNF-α which are responsible for macrophage activation, protection against intracellular pathogens and perpetuating autoimmune responses. Thus, they are effective inducers of cell-mediated immune responses. In contrast, Th-2/Tc-2 cells secrete interleukins such as IL-4, IL-5, IL-6, IL-10 and IL-13 which are essential in promoting antibody secretion, immunoglobin differentiation and protection against extracellular infections (Stockinger *et al.,* 2004). Thus, they stimulate the humoral immune response. This is shown in figure 51, which provides a brief overview of the immune system

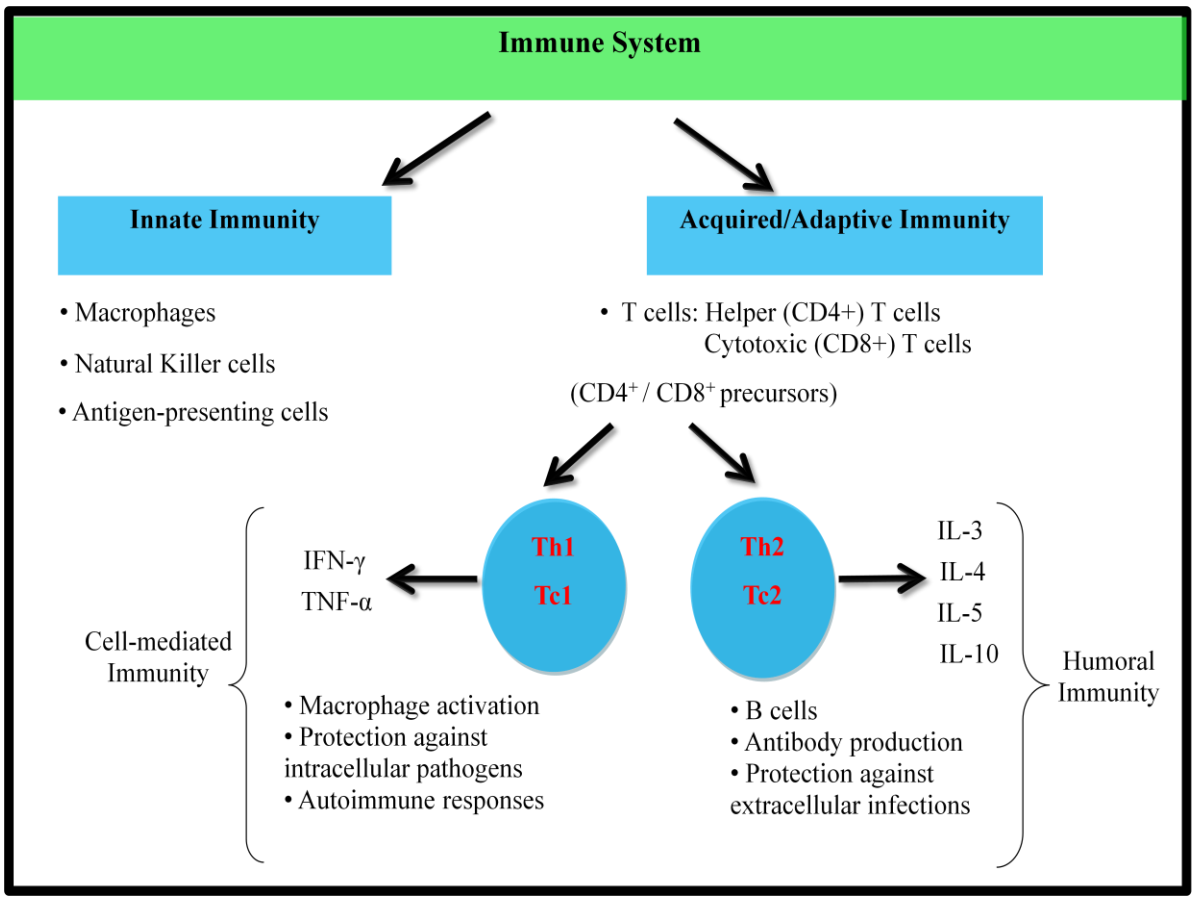


Figure 51: Brief overview of the immune system

Apart from these natural mechanisms, there are other factors that stimulate and suppress host immunity. These also heighten humoral and cellular immune responses, by either enhancing cytokine secretion, or by directly stimulating B- or T lymphocytes. In this study two models were used to evaluate the immunomodulatory response of each plant on lymphocytes. These are determined by the type of cytokine and the cell subsets which are released by the extract-stimulated lymphocytes: On the basis of the responses that the plants exert, we can associate the production of cytokines (IFN-γ or IL-10) with either the stimulation of cell-mediated or humoral immunity.

The first model is a cytokine response model (CRM) proposed by Cherng *et al.*,(2008). This is based on the cytokine profile and categorises the immune response into three types:

- Type 1 stimulates lymphocyte proliferation and secretion of IFN-γ
- Type 2 augments lymphocyte activation and inhibits IFN-γ secretion
- Type 3 stimulates secretion of IFN-γ

Another model that is used to evaluate the type of immune response, predicts the branch of immunity that is followed i.e. cell-mediated or humoral, is the acquired immunity model (AIM) and is proposed by Mosmann and Sad, (1996) and Vukmanovic-Stejic *et al*., (2000). In this model, $CD8^+$ cytotoxic T or $CD4^+$ helper T precursor cells will give rise to a cell-mediated immune response if there is a release of IFN-γ and this is termed a Tc1 or Th1 response. Or $CD8^+$ cytotoxic T or $CD4^+$ helper T precursor cells will induce a humoral immune response if there is release of IL-10 and this is known as a Tc2 or Th2 response (Fig. 3 and Fig. 4).

Each plant showed a different immune response which is categorised according to these two models. They are discussed in the sections (5.1.-5.14).

5.1. *A. dubius*

The aqueous extract of *A. dubius* did not cause the proliferation of PBMC but showed an increase in the secretion of IFN-γ and the $CD8^+$ cytotoxic T cells and B cells, thus indicating a Type 3 immunomodulatory response according to the CRM model (Cherng *et al.*,2008). The increased secretion of IFN-γ might be due to the stimulation of the $CD8^+$ cytotoxic T cells since it is significantly elevated in the PHA and Con A activated lymphocytes and because the $CD8^+$ cytotoxic T cell is noted to be the main cell type to secrete IFN-γ (Cherng *et al*., 2008). According to the AIM model (Mosmann and Sad, 1996; Vukmanovic-Stejic *et al*., 2000), the exposure of PBMC's to the aqueous extract of *A. dubius* resulted in an increase in IFN-γ and the $CD8^+$ cytotoxic T cells thus suggesting the Tc1 response on lymphocytes which is responsible for the stimulation of cell-mediated immunity. The aqueous extract also showed a

significant increase in B cells which could also be classified as a Tc2 response but for the lack of IL-10 secretion. This therefore highlights the fact that although cell numbers increase this does not translate to function i.e. the numbers may be high but this doesn't mean they are functional.

The methanolic extract did not affect PBMC's nor did they show an effect on the release of IFN-γ and IL-10. They caused an increase in the $CD8^+$ cytotoxic T cells and B cells, a decrease in the $CD4^+$ helper T cells and no change in the NK cells. However, this profile cannot be categorised into a definitive immune response as there was no cytokine secreted.

A. dubius is used traditionally for feeding young children and patients with haemorrhage, anaemia, constipation, stomach pains and kidney complaints (Grubben and Denton, 2004). Our studies show that the aqueous extract of this plant may enhance cell mediated immunity, thus highlighting the compounds from this plant which can be isolated to provide further information. Thus we can postulate a link between its traditional usage and scientific findings. However it is not that popular because of the presence of a high content of hydrocyanic acid and oxalic acid (Grubben and Denton, 2004) which are generally regarded as anti-nutrients.

5.2. *A. hybridus*

The methanolic extract of *A. hybridus* stimulated PBMC's and showed an increase in the levels of IFN-γ and the $CD8^+$ cytotoxic T cells and B cells. There was no significant change in the NK cells or the $CD4^+$ helper T cells. Thus, according to the CRM model, the immunomodulatory pathway followed by the methanolic extract is the Type 1 immunomodulatory response. The same trend was seen with the aqueous extract except for their suppression of PBMC's. The aqueous extract showed the Type 3 immunomodulatory response as they did not stimulate PBMC's but did cause the release of IFN-γ.

The increased secretion of IFN-γ caused by the extracts might be attributed to the increase in the $CD8^+$ cytotoxic T cells. This is in accordance with the research of Cherng *et al.*, (2008) who noted that the $CD8^+$ cytotoxic T cells were the main cell type to secrete IFN-γ. To elucidate which type of $CD8^+$ cytotoxic T cells regulated the patterns of immunomodulation, the results indicated that the immunostimulation of *A. hybridus* might be due to the Tc1 cells due to the fact that they stimulated IFN-γ secretion but suppressed IL-10 secretion. Thus, according to the AIM model, this indicates the Tc1 response by both the methanolic and the aqueous extracts, therefore leading to a possible stimulation of cell mediated immunity.

Traditionally *A. hybridus* is used to treat many conditions such as anaemia, chronic fatigue, heavy menstrual bleeding, intestinal bleeding, diarrhoea and coughs (Beekrum, 2003) in which the immune system is not defined. The only correlation between our results which indicate a cell-mediated response by the extracts and the plant's traditional uses is the utilisation of the poultice for bleeding wounds and infusions which is splashed onto the skin to alleviate burning and itchy skin (Grubben and Denton, 2004). This alludes to a pro-inflammatory response which the methanolic and aqueous exracts of *A. hybridus* have shown to stimulate as indicated by their stimulation of the pro-inflammatory cytokine IFN-γ.

5.3. *A. spinosus*

The methanolic extract of *A. spinosus* suppressed PBMC's but caused an increase in the secretion of both IFN-γ and IL-10, and the $CD8^+$ cytotoxic T cell and B cell populations. This response is typical of the Type 3 pathway of immunomodulation according to the CRM model. The release of IFN-γ can be attributed to the increased stimulation of the $CD8^+$ cytotoxic T cells since IFN-γ is known to promote cytotoxic T lymphocyte activity while the release of IL-10 might be due to rise in B cells or $CD8^+$ cytotoxic T cells. According to the AIM model, the

increase in both IFN-γ and IL-10 secretion alludes to the Tc1 and Tc2 responses respectively, indicating the possible promotion of both cell-mediated immunity and humoral immunity by the methanolic extract.

Previous research has shown that the methanolic extract of the leaves of *A. spinosus* display anti-inflammatory activity by inhibiting the synthesis of highly specific prostaglandin activity *in vitro* (Zeashan *et al.*, 2009). Our results have shown the ability of *A. spinosus* to promote this response due to its effect on the anti-inflammatory cytokine, IL-10.

The aqueous extract stimulated PBMC's and showed an increase in the $CD8^+$ cytotoxic T cells, B cells and IL-10. According to the CRM model, their stimulation of PBMC's and inhibition of IFN-γ is typical of the Type 2 immunomodulation response. The increase in IL-10 secretion can be attributed to the B cells or $CD8^+$ cytotoxic T cells since IL-10 was found to be produced by all helper T cell subsets and $CD8^+$ cytotoxic T cells (Aman *et al.,* 1996). According to the AIM model, this indicates the Tc2 response and the possible stimulation of humoral immunity by the aqueous extract.

Previous research by Lin *et al*., (2005) showed that the aqueous extract of this plant exerted significant immunomodulating effects on T and B lymphocytes in a dose-dependent manner, and also stimulated the production of both Th1 and Th2 cytokines with Th2 (IL-5, IL-6, IL-10) cytokine secretion being much higher than that of Th1 cytokines (IL-2, IFN-γ). The immunostimulating activity of *A. spinosus* was attributed to a novel proteinaceous compound with a high molecular weight of 313 kDa. Their findings correlate with our research which shows that the aqueous extract stimulated the release of IL-10, possibly due to its effect on the increase of the T- and B cell counts. IL-10 is known to promote humoral immunity and stimulate antibody responses.

A. spinosus has been used extensively in traditional medicine to treat a variety of illnesses and it also has many bioactivities including anti-inflammatory, anti-malarial, anti-bacterial and anti-diuretic (Hussain *et al.,* 2009). There are also a vast range of active compounds in the plant including alkaloids, flavonoids, phenolic acids, steroids and terpernoids, accounting for its wide use in medicine. It can be postulated that there is a link between the results of our study and other studies, which indicate the potential use of *A. spinosus* in both the cell-mediated and humoral immune responses and its usage in traditional medicine for blood diseases and inflammation (Kirtikar and Basu, 2001). The immunostimulating activity may be due partly to the phenolic compounds and flavonoids, which have been shown to possess this activity (Cherng *et al.*, 2008).

5.4. *A. gangetica*

The methanolic extract of *A. gangetica* did not stimulate PBMC's or the secretion of IFN-γ but did stimulate the secretion of IL-10. They also showed an increase in the $CD8^+$ cytotoxic T cells and B cells. Thus they show a different type of response that does not correspond with the CRM model proposed by Cherng *et al*., (2008) since they did not affect lymphocyte proliferation or IFN-γ. This can be regarded as a Type 4 immunomodulatory response. According to the AIM model, the methanolic extract caused the Tc2 response, as indicated by the increase in $CD8^+$ cytotoxic T cells and secretion of IL-10 suggesting the possible stimulation of humoral immunity.

The aqueous extract stimulated PBMC's and showed an increase in the levels of IL-10 and the $CD8^+$ cytotoxic T cells and B cells and a decrease in the levels of IFN-γ. Therefore, according to the CRM model the aqueous extract show the Type 2 immunomodulatory response. According to the AIM model, the aqueous extract also caused the Tc2 response, as indicated by

the increase in $CD8^+$ cytotoxic T cells and secretion of IL-10 suggesting the possible stimulation of humoral immunity.

An important active compound in *A. gangetica* is the flavonoid apigenin (Anderson, 2007). Flavonoids form an intrinsic component of fruits and vegetables and are widely found in the plant kingdom. Three of their major subclasses are flavones, flavonoles and flavonones. Verbeek *et al.,* (2004) evaluated the *in vitro* effects of flavonoids on T cell functions, which were relevant to autoimmune disease. Among those tested was the flavone, apigenin. Their research showed that apigenin strongly inhibited both murine lymph node T cell and human T cell responses. Moreover, when investigated for their effects on T cell proliferation and antigen-specific IFN-γ release, apigenin was found to reduce the production of IFN-γ more effectively than T cell proliferation. The more pronounced impact of flavonoids on IFN-γ production as compared to proliferative responses is linked to the intracellular pathway of IFN-γ, which could be relatively sensitive to flavone inhibition (Verbeek *et al.,* 2004).

This is in accordance with our results which show a decrease in IFN-γ secretion caused by the extracts of *A. gangetica* possibly due to their active compound flavonoid, in particular flavone, which Verbeek *et al.,* (2004) emphasized are potent inhibitors of IFN-γ production and antigen triggered proliferative responses by T cells. Since flavonoids form an active part of *A. gangetica* and the results presented here show the ability of the leaf extracts to stimulate the immunosuppressive cytokine IL-10, a focus on this type of plant provides and highlights information on the possible therapeutic application of flavonoids in human autoimmune diseases.

5.5. *B. pilosa*

The methanolic extract of *B. pilosa* showed an increase in the number of PBMC's and in the secretion of IL-10 and the $CD8^+$ cytotoxic T cells. According to the CRM model, this is typical of the Type 2 immunomodulatory response since they stimulated PBMC's and suppressed IFN-γ secretion. According to the AIM model, the methanolic extract stimulates the Tc2 response of the $CD8^+$ cytotoxic T cells due to their effects on IL-10, leading to the possible stimulation of humoral immunity.

Previous research by Chiang *et al.,* (2007) has shown that compounds from *B. pilosa* inhibit the differentiation of Tho cells into Th1 cells but promote the differentiation of Tho cells into Th2 cells. Further research has demonstrated that cytopiloyne, a novel bioactive polyacteylenic glucoside of *B. pilosa* inhibited the differentiation of Th0 cells into Th1 cells but stimulated the differentiation of Th0 cells into Th2 cells, thus it was able to up-regulate IL-4 expression and down-regulate IFN-γ expression (Chiang *et al.,* 2007). Our findings correlate with their research since it was showed that the methanolic extracts inhibited the release of IFN-γ and stimulated the release of IL-10, a Th2 cytokine thus promoting the humoral immune response.

The aqueous extract showed a decrease in the number of PBMC's but an increase in the secretion of IFN-γ and IL-10 and the $CD8^+$ cytotoxic T cells and B cells. This indicates a Type 3 immunomodulatory response according to the CRM model. The augmentation of IFN-γ and IL-10 secretion after the treatment of PBMC's with the aqueous extract might be due mainly to the $CD8^+$ cytotoxic T cells. Thus according to the AIM model, the induction of both multi-potent cytokines IFN-γ and IL-10 by the aqueous extract indicate that it might be able to activate the Tc1 and Tc2 response of $CD8^+$ cytotoxic T cells, possibly leading to the stimulation of cell-mediated and humoral immunity.

Previous research by Chang *et al*., (2007) has shown that two bioactive flavonoids namely centaurein and centaureidin isolated from the water extracts of *B. pilosa* were able to enhance the transcription of interferon-γ, thereby significantly promoting IFN-γ promoter activity. This validates the results presented here which show a considerable increase in IFN-γ caused by the aqueous extracts. Chang *et al*., (2007) confirmed the efficacy of both flavonoids in being able to modulate IFN-γ expression by activating the enhancer activity of nuclear factors such as NFAT and NF_KB, which are involved in the transcription.

Bidens pilosa is well known for its use in folk medicine in various parts of the world for improving immunity by acting as an immunomodulatory agent. Since cytokines such as IL-4 and IFN-γ play key roles in the modulation of T cell differentiation, it is very likely that cytopiloyne promotes the Th2 cell differentiation. Therefore, this compound plays a significant role in the modulation of T cell differentiation. The function of cytopiloyne as an effective T cell modulator is thought to contribute to the ethnopharmocological effect of the *B. pilosa* extracts in treating Th1 cell mediated autoimmune diseases such as diabetes (Chiang *et al*., 2007).

5.6. *C. asiatica*

The methanolic extract of *C. asiatica* stimulated PBMC's and showed an increase in the levels of IFN-γ and the $CD8^+$ cytotoxic T cells and B cells. Thus, they follow the Type 1 pathway of immunomodulation according to the CRM model. According to the AIM model, the increase in IFN-γ can be attributed to the rise in the $CD8^+$ cytotoxic T cells, therefore indicating the Tc1 response and the stimulation of cell-mediated immunity.

Jayathirtha and Mishra, (2004) investigated the immunomodulatory activities of *C. asiatica* by testing carbon clearance and antibody titer parameters. They showed that the methanolic extract

caused an increase in the phagocytic index and no rise in the antiboder titer, indicating a suppressed humoral response, which was also noted in our findings as indicated by the suppressed IL-10 secretion.

The aqueous extract did not stimulate PBMC's but showed an increase in the levels of IFN-γ and the $CD8^+$ cytotoxic T cells and B cells. Thus, according to the CRM model they follow the Type 3 pathway of immunomodulation. According to the AIM model, the increased secretion of IFN-γ by the aqueous extract might be due to the stimulation of the $CD8^+$ cytotoxic T cells hence it induces the Tc1 response and possibly stimulates cell-mediated immunity.

Punturee *et al.,* (2005) explored the immunomodulatory effects of the aqueous extracts of *C. asiatica* on mitogen induced proliferation of lymphocytes. They showed that the extracts exerted an immunostimulatory effect on human peripheral blood mononuclear cells (PMBC). They related the possible actions of the plant to interference with cell signalling and cytokine production (Punturee *et al*., 2005).

C. asiatica is of great medicinal importance especially in the Indian traditional systems of medicine such as diuretic, alterative and tonic. In India and Madagascar, infusions of the plant are used to treat leprosy and to improve the general health of the patient. The leaves are used for improving memory and for curing skin diseases internally as well as externally. The plant also has many notable pharmacological activities such as; hepatoprotective (Antony *et al.,* 2006), anti-tumour (Babu *et al.,* 1995), antimicrobial (Mamtha *et al*., 2004), anti-inflammatory (Somchit *et al.,* 2004) and anti-oxidant (Jayashree *et al.,* 2003) activities. Earlier immunological studies have demonstrated promising immunomodulatory activity of *C. asiatica*. The immuostimulating activity of the extracts may be due partly to the triterpenoid saponins and

pectins which are present in the plant and widely distributed in the plant kingdom and are also reported to exert immunomodulatory activity (Wang *et al.*, 2005; Mali and Hatipakki, 2008).

5.7. *C. triloba*

The methanolic extract of *C. triloba* stimulated PBMC's and caused an increase in the levels of IFN-γ and IL-10 while also increasing the $CD8^+$ cytotoxic T cells and B cells. According to the CRM model, this is typical of the Type 1 immunomodulatory response. The augmentation of IFN-γ and IL-10 secretion might be due mainly to the $CD8^+$ cytotoxic T cells and B cells. Thus the methanolic extract drives the Tc1 and the Tc2 responses, as indicated by the AIM model, due to their stimulation of both multi-potent cytokines IFN-γ and IL-10. Therefore, they have shown the ability to stimulate both the cell-mediated and humoral immune responses.

The aqueous extract suppressed PBMC's but caused an increase in the release of IL-10 and the $CD8^+$ cytotoxic T cells and B cells. They demonstrated similar results to *A. gangetica* (refer to section 5.4.) and thus show the Type 4 immunomodulatory response. According to the AIM model, the aqueous extract influences the Tc2 response, due to their stimulation of IL-10 and the $CD8^+$ cytotoxic T cells. Therefore, they influence the humoral immune response.

Previous research has shown that methanolic extracts of *C. triloba* exhibits anti-oxidant activity due to the presence of phenolic compounds in the plant (Akula and Odhav 2008). Mohanlall (2010) also reported a ± 50% free radical scavenging capacity by anthraquinones of *C. triloba* The present study has shown the immunostimulatory activity of *C. triloba* by potentiating humoral as well as cellular immunity. The immunomodulatory activity can be attributed to the active compounds: anthraquinones, saponins, steroids and terpenoids and phenols that are known to be present in *C. triloba* and have shown remarkable effects on the immune system (Fereira *et al*., 2003; Cherng *et al*., 2008).

5.8. *E. australis*

The methanolic extract of *E. australis* suppressed PBMC's but showed an increase in the secretion of IL-10 and the $CD8^+$ cytotoxic T cells and B cells. They show the Type 4 immunomodulatory response. According to the AIM model, the methanolic extract shows the Tc2 response as indicated by the increase in IL-10 and $CD8^+$ cytotoxic T cells, possibly causing a stimulation of the humoral immune response.

The aqueous extract stimulated PBMC's and showed an increase in the secretion of IL-10 and the $CD8^+$ cytotoxic T cells, $CD4^+$ helper T cells and B cells. Their stimulation of PBMC's and suppression of IFN-γ is is indicative of the Type 2 immunomodulatory response according to the CRM model. The increase in IL-10 secretion can be attributed to the Tc2 response of the $CD8^+$ cytotoxic T cells or the Th2 response of the $CD4^+$ helper T cells, resulting in the possible stimulation of humoral immunity by the aqueous extracts (Cherng *et al.,* 2008).

Traditionally, the plant has been used to treat gastrointestinal disorders, biliousness and dyspepsia (Beekrum, 2003). There has also been no previous literature conducted on the immunomodulatory function of *E. australis.* Although the findings of this study do not indicate any relationship to its traditional usage it provides valuable information relating to the immunostimulatory activity of *E. australis* by potentiating humoral immunity.

5.9. *J. flava*

The methanolic extract of *J. flava* stimulated the proliferation of PBMC's and showed an increase in the secretion of IL-10 and the $CD8^+$ cytotoxic T cells and B cell and a decrease in the secretion of IFN-γ. Thus, according to the CRM model the methanolic extract followed the Type 2 pathway of immunomodulation. The increase in IL-10 secretion can be attributed to the increase in the B cells and also to the $CD8^+$ cytotoxic T cells. According to the AIM model, this

indicates the Tc2 response and the possible stimulation of humoral immunity by the methanolic extract.

The aqueous extract showed the same trend as the methanolic extract except that it increased the secretion of IFN-γ. According to the CRM model the aqueous extract showed the Type 1 immunomodulatory response as indicated by their stimulation of PBMC's and secretion of IFN-γ. According to the AIM model, the increase in the secretion of IFN-γ and IL-10 can be attributed to the Tc1 and Tc2 responses of the $CD8^+$ cytotoxic T cells. Hence, the results show that the aqueous extract seems to stimulate both cell-mediated and humoral immunity.

J. flava is used in traditional medicine in many parts of Africa to treat convulsions, fever and diarrhoea (Grubben and Denton, 2004). Our findings have shown that *J. flava* may be of use in modulating the immune system by promoting both the cell-mediated and humoral immune responses, as suggested by their stimulation of both the pro-inflammatory cytokine IFN-γ and anti-inflammatory cytokine IL-10.

5.10. *M. balsamina*

The methanolic extract stimulated PBMC's and showed an increase in IL-10 secretion, $CD8^+$ cytotoxic T cells and B cells and a decrease in IFN-γ secretion. Thus, according to the CRM model, they show the Type 2 immunomodulatory response. According to the AIM model the methanolic extract follows the Tc2 response of the $CD8^+$ cytotoxic T cells, leading to the stimulation of humoral immunity.

The aqueous extract of *M. balsamina* suppressed the proliferation of PBMC's and secretion of IFN-γ but showed an increase in the secretion of IL-10, $CD8^+$ cytotoxic T cells, $CD4^+$ helper T cells and B cells. Thus, they show the Type 4 immuomodulatory response. According to the

AIM model, the increase in IL-10 secretion could be due to the Tc2 response of the $CD8^+$ cytotoxic T cells or the Th2 response of the $CD4^+$ helper T cells. Therefore, the aqueous extract seems to stimulate humoral immunity.

The isolation of many cucurbitacins from the *Momordica* species has been reported in recent literature. Interest in cucurbitacins, which are highly oxygenated triterpenes, is due mainly to their vast range of biological activities such as cytotoxic, anti-inflammatory, anti-diabetic, cardiovascular and hepatoprotective activities (Ramalhete *et al.*, 2009). Plants of the genus *Momordica* also produce a number of related type I ribosome inactivating proteins known as momordins or momorcharins. These proteins inhibit eukaryote protein synthesis (Ortigao and Better, 1992) and momordin I, specifically, inhibits the function of the activator protein-1 (AP-1), which leads to an inhibition of cellular proliferation (Park *et al*., 2000).

One of the active ingredients of this plant, rosmarinic acid, has shown a multitude of biological activities. The main bio-activities are; anti-oxidative, anti-inflammatory, anti-bacterial and anti-viral (Parnham and Kesselring, 1985) activities. The anti-inflammatory properties arise from the compound's inhibition of lipo-oxygenases and cyclo-oxygenases as well as the interference of rosmarinic acid with the complement cascade. The results presented in our study show a decrease in the proliferation of lymphocytes by the extracts of *M. balsamina*, possibly due to the momordin proteins present in the plant. There was also a strong increase in the secretion of IL-10 caused by the extracts, which can be attributed to the anti-inflammatory activities of the plants active ingredients. The presence of phenolic compounds, such as rosmarinic acid in medicinal plants and leafy vegetables such *as M. Balsamina* provide beneficial and health promoting effects. Thus, this plant and its active compounds have great potential in modulating the immune system.

5.11. *O. sinuatum*

Both the methanolic and aqueous extracts of *O. sinuatum* suppressed PBMC's and inhibited the secretion of IFN-γ whilst stimulating the release of IL-10 and the $CD8^+$ cytotoxic T cells and B cells. They show the Type 4 response of immune modulation as they did not stimulate PBMC's or IFN-γ but stimulated IL-10. The increased production of IL-10 can be attributed to the rise in B cells and the $CD8^+$ cytotoxic T cells. According to the AIM model, both the methanolic and aqueous extracts cause the Tc2 response and therefore, stimulates humoral immunity.

O. sinuatum has been documented as an ethnobotanical treatment in Kenya for several disorders. It recently yielded a single bioactive compound whose molecular formula was determined to be C15H10O5 based on the structure suggesting 6-methyl-1,3,8-trihydroxyanthraquinone (emodin) – a known product of several other Polygonaceae species. Emodin was the primary constituent found to be responsible for the plant's bioactivity, most notably its inhibition of vascular development, indicating anti-angiogenic activity (Crawford *et al*., 2011). Emodin was also found to inhibit the proliferation, migration and tube formation of mammalian endothelial cells and in addition, it was shown to be an inhibitor of protein kinases (Crawford *et al.*, 2011).

Lin *et al.*,(2010) investigated the mechanism of action of emodin for suppressing acute allograft rejection in rat models of liver transplantation. Their research revealed that the emodin treated group markedly down-regulated plasma levels of the Th1 cytokines TNF-α and Il-2 while it highly up-regulated levels of the Th2 cytokine IL-10. They related the inhibitory effects of emodin to the deviation of the Th1/Th2 paradigm to Th2, thereby inducing immune tolerance in the transplanted liver (Lin *et al*., 2010).

Furthermore, it is known that the activation and proliferation of $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells play a vital role in acute allograft rejection following liver transplantation. Therefore, it is expected that the plasma levels of $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells as well as the $CD4^+/CD8^+$ ratio would increase considerably during allograft rejection. The research of Lin *et al*., (2010) revealed that the emodin-treated group reduced the levels of $CD4^+$ helper T cells and the $CD4^+/CD8^+$ ratio as compared to those in the allograft group, indicating its immunosuppressive effects, caused in part by its inhibition of $CD4^+$ helper T cell proliferation. Kuo *et al*., (2001) also reported on the immunosuppressing effects of emodin, which might be mediated by its effects on the reduction of cytokine activity.

Therefore, our findings which include the decrease in the proliferation of PBMC's and the $CD4^+$ helper T cells and the increased secretion of IL-10 caused by methanolic and aqueous extracts of *O. sinuatum* are in accordance with the research of Crawford *et al.,* (2011) and Lin *et al.,* (2010). As mentioned above, the effects of this plant on the immunosuppressive cytokine IL-10 is caused by the stimulation of a predominantly Tc2 response, which could be explained by the actions of one of the active ingredients in this plant– emodin.

5.12. *P. viscosa*

The aqueous extract of *P. viscosa* inhibited PBMC's and the levels of IFN-γ but augmented the levels of IL-10 and the $CD8^+$ cytotoxic T cells and B cells. Thus, they show the Type 4 immunomodulatory response. The increased secretion of IL-10 by the extract can be correlated to their effect on the T cell subsets. Hence the aqueous extracts follow the Tc2 response indicating the stimulation of the humoral immune response on lymphocytes.

P. viscosa is known to contain the phytoactive ingredient, withanolide (Silva *et al.,* 1993). Withanolides belong to a group of naturally occurring, oxygenated ergostane type steroids that

are known for their broad spectrum of biological activity. Among their wide range of pharmacological activities, including anti-inflammatory and anti-oxidant activity, they have shown immunomodulatory properties, specifically immunosuppression (Huang *et al.,* 2009). Six withanolides from the aerial parts of *Withania coagulans* exhibited suppressive activity against B and T-cell proliferation (Huang *et al*., 2009).

The mechanism for the induced immunosuppression and anti-proliferative effects of this compound was discovered by Ichikawa *et al.,* (2006). They found that withanolides potently suppress the activation of the nuclear factor NF-$_K$B, which is induced by inflammatory and carcinogenic agents. This activity accounts for the ability of withanolides to suppress the expression of gene products that regulate the expression of apoptosis, proliferation and angiogenesis (Ichikawa *et al.,* 2006). This is indicated by our results which show a predominant anti-inflammatory, immunosuppressive response by the aqueous extract of *P. viscos*a, possibly due to the presence of these compounds.

5.13. *S. occidentalis*

The methanolic extract of *S. occidentalis* stimulated PBMC's and showed an increase in the levels of IL-10 and $CD8^+$ cytotoxic T cells and B cells and a decrease in the levels of IFN-γ. According to the CRM model, this is typical of the Type 2 response of immunomodulation. The increased levels of IL-10 can be correlated to the increased B cell and $CD8^+$ cytotoxic T cell counts indicating the Tc2 response by the extract and the promotion of humoral immunity.

The aqueous extract inhibited PBMC's but showed an increase in the levels of IL-10 and a decrease in all the cell subsets. This profile cannot be categorised according to the CRM and AIM models as it cannot be said for certain which type of immune response the extract exerts since none of the B- or T cell subset populations were affected.

Senna occidentalis, also known as fedegoso has been used in traditional medicine for many years and has been used clinically to enhance immune function. Research has demonstrated that the leaf extracts show significant immunostimulant activity by increasing humoral immunity and bone marrow immune cells in mice and protecting them from chemically-induced immunosuppression (Rain-tree, 2002). This correlates with the findings presented here which predominantly show the Tc2 response of the methanolic extracts, thus promoting the humoral immune response.

5.14. *S. oleraceous*

Both the methanolic and aqueous extracts of *S. oleraceous* decreased the number of PBMC's and secretion of IFN-γ but increased the secretion of IL-10 and $CD8^+$ cytotoxic T cells and B cells. Thus, they show the Type 4 immunomodulatory response. The increase in IL-10 secretion can be attributed to the rise in B cells and to $CD8^+$ cytotoxic T cells, inducing the Tc2 response and humoral immunity by the extracts.

Research has shown that one of the active compounds of *S. oleraceous*, loliolide, showed immunosuppressive properties against T lymphocytes (Okada *et al.,* 1994). The results presented here show an inhibition of proliferation on human lymphocytes by extracts of *S. oleraceous* and an upregulation in the release of cytokine IL-10. When considering previous research of *S. oleraceous* and the fact that IL-10 is regarded as an important suppressor of immune functions (Aman *et al*., 1996), it can be noted that *S. oleraceous* shows strong immunosuppressive potential and a deviation towards the Tc2 response, indicating the stimulation of humoral immunity.

CHAPTER SIX: CONCLUSION

There are more than 45,000 plant species in Africa and about 1000 are edible as green vegetables (Habwe *et al.*, 2008). Apart from being consumed as staple diets, leafy vegetables also have many positive effects on human health since they are rich in nutrients and contain many bioactive components with medicinal properties that prevent a number of health conditions. Epidemiological studies have indicated a relationship between the consumption of vegetables and chronic diseases such as cancer, hypertension and heart diseases as well as debilitating nutritional disorders and weakened immune systems. Increased consumption of traditional leafy vegetables can have a positive effect on nutrition, health and the economic well being of both urban and rural populations (Mwangi and Kimathi, 2006).

In Africa especially, vegetables have strongly been acknowledged to offer health protecting properties and benefits to the immune system. In addition, these vegetables contain several active biochemical constituents including, flavonoids, alkaloids, triterpenoids and anthraquinones that have been reported to have anti-bacterial, cytotoxic (Elkhayat, 2009), anti-inflammatory (Chiang *et al.*, 2007; Somchit *et al*., 2004; Zeashan *et al.*, 2009;), immunosuppressive (Chiang *et al.*, 2007; Elkhayat, 2009; Pereira *et al*., 2009), anti-asthmatic (Akah *et al*., 2003), anti-angiogenic (Crawford *et al*., 2011) and anti-oxidant activity (Akula and Odhav, 2008). A number of disorders can be treated by biological response modifying (BRM's) compounds (immunomodulators). These include immunodeficiency diseases and autoimmune disorders. These compounds may work on the cellular or humoral immune system or both (Mali and Hatipakki, 2008).

We investigated the immunomodulatory effects of leafy vegetables by testing for lymphocyte proliferation, cytokine secretion and T cell subset responses on lymphocytes. Hence we were able to establish the specific effect that the plants have on the immune system based on the cytokine response, according to the CRM model (Cherng *et al.*, 2008) and, the Th1/Th2 and Tc1/Tc2 paradigm, according to the AIM model (Mosmann and Sad, 1996; Vukmanovic-Stejic *et al*., 2000). A summary of the results is presented in Table 9.

Our results indicated that most of the methanolic extracts and a few of the aqueous extracts, at 1000 μg/ml stimulated the proliferation of PBMC and cell subsets and/or the secretion of potent immunomodulators IFN-γ or IL-10. According to the CRM model (Cherng *et al*., 2008), the methanolic extracts of *A. hybridus*, *C. asiatica*, *C. triloba* and the aqueous extract of *J. flava*, showed the Type 1 immunostimulation response as they significantly stimulated the proliferation of resting PBMC and caused an increase in the secretion of IFN-γ. The methanolic extracts of *B. pilosa, J. flava, M. balsamina* and *S. occidentalis* and the aqueous extracts of *A. spinosus, A. gangetica* and *E. australis* showed the Type 2 response because they stimulated PBMC's and showed a decrease in IFN-γ secretion. The methanolic extract of *A. spinosus* and the aqueous extracts of *A. dubius, A. hybridus, B. pilosa* and *C. asiatica* showed the Type 3 response as they did not cause proliferation of PBMC but augmented the secretion of IFN-γ was shown by (Table 7). A Type 4 response of immunomodulation in which there was no proliferation of PBMC's and secretion of IFN-γ but an increase in the secretion of IL-10 was shown by the methanolic extracts of *A. gangetica* and *E. australis, O. sinuatum, S. oleraceous* and the aqueous extracts of *C. triloba, M. balsamina, P.viscosa, O. sinuatum* and *S. oleraceous.*

According to the AIM model (Mosmann and Sad, 1996; Vukmanovic-Stejic *et al*., 2000), the methanolic extracts of *A. hybridus* and *C. asiatica* and the aqueous extracts of *A. dubius, A. hybridus* and *C. asiatica* induced the Tc1 response on human lymphocytes mediated by the

secretion of IFN-γ, thus leading to the stimulation of cell-mediated immunity. IFN-γ is a pro-inflammatory cytokine and a multi-potent immune activator, therefore the stimulation of this cytokine leads to the modulation of nearly all phases of immune and inflammatory responses and is associated with effective host defense mechanisms against intracellular pathogens and viruses (Cherng *et al*., 2008).

Other extracts especially the methanolic extracts of *A. gangetica, B. pilosa, E. australis, J. flava, M. balsamina, O. sinuatum, S. occidentalis* and *S. oleraceous* and the aqueous extracts of *A. spinosus, A. gangetica, C. triloba, O. sinuatum, P. viscosa* and *S. oleraceous* stimulated the Tc2 response and release of IL-10, thus regulating humoral immunity. IL-10 is regarded as an important suppressor of immune functions mediated by the macrophages, T cells and natural killer cells therefore the stimulation of this cytokine leads to the anti-inflammatory reactions responsible for antibody production and antigen recognition (Aman *et al.,* 1996). Furthermore, the methanolic extracts of *A. spinosus,* and *C. triloba* and the aqueous extracts of *B. pilosa, J. flava* stimulated both Tc1 and Tc2 cytokine profiles mediated by the secretion of IFN-γ and IL-10. Hence, they were able to stimulate cell-mediated and humoral immune responses. The immunostimulating activity of the plants may be attributed to the compounds such as flavonoids, alkaloids, anthraquinones, steroids which have been shown to possess this activity and to be present in the above-mentioned leafy vegetables.

Concerns have been raised regarding the lack of documentation of traditional and indigenous food cultures. There has been a great demand in research for locating natural resources that show immunomodulatory activity since most of the chemotherapeutic agents available today have cytotoxic effects and exert a variety of side effects. We have demonstrated in our study that the extracts from leafy vegetables display immunomodulatory activities based on their pro-

inflammatory and anti-inflammatory properties. Hence these extracts can produce the adaptive and humoral immune responses that are needed to combat many diseases and pathologies.

Both anti-inflammatory (immunosuppressive) and immunostimulatory agents are needed to achieve the pharmacological modulation of the immune system. The modulation of the immune response through stimulation or suppression may help in maintaining a disease-free state. There is great potential for the discovery of more specific immunomodulators which imitate or antagonize the biological effects of cytokines and interleukins; and furthermore agents that activate host defense mechanisms in the presence of a weakened immune response could provide support to conventional chemotherapy. Thus, natural remedies should be revisited as important sources of novel compounds capable of targeting specific cellular receptors.

This study contributes to the much needed documentation of traditional leafy vegetables in South Africa especially to explore the potential health benefits for treatment of immune related illnesses and provides a scope for further detailed investigation on the active ingredients which stimulate their activity.

Table 9: Summary of results showing the effect of methanolic and aqueous extracts of the leafy vegetables on cytokine production, T cell subsets and, the types of immune responses elicited

Plant	Type of immunomodulatory response	Cytokine produced	Predominant cell subset populations	Immune response stimulated
A. dubius	No response	None	$CD8^+$ cytotoxic T cell, B cell	No defined response
	Type 3	IFN-γ	$CD8^+$ cytotoxic T cell, B cell	Cell Mediated (Tc1)
A. hybridus	Type 1	IFN-γ	$CD8^+$ cytotoxic T cell, B cell	Cell Mediated (Tc1)
	Type 3	IFN-γ	$CD8^+$ cytotoxic T cell	Cell Mediated (Tc1)
A. spinosus	Type 3	IFN-γ and IL-10	$CD8^+$ cytotoxic T cell, B cell	Cell-Mediated/Humoral (Tc1 or Tc2)
	Type 2	IL-10	$CD8^+$ cytotoxic T cell, B cell	Humoral (Tc2)
A. gangetica	Type 4	IL-10	$CD8^+$ cytotoxic T cell, B cell	Humoral (Tc2)
	Type 2	IL-10	$CD8^+$ cytotoxic T cell, B cell	Humoral (Tc2)
B. pilosa	Type 2	IL-10	$CD8^+$ cytotoxic T cell, B cell	Humoral (Tc2)
	Type 3	IFN-γ and IL-10	$CD8^+$ cytotoxic T cell, B cell	Cell-Mediated/Humoral (Tc1 or Tc2)
C. asiatica	Type 1	IFN-γ	$CD8^+$ cytotoxic T cell, B cell	Cell Mediated (Tc1)
	Type 3	IFN-γ	$CD8^+$ cytotoxic T cell, B cell	Cell Mediated (Tc1)
C. triloba	Type 1	IFN-γ and IL-10	$CD8^+$ cytotoxic T cell, B cell	Cell-Mediated/Humoral (Tc1 or Tc2)
	Type 4	IL-10	$CD8^+$ cytotoxic T cell, B cell	Humoral (Tc2)
E.australis	Type 4	IL-10	$CD8^+$ cytotoxic T cell, B cell	Humoral (Tc2)
	Type 2	IL-10	$CD8^+$ cytotoxic T cell, $CD4^+$ helper T cell, B cell, NK cell	Humoral (Th2 or Tc2)

J.flava	Type 2	IL-10	$CD8^+$ cytotoxic T cell, B cell	Humoral (Tc2)
	Type 1	IFN-γ and IL-10	$CD8^+$ cytotoxic T cell, B cell, NK cell	Cell-Mediated/Humoral (Tc1 or Tc2)
M.balsamina	Type 2	IL-10	$CD8^+$ cytotoxic T cell, B cell	Humoral (Tc2)
	Type 4	IL-10	$CD8^+$ cytotoxic T cell, $CD4^+$ helper T cell, B cell	Humoral (Th2 or Tc2)
O.sinuatum	Type 4	IL-10	$CD8^+$ cytotoxic T cell, B cell	Humoral (Tc2)
	Type 4	IL-10	$CD8^+$ cytotoxic T cell, B cell	Humoral (Tc2)
P.viscosa	n/a	n/a	n/a	n/a
	Type 4	IL-10	$CD8^+$ cytotoxic T cell, B cell	Humoral (Tc2)
S.occidentalis	Type 2	IL-10	$CD8^+$ cytotoxic T cell, B cell	Humoral (Tc2)
	-	IL-10	-	No defined response
S.oleraceous	Type 4	IL-10	$CD8^+$ cytotoxic T cell, B cell	Humoral (Tc2)
	Type 4	IL-10	$CD8^+$ cytotoxic T cell, B cell	Humoral (Tc2)

Orange indicates methanolic extracts

Blue indicates aqueous extracts

*n/a = not applicable (Methanolic extract of *P. viscosa* not tested)

CHAPTER SEVEN: REFERENCES

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CHAPTER SEVEN: APPENDIX

APPENDIX A

PREPARATION OF REAGENTS

SECTION 1

Media and Reagents for Continuous Cell Culture

1. **70% ethanol**

   Contained 70ml Analar grade ethanol (100%) and 30ml distilled water was combined to provide general tissue culture disinfectant (Merck NT, South Africa).

2. **Complete Culture Media (CCM)**

   Comprised 10 % FCS, 1 % antibiotics (Pen/Strep) and RPMI 1640 media stored in a sterile bottle at 4 °C.

3. **Dimethyl Sulfoxide (DMSO)**

   Was pharmaceutical grade (tested in tissue culture) was filter sterilized and aliquoted into 100ml sterile Dark Bottles and kept away from direct sunlight and extreme temperature until use. (Highveld Biological, South Africa).

4. **Fetal Calf Serum (FCS)**

   Was filter sterilized and gamma irradiated at 25 to 28 kGy under conditions, which preserve the biological integrity of serum. The serum was aliquoted into 10ml sterile flasks and kept frozen (-20º) until use. (Highveld Biological, South Africa).

5. **Roswell Park Memorial Institute (RPMI) 1640 Medium –** Invitrogen, USA

6. **Penicillin/Streptomycin (Pen/Strep)**

Mixture contained 100mg/ml penicillin G sodium and 100mg/ml streptomycin sulphate indouble reverse osmosis water. One ml aliquots were frozen (-20ºC) until use (Highveld Biological, South Africa).

Reagents for Microscopy

0.2 % Trypan Blue Solution (w/v) was made in PBS pH 7.4. Biowhittaker, Walkersville, USA.

SECTION 2

Reagents for Blood Isolation

1. Histopaque 1077 – Sigma, USA

SECTION 3

Reagents for Proliferation assay

1. **MTT Reagent**

1. 3-{4,5-dimethylthylthiazol-2-yl}-2,5 diphenyl tetratrazolium bromide (MTT) reagent was prepared by dissolving 5 mg MTT in 1 ml PBS (pH 7.5). The solution was filter sterilizedand stored in the fridge. (Sigma –Aldrich, South Africa)

SECTION 4

Standards

1. Concanavalin A (ConA)

ConA (purchased from Sigma) is lectin protein originally extracted from the jackbean, *Canavalia ensiformis*. Lectin has a number of effects on cell metabolism: it induces mitosis and affects the cell membrane in regard to transport and permeability to proteins and thus serves as a positive control. 1mg of lyophilized ConA powder was added to 1 ml of phosphate buffered saline to make the stock of 1mg/ml. This was further diluted to in RPMI-1640 (with antibiotics) to make the working stocks of 20µg/ml, 10µg/ml, 5µg/ml.

2. Lipopolysaccharide (LPS)

LPS (purchased from Sigma) are a major constituent of the cell wall of gram negative bacteria, *Escherichia coli*. LPS directly activate B cells, regardless of their antigenic specificity. Plasma B cells are terminally differentiated and therefore cannot undergo mitosis. Memory B cells can proliferate to produce more memory cells or plasma B cells, hence LPS induces mitosis in B cells. 1mg of lyophilized LPS powder was added to 1 ml of phosphate 160 buffered saline to make the stock of 1mg/ml. This was further diluted to in RPMI-1640 (with antibiotics) to make the working stocks of 20 µg/ml, 10 µg/ml, 5 µg/ml.

3. Cyclosporin A (CSA)

CSA (purchased from Sigma) is a non-polar cyclic oligopeptide produced by the fungus, *Tolypocladium inflatum*. It is a potent immunosuppressive agent affecting primarily Tlymphocytes. Hence it is used as a negative control. CSA is an extremely hydrophobic substance. 1 mg CSA was added to 100 µl of absolute ethanol and 20 µl of Tween 80 respectively. This was made up to a final volume of 1 ml with RPMI-1640 to give a stock

solution of 1 mg/ml. This was further diluted to in RPMI-1640 (with antibiotics) to make the working stocks of 20 μg/ml, 10 μg/ml, 5 μg/ml.

4. Phytohaemagglutinin (PHA)

PHA (purchased from Sigma) is an extract of *Paseolus vugaris* seed. It stimulates progressive non-specific lymphocyte mitosis in cell culture and thus serves as a positive control. It is stored lyophilized at 2-8 ºC, however if reconstituted PHA can be stored at – 80 ºC for 6- 12 months or stored at 4 ºC for 4 weeks. 2mg of lyophilized PHA powder was added to 2 ml of phosphate buffered saline (pH) to make the stock of 1mg/ml. This was further diluted to a 1: 4 dilution of 2 ml of stock solution in 6 ml of RPMI-1640(with antibiotics) to make the working stock of 250μg/ml

APPENDIX B

RESULTS

SECTION ONE: MTT Proliferation assay

Table 1: Effect of methanolic and aqueous extracts of leafy vegetables on PBMC proliferation activity (Absorbances at 570 nm)

***A.hybridus* methanolic**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.242	0.22	0.212	263.0435	194.6903	238.2022	231.9787	34.5990
500	0.232	0.176	0.182	252.1739	155.7522	204.4944	204.1402	48.2118
250	0.205	0.179	0.186	222.8261	158.4071	208.9888	196.7406	33.9111
100	0.207	0.173	0.175	225.0000	153.0973	196.6292	191.5755	36.2167
50	0.231	0.242	0.27	251.0870	214.1593	303.3708	256.2057	44.8255
25	0.245	0.194	0.211	266.3043	171.6814	237.0787	225.0215	48.4500
1	0.263	0.204	0.228	285.8696	180.5310	256.1798	240.8601	54.3146
PHA	0.189	0.182	0.179	205.4348	161.0619	201.1236	189.2068	24.4693
DMSO	0.089	0.078	0.091	96.7391	69.0265	102.2472	89.3376	17.8042
Cells only	0.092	0.113	**0.089**	100	100	100	100	0
***A.hybridus* aqueous**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.048	0.055	0.052	48.3871	54.9649	50.1458	51.1659	3.4055
500	0.069	0.073	0.072	69.5565	73.1194	69.9708	70.8822	1.9485
250	0.065	0.068	0.076	65.3226	67.8034	73.9553	69.0271	4.4445
100	0.067	0.066	0.078	67.2379	66.1986	75.6074	69.6813	5.1584
50	0.074	0.086	0.077	74.6976	86.1585	74.5384	78.4648	6.6634
25	0.078	0.073	0.075	79.0323	73.6209	73.0807	75.2446	3.2913
1	0.121	0.124	0.093	121.9758	124.3731	90.3790	112.2426	18.9724
PHA	0.152	0.153	0.159	153.2258	69.5455	154.5190	125.7634	48.6905
DMSO	0.062	0.059	0.0614	62.5000	59.1775	59.6696	60.4490	1.7931
Cells only	0.0992	0.0997	**0.1029**	100	100	100	100	0
***A.dubius* methanolic**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.161	0.185	0.161	91.71429	110.4949	80.01993	94.07638	15.3742
500	0.166	0.175	0.178	95.08571	104.4126	88.83906	96.11247	7.837395
250	0.149	0.145	0.132	85.02857	86.70244	65.96911	79.23337	11.51764
100	0.138	0.142	0.137	78.97143	84.67501	68.36074	77.33573	8.279224
50	0.148	0.148	0.142	84.51429	88.31246	70.85202	81.22626	9.182872
25	0.134	0.140	0.136	76.4	83.30352	67.86248	75.85533	7.734914
1	0.139	0.145	0.148	79.25714	86.70244	73.84155	79.93371	6.457084

PHA	0.171	0.178	0.182	97.71429	106.1419	90.68261	98.17961	7.740152
DMSO	0.192	0.196	0.210	109.7143	116.8754	104.6338	110.4078	6.150193
Cells only	0.175	0.168	0.201	100	100	100	100	0
A.dubius **aqueous**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.128	0.131	0.124	73.25714	78.29457	61.93323	71.16165	8.379539
500	0.147	0.161	0.151	84	95.82588	74.98754	84.93781	10.45077
250	0.146	0.139	0.151	83.48571	82.58795	75.18685	80.42017	4.554368
100	0.139	0.133	0.148	79.31429	79.1294	73.7419	77.3952	3.165194
50	0.139	0.143	0.165	79.48571	85.15206	82.26208	82.29995	2.833361
25	0.174	0.145	0.171	99.37143	86.2254	85.10214	90.23299	7.934022
1	0.136	0.139	0.149	77.71429	82.76685	74.14051	78.20721	4.334242
PHA	0.198	0.192	0.220	113.1429	114.4902	109.6163	112.4165	2.516798
DMSO	0.128	0.131	0.127	73.14286	78.17531	63.0294	71.44919	7.713694
Cells only	0.175	0.168	0.201	100	100	100	100	0
A.spinosus **methanolic**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.025	0.03	0.029	34.72222	50.84746	42.02899	42.53289	8.074419
500	0.043	0.049	0.045	59.72222	83.05085	65.21739	69.33015	12.196
250	0.039	0.023	0.029	54.16667	38.98305	42.02899	45.05957	8.032675
100	0.029	0.03	0.031	40.27778	50.84746	44.92754	45.35092	5.297544
50	0.028	0.035	0.033	38.88889	59.32203	47.82609	48.679	10.24324
25	0.011	0.014	0.024	15.27778	23.72881	34.78261	24.5964	9.781316
1	0.067	0.049	0.058	93.05556	83.05085	84.05797	86.72146	5.508554
PHA	0.091	0.104	0.107	126.3889	176.2712	155.0725	152.5775	25.03457
DMSO	0.053	0.059	0.049	73.61111	100	71.01449	81.54187	16.03785
Cells only	0.072	0.059	0.069	100	100		100	0
A. spinosus **aqueous**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.078	0.063	0.071	108.3333	106.7797	102.8986	106.0038	2.799219

500	0.071	0.059	0.068	98.61111	100	98.55072	99.05395	0.819864
250	0.066	0.066	0.088	91.66667	111.8644	127.5362	110.3558	17.98231
100	0.087	0.07	0.078	120.8333	118.6441	113.0435	117.507	4.017489
50	0.068	0.061	0.071	94.44444	103.3898	102.8986	100.2443	5.028804
25	0.08	0.074	0.085	111.1111	125.4237	123.1884	119.9077	7.699662
1	0.076	0.081	0.092	105.5556	137.2881	133.3333	125.3923	17.29259
PHA	0.091	0.104	0.107	126.3889	176.2712	155.0725	152.5775	25.03457
DMSO	0.053	0.059	0.049	73.61111	100	71.01449	81.54187	16.03785
Cells only	0.072	0.059	0.069	100	100	100	100	0
***A.gangetica* methanolic**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.049	0.036	0.039	68.05556	61.01695	56.52174	61.86475	5.813459
500	0.041	0.04	0.039	56.94444	67.79661	56.52174	60.42093	6.391021
250	0.036	0.028	0.034	50	47.45763	49.27536	48.911	1.309766
100	0.021	0.025	0.029	29.16667	42.37288	42.02899	37.85618	7.527302
50	0.02	0.038	0.025	27.77778	64.40678	36.23188	42.80548	19.1789
25	0.014	0.019	0.013	19.44444	32.20339	18.84058	23.49614	7.546744
1	0.029	0.027	0.033	40.27778	45.76271	47.82609	44.62219	3.90126
PHA	0.091	0.104	0.107	126.3889	176.2712	155.0725	152.5775	25.03457
DMSO	0.053	0.059	0.049	73.61111	100	71.01449	81.54187	16.03785
Cells only	0.072	0.059	0.069	100	100	100	100	0
***A. gangetica* aqueous**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.069	0.077	0.076	116.9492	135.0877	111.7647	121.2672	12.24642
500	0.049	0.062	0.066	83.05085	108.7719	97.05882	96.29387	12.87759
250	0.052	0.06	0.062	88.13559	105.2632	91.17647	94.85841	9.138154
100	0.032	0.049	0.055	54.23729	85.96491	80.88235	73.69485	17.04129
50	0.05	0.054	0.06	84.74576	94.73684	88.23529	89.2393	5.070645
25	0.054	0.036	0.06	91.52542	63.15789	88.23529	80.97287	15.51568
1	0.073	0.059	0.07	123.7288	103.5088	102.9412	110.0596	11.8413
PHA	0.091	0.079	0.094	154.2373	138.5965	138.2353	143.6897	9.136272

DMSO	0.061	0.055	0.064	103.3898	96.49123	94.11765	97.99957	4.816603
Cells only	0.059	0.057	0.068	100	100	100	100	0
***Bidens pilosa* Methanolic**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.273	0.25	0.272	296.7391	221.2389	305.618	274.532	46.36618
500	0.248	0.221	0.233	269.5652	195.5752	261.7978	242.3127	40.66177
250	0.236	0.212	0.234	256.5217	187.6106	262.9213	235.6846	41.75604
100	0.219	0.192	0.229	238.0435	169.9115	257.3034	221.7528	45.91704
50	0.228	0.203	0.223	247.8261	179.646	250.5618	226.0113	40.1768
25	0.218	0.188	0.217	236.9565	166.3717	243.8202	215.7161	42.87114
1	0.102	0.104	0.101	110.8696	92.0354	113.4831	105.4627	11.70158
PHA	0.189	0.182	0.179	205.4348	161.0619	201.1236	189.2068	24.46927
DMSO	0.089	0.078	0.091	96.73913	69.02655	102.2472	89.33762	17.8042
Cells only	0.092	0.113	**0.089**	100	100	100	100	0
***Bidens pilosa* Aqueous**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.051	0.046	0.049	51.00806	46.43932	47.3275	48.25829	2.422425
500	0.071	0.069	0.072	71.47177	68.70612	69.97085	70.04958	1.384508
250	0.070	0.070	0.069	70.76613	70.41123	66.56948	69.24895	2.327259
100	0.070	0.077	0.077	70.3629	77.13139	74.92711	74.14047	3.452133
50	0.0658	0.0754	0.0719	66.33065	75.62688	69.87366	70.6104	4.691703
25	0.0734	0.0804	0.0828	73.99194	80.64193	80.46647	78.36678	3.78974
1	0.075	0.077	0.063	75.60484	77.2317	61.22449	71.35367	8.809765
PHA	0.124	0.132	0.120	125	132.3972	116.6181	124.6718	7.894677
DMSO	0.062	0.059	0.0614	62.5	59.17753	59.66958	60.44904	1.793143
Cells only	0.0992	0.0997	0.1029	100	100	100	100	0
***C. asiatica* methanolic**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.425	0.341	0.333	277.7778	431.6456	378.4091	362.6108	78.14098
500	0.274	0.203	0.151	179.085	256.962	171.5909	202.546	47.27442

250	0.268	0.251	0.068	175.1634	317.7215	77.27273	190.0525	120.9139
100	0.226	0.192	0.124	147.7124	243.038	140.9091	177.2198	57.1016
50	0.152	0.159	0.168	99.34641	201.2658	190.9091	163.8404	56.09301
25	0.128	0.134	0.08	83.66013	169.6203	90.90909	114.7298	47.67448
1	0.152	0.152	0.09	99.34641	192.4051	102.2727	131.3414	52.90292
PHA	0.179	0.154	0.143	116.9935	194.9367	162.5	158.1434	39.15383
DMSO	0.058	0.06	0.084	37.9085	75.94937	95.45455	69.7708	29.26633
Cells only	0.153	0.079	**0.088**	100	100	100	100	0
***C. asiatica* aqueous**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.058	0.059	0.056	58.56855	58.97693	54.61613	57.3872	2.40849
500	0.073	0.074	0.075	73.4879	74.22267	72.8863	73.53229	0.66929
250	0.080	0.072	0.075	80.24194	72.21665	72.40039	74.95299	4.581281
100	0.072	0.071	0.071	72.58065	70.81244	68.6103	70.66779	1.98912
50	0.070	0.072	0.069	70.76613	72.11635	67.44412	70.10887	2.40446
25	0.0651	0.0637	0.0705	65.625	63.89168	68.51312	66.00993	2.334645
1	0.075	0.073	0.074	75.80645	73.62086	72.30321	73.91017	1.769451
PHA	0.096	0.125	0.132	96.77419	125.3761	128.2799	116.8101	17.41221
DMSO	0.062	0.059	0.0614	62.5	59.17753	59.66958	60.44904	1.793143
Cells only	0.0992	0.0997	**0.1029**	100	100	100	100	0
***C. triloba* methanolic**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.248	0.24	0.202	344.4444	406.7797	292.7536	347.9926	57.09576
500	0.181	0.179	0.16	251.3889	303.3898	231.8841	262.2209	36.96307
250	0.123	0.121	0.118	170.8333	205.0847	171.0145	182.3109	19.72297
100	0.099	0.09	0.094	137.5	152.5424	136.2319	142.0914	9.072974
50	0.071	0.077	0.081	98.61111	130.5085	117.3913	115.5036	16.03225
25	0.076	0.086	0.092	105.5556	145.7627	133.3333	128.2172	20.58604
1	0.089	0.085	0.092	123.6111	144.0678	133.3333	133.6707	10.23252
PHA	0.091	0.104	0.107	126.3889	176.2712	155.0725	152.5775	25.03457
DMSO	0.053	0.059	0.049	73.61111	100	71.01449	81.54187	16.03785
Cells only	0.072	0.059	**0.069**	100	100	100	100	0

***C.triloba* aqueous**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.021	0.018	0.026	29.16667	30.50847	37.68116	32.4521	4.577926
500	0.032	0.033	0.039	44.44444	55.9322	56.52174	52.29946	6.809028
250	0.023	0.031	0.026	31.94444	52.54237	37.68116	40.72266	10.63046
100	0.087	0.064	0.065	120.8333	108.4746	94.2029	107.8369	13.32666
50	0.067	0.052	0.058	93.05556	88.13559	84.05797	88.41637	4.505359
25	0.042	0.038	0.063	58.33333	64.40678	91.30435	71.34815	17.54733
1	0.022	0.025	0.032	30.55556	42.37288	46.37681	39.76842	8.225902
PHA	0.091	0.104	0.107	126.3889	176.2712	155.0725	152.5775	25.03457
DMSO	0.053	0.059	0.049	73.61111	100	71.01449	81.54187	16.03785
Cells only	0.072	0.059	**0.069**	100	100	100	100	0
***E. australis* methanolic**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.029	0.033	0.032	49.15254	57.89474	47.05882	51.3687	5.747849
500	0.023	0.021	0.025	38.98305	36.84211	36.76471	37.52995	1.259014
250	0.013	0.019	0.016	22.0339	33.33333	23.52941	26.29888	6.137734
100	0.028	0.047	0.039	47.45763	82.45614	57.35294	62.42224	18.04154
50	0.042	0.035	0.036	71.18644	61.40351	52.94118	61.84371	9.130594
25	0.032	0.036	0.028	54.23729	63.15789	41.17647	52.85722	11.05551
1	0.038	0.057	0.05	64.40678	100	73.52941	79.31206	18.4878
PHA	0.091	0.079	0.094	154.2373	138.5965	138.2353	143.6897	9.136272
DMSO	0.061	0.055	0.064	103.3898	96.49123	94.11765	97.99957	4.816603
Cells only	0.059	0.057	**0.068**	100	100	100	100	0
***E. australis* aqueous**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.097	0.087	0.095	164.4068	152.6316	139.7059	152.2481	12.35491
500	0.055	0.075	0.065	93.22034	131.5789	95.58824	106.7958	21.49543
250	0.05	0.057	0.058	84.74576	100	85.29412	90.01329	8.653086
100	0.064	0.075	0.08	108.4746	131.5789	117.6471	119.2335	11.6336
50	0.066	0.068	0.064	111.8644	119.2982	94.11765	108.4268	12.93749

25	0.044	0.045	0.048	74.57627	78.94737	70.58824	74.70396	4.181029
1	0.015	0.013	0.015	25.42373	22.80702	22.05882	23.42986	1.766803
PHA	0.091	0.079	0.094	154.2373	138.5965	138.2353	143.6897	9.136272
DMSO	0.061	0.055	0.064	103.3898	96.49123	94.11765	97.99957	4.816603
Cells only	0.059	0.057	**0.068**	100	100	100	100	0
***J.flava* methanolic**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.104	0.102	0.113	221.2766	242.8571	198.2456	220.7931	22.30969
500	0.095	0.083	0.091	202.1277	197.619	159.6491	186.4653	23.33263
250	0.074	0.034	0.027	157.4468	80.95238	47.36842	95.25587	56.41591
100	0.052	0.050	0.049	110.6383	119.0476	85.96491	105.2169	17.19476
50	0.080	0.054	0.051	170.2128	128.5714	89.47368	129.4193	40.37622
25	0.113	0.076	0.086	240.4255	180.9524	150.8772	190.7517	45.57133
1	0.030	0.027	0.072	63.82979	64.28571	126.3158	84.81043	35.94542
PHA	0.065	0.070	0.056	138.2979	166.6667	98.24561	134.4034	34.37638
DMSO	0.043	0.034	0.03	91.48936	80.95238	52.63158	75.02444	20.0957
Cells only	0.047	0.042	**0.057**	100	100	100	100	0
***J.flava* aqueous**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.034	0.054	0.042	178.9474	168.75	247.0588	198.2521	42.5743
500	0.044	0.045	0.036	231.5789	140.625	211.7647	194.6562	47.8297
250	0.017	0.050	0.017	89.47368	156.25	100	115.2412	35.90251
100	0.020	0.019	0.017	105.2632	59.375	100	88.21272	25.11246
50	0.032	0.023	0.029	168.4211	71.875	170.5882	136.9614	56.37692
25	0.030	0.045	0.043	157.8947	140.625	252.9412	183.8203	60.48001
1	0.023	0.017	0.025	121.0526	53.125	147.0588	107.0788	48.50094
PHA	0.043	0.039	0.051	226.3158	121.875	300	216.0636	89.50396
DMSO	0.023	0.021	0.013	121.0526	65.625	76.47059	87.71607	29.37518
Cells only	0.019	0.032	**0.017**	100	100	100	100	0
***M.balsamina* methanolic**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**

1000	0.042	0.036	0.037	221.0526	112.5	217.6471	183.7332	61.71328
500	0.031	0.02	0.026	163.1579	62.5	152.9412	126.1997	55.40156
250	0.033	0.023	0.03	173.6842	71.875	176.4706	140.6766	59.60022
100	0.04	0.031	0.032	210.5263	96.875	188.2353	165.2122	60.22211
50	0.044	0.036	0.03	231.5789	112.5	176.4706	173.5165	59.59441
25	0.033	0.04	0.04	173.6842	125	235.2941	177.9928	55.27315
1	0.037	0.043	0.041	194.7368	134.375	241.1765	190.0961	53.55176
PHA	0.053	0.051	0.03	278.9474	159.375	176.4706	204.931	64.66748
DMSO	0.023	0.021	0.013	121.0526	65.625	76.47059	87.71607	29.37518
Cells only	0.019	0.032	**0.017**	100	100	100	100	0
***M.balsamina* aqueous**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.022	0.015	0.035	37.28814	26.31579	51.47059	38.35817	12.61149
500	0.033	0.03	0.033	55.9322	52.63158	48.52941	52.3644	3.708621
250	0.038	0.032	0.047	64.40678	56.14035	69.11765	63.22159	6.569327
100	0.121	0.143	0.09	205.0847	250.8772	132.3529	196.105	59.7702
50	0.046	0.078	0.049	77.9661	136.8421	72.05882	95.62234	35.81935
25	0.045	0.087	0.049	76.27119	152.6316	72.05882	100.3205	45.35163
1	0.09	0.113	0.073	152.5424	198.2456	107.3529	152.7136	45.44658
PHA	0.091	0.079	0.094	154.2373	138.5965	138.2353	143.6897	9.136272
DMSO	0.061	0.055	0.064	103.3898	96.49123	94.11765	97.99957	4.816603
Cells only	0.059	0.057	**0.068**	100	100	100	100	0
***O. sinuatum* methanolic**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.066	0.057	0.069	66.53226	57.17151	67.05539	63.58639	5.561598
500	0.071	0.072	0.076	71.57258	72.21665	73.85811	72.54912	1.17848
250	0.078	0.067	0.074	78.62903	67.2016	71.91448	72.58171	5.742858
100	0.075	0.07	0.035	75.60484	70.21063	34.01361	59.94303	22.61693
50	0.074	0.069	0.067	74.59677	69.20762	65.11176	69.63872	4.75718
25	0.074	0.067	0.069	74.59677	67.2016	67.05539	69.61792	4.31243
1	0.077	0.089	0.106	77.62097	89.2678	103.0126	89.96713	12.71027
PHA	0.121	0.125	0.119	121.9758	125.3761	115.6463	120.9994	4.937876

DMSO	0.062	0.059	0.0614	62.5	59.17753	59.66958	60.44904	1.793143
Cells only	0.0992	0.0997	**0.1029**	100	100	100	100	0
***O. sinuatum* aqueous**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.066	0.068	0.064	66.53226	68.20461	62.19631	65.64439	3.100994
500	0.077	0.079	0.072	77.62097	79.23771	69.97085	75.60984	4.949968
250	0.075	0.078	0.075	75.60484	78.2347	72.8863	75.57528	2.674326
100	0.077	0.102	0.072	77.62097	102.3069	69.97085	83.29958	16.89942
50	0.078	0.077	0.076	78.62903	77.2317	73.85811	76.57295	2.452728
25	0.072	0.068	0.067	72.58065	68.20461	65.11176	68.63234	3.752769
1	0.079	0.078	0.077	79.6371	78.2347	74.82993	77.56724	2.472112
PHA	0.121	0.125	0.119	121.9758	125.3761	115.6463	120.9994	4.937876
DMSO	0.062	0.059	0.0614	62.5	59.17753	59.66958	60.44904	1.793143
Cells only	0.0992	0.0997	**0.1029**	100	100	100	100	0
***P.viscosa* aqueous**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.053	0.053	0.054	53.32661	53.36008	52.08941	52.92537	0.724156
500	0.073	0.064	0.073	73.4879	63.89168	71.33139	69.57032	5.034671
250	0.078	0.074	0.083	78.22581	74.02207	80.46647	77.57145	3.271656
100	0.074	0.076	0.073	74.29435	76.12839	70.65112	73.69129	2.787989
50	0.068	0.066	0.068	68.95161	66.5998	65.88921	67.14688	1.602823
25	0.074	0.075	0.079	74.49597	75.02508	76.87075	75.46393	1.246732
1	0.069	0.071	0.070	69.65726	71.51454	68.22157	69.79779	1.650977
PHA	0.098	0.124	0.112	98.79032	124.3731	108.8435	110.669	12.88872
DMSO	0.062	0.059	0.061	62.5	59.17753	59.66958	60.44904	1.793143
Cells only	0.099	0.100	**0.103**	100	100	100	100	0
***S.occidentalis* methanolic**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.048	0.042	0.031	252.6316	131.25	182.3529	188.7448	60.94271
500	0.042	0.031	0.022	221.0526	96.875	129.4118	149.1131	64.39044
250	0.012	0.028	0.015	63.15789	87.5	88.23529	79.63106	14.27092

100	0.027	0.025	0.013	142.1053	78.125	76.47059	98.90028	37.42575
50	0.063	0.075	0.051	331.5789	234.375	300	288.6513	49.58575
25	0.064	0.082	0.062	336.8421	256.25	364.7059	319.266	56.3237
1	0.046	0.059	0.047	242.1053	184.375	276.4706	234.317	46.53915
PHA	0.040	0.033	0.034	210.5263	103.125	200	171.2171	59.2039
DMSO	0.019	0.019	0.016	100	59.375	94.11765	84.49755	21.95467
Cells only	0.019	0.032	**0.017**	100	100	100	100	0
***S.occidentalis* aqueous**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.0512	0.0482	0.0429	51.6129	48.34504	41.69096	47.2163	5.056358
500	0.0744	0.0734	0.069	75	73.62086	67.05539	71.89209	4.245079
250	0.0786	0.0753	0.0672	79.23387	75.52658	65.30612	73.35552	7.213228
100	0.0764	0.0774	0.0757	77.01613	77.6329	73.56657	76.07187	2.191457
50	0.0741	0.0726	0.076	74.69758	72.81846	73.85811	73.79138	0.941338
25	0.0737	0.079	0.0714	74.29435	79.23771	69.38776	74.30661	4.92499
1	0.123	0.095	0.108	123.9919	95.28586	104.9563	108.078	14.60543
PHA	0.121	0.125	0.115	121.9758	125.3761	111.759	119.7036	7.087219
DMSO	0.062	0.059	0.0614	62.5	59.17753	59.66958	60.44904	1.793143
Cells only	0.0992	0.0997	**0.1029**	100	100	100	100	0
***S. oleraceous* methanolic**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.055	0.062	0.063	55.44355	62.18656	61.22449	59.6182	3.647215
500	0.077	0.075	0.073	77.62097	75.22568	70.94266	74.59644	3.383326
250	0.066	0.077	0.065	66.53226	77.2317	63.16812	68.97736	7.343698
100	0.069	0.075	0.064	69.55645	75.22568	62.19631	68.99281	6.532946
50	0.067	0.072	0.066	67.54032	72.21665	64.13994	67.96564	4.055117
25	0.070	0.074	0.075	70.56452	74.22267	72.8863	72.55783	1.851064
1	0.073	0.072	0.066	73.58871	72.21665	64.13994	69.98177	5.105471
PHA	0.135	0.126	0.132	136.0887	126.3791	128.2799	130.2492	5.145652
DMSO	0.062	0.059	0.061	62.5	59.17753	59.66958	60.44904	1.793143
Cells only	0.099	0.100	**0.103**	100	100	100	100	0

***S. oleraceous* aqueous**	**1**	**2**	**3**	**CV1**	**CV2**	**CV3**	**AVG**	**STD DEV**
1000	0.047	0.048	0.047	46.875	48.04413	45.67541	46.86485	1.184392
500	0.060	0.066	0.068	60.68548	65.89769	66.08358	64.22225	3.06434
250	0.062	0.065	0.063	62.5	65.19559	61.22449	62.97336	2.027426
100	0.068	0.076	0.075	68.34677	75.72718	72.8863	72.32008	3.72264
50	0.067	0.077	0.077	67.43952	76.83049	74.53839	72.93613	4.896225
25	0.065	0.073	0.073	65.32258	72.81846	71.13703	69.75935	3.933258
1	0.063	0.076	0.074	63.00403	76.32899	71.91448	70.41583	6.787714
PHA	0.121	0.115	0.112	121.9758	115.346	108.8435	115.3885	6.566237
DMSO	0.062	0.059	0.061	62.5	59.17753	59.66958	60.44904	1.793143
Cells only	0.099	0.100	**0.103**	100	100	100	100	0

SECTION TWO: Immunomodulatory activity of plants evaluated by the lymphocyte secretion of cytokines IFN-γ and IL-10 using ELISA

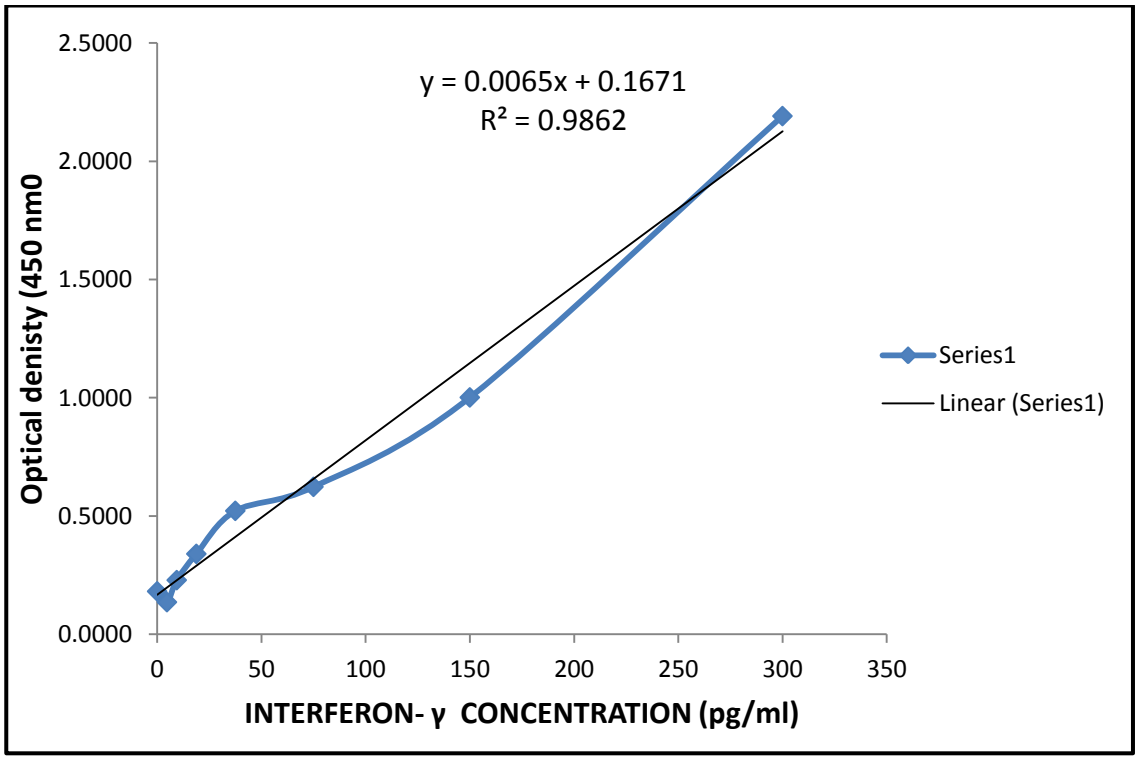

Figure 1: Standard graph of Interfeon-gamma (pg/ml).

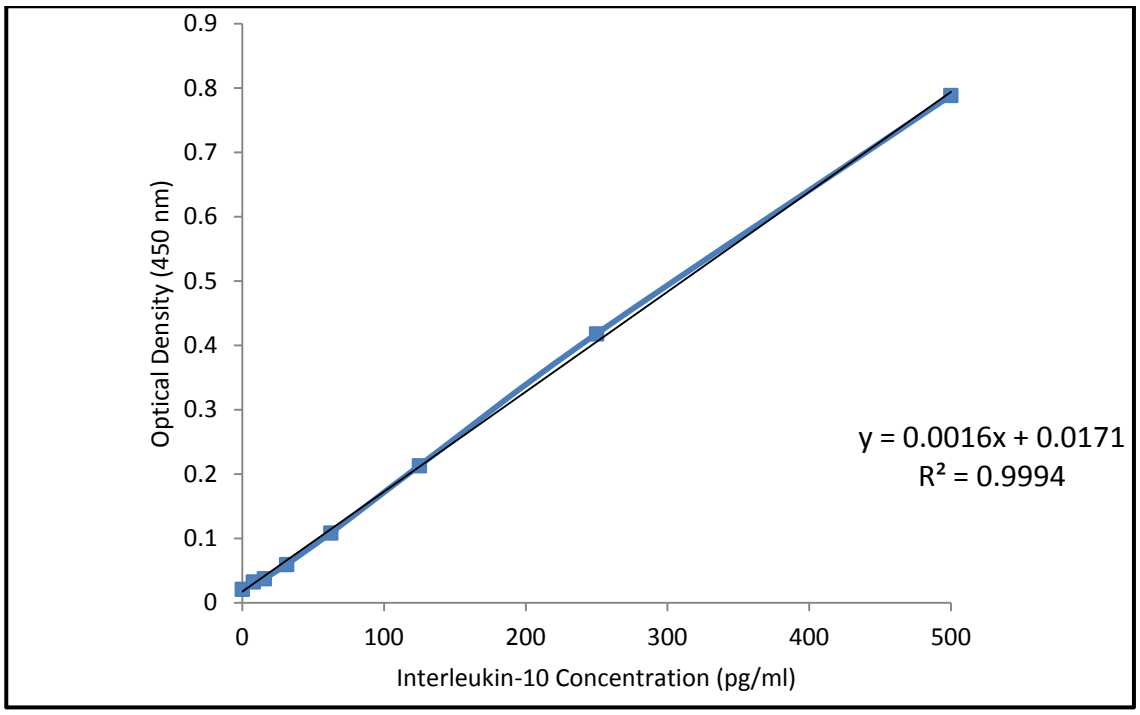

Figure 2: Standard graph of Interleukin-10 (pg/ml).