The immune-modulating activity of *Artemisia afra*

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<u>Key Words</u>

Artemisia afra

Cell-mediated immunity

Cytotoxicity

ELISA

IFN- γ

IL-6

IL-10

Inflammatory activity

Humoral immunity

Human whole blood cultures

LDH assay

Abbreviations

ADCC-	Antibody-dependent cellular cytotoxicity
APCs-	Antigen presenting cells
B cells-	Bone marrow cells
C-	Complement
CD (cells)-	Cluster of differentiation (cluster of designation)
c-GMP-	Cyclic guanosine monophospahate
COX-2-	Cyclooxygenase-2
(DAS)-ELISA-	Double antigen sandwich ELISA
DMSO-	Dimethyl sulfoxide
ELISA-	Enzyme-linked immunosorbent assay
GC-MS-	Gas chromatography-mass spectrometry
GIT-	Gastro-intestinal tract
HPLC-	High-performance liquid chromatography
IFNs-	Interferons
IFN-α-	Interferon-alpha
IFN-β-	Interferon-beta
IFN-γ-	Interferon-gamma
Ig-	Immunoglobin
IL-1β-	Interleukin 1 beta
IL-4-	Inteleukin-4
IL-6-	Interleukin-6
IL-10-	Interleukin-10
iNOS-	Inducible nitric oxide synthase
K cells-	Killer cells
KAL-	Killer activating ligand
KAR-	Killer activating receptor
KIR-	Killer inhibiting receptor

LAK-	Lymphokine activated killer (cells)
LDH-	Lactate dehydrogenase
LGL-	Large granular lymphocytes
LPS-	Lipopolysaccharide
MAC-	Membrane attack complex
MAPK-	Mitogen-activated protein kinases
MASP-	MBL-associated serine proteases
MBL-	Mannose-binding lectin
mRNA-	Messenger ribonucleic acid
NADPH-	Reduced nicotinamide adenine dinucleotide phosphate
NF-κβ-	Nuclear factor kappa beta
NK cells-	Natural killer cells
NO-	Nitric oxide
PAMPS-	Pathogen associated molecular patterns
PG-E2-	Prostaglandin E2
pH-	Potential of Hydrogen
PHA-	Phytohaemagglutinin
PMNs-	Polymorphonuclear cells
PRRs-	Pattern recognition receptors
TB-	Tuberculosis
T cells-	Thymus cells
Tf-	Transferrin
Th cells-	Thymus helper cells
TNF-α	Tumour necrosis factor alpha
tPA-	Tissue plasminogen activator
Tregs-	Thymus regulatory cells
WHO-	World Health Organization

Abstract

The human immune system consists of innate and adaptive mechanisms of defence that protect the host from harmful substances. Cytokines and other immune components play an important role in the induction and regulation of these defence mechanisms. Despite these mechanisms, sometimes pathogens still manage to evade the immune system causing disease; or allergens result in hypersensitive reactions; or the immune system becomes overly sensitive and starts attacking the "self". Irrespective of the cause, despite its best efforts, the immune system sometimes needs help regulating its defences.

Artemisia afra is an indigenous member of the daisy or Asteraceae family. It is one of the oldest and most common plants used as a traditional medicine in South Africa. Because of the great diversity of ailments traditionally treated with *Artemisia afra*, it is considered a "cure-all". The aerial parts contain various phenolic compounds that have anti-bacterial and anti-fungal activity. This helps to explain its popularity in treating bacterial infections such as sore throats, ear infections and various bronchial diseases. Other traditional uses include viral infections such as measles and influenza, and parasitic infections such as malaria and intestinal parasites. Non-pathogenic conditions traditionally treated with *Artemisia afra* include diabetes mellitus, gout and neuraligia, autoimmune conditions like rheumatoid arthritis and allergic conditions such as asthma. Due to this wide range of traditional indications, *Artemisia afra* is thought to have immune regulating effects.

Herbal medications are becoming increasingly popular with the general public. Knowledge regarding indigenous medicines is very limited, while the need for such knowledge is becoming more essential. It is estimated that in Africa approximately 80 % of people rely on herbal medicine for primary health care. Most literature sources focus on the study of European herbs and medical practitioners are not well equipped to guide the public on issues

relating to herbal medicines. Adverse and side effects of herbs become common when the herb is taken incorrectly or together with certain medications. Despite the wide variety of conditions traditionally treated with *Artemisia afra*, limited literature exists regarding the bio-activities on the species, and no immune studies have been done until now. The aim of this study was to use human whole blood cultures to examine *Artemisia afra's* immunomodulating effects *in vito.*

A 20 % (w/v) *Artemisia afra* extract was prepared using 94.4 % ethanol and milled aerial herb organs. The extract was air dried and re-suspended in DMSO to obtain a 50 % (wet leaf w/v) extract. Blood was collected from healthy male volunteers and diluted with RPMI-1640. To measure inflammatory activity and cytotoxicity, stimulated blood contained 1 volume of 10 μg/ml LPS in DMSO, 10 volumes of blood and 89 volumes of RMPI-1640. Blood (200 μl/well) was added to various concentrations of *Artemisia afra*. This was incubated at 37 ºC for 24 hours. For cell-mediated and humoral immunity, stimulated blood contained 10 volumes of blood and 89 volumes of RPMI-1640 medium and 1 volume of 1.6 mg/ml PHA in RPMI-1640 and incubated for 48 hours. An LDH assay was used to analyse the herb for cytotoxicity and various ELISAs for cytokine analysis. IL-6 was used as a biomarker for inflammatory activity, IL-10 for humoral immunity and IFN-γ for cell-mediated immunity. Results were statistically analysed using ANOVA tests.

Results showed that *Artemisia afra* was significantly cytotoxic ($P<0.050$) at 5 000 μg/ml. IL-10 production was significantly suppressed ($P<0.001$). This result indicates down regulation of the Th2 pathways, coinciding with its traditional use in allergic conditions. IFN-γ production was also significantly suppressed ($P<0.001$). This implies that although no direct immune defences are offered against bacterial, viral and parasitic infections, they may still provide symptomatic relief by decreasing fever as it also significanlty decreased IL-6 release ($P<0.001$). Direct anti-microbial effects of *Artemisia afra* on various pathogenic agents

such as *Plasmodium* and *Influenza* were not tested. The decrease of IL-6 production occurred at high concentrations (1 666 and 5 000 µg/ml). This demonstrates a potential ability to reduce the effects of conditions associated with abnormally high levels of IL-6 such as rheumatoid arthritis and arteriosclerosis.

This study shows that herbs can be effectively screened for potiential bio-activity using *in vitro* methods. Further studies will be needed to better explore *Artemisia afra's* effect on immunoregulation, particularly long term effects of the herb on the immune system and its effect on other disease states.

Declaration

I declare that this is my own work; that it has not been submitted before for any degree or examination in any other university and that all the sources I have used or quoted from have been indicated and acknowledged by complete references.

Yusra Kriel   March 2010

Signed……………………….

Acknowledgments

<div dir="rtl">بسم الله الرحمان ارحيم</div>

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

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Chapter 1: The bio-activities of *Artemisia afra*

1.1 Introduction

Artemisia afra (Asteraceae/Daisy) is one of the oldest and best known of South Africa's indigenous medicines. The plant may also be found in Eastern parts of Africa and is used as a medicine in Ethiopia (van Wyk and Gericke, 2005; Van Wyk, 2008). Wormwood, the common name, is derived from the Old English word 'wermode', meaning "mind preserver" (Mars, 2007). African tribes like the Zulu, Xhosa, and Sotho all have different traditional names for *Artemisia afra*. Popular names include 'wilde als' or 'als' in Afrikaans, 'mhlanyane' in Zulu, 'umhlonyane' in Xhosa, 'zengana' in Southern Sotho and 'lengana' in Pedi and Tswana (van Wyk and Gericke, 2005, Mukinda, 2007).

1.2 Botany

Artemisia afra is classified under the Magnoliphyt division, Mognoliphyta class (Asteridae subclass) and the Asterales order (Mukinda, 2007). The species is graphically represented in figure 1.1 below. It is an aromatic medium sized clump-forming, multi-stemmed perennial herb, growing up to two meters in height. It has a ribbed stem, multiple branches and a short rhizome. Silver-grey leaves are finely divided. The characteristic colour is due to the presence of fine leaf hairs, growing up to 80 mm long and 40 mm wide. The leaves are alternative and oval. Pale yellow or cream coloured tubular florets, with few outer female and inner bisexual florets, occur in an elongated racemose panicle during summer. The capitula are all small, and the receptacle is flat and naked. Fruits produced from the flowers are 1 mm in length, 3-angled and slightly curved with a silver-white coat. The aerial parts have a characteristic 'wormwood' smell and a bitter taste. *Artemisia afra* is drought resistant, hardy,

and easy to propagate. It grows well in any soil, needing only occasional watering and pruning (van Wyk and Gericke, 2005; Mukinda, 2007).

Figure: 1.1: Aerial parts (traditional parts used) of *Artemisia afra* (van Wyk and Gericke, 2005: 45)
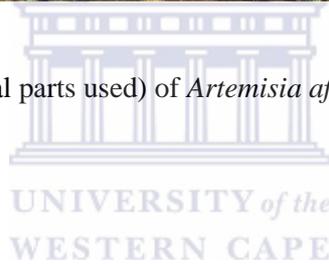
1.3 Geographical distribution

Artemisia afra grows naturally in Eastern and Southern Africa at altitudes ranging between 1500 and 3000 m and in a wide variety of soils. In South Africa, it normally grows in the mountainous areas along forest margins and streams. The natural distribution extends from the Northern and Eastern Mpumalanga, Limpopo and the North West Province to the Western Cape. It does not occur naturally in the Northern Cape. It also occurs northwards into tropical east Africa as far north as Ethiopia (van Wyk and Gericke, 2005; Mukinda, 2007). This natural area of distribution can be seen in figure below in figure 1.2.
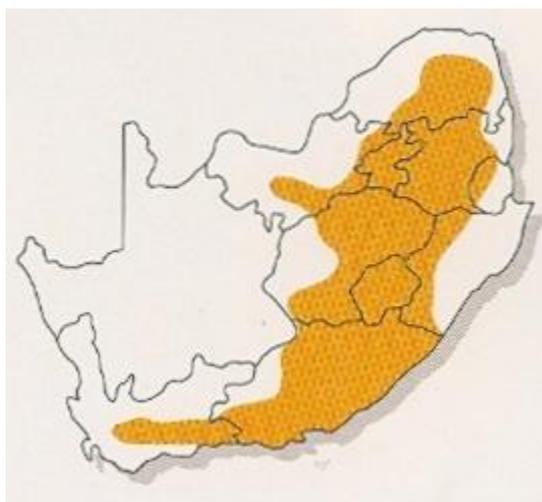
Figure 1.2: Natural distribution of *Artemisia afra* in South Africa (van Wyk and Gericke, 2005: 44)

1.4 Traditional medicinal uses of *Artemisia afra*

Therapeutically *Artemisia afra* is used as an analgesic and anthelmintic. Other traditional uses include a variety of ailments. These include coughs, colds, sore throats, gumboils, influenza, asthma, whooping cough, bronchial complaints, diabetes mellitus, stomach ailments, measles, malaria, wounds, indigestion, heartburn, flatulence, colic, headache, earache, constipation, haemorrhoids and gout. In the Cape region of South Africa it is popularly used as a bitter tonic and to help stimulate a poor appetite. The plant is also said to be an anti-histaminic and is used to treat allergies. The fresh or dried leaves and stems are used to make medicinal preparations in the form of teas, decoctions and tinctures. The steam and fumes are inhaled to clear headaches, congestion, asthma, hay fever and sinusitis (Mukinda, 2007; van Wyk and Gericke, 2005; van Wyk, 2008). The leaves may also be rolled and stuffed into the nostril to kill inhaled bacteria when visiting the ill. Because of the great diversity of ailments treated with *Artemisia afra*, it is widely considered a "cure all" remedy (Mukinda, 2007).

The leaves and flowers are the main parts used in traditional medicine. This is normally administered orally as a tea or a decoction. Alcoholic extracts or tinctures are also a popular dosage form. The roots may also be used medicinally, although this is not commonly practised (Mukinda, 2007; van Wyk and Gericke, 2005).

1.5 Traditional dosage forms of *Artemisia afra*

Artemisia afra is mostly taken as a tea or decoction. It may also be taken as a 'steam', where the vapours arising from the leaves in boiling water are inhaled. Infusions or decoctions are made with variable quantities of fresh or dried leaves and is used to treat various ailments (Mukinda, 2007).

The most common method of preparing a tea is to add a quarter cup of fresh leaves to one cup of boiling water, allow it to steep for five minutes, and strain before drinking. One tablespoon of dried leaves could alternatively be added to one cup of boiling water, and allowed to steep for twenty minutes (Mukinda, 2007).

A decoction can be prepared by pouring two litres of boiling water over one cup of fresh leaves and stems. This is then allowed to stand for an hour before straining. The filtrate can then be used as a wash for haemorrhoids and in a bath for measles, to treat fevers, as a wound-wash for sores, rashes, bites, stings, and as an eyebath diluted with warm water to soothe red, irritated eyes. An enema may be made with ground leaves and hot water and given to children suffering from intestinal worms or constipation (Mukinda, 2007).

A *'strong brew'* that is used as a mouthwash for gumboils and oral ulcers, or as eardrops for earache can be made by adding one and a half or two cups of boiling water to a quarter cup of

Artemisia afra leaves. The leaves are allowed to draw for ten minutes before it is strained and used (Mukinda, 2007).

Another popular preparation is '*Wilde als brandy,*' which is an old remedy for colds, coughs, chest ailments, indigestion and stomach cramps. One bottle of brandy is mixed with one cup of *Artemisia afra* leaves, a quarter cup thyme, half a cup of mint leaves, one cup of sugar, one thumb-length piece of ginger and a quarter cup of rosemary. All the ingredients are pushed into the brandy bottle and shaken every day for a month. It is then either strained or allowed to age by adding more herbs. One tablespoon is then mixed with water and taken as needed (Mukinda, 2007).

For inhalation purposes, two or three cups of leaves are placed in a bowl with enough boiling water to cover. A towel is draped over the patient's head, with the bowl held under the nose. The steam is then deeply inhaled until the brew cools. This traditional method of administering *Artemisia afra* is used to treat bronchitis, a blocked nose or tight chest, asthma and chest colds (Mukinda, 2007).

A wash can also be made for skin ailments, and warm leaves can be used as a poultice to draw out pimples and boils. The poultice may also be used for neuralgia, mumps, painful and tired muscles, or bound over the abdomen to treat colic (Mukinda, 2007).

Leaves may be rolled and inserted into the nostrils to alleviate headache and a blocked nose, or a fresh leaf may be packed in the gaps of the teeth to relieve toothache. The dried leaf may also be smoked to relieve a sore throat and tight chest, to expel phlegm and to treat coughs (Mukinda, 2007).

1.6 The chemical constituents of *Artemisia afra*

Artemisia afra contains a high content of volatile oils, which varies depending on the geographical origin of the plant. The volatile oils are responsible for the ant-microbial and anti-oxidant activity associated with the plant. Often present are 1.8-cineole (eucalyptol), alpha-thujone, beta-thujone, camphor, borneol and sesquiterpenoids such as chrysanthenyl acetate and devannone. Sesquiterpene lactones (guaianolides and glaucolides) have been isolated from the aerial parts, and are responsible for the digestive effects such as its anti-spasmodic and appetite stimulating properties. Non-volatile constituents include triterpenes (amyrin and friedelin), alkanes (ceryl cerotinate and N-nonacosane), and flavonoids (methyl ethers of luteolin), coumarins (scopoletin) and plyacetylens (Addea-Nensai, 1999; Mukinda, 2007; van Wyk and Gericke, 2005 and van Wyk, 2008).
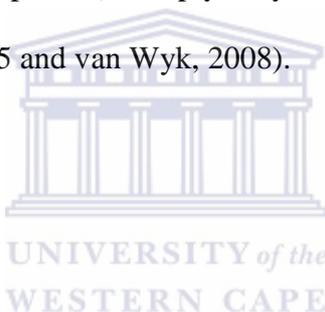
1.6.1 Non-volatile Constituents

1.6.1.1 *Flavonoids*

Flavonoids are phenolic compounds widely present in the plant kingdom. They are responsible for the yellow colour of certain flowers and fruit. Flavonoids occur either as free aglycones or bound to a sugar as a glycoside. Flavonoid glycosides may be either an O- or a C-glycosides, depending on the type of bond existing between the sugar and aglycone region (Evans, 2005).
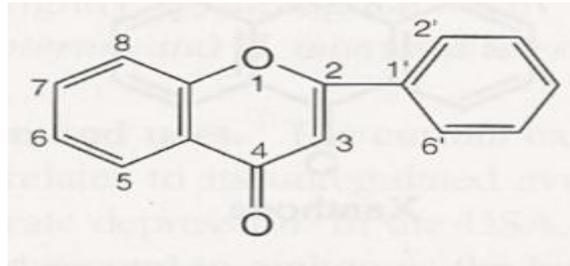
Figure 1.3: Flavonoid structure (Evans, 2005: 246)

1.6.1.1.1 Classification of flavonoids

All flavonoids are polyphenols consisting of a basic C6-C3-C6 skeleton. They are yellow pigments occurring in the cell sap of higher plants and young tissue. Depending on the variation of their basic carbon skeleton structure, flavonoids will be classified into a specific sub-group. Classification can be done using one of four systems. These systems include: classification according to the degree of saturation, the biosynthetic origin of the flavonoid, the molecular size of the flavonoid, and flavonoid conjugates. Discrepancies exist regarding the classification of flavonoids, but the major sub-groups are normally considered as the flavonols, flavanones, flavones and the chalcone flavonoids (Evans, 2005). Others may include isoflavonoids and anthrocyanadins. Luteolin, most commonly found in the Asteracea family, is a flavonoid known as a flavone, (Mukinda, 2007).

1.6.1.1.2 Biological effect of flavonoids

Flavonoids display a wide range of biological activities. These include anti-oxidation, anti-inflammation, anti-platelet, anti-thrombotic, anti-allergenic, anti-carcinogenic, cardio-protective, anti-proliferative, anti-microbial, anti-mutagenic, enzyme inhibitory, immune-modulating and biological modifying actions (Bone and Mills, 1999).

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Luteolin is a yellow crystalline compound found in *Artemisia afra* and in foods like parsley, artichokes, celery, peppers, olive oil, rosemary, citrus fruits, sage and thyme. It normally occurs as luteolin-7-O-glucuronide, luteolin-5-O-glucuronide or luteolin-3'-glucuronide. It is reported to have platelet aggregation effects (Mukinda, 2007).
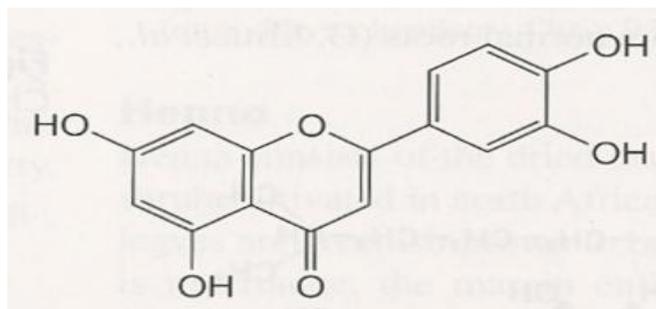
Figure 1.4: Luteolin (flavone) structure (Evans, 2005: 246)

Luteolin is an anti-oxidant and vasodilator, anti-inflammatory, anti-allergenic, anti-carcinogenic, immune-modulating and anti-spasmodic. It also appears to be hypoglycaemic agent and to aid in weight management. It also helps the body to better tolerate radiation and chemotherapy (Mukinda, 2007).

1.6.1.2 Scopoletin

Socopoletin is a yellow-beige crystalline powder, also known as 7-hydroxy-6-methylcoumarin, gelseminic acid, chrysatropic acid or esculetin-6-methyl ether. It belongs to the group of coumarines, which are commonly used to treat clotting disorders such as thrombophlebitis, pulmonary embolism and certain cardiac conditions. Scopoletin may act as both a hyper- and hypotensive agent (Kim, et al., 2004). It is a nonspecific anti-spasmodic agent, due to its ability to inhibit the intracellular calcium mobilization from the noradrenaline-sensitive stores, helping to keep blood vessels dilated (Obidoa et al., 2005;

Oliveira et al., 2001). However, it also appears to increase total serum cholesterol levels, free cholesterol levels and serum phospholipid levels, but decreases the levels of the serum esterified cholesterol (Oliveira et al., 2001). In this way it appears to act as both a hyper- and hypotensive agent (Kim et al., 2004).

The phytochemical webpage describes scopoletin as having bacteriostatic activity against *Escherichia coli, Staphylococcus aureus, Streptococcus species, Klebsiella pneumoniae* and *Psuedomonas aeruginosa*. It is anti-inflammatory and can be used to treat bronchial illnesses and asthma. It regulates serotonin levels/, helping to reduce anxiety and depression (Duke, 2008). Scopoletin suppresses pro-inflammatory cytokines and prostaglandin E2 (PGE2) (Kim et al., 2004).

Figure 1.5: Structure of scopoletin (Phytochemicals 2009)

Scopoletin occurs in oats, horse chestnut, lavender, strawberries, cinnamon, and various *Artemisia* species (*Artemisia annua, Artemisia capillaries, Artemisia afra* and *Artemisia dracunculus*). Biological activities associated with scopoletin include being anti-septic, diuretic, anti-asthmatic, anti-arrhythmic and anti-bronchoconstrictor. It is also an analgesic by blocking inflammation through inhibiting inducible nitric oxide synthase (iNOS) (Obasi et al., 1994; Duke, 2008; Monoforte, 1995).

Scopoletin has a dual action on tumoural lymphocytes, exhibiting both a cytostatic and a cytotoxic effect, indicating that it could be a potential anti-tumour compound to be used for cancer treatment (Kang et al., 1999). Scopoletin decreases the levels of serum thyroid

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hormones and glucose. It also decreases hepatic glucose-6-phosphatase activity, demonstrating a potential to regulate both hyperthyroidism and hyperglycemia. It inhibits hepatic lipid peroxidation and also increases the activity of antioxidants, superoxide dismutase and catalase (Manuele et al., 2006).

1.6.1.3 Triterpene Constituents
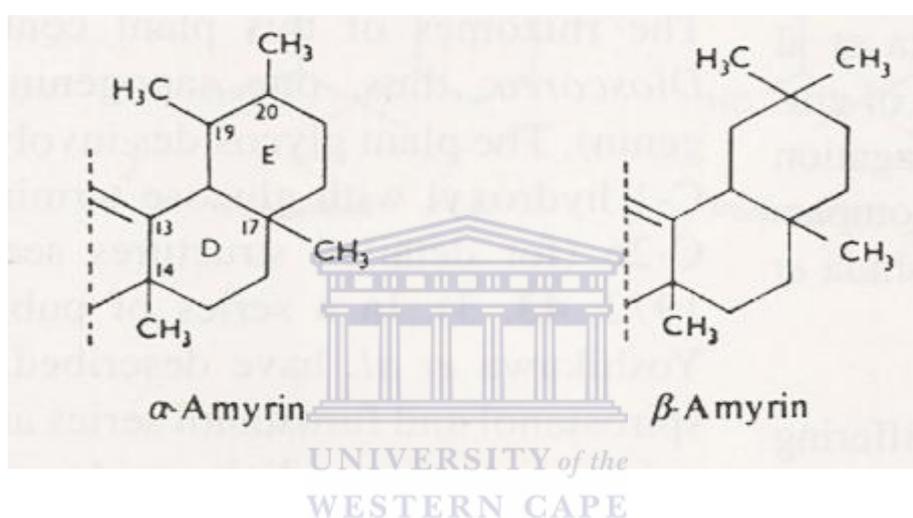
Figure 1.6: α- and β-Amyrin (Evans, 2005: 298)

Amyrin and friedelin are triterpene compounds present in *Artemisia afra*. Amyrin is known to be anti-depressant and anxiolytic (Aragão et al., 2006). It has also been found to inhibit inflammation, (Medeiros et al., 2007).

1.6.2 Volatile Compounds

1.6.2.1 Volatile Oils

Most volatile oils are mixtures of hydrocarbons and oxygenated compounds derived from these hydrocarbons. These oxygenated compounds are responsible for the characteristic odour of many plants.   Most volatile oils are terpenoid, while some are aromatic (benzene) derivatives mixed with terpenes (Evans, 2005).
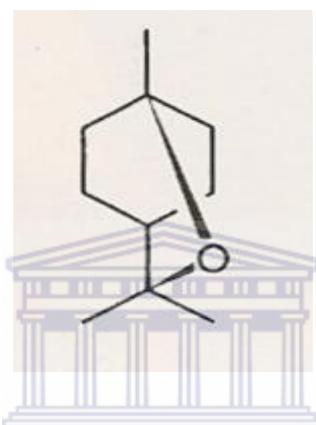
Figure 1.7: Eucalyptol (1,8-Cineole) (van Wyk and Gericke, 2005: 44)

Eucalyptol is a volatile oil also known as 1.8 cineol. It is mainly found in *Eucalyptus* species, but is also present in *Artemisia afra.* It is a powerful anti-inflammatory and inhibits tumour necrosis factor-alpha (TNF-α) and interleukin 1beta (IL-1β). It is thought to be useful in reducing the severity of asthma, sinusitis and chronic obstructive pulmonary disorders (COPD) (Jeurgens et al., 2004).   It also helps prevent gastrointestinal inflammation and ulceration (Santos et al., 2004).
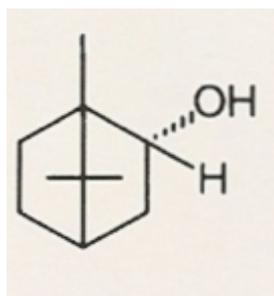
Figure 1.8: Borneol (van Wyk and Gericke 2005: 44)

Borneol is a bicyclic monoterpene volatile oil used for analgesia and anaesthesia in traditional Asian medicine. It is found in many medicinal herbs, including *Artemisia afra*. Borneol has been found to have a positive modulating action at gamma aminobutyric acid (GABA) receptors (Granger et al., 2005).
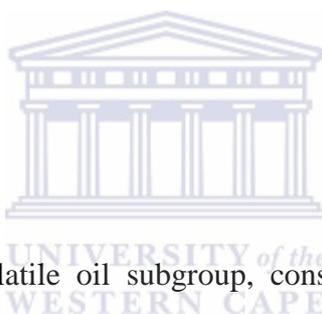
1.6.2.2 Sesquiterpene lactones

Sesquiterpene lactones are a volatile oil subgroup, consisting of the most volatile plant constituents. They are all formed from acetyl co-enzyme A (acytyl CoA) or glycolytic intermediates. They consist of 15 carbon atoms with isoprene as the basic structural unit (Evans, 2005). Sesquiterpene lactones are the main active ingredients in *Chrysanthemum parthenium*, commonly known as feverfew. These lactones are responsible for inhibiting platelet aggregation and the formation of prostaglandins and leucotrines. Sesquiterpene lactones are also anti-inflammatory, spasmolytic, anti-microbial and cytotoxic (van Wyk and Gericke, 2005). Chrysanthenyl acetate is a sesquiterpene lactone found in *Artemisia afra.*

1.7 Pharmacology and toxicology

Few studies have been conducted on indigenous herbs, including *Artemisia afra*. Due to the harmful effects of thujone (possible addiction and cerebral dysfunction resulting from neurotoxicity), high doses and chronic use of *Artemisia afra* is discouraged. Sporadic ingestion of small amounts of thujone in medicinal preparations and in food products does not, however, appear to be a health risk (van Wyk and Gericke, 2005).

Aqueous extracts appear to be non-toxic when administered acutely, and there is a low chronic toxicity potential. Hepatotoxic effects could result from high doses (Mukinda, 2007).

1.8 Side effects of *Artemisia afra*

In herbal medicine, *Artemisia* is considered as one of the great bitters. All species of *Artemisia* should be avoided during pregnancy. Thujone encourages menstruation and acts as an abortifacient. It should not be given to children, epileptics and to patients suffering from duodenal ulceration. According to herbal medicine lore, patients with a dry cough and severe kidney pathology should also avoid using *Artemisia* species (Kim et al., 2005).
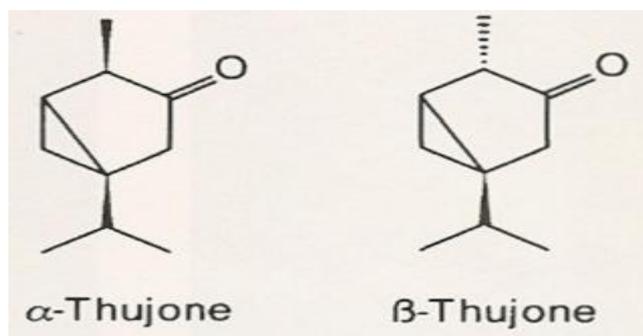
Figure 1.9: α and β thujone (van Wyk and Gericke, 2005: 44)

Commonly known as absinthism, the effects of excessive or prolonged ingestion include restlessness, vomiting, vertigo, tremor, convulsions and fatty degeneration of the liver (van Wyk and Wink, 2004). The effects of thujone are mediated by modulation of the gamma-Aminobutyric acid (GABA)A receptors. Inhibition of the serotonin 5-HT3 receptors is also thought to be involved, (Deiml et al, 2004). Published data from clinical trials in humans is very limited in this field.

1.9   Studies involving *Artemisia*

Table 1.1 summarizes the various *Artemisia* species previously studied as well as their traditional uses. Scientifically validated uses are briefly discussed in the section below.

Table 1.1: A brief overview of some *Artemisia* species studied

Botanical Name	Synonyms	Geographical Source	Traditional Uses	Reference
Artemisia afra	Wilde als, African wormwood	Southern and Eastern Africa	Colds, flu, fevers, diabetes	van Wyk and Gericke, 2004
Artemisia annua	Sweet wormwood, Qing Hao	South East Europe, Western Asia	Anti-malarial, fevers, colds, antibiotic	van Wyk and Gericke, 2004
Artemisia asiatica	Asian wormwood	Eastern Asia	Inflammation, gastric ulcers	Reddy et al., 2006; Seo et al., 2001
Artemisia capillaris	Capillary Artemisia, Yin Chen Hao	Eastern Asia	Hepatitis, jaundice, biliary infection and fevers	Jang, 2005
Artemisia drcunculus	Tarragon, Russian tarragon	Russia, Western Asia, Himalyas.	Intestinal worms, stomach cramps, sleep disorders, toothache	Mars, 2007
Artemisia iwayomogi	Russian wormwood	Eastern Asia: Afghanistan, India, Tibet, China &	Traditional Korean medicine, anti-inflammatory	Kim et al., 2005

Botanical Name	Synonyms	Geographical Source	Traditional Uses	Reference
		Mongolia		
Artemisia princeps	*Artemisia indica*	Eastern China, Japan, Korea	Digestive complaints	Baek et al., 2007
Botanical Name	**Synonyms**	**Geographical Source**	**Traditional Uses**	**Reference**
Artemisia verlotorum	None	Northern United States and Europe	Hypertension	Martinotti et al., 1999
Artemisia vulgaris	Mugwort	Mainly Britain and other northern temperate regions. Also found in Japan & Philippines	Anti-septic, anti-inflammatory, intestinal worms, insomnia	Mars, 2007; Tingo et al., 2000; van Wyk and Gericke, 2004

1.9.1 Studies involving *Artemisia afra*

1.9.1.1. Anti-microbial studies

Focus has been mainly placed on *Artemisia afra's* anti-microbial effects, especially that of the essential oils, although studies have been done using alcoholic and aqueous extractions. *Artemisia afra* has been found to be active against amoeba, *Streptococcus pyogenes, Listeria monocytogenes* and *Acinetobacter johnsonil* (Gundidza, 1993). Methanol extracts have shown activity against *Staphylococcus aureus, Staphylococcus epidermis* and *Bacillus subtilis.* No activity was found against *Escherichia coli and Klebsiella pneumoniae* (Rabe and van Staden, 1997).

1.9.1.2 Cardiovascular studies

The cardiovascular effects of aqueous extracts of *Artemisia afra* as well as a mixture of long chain fatty esters and scopoletin (isolated from *Artemisia afra*) were investigated by Addea-Nensia and Guantai in 1999. The long chain fatty esters induced hypotensive effects. The

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diastolic pressure was affected more than the systolic pressure. The aqueous extract had a hypotensive effect *in vivo* and a dose-dependent biphasic effect on the heart *in vitro*. Lower doses induced an initial cardio-stimulation followed by cardio-depression. Higher doses were mainly cardio-depressant. Scopoletin induced a dose-dependent decrease in inotropic activity and decreased the chronotropic effects, suggesting that *Artemisia afra* is potentially useful in the management of hypertensive conditions.

1.9.2 Biological studies involving other *Artemisia* species

1.9.2.1 Anti-inflammatory studies

Artemisia iwayomogi, commonly used as a traditional medicine in Korea, has a known anti-inflammatory effect. One study found that *Artemisia iwayomogi* inhibited plasma histamine release. It was also found to decrease Immunoglobulin (Ig) E–mediated local allergic reactions, as well as passive cutaneous anaphylaxis reactions. A dose dependent decrease in histamine release from peritoneal mast cells activated by IgE was also noted (Ahn et al;, 2003; Kim et al., 2005 and Shin et al., 2006). It also decreased calcium ionophore-stimulated tumour necrosis factor-alpha (TNF-α) and interleukin (IL)-6 gene expression and protein synthesis in human mast cells. The inhibitory effect of *Artemisia iwayomogi* on the pro-inflammatory cytokines were found to be mitogen-activated protein kinases (MAPK) and nuclear factor-kappa beta (NF-κβ) dependent (Ahn et al., 2003, Kim et al., 2005 and Shin et al., 2006). Similar results were also found for *Artemisia sylvatica* (Jin et al., 2004).

Artemisia capillaris is traditionally used in traditional Asian Medicine for the treatment of hepatitis and biliary tract infection. It exhibited no cytotoxic effects in unstimulated macrophages, but reduced the release of nitric oxide (NO) and prostaglandin (PG) E2 when

stimulated by interferon-gamma/lipopolysaccharide (IFN-γ/LPS) or LPS. The inhibitory effects were found to be due to the suppression of inducible nitric oxide synthase (iNOS) and cylooxygenases-2 (COX-2) in IFN-γ/LPS stimulated cells. It also decreased the production of TNF-α, interleukin (IL) -1β and IL-6 in LPS-stimulated cells, suggesting that it decreases the production of the inflammatory mediators (such as NO and PGE2) in macrophages by inhibiting iNOS and COX-2 expression (Jang et al., 2005).

Artemisia asiatica is commonly used in oriental medicine to treat inflammation. Ethanolic extracts of *Artemisia asiatica* inhibited histamine and leukotriene release in guinea pig lung mast cells activated with specific antigen/antibody complexes. Administered orally, it suppressed allergy-associated inflammation of the airways by regulating various cellular molecules expressed by the MAPK/NF-κβ pathway (Kim et al., 2006).

Sesquiterpene lactones isolated from *Artemisia leucodes* also showed anti-inflammatory action *in vivo* (Schmidt et al., 2008). *Artemisia princeps* reduced the IgE levels in the blood of asthmatic mice. It also reduced IL-6 and IL-4 levels in the trachea and lungs. The transcription and translation of IL-6 and the TNF-α levels in cells that were Ig-E induced were also reduced (Beak et al., 2007).

1.9.2.2 Anti-tumour studies

Artemisia asiatica has been studied as a potential anti-cancer agent. Ethanolic extracts inhibit tissue plasminogen activator (tPA)-induced COX-2 expression and iNOS (by blocking the NF-κβ signalling cascades) in a dose-dependant manner, indicating its use in depressing both inflammation and tumour promotion (Seo et al., 2001). It has also been found to inhibit the NF-κβ transcriptional activity in LPS-stimulated macrophages (Reddy et al., 2006).

1.9.2.3 Cardiovascular studies

Artemisia verlotorum, found in North America and Europe, is used as a folk medicine in some European countries as a hypertension remedy. The dried aqueous extract has a significant but transient hypotensive effect *in vitro*. The hypotensive effect is mediated by a strong vasodilator action, and closely linked to the release of endothelial NO and to the cyclic guanosine monophosphate (cGMP) pathway, caused by muscarinic receptor agonims (Martinotti et al., 1999).

A hydro-methanolic extract of *Artemisia scoparia* produced hypotensive and bradycardiac effects when administered intravenously. These effects were not mediated through the activation of the muscarinic receptors or adrenoreceptor blockade, because they remained constant in the presence of atropine, and did not modify the vascoconstrictor response of norepinephrine. It also inhibited K^+-induced tonic contraction in a manner similar to that of a calcium channel blocker such as verapamil (Gilani et al., 1994).

Artemisia vulgaris is widely used in the Philippines for its anti-inflammatory properties. It reverses the hypertensive action induced by norepinephrine. In another study, it did not significantly alter baseline blood pressures, but decreased the mean systolic and diastolic pressures induced by norepinephrine, without altering the heart rate in either normotensive or hypertensive states. This study indicated that *Artemisia vulgaris* has an anti-hypertensive action but has no significant effects on cardiovascular hemodynamics under basal conditions (Tingo et al., 2000).

Artemisia dracunculus is traditionally used in Iranian folk medicine as an anti-coagulant. Studies done with this species of *Artemisia* showed an inhibition in platelet adhesion. Methanol extracts also affected the self aggregation and protein secretion of the cells,

supporting its traditional use in the treatment of cardiovascular diseases and thrombosis (Shahriyary and Yazdanparast, 2008).

1.9.2.4 Immunological studies

The immunosuppressive activity and the inhibitory effect on the inflammatory and immune mediator, calmodulin, from artemisinin (extracted from *Artemisia annua*) was studied. Results showed an inhibitory effect on the activity of calmodulin. Both *in vivo* and *in vitro* studies indicated that artemisinin acted as an immunosuppressive agent (Noori, et al., 2004).

1.9.2.5 Anti-diabetic studies

Artemisia dracunculus, known for its antidiabetic activity, was examined as a possible aldose reductase inhibitor. Aldose reductase is involved in carbohydrate metabolism, and is though to be responsible for diabetic complications involving a number of organs, such as the liver, eyes and nerves. The enzyme activity was inhibited by 40 %, justifying its traditional use to help reduce diabetic complications (Logendra et al., 2006).
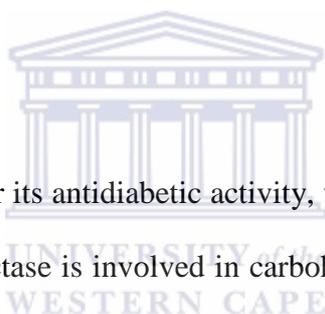
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Chapter 2: *Artemsia afra* and the immune system

2.1 Introduction

The immune system is comprised of Th1 and Th2 pathways. If these pathways are imbalanced, inflammatory conditions such as asthma (initiated by a type I hypersensitivity reaction and sustained by a dominant Th2 pathway) and rheumatoid arthritis (resulting from a dominant Th1 pathway) may result. A dominant Th1 pathway may also result from suppressing humoral pathways in conditions where the host is susceptible to bacterial, parasitic and extracellular infections. Th2 pathway dominance suppresses cell-mediated immunity in conditions where the host is susceptible to viral invasion and other intracellular microbes such as *Mycobacterium tuberculosis* (Clark, 2009; Underwood, 2004).

Artemisia afra is traditionally used to treat colds, influenza, asthma, allergies, rheumatic pains, tuberculosis (TB), and malaria (Mukinda, 2007; van Wyk and Gericke, 2005). This suggests that it has a regulating effect on the immune system, potentially being able to address all aspects of immune dysfunction.

2.2 Research problem

In 2005, Rai described herbal forms of medicines becoming increasingly popular with the general public. He also stated that medical practitioners were not well-equipped to guide the public on issues relating to herbal medicines as they have never seriously considered the idea that a herb could work as well as a pharmaceutical agent. On their website, The World Health Organization (WHO) reports that in some African countries, up to 80 % of the population depend on traditional medicine for primary health care.

The WHO continues to describe some of the challenges facing traditional herbal medicines and those who use them. The main one remains the lack of scientific data to critically evaluate the safety and efficacy of these medicines. Many patients also believe that because herbal medicines are natural, they are safe and free of any side or adverse effects. Adverse effects are common when the herb is taken incorrectly or together with other medications. This makes it necessary for increased training, collaboration and communication to exist between practitioners of herbal medicine and traditional Western medicine. Methods of researching herbal products are complex, due to the hundreds of phytochemicals occurring in a single plant. For every constituent to be properly evaluated, much time and resources would be required. This is in turn closely related to the quality control involved in producing herbal medications, which is proving difficult to monitor. Another key challenge is the lack of knowledge about herbal medicines within national drug authorities. This leads to a delay in the creation of national policies, laws and regulations for traditional medicines. In the July 2008 edition of the *Government Gazette*, the South African minister of health, Dr Metshabalala-Msimang issued a draft policy concerning the incorporation of African herbal medicine into the health care system.

In short, knowledge surrounding indigenous medicines is very limited, while the need for such knowledge is becoming more essential. Most literature sources focus on the study of European herbs. When indigenous herbs are researched, they tend to include pharmacokinetic studies involving HPLC, GC-MS and microbial assays. These are often expensive, complicated and time consuming. Another draw-back involving herbal research relates to the dosage and concentration of the herb used. Most studies involve freeze-dried extracts or encapsulated samples, when most herbs are traditionally taken as an infusion (van Wyk and Gericke, 2005). Long-term exposure of the herb also cannot be monitored using these procedures.

Biological studies also tend to be animal-related. Although animal studies play a key role in understanding herbal pharmacology, interspecies differences regarding physiology may lead to the misunderstanding of certain herbal compounds, as they would not be metabolised the same way (Collins, 2001). Most of these problems are eliminated when *in vitro* bioassays are employed, as they allow for quick and effective determination of the herb's effects on human physiology. Rapid screens to asses the immune-modulating activity of indigenous herbs are required. Running these assays via whole blood cell cultures is preferred as only small amounts of blood are required and tests are done *in vitro.* They can also be conducted quickly and represent the *in vivo* environment more accurately than isolated monocytes (Pool and Bouic, 2001).

The following study aims to validate *in vitro* methods to monitor immune functions. A whole blood culture assay will be used. Inflammatory effects will be studied using IL-6, while immune function via Th1 and Th2 pathways will be monitored using IL-10 and IFN-γ cytokines.
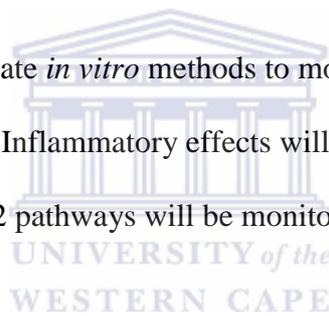
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World Health Organization Media Centre. Traditional Medicines. Available from: http://www.who.int/mediacentre/factsheets/fs134/en/ (Accessed 12 March 2010)

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

3.1 The immune system

3.1.1 Introduction

The immune system functions by protecting the body from invading pathogens that are potentially harmful. Normal individuals have two levels of defence against these foreign agents. The first level is referred to as natural or innate immunity. It presents in neonatal animals and invertebrates. The second type of immunity is adaptive or acquired immunity. Adaptive immunity is specific to vertebrates (Clark, 2009).

Despite the distinct functions each of these immune components, interplay between these systems does exist. Each system also consists of both cellular and humoral components (Mayor, 2000). An overview of the immune system is graphically represented below in figure 3.1. Comparisons between the innate and acquired immunity are briefly represented in table 3.1.
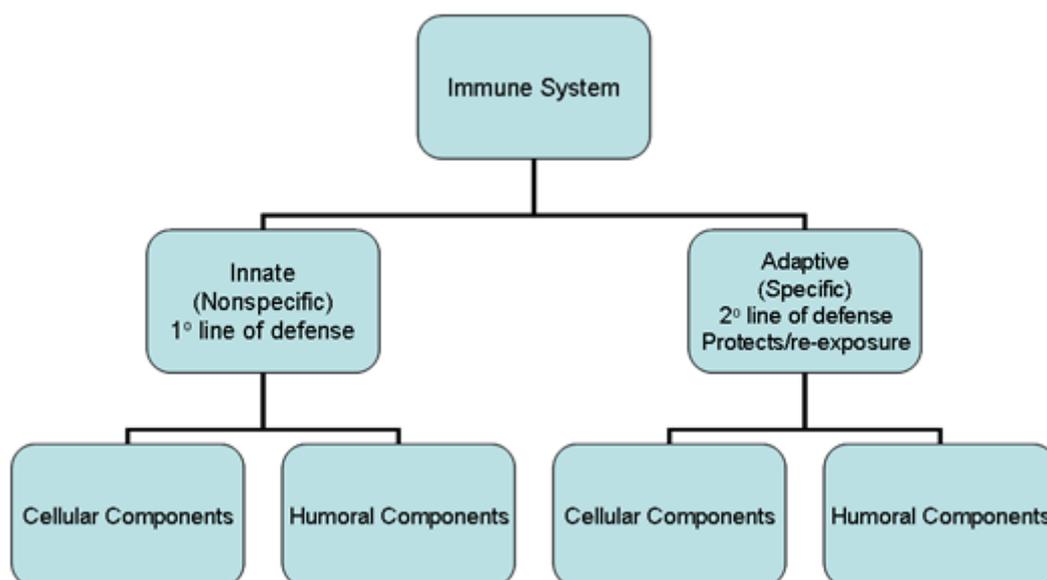
Figure 3.1: Immune system overview (Mayor, 2000)

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Table 3.1: Comparisons between innate and adaptive immunity (Mayor, 2000)

Innate Immunity	Adaptive Immunity
Response is antigen independent	Response is antigen dependent
Immediate maximal response	Lag time between exposure and maximal response
Not antigen-specific	Antigen specific
No immunological memory	Immunological memory
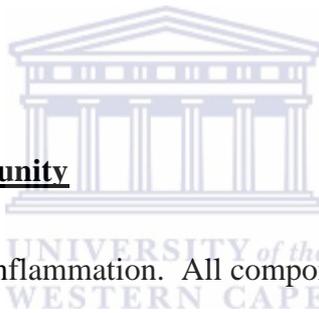
3.1.2 Non-specific (innate) immunity

Innate immunity involves acute inflammation. All components of the innate immune system are regulated by mediators of the adaptive immune system, such as interleukins, IFN-γ and antibodies (Mayor, 2000).

3.1.2.1 Anatomical barriers of the innate immune system

The innate immune system consists of anatomical barriers, cellular components and secretory molecules. The anatomical barriers themselves can be divided into three groups of defences: mechanical defences, chemical defences and biological defences. Mechanical defences involve epithelial layers. The skin serves as the initial defence.   Pathogens that adhere to epithelial surfaces are removed by desquamation.   In the lungs, cilia keeps air passages free from microbes, while in the gastrointestinal tract (GIT), peristalsis serves a similar function.

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Tears and saliva help flush out the eyes and mouth. Mucous linings in the GIT and respiratory tract provide additional protection (Mayor, 2000).

Chemical defences consist of fatty acids in sweat that inhibits bacterial growth. Tears, saliva and nasal secretions contain lysozyme and phospholipase. This damages bacterial walls and membranes. The low pH of sweat and gastric secretions prevents bacterial growth. Low molecular weight proteins called defensins occur in the lungs and GIT. These exert antimicrobial activity. Lung surfactants act as opsonins and promote phagocytosis (Mayor, 2000).

Biological factors include non-pathogenic flora occurring on the skin and in the GIT. These prevent pathogenic colonization by secreting toxins or through competitive inhibition (Mayor, 2000).
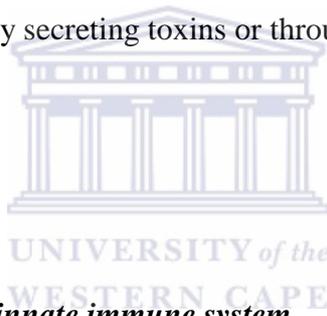
3.1.2.2 Humoral barriers of the innate immune system

Acute inflammation results when mechanical barriers are ineffective. Humoral factors then initiate oedema and an influx of phagocytes. These humoral factors are found in the serum, while some are formed on demand at the infection site (Mayor, 2000).

The complement system is the primary humoral mechanism of innate immunity. It is responsible for increased vascular permeability, lysis and opsonisation of bacteria and the recruitment of phagocytes. Tissue damage may activate the coagulation system. This results in increased vascular permeability (Mayor, 2000).

Bacterial cells require iron to grow. They acquire this iron directly from the host iron-binding glycoprotein, transferrin (Tf) (Moraes et al., 2009). Lactoferrin, a humoral

component of the immune system produced by mucosal secretions, chelates iron molecules, preventing bacterial infection (Singh, et al., 2002). Other humoral factors include interferons (IFNs), proteins produced by immune cells to limit viral replication in the host, and lysosomes which are released to destroy bacterial cell walls (Mayor, 2000).

3.1.2.3 Cellular barriers of the innate immune system

Macrophages and polymorphonuclear eosinophiles are vital to innate immune responses and inflammation. Polymorphonuclear cells (PMNs), or neutrophils, are recruited to the infection site. They phagocytose invading pathogens and kill them intracellularly. During inflammation, PMNs also cause collateral tissue damage. Macrophages and monocytes (which differentiate into macrophages) act as additional phagocytes. Macrophages are capable of both intracellular and extracellular killing of infected cells. Unlike PMNs, macrophages contribute to tissue repair and act as antigen-presenting cells (APCs). These APCs induce specific immune responses (Mayor, 2000).

NK cells and lymphokine activated killer (LAK) cells non-specifically kill virus infected and tumour cells. They do not form part of the inflammatory response. Eosinophils contain granular proteins responsible for eliminating various parasites (Mayor, 2000).

3.1.2.4 Phagocytosis and intracellular immunity

PMNs are motile phagocytic cells identified by their nucleus or the CD66 antigen present on their surface. Primary granules occur in newly formed PMNs. These contain defensins to kill bacteria, lysozyme to break down bacterial cell walls and myeloperoxidase which produces bacteriocidal compounds. Mature PMNs contain granules consisting of lysozyme, reduced

nicotinamide adenine dinucleotide phosphate (NADPH) oxidase components (these generate toxic oxygen products) and lactoferrin (responsible for chelating iron). Monocytes (macrophages) are phagocytic cells that have a kidney-shaped nucleus and can be identified by their CD14 cell surface marker. They contain numerous lysosomes that have contents similar to PMN granules (Mayor, 2000).

Once the PMNs are recruited, ingestion (phagocytosis) and intracellular killing of the pathogen and clearing of cellular debris occurs. Phagocytosis occurs by pseudopodia formation (cytoplasmic membrane projections) around the pathogen. The podia eventually fuse to form a membrane-bound vesicle (phagosome). This then fuses with the neutrophil cytoplasmic granules (phago-lysosome). It is within this localized environment that the pathogen is killed, while the remaining cytoplasm remains protected (Kumar and Clark, 1999).

In this phago-lysome, killing occurs via one of two methods. An oxygen-dependant response or "respiratory burst" results in the production of reactive oxygen metabolites, by reducing an NADPH oxidase present in the phagocyte cytoplasm. An oxygen-independent response results due to the toxic action of preformed cationic proteins and enzymes found in the neutrophil cytoplasmic granules (Kumar and Clark, 1999).

3.1.2.5 *Complement*

The term complement (C) was traditionally used to refer to a heat-sensitive serum compound capable of lysing bacteria, but compliment is also responsible for other types of defences. These include eradicating immune complexes and pathogenic organisms and immunoregulation. Effects detrimental to the host may also result from complement as it

induces inflammation, tissue damage and may trigger anaphylaxis (Kumar and Clark, 1999; Mayor, 2000).

Complement consists of more than 20 serum proteins. These are produced by hepatocytes, macrophages and GIT epithelial cells. When cleaved, certain complement proteins produce fragments that activate cells, increase vascular permeability or opsonize bacteria (Mayor, 2000). Complement can be activated by one of four pathways. Primary pathways are represented in figure 3.2 below.

3.1.2.5.1 *The classical pathway*

The classical pathway consists of four stages. In the first stage, C1, a multi-protein containing various proteins (C1q, C1r and C1s), binds to the F*c* region of an IgG and IgM antibody (that have interacted with an antigen). This binding of C1 to the antibody occurs via C1q and C1q and must cross link at least two IgG antibody molecules before it is firmly fixed. The binding of C1q results in the activation of C1r which in turn activates C1s. The result is the formation of an activated "C1qrs", an enzyme that cleaves C4 into two fragments; C4a and C4b (Kumar and Clark, 1999; Mayor, 2000).

C4b (promotes phagocytosis) binds to the pathogen membrane while C4a is released. Activated "C1qrs" also cleaves C2 into C2a and C2b. C2a binds to the membrane in relation with C4b. C2b is released (results in oedema). This forms a C4bC2a complex, which is a C3 convertase. C3 convertase cleaves C3 into C3a and C3b (Mayor, 2000).

C3b (activates phagocytosis) binds to the membrane in relation with C4b and C2a, while C3a is released (may result in anaphylaxis). This results in a C4bC2aC3b molecule, which is a C5 convertase. C5 convertase production marks the end of the classical pathway. Various

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regulators control the activity of the classical pathway (Kumar and Clark, 1999; Mayor, 2000).

3.1.2.5.2 *The lectin pathway*

The lectin pathway is similar to the classical pathway. It is initiated by mannose-binding lectin (MBL). MBL is a circulating protein that binds to carbohydrate molecules on the surface of certain microbes. As MBL is structurally related to C1q, it activates complement through MBL-associated serine proteinase (MASP), which is similar to C1r and C1s of the classical pathway. The biological activities and regulatory proteins of the lectin pathway are similar to that of the classical pathway. This pathway also contributes to the regulation of the innate immune system (Kumar and Clark, 1999).
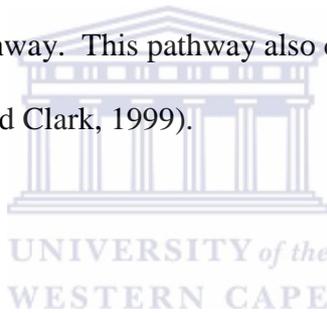
3.1.2.5.3 *The alternative pathway*

The alternative pathway is considered to be the more primitive pathway, while the classical and lectin pathways are thought to have developed from it. It begins with an activated C3 and requires Factor B, Factor D and Mg^{++} cations, (all of which occur in healthy serum). Serum normally has low levels of spontaneous hydrolysis of C3 to produce C3i. Factor B binds to C3i and becomes susceptible to Factor D. This cleaves Factor B into Bb. The C3iBb complex serves as a C3 convertase. It cleaves C3 into C3a and C3b. When C3b is formed, it binds to Factor B and becomes susceptible to cleaving by Factor D. The resulting C3bBb complex is a C3 convertase. This will generate more C3b, amplifying C3b production (Mayor, 2000).

When bound to an appropriate activator, C3b binds Factor B, which is cleaved by Factor D to produce C3 convertase (C3bBb). C3b is resistant to Factor I degredation. C3 convertase is degraded slowly, as it is stabilized by the activator surface. The complex is further stabilized by properdin binding to C3bBb. Surface components of various pathogens act as activators in this pathway. They include lipopolysaccharide (LPS) from Gram-negative bacteria and the cell walls of certain yeasts and bacteria. When C3b binds to an activator, the C3 convertase formed is stable and continues to generate additional C3a and C3b by cleavage of C3 (Mayor, 2000).

Some of the C3b generated by stabilized C3 convertase associates with the C3bBb complex. This forms a C3bBbC3b complex - the C5 convertase of the alternative pathway. The production of C5 convertase marks the end of this pathway. It may be activated by numerous Gram-negative bacteria (*Neisseria meningitidis* and *Neisseria gonorrhoea*), some Gram-positive bacteria, aggregated immunoglobulins (especially IgA) and certain viruses and parasites. This results in the lysis of these pathogens. The alternative pathway functions as an additional defence mechanism against pathogens before an antibody response is initiated. A deficiency of C3 results in an increased susceptibility to microbes (Mayor, 2000).

3.1.2.5.4 *The membrane attack (lytic) pathway*

C5 convertase can be produced from either the classical (C4b2a3b), lectin (C4b2a3b) or alternative (C3bBb3b) pathways, to cleave C5 into C5a and C5b. C5a remains in the serum. C5b associates with C6 and C7 and inserts into the pathogen membrane. As a result, C8 binds, followed by several molecules of C9. The C9 molecules form a pore in the membrane. Cellular contents leak through the pore. Membrane damage results in lysis. The complex

consisting of C5bC6C7C8C9 is referred to as the membrane attack complex (MAC) (Mayor, 2000).

C5a generated in this pathway has numerous biological activities. C5a is the most powerful anaphylotoxin. It is a chemotactic factor for neutrophils and initiates the respiratory burst in these cells. Inflammatory cytokine production by macrophages is also stimulated (Mayor, 2000; Underwood, 2004).
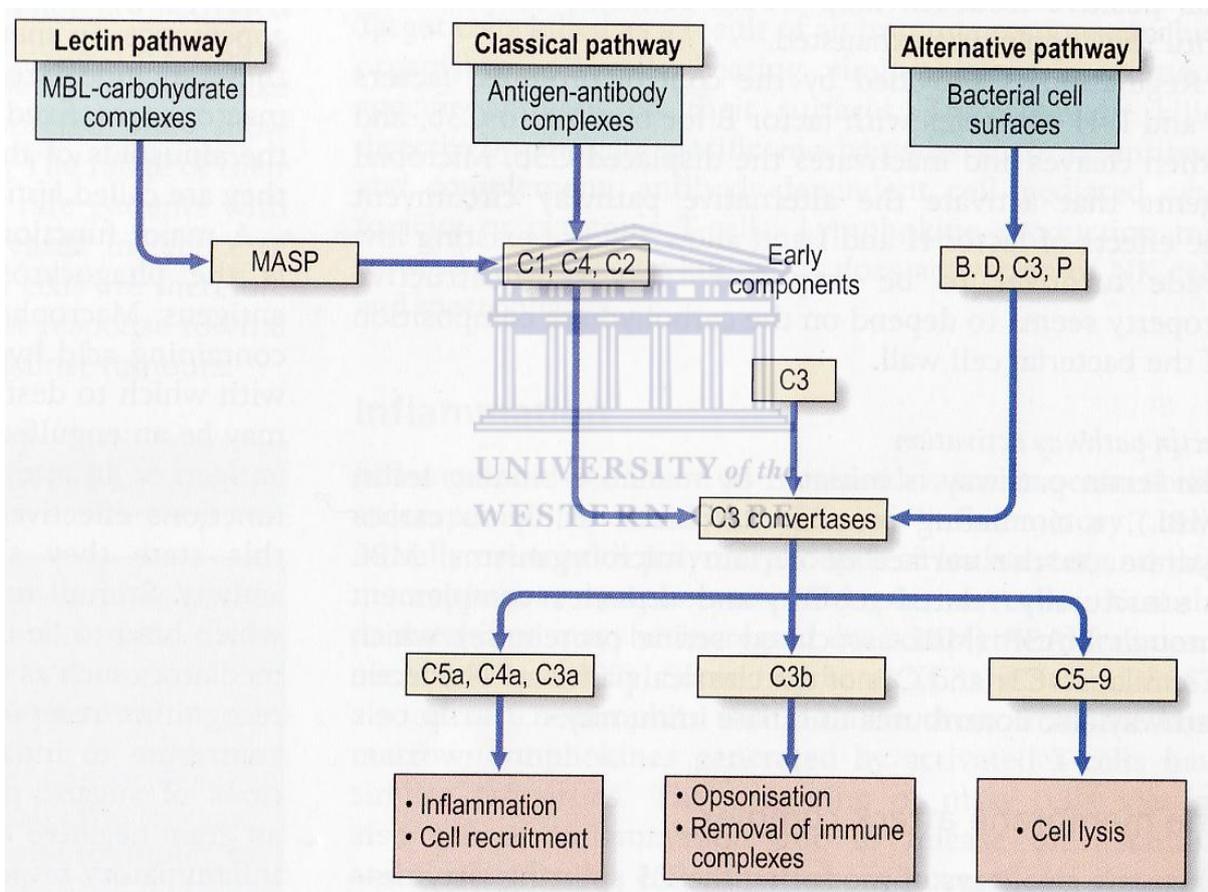
Figure 3.2: Compliment activation via the lectin, classical and alternate pathways (Underwood, 2004: 175)

3.1.3 Immune cells

Immune cells all originate in the bone marrow. These cells include lymphoid cells and myeloid cells. Each of these progenitor groups differentiate to form distinct cell types (Underwood, 2004).

Myeloid progenitor stem cells in the bone marrow differentiate into erythrocytes, platelets, neutrophils, monocytes (macrophages) and dendritic cells.   Stem cells from the lymphoid progenitor differentiate into natural killer (NK) cells, thymus (T) cells and bone marrow (B) cells. For T cells to develop, the precursor T cells migrates to the thymus, where they differentiate into two distinct types of T cells; the CD4+ Th cells and the CD8+ pre-cytotoxic T cells. In the thymus, two types of T helper cells are produced. The first is the Th1 cells which help CD8+ pre-cytotoxic cells differentiate into cytotoxic T cells. The second group of cells are the Th2 cells. These help B cells differentiate into plasma cells, which secrete antibodies (Mayor, 2000). Figure 3.3 below represents the various immune cells and how they develop within the body.

Figure3.3: T and B cell development (Underwood, 2004: 170)

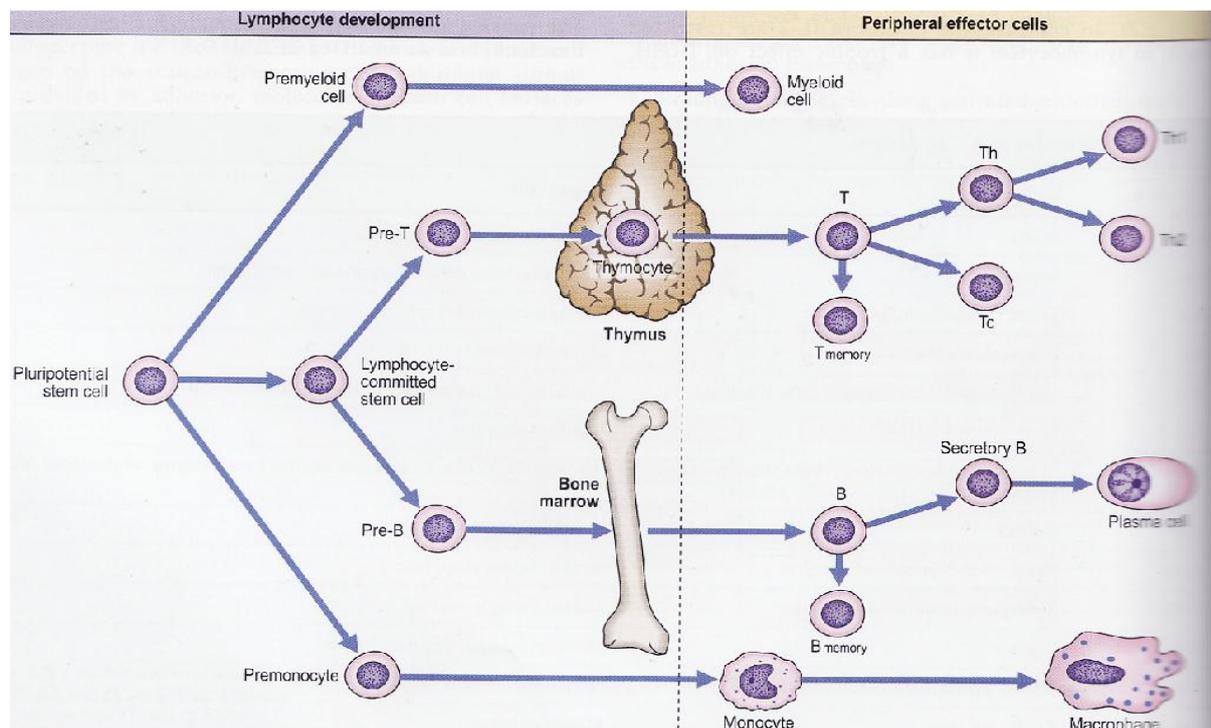### *3.1.3.1 Cells of the innate immune system*

These include phagocytic cells (monocyte/macrophages and PMNs), NK cells, basophils, mast cells, eosinophiles and platelets. They have pattern recognition receptors (PRRs) that recognize broad molecular patterns found on pathogens (pathogen associated molecular patterns, PAMPS). Antigen presenting cells (APCs) are heterogenous leukocytes vital in innate immunity. They include macrophages and dentritic cells. They link the innate immune system to the adaptive immune system by participating in the activation of helper T cells. APCs all express class II MHC molecules. Although B lymphocytes also express class II MHC molecules and also function as APCs, they are not considered part of the innate immune system. In addition, certain other cells (thymic epithelial cells) can express class II MHC molecules and can function as APCs (Mayor, 2000).

3.1.3.2 Cells of the acquired immune system

B and T lymphocytes make up the acquired immune system. After antigen exposure, B cells differentiate into plasma cells. Their main function is now antibody production. T cells differentiate into either T cytotoxic (Tc) or T helper (Th) cells of which there are two types, Th1 and Th2 cells (Mayor, 2000).

3.1.3.3 Non-specific immune cells

Large granular lymphocytes (LGL) or NK cells are identified by the CD56 and CD16 on the

cell surface. They are responsible for targeting virus-infected cells. When exposed to IL-2 and IFN-γ, NK cells become lymphokine-activated killer (LAK) cells which effectively eliminate malignant cells. NK and LAK cells contain both a killer activating receptor (KAR) and a killer inhibiting receptor (KIR). When the KAR encounters its ligand, a killer activating ligand (KAL), the NK or LAK cell eliminates the pathogen. When the KIR also binds to its ligand then killing is inhibited even if KAR is bound to a KAL. Ligands for KIR are termed MHC-class I molecules. Healthy cells express MHC class I molecules on their surface, while malignant and virus infected cells down regulate this expression, resulting in the selective elimination of cells (Mayor, 2000).

Killer (K) cells are any cells that mediate antibody-dependent cellular cytotoxicity (ADCC). In ADCC, antibody acts as a link between the K cell (containing an F*c* recptor) and the pathogenic cell. Killer cells include NK, LAK and macrophages (have an F*c* receptor for IgG antibodies) and eosinophils (have an F*c* receptor for IgE antibodies) (Mayor, 2000).
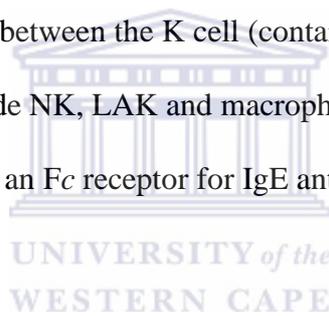
3.1.4 Cytokines and immune regulation

3.1.4.1 Cytokines

Cytokines are a diverse group of non-antibody proteins that act as mediators between cells. Originally thought to be products of immune cells to mediate and regulate immune activity, they are also produced by cells other than those found in the immune system. They may also have non-immune related functions. Three main types of cytokines exist: monokines, which are produced by mononuclear phagocytic cells; lymphokines, produced by activated lymphocytes (mainly Th cells) and interleukins that act as mediators between leukocytes,

(Mayor 2000). Figure 3.4 indicates the various cytokines and the cells they are released from.

Cytokines produce cascades that up or down regulate the production of other cytokines. They influence the action of other cytokines by acting either additively, antagonistically or synergistically. Cellular responses to cytokines normally take a few hours as they require new mRNA and protein synthesis (Mayor, 2000).
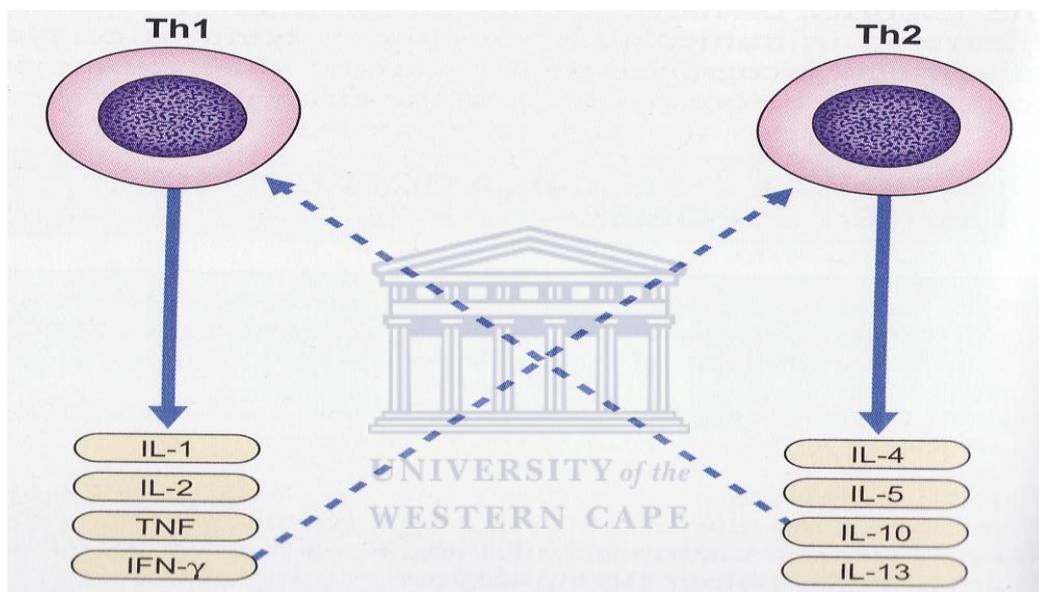
Figure 3.4 Various cytokines secreted by Th1 and Th2 cells (Underwood, 2004: 174)

IL-10 is produced by activated macrophages and Th2 cells. It functions mainly as an inhibitory cytokine in the acquired immune system. It inhibits the production of IFN-γ by Th1 cells. This shifts immune responses toward a Th2 pathway. It also inhibits cytokine production by activated macrophages. This results in a down regulation of the immune response, inhibiting APC activity and cytokine production. IFN-γ may also be produced by Tc and NK cells. It has numerous functions in both the innate and adaptive immune systems. The anti-proliferation activity of IFN-γ is weaker than that of INF-α and IFN-β, but it is most

effective at activating macrophages and inducing class I and II MHC expression (Mayor, 2000). Additional immune-regulating functions of IFN-γ are represented in figure 3.5 below.
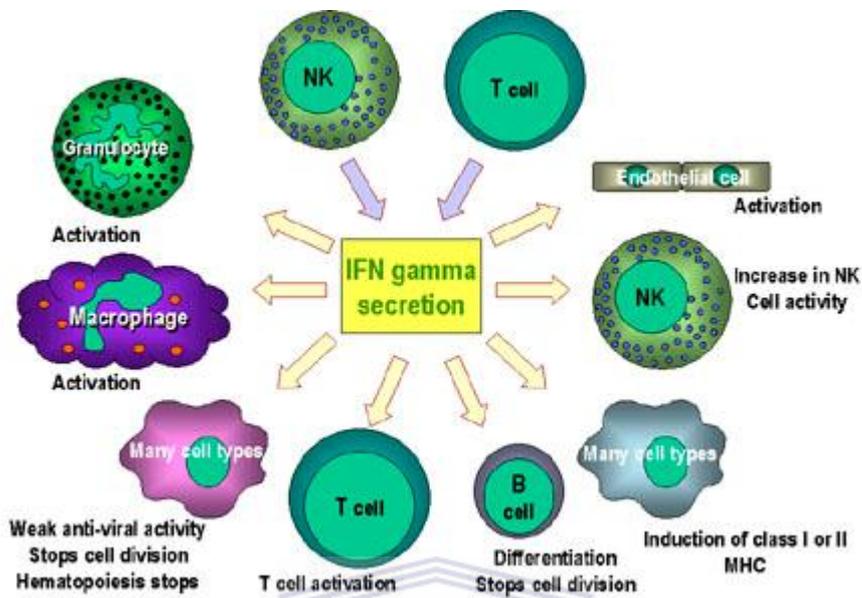
Figure3.5: Immunoregulatory effects of IFN-γ (Mayor, 2000)
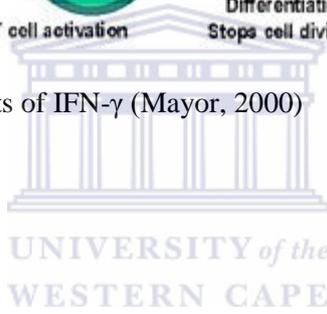
3.1.4.2 Immune regulation

Immune activity is regulated by the balance between the antigen-driven activation of lymphocytes and the negative regulatory influences that prevent or down regulate the immune response. Immune responses can also be regulated by antibodies. This happens either by antibodies competing with antigen receptors on B cells, preventing B cell activation, or by the antigen-antibody complex binding to the Fc receptor of the B cell, sending an inhibitory signal to the B cells. Regulatory T cells (Tregs) inhibit a sustained Th1 and Th2 cell response and prevent chronic (potentially damaging) responses. They do not prevent initial T cell activation, (Mayor, 2000). By avoiding a chronic inflammatory response and by maintaining Th1 and Th2 balance, both innate and adaptive immune systems remain balanced.

3.1.4.3 Th1/Th2 balance

After recognizing a specific antigen presented by an APC, Th cells initiate several processes vital to the immune response. These include the selection of appropriate effector mechanisms (B cell activation or Tc generation), inducing the proliferation of appropriate effector cells and enhancing the activities of other cells (macrophages and NK cells) (Mayor, 2000).

When naïve Th0 cells encounter antigen in secondary lymphoid tissues, they may differentiate into either inflammatory Th1 cells or a helper Th2 cells (each of which produce different cytokines). This differentiation depends on the surrounding cytokines, which is in turn influenced by the invading pathogen. Some antigens therefore stimulate IL-10 production (resulting in the production of Th2 cells) while other antigens stimulate IFN-γ production (which results in the production of Th1 cells). These Th1 and Th2 cells affect numerous other cells and influence the type of immune response. Cytokines that are produced by Th1 cells activate macrophages and Tc cell production, causing a cell-mediated immune response. Cytokines that are produced by Th2 cells activate B cells, resulting in antibody production (humoral immune responses). Th2 cytokines also activate granulocytes. Each pathway also inhibits the initiation of the other pathway. IFN-γ, for example, produced by Th1 cells inhibits the proliferation of Th2 cells while IL-10 produced by Th2 cells inhibits production of IFN-γ by Th1 cells. The immune response is therefore always directed to the type of response that is needed to deal with the specific pathogen encountered. Cell-mediated responses are induced in cases of intracellular pathogens while antibody responses result due to extracellular pathogens (Mayor, 2000).

3.1.5 Adaptive (acquired) immunity

Acquired immunity is recognized by three key features: the ability to discriminate between self and non-self molecules, the ability to memorize and recognize specific antigens and this recognition results in a very specific immune reaction (Mayor, 2000).

Adaptive immunity allows for the immune system to acquire a memory towards a specific pathogen. The initial encounter with an antigen results in the primary immune response. A repeated encounter with the same antigen results in the secondary response. Secondary responses occur more rapidly and more efficiently due to the presence of pre-formed antigen specific lymphocytes (Clark, 2009). This process is represented in figure 3.6.

Antigen specificity occurs when the lymphocyte surface receptor recognizes specific parts of the antigen (antigenic epitopes). The cell surface receptor of B lymphocytes is an immunoglobulin molecule. When this immunoglobulin is secreted by the B cell, it is known as an antibody (Clark, 2009).
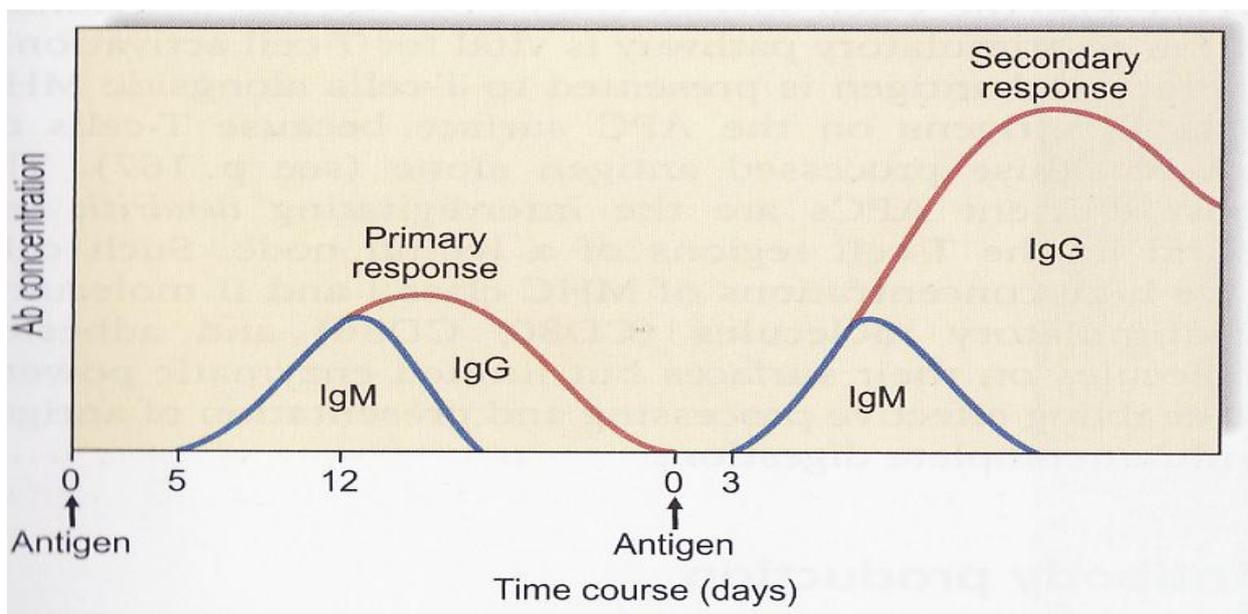
Figure 3.6: Primary and secondary immune responses (Underwood, 2004: 174)

3.1.5.1 Antibody molecules (immunoglobulins)

The basic structure of an antibody is made up of glycoproteins consisting of 2 heavy chains and 2 light chains. This leads to the formation of a molecule that has two "arms" with antigen-binding sites, and a single stem that has the F*c* portion which is not antigen specific and attaches to cellular immunoglobulin receptors.

3.1.5.2 Extracellular recognition

Antibodies are the primary defense against extracellular pathogens and result in immune responses through one of three mechanisms.

Neutralisation occurs by binding to the pathogen. The antibody blocks the link between the pathogen and their targets. Antibodies to bacterial toxins can prevent toxins from binding to the host cell by rendering the toxin ineffective (Clark, 2009; Mayor, 2000).

Opsonisation allows for specific receptors on various cells, including macrophages, neutrophils, basophils and mast cells to recognize and respond to a certain antigen. The F*c* region of the antibody interacts with F*c* receptors of the phagocytes, enhancing phagocytosis (Clark, 2009; Mayor, 2000).

Antibody binding to pathogens can also activate the complement system. Activation of complement results in pathogen lysis and enhances phagocytosis (Clark, 2009; Mayor, 2000).

3.1.6 *In vitro* and *in vivo* methods to monitor immune function

To thoroughly investigate the immune system, both *in vitro* and *in vivo* experimentation is required. *In vitro* investigation plays an important role in evaluating the release of various immune products which may increase antimicrobial potential. However, presumption based solely on *in vitro* findings may lead to incorrect beliefs regarding immune function as they lack the complexity of specific microenvironments and homeostatic controls found in humans. Such *in vitro* models may also be limited due to the use of the use of non-physiological target cells. After completing the *in vitro* model, it is largely required that the experiment be repeated in an animal model, to ensure that results have *in vivo* relevance and are not merely a by-product of the isolation process or the *in vitro* environment as these are overly simplistic (Silliman and Wang, 2006).

Due to ethical and moral concerns regarding animal testing, many immune assays are done *in vitro*. Animal models are useful *in vivo* to represent the human physiology. Due to the complexity of the immune system, especially with regards to issues relating to the discovery of different lymphocytes, proper distinction between T and B cells can only be properly recognized using animal models with intact immune systems with selective gene knockouts and chimeras. Reconstituting lymphocyte development and immune responses *in vitro* has proven to be problematic. Meaningful studies, particularly relating to the adaptive immune system, cannot be properly conducted outside an intact animal. This fact has lead to the development of many genetically modified animals to improve understanding of the basic mechanism of adaptive immune activity. Moreover, the role of complement in host protection can not be completed without using gene-targeting technology in mice (Silliman and Wang, 2006).

In vivo models also have their drawbacks. One such limitation is that animal models do not accurately represent various human states, especially human pathology. This increases the need for *in vitro* data to better correlate with *in vivo* function (Silliman and Wang, 2006). To fully understand human immunity, animal models are essential, as well as germ-line mutations and gene-targeting technologies. *In vitro* models are however useful as screening profiles, to assess the selectivity of certain herbs and drugs, and determine certain immune-side effects of various compounds. *In vivo* models, on the other hand, are more specific to asses the therapeutic efficacy of these various drugs and herbal compounds and to help discover previously unknown disease indications for these compounds (Calipher Life Sciences, 2008).

As far as we are aware, this is the first study of its kind used to examine *Artemisia afra*. *In vitro* methods were employed as they offer a quick and effective method to determine immunological effects. Running *in vitro* assays via whole blood cell cultures was preferred as they represent the *in vivo* environment more accurately than isolated monocytes in terms of the immune system (Pool and Bouic, 2001).

The following study aims to validate *in vitro* methods to monitor immune functions. A whole blood culture assay will be used. Inflammatory effects will be studied using IL-6, while immune function via Th1 and Th2 pathways will be monitored using IL-10 and IFN-γ cytokines.

3.2 Methods

3.2.1 Inflammatory activity

Blood samples were collected from healthy male donors by venous puncture into citrate-containing sterile blood collecting tubes (after informed consent). Whole blood cultures were

conducted within 4 hours after collection. All methods were performed under sterile conditions. Stimulated whole blood cultures contained 1 volume of 10 µg/ml endotoxin in dimethyl sulfoxide (DMSO), 10 volumes of blood and 89 volumes of RPMI-1640 medium. Unstimulated blood contained 1 volume DMSO, 10 volumes of blood and 89 volumes of RPMI-1640. The blood (stimulated or unstimulated; 200 µl/well) was incubated at 37 ˚C for 24 hours. Culture supernatants were then collected and assayed for LDH and IL-6.

3.2.2 Th1/Th2 activity

Stimulated whole blood cultures contained 10 volumes of blood, 89 volumes of RPMI-1640 medium and 1 volume of 1.6 µg/ml phytohaemagglutinin (PHA) in RPMI. The final concentration of PHA in the stimulated blood mixture was 1.6 µg/ml. For unstimulated whole blood cultures, no additions were made to the diluted blood. Stimulated or unstimulated blood (200 µl/well) cultures were incubated for 48 hours. Culture supernatants were then used for IL-10 and IFN-g assays.

3.2.3 Cytotoxicity assay

A commercially available chromogenic lactate dehydrogenase (LDH) assay kit (Biovision, USA) was used to determine the cytotoxicity of samples. Cell culture supernatants were collected from blood samples incubated for inflammatory activity. Samples were transferred to a 96-well plate. For the 100 % toxicity standard a control blood sample (250 µl) received 2.5 µl of a 10 % volume/volume Triton X 100 solution. The blood immediately lysed. This lysed sample was mixed and an aliquot of the lysed blood was diluted in saline at a ratio of 1:10. The lysed blood sample was used as the 100 % cytotoxicity control. Dilutions of this sample were used to construct a standard curve. The standards were also transferred to the

48

96-well plate. LDH substrate was added to samples. Well contents were mixed (by tapping the plate). Absorbance readings were taken at 10 minute intervals at 592 nm. Between readings the plate was incubated away from light at ambient temperature. The cytotoxicity of the samples were read off a standard curve constructed using the standards.

3.2.4 Cytokine analysis

Cytokine concentrations were determined using ELISA's . ELISA kits were purchased from e-Bioscience, Denmark. Kits contained all the reagents required for the assays. The plates were coated with capturing antibody (50 µl/well) diluted appropriately with coating buffer. This was incubated overnight at 4 ˚C. The plates were washed 5 times with wash buffer consisting of autoclaved PBS containing 0.05 % Tween-20. Non-specific binding sites were blocked using assay diluent (100 µl/well) for 1 hour at room temperature. Plates were again washed as before. Cell free supernatants (50 µl/well) were added to the each plate. The assay was standardized using a 2-fold serial dilution of a cytokine standard on each plate. The plates were sealed and incubated for 2 hours at room temperature. The plates where then washed as before after which detection antibody (50 µl/well) was added. The plates were incubated for 1 hour at room temperature. After washing the plates five times, avidin-horseradish peroxidise (HPR) was added (50 µl/well) to detect bound cytokines. Plates were incubated for 30 minutes at room temperature and then washed 7 times with wash buffer. Substrate solution was added (50 µl/well) and incubated for 15 minutes in a dark area. The reaction was stopped by adding 50 µl/well stop solution. Absorbances were read on a plate reader at 450 nm.

3.3 Results and discussion

3.3.1 Cytotoxicity

Total LDH in the blood culture was obtained by lysing diluted whole blood with Triton X100 % detergent. The total LDH from the blood cultures was considered to be 100 % toxicity (after being released into the medium). A standard curve was constructed from dilutions of the 100 % toxicity sample. This curve (Figure 3.7) indicates good correlation between the absorbance values and the % toxicity (R^2=0.983).

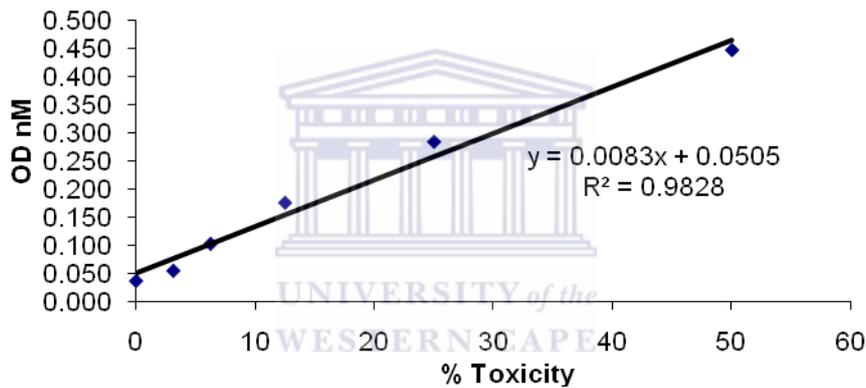**Figure 3.7:** Standard curve of toxicity.

3.3.2 Inflammatory activity

Culture supernatants from LPS stimulated cultures were analysed for inflammatory activity using IL-6 as a biomarker. Figure 3.8 is a representative example of a standard curve obtained using the IL-6 ELISA kit. It shows that there is good correlation between the absorbance values and the cytokine concentration (R^2=0.993).
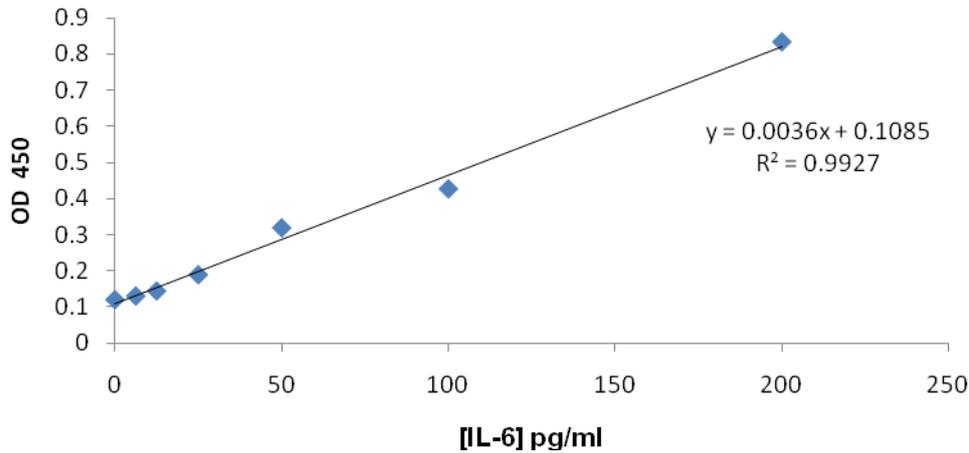
Figure 3.8: Standard curve for IL-6. The kit standards were assayed by DAS ELISA according to the manufacturer's instructions.

The IL-6 by stimulated whole blood cultures was 3418.3±4588.1 pg/ml while the absorbance for unstimulated blood was 31.9±35.9 pg/ml (n=9±SD). The data shows that there is statically significant difference between IL-6 secretions of the stimulated and unstimulated whole blood cell cultures ($P<0.042$) showing that this biomarker assay can be used to monitor inflammatory activity.

3.3.3 Humoral immunity

Culture supernatants from PHA stimulated cultures were analysed for humoral immune activity using IL-10 as a biomarker. Figure 3.9 is a representative example of a standard curve obtained using the IL-10 ELISA kit. It shows that there is good correlation between the absorbance values and the cytokine concentration ($R^2=0.999$).

The figure shows a standard curve plotting OD 450 on the y-axis (0 to 0.8) against [IL-10] pg/ml on the x-axis (0 to 400), with the equation:

$$y = 2\text{E-}06x^2 + 0.0015x + 0.1359$$
$$R^2 = 0.9999$$

Figure 3.9: Standard curve for IL-10. The kit standards were assayed using the DAS ELISA according to manufacturer's instructions.

The IL-10 released by stimulated whole blood cultures was 102.1±3.5 pg/ml while the absorbance for unstimulated blood was 2.0±4.9 pg/ml (n=9±SD). The data shows that there is statically significant difference between IL-10 secretions of the stimulated and unstimulated whole blood cell cultures (P<0.001) showing that this biomarker assay can be used to monitor inflammatory activity.

3.3.4 Cellular immunity

Culture supernatants from PHA stimulated cultures were analysed for cellular immune activity using IFN-γ as a biomarker. Figure 3.10 is a representative example of a standard curve obtained using the IFN-γ ELISA kit. It shows that there is good correlation between the absorbance values and the cytokine concentration (R^2=0.999).
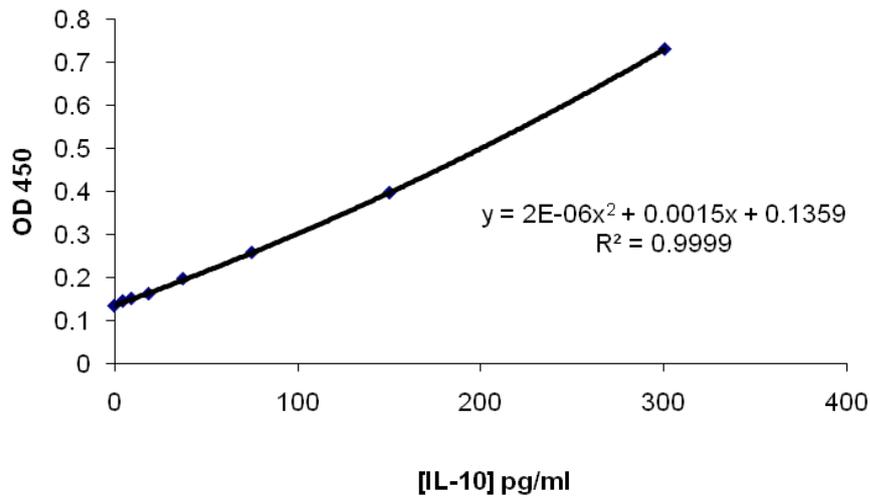
The chart shows OD 450 on the y-axis (0 to 0.5) versus [IFN-γ] pg/ml on the x-axis (0 to 600), with the equation:

$$y = -3E\text{-}07x^2 + 0.0009x + 0.0882$$
$$R^2 = 0.9991$$
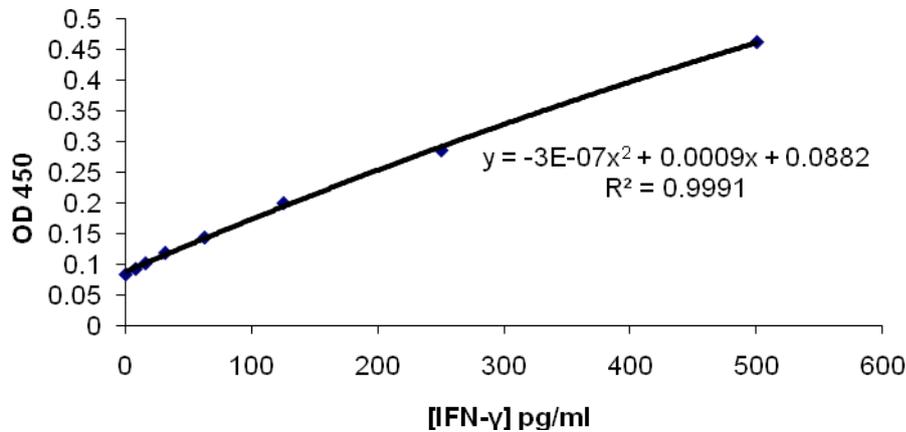
Figure 3.10: Standard curve obtained for IFN-γ. The kit standards were assayed by the DAS ELISA according to the manufacturer's instructions.

The IFN-γ released by stimulated whole blood cultures was 3540.5 ± 2126.8 pg/ml while the absorbance for unstimulated blood was 23.2 ± 15.4 pg/ml ($n=9\pm SD$). The data shows that there is statically significant difference between IFN-γ secretions of the stimulated and unstimulated whole blood cell cultures ($P<0.001$) showing that this biomarker assay can be used to monitor inflammatory activity.

3.4. Conclusion

These results show that mitogen activated whole blood cultures can be used as an effective method to monitor immune function. This study confirms earlier work that shows that cytokines are a sensitive indicator of immune modulation (Pool and Magcwebeba, 2008).

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Chapter 4: The immune-modulating activity of *Artemisia afra,* one of the indigenous herbs of South Africa

4.1 Introduction

Artemisia afra (Asteraceae) is one of the oldest and best-known of South Africa's indigenous medicines. The plant is also found in parts of Eastern Africa and is used as a medicine in Ethiopia (van Wyk and Gericke, 2005). Wormwood, its common name, is derived from the Old English word 'wermode', meaning "mind preserver" (Mars, 2007). African tribes like the Zulu, Xhosa, and Sotho all have different traditional names for *Artemisia afra*. One of these popular names include 'wilde als' or 'als' in Afrikaans, translating as 'all' or 'everything' as the herb is a common folk remedy for nearly every ailment (Mukinda, 2007).

Therapeutically *Artemisia afra* is used as an analgesic and anthelmintic. Because of the great diversity of ailments treated with *Artemisia afra*, it is widely considered a "cure all" remedy in South Africa and its neighbouring countries (Mukinda, 2007; van Wyk and Gericke, 2005; van Wyk and Wink, 2004).

Artemisia afra contains various phenolic compounds such as borneol and other volatile oils that have anti-bacterial and anti-fungal action (van Wyk and Wink, 2004). This helps to explain its popularity to help treat bacterial infections. Bacterial infections traditionally treated with *Artemisia afra* include gumboils, sore throats, ear infections, whooping cough and certain bronchial complaints (Mukinda, 2007; van Wyk and Gericke, 2005). The scopoletin (coumarin) compound found in *Artemisia afra* has been attributed with anti-viral, hypotensive and hypocholesterolaemic effects, (Manuele et al., (2006). The presence of scopoletin helps justify traditional uses relating to viral conditions such as measles, mumps, colds and influenza.

In 2004 van Wyk and Wink reported that *Artemisia afra* has antihistamine activity. This potentially explains its traditional use in the treatment of asthma and other allergies. A possible mechanism of action in this regard is by down regulating the Th2 pathway. Should *Artemisia afra* be capable of doing so by up regulating cell-mediated immunity, viruses and infections such as tuberculosis (TB) could be potentially treated using this herb. Parasites (popularly treated using *Artemisia afra*) such as intestinal worms and malaria, are addressed via the humoral immune system. Literature therefore suggests that *Artemisia afra* should have immune modulating activity; being able to suppress and activate both Th1 and Th2 pathways, depending on the stimulus encountered by the host, rather than acting on only one of the pathways.

Few studies have been conducted on indigenous herbs, including *Artemisia afra.* Due to the harmful effects of thujone (possible addiction and cerebral dysfunction resulting from neurotoxicity), high doses and chronic use of *Artemisia afra* are discouraged. Sporadic ingestion of small amounts of thujone in medicinal preparations and in food products does not appear to be a health risk (van Wyk and Gericke, 2005). Aqueous extracts appear to be non-toxic when administered acutely and there is a low chronic toxicity potential. Hepatotoxic effects could result from high doses (Mukinda, 2007).

The aim of this study is to investigate if *Artemisia afra* has immunomodulating activity *in vitro*, using whole blood culture assays as described in the previous chapter.

4.2 Materials and Methods

4.2.1 Plant Extraction

Artemisia afra samples were obtained and prepared for analysis. A 20 % (w/v) extract was prepared using 94.4 % ethanol by Parceval (Pty) Ltd pharmaceuticals (South Africa). Aerial parts of the plant were milled (sieve size~ 2 - 3 mm) and mixed with 94.4 % ethanol at 20 g *Artemisia afra* per 100 ml ethanol. The mixture was shaken for brief periods during overnight extraction. The extracts were then pressed to separate the resulting tincture from the milled leaves. The tincture was filtered and the extracts were stored at 20 ˚C. The extract was air dried and re-suspended in DMSO to obtain a 50 % (wet leaf w/v) extract.

4.2.2 Anti-inflammatory activity

Blood samples were collected from healthy male donors by venous puncture into citrate-containing sterile blood collecting tubes (Lasec, SA). Consent was obtained from all volunteers. Whole blood cultures were conducted within 4 hours after blood collection. All methods where performed under sterile conditions.   Stimulated whole blood cultures contained 1 volume of 10 µg/ml endotoxin in dimethyl sulfoxide, 10 volumes of blood and 89 volumes of RPMI-1640 medium (Sigma, USA). Unstimulated blood contained 1 volume DMSO, 10 volumes of blood and 89 volumes of RPMI-1640. Replicate samples of various dilutions of *Artemisia afra* were transferred to wells of a 96-well plate (2µl/well). The (stimulated or unstimulated) blood (200 µl/well) was added to the *Artemisia afra* samples and incubated at 37 ˚C for 24 hours. Culture supernatants were then collected and assayed for LDH and IL-6.

4.2.3 Th1/Th2 activity

Blood samples were prepared in a manner similar to the preparation using endotoxin (LPS). Stimulated whole blood cultures contained 10 volumes of blood and 89 volumes of RPMI-1640 medium (Sigma, USA) and 1 volume of 1.6mg/ml PHA (Sigma, USA) in RPMI (Sigma, USA). For unstimulated whole blood cultures, no additions were made to the diluted blood. Various concentrations of *Artemisia afra* extracts (2 µl/well) were added to each well. Stimulated or unstimulated blood was added to wells (200 µl/well). Cultures were incubated for 48 hours. Culture supernatants were then used to perform IL-10 and IFN-γ assays.

4.2.4 Cytotoxicity

A commercially available chromogenic LDH assay kit (Biovision, USA) was used to determine the cytotoxicity of *Artemisia afra* samples. Cell culture supernatants were collected from blood samples incubated for inflammatory activity. Samples were transferred to a 96-well plate. For the 100 % toxicity standard a control blood sample (250 µl) received 2.5 µl of a 10 % volume/volume Triton X 100 solution. The blood immediately lysed. An aliquot of the lysed blood was diluted in saline at a ratio of 1:10. The lysed blood sample was used as the 100 % cytotoxicity control. Dilutions of this sample were used to construct a standard curve. The standards were also transferred to the 96-well plate. LDH substrate was added to samples. Well contents were mixed to produce a homogenous solution by tapping the plate. Absorbance readings were taken at 10 minute intervals at 592 nm. Between readings the plate was incubated in a dark area at ambient temperature. The cytotoxicity of the samples were read off a standard curve constructed using the standards.

4.2.5 Cytokine analysis

Double antibody sandwhich (DAS) ELISAs (e-Bioscience, Germany) were used to quantify cytokine production from *Artemisia afra*-containing supernatants of the whole blood cultures. Nunc maxisorp (NuncTM, Denmark) 96-well plates were used for the assays. The kit contained all diluents and reagents required. The assay was performed according to manufacturer protocol. Primary antibody (50 µl/well) against the respective cytokine was coated onto the plate. The coat was incubated at 4 ˚C overnight. Plates were then washed 5 times with phosphate buffered saline (PBS) containing 0.05 % Tween. Plates were blocked with an assay buffer (100 µl/well) for 1 hour and washed as before. Cytokine standards and samples were added to the wells (50 µl/well). The plate was incubated on a shaker at ambient temperature for 2 hours. Plates were washed 5 times with wash buffer. Biotinylated secondary (detection) antibody against the respected cytokine was added (50µl/well). Plates were incubated on a shaker at ambient temperature for 1 hour and washed 5 times. Avidin-perioxidase conjugate (50 µl/well) was added to the wells and incubated for 30 minutes on a shaker at ambient temperature. The plates were washed 7 times with buffer and substrate was added (50 µl/well). The plates were incubated in a dark area for 15 minutes. The reaction was stopped using 2M H_2SO4 as stop solution (50 µl/well). Plates were then read on a plate reader spectrophotometer at 450 nm. Standard curves for each ELISA plate were generated using Excel. The standard curve was used to determine the amount of cytokines produced by the blood cultures.

4.2.6 Statistical analysis

Experiments were all performed thrice, each in triplicate. Data was statistically analysed using one-way ANOVA (P<0.05) and regression analysis. All data is presented as a mean ± standard deviation.

4.3 Results and discussion

4.3.1 Cytotoxicity of *Artemisia afra*

Based on the results of previous studies (Mukinda, 2007), no toxicity of *Artemisia afra* was expected. Figure 4.1 is a diagrammatical representation of the data obtained for the LDH assay. Data shows that *Artemisia afra* was significantly toxic (P<0.050) at 5 000 µg/ml, the highest concentration tested. At this concentration, 39.9 % of cellular LDH was leached out of cells. This was much higher than the level found in the control (DMSO) cultures. At lower concentrations *Artemisia afra* is not cytotoxic.
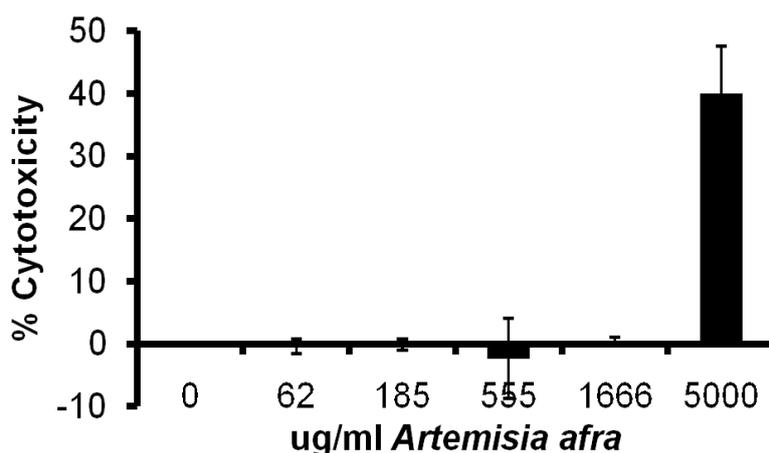

Figure 4.1 Cytotoxicity of *Artemisia afra.* Each point represents the mean and standard deviation of triplicate assays. Data is expressed as a % of the total cellular LDH. *Artemisia afra* is significantly toxic (P<0.050) at 5000µg/ml.

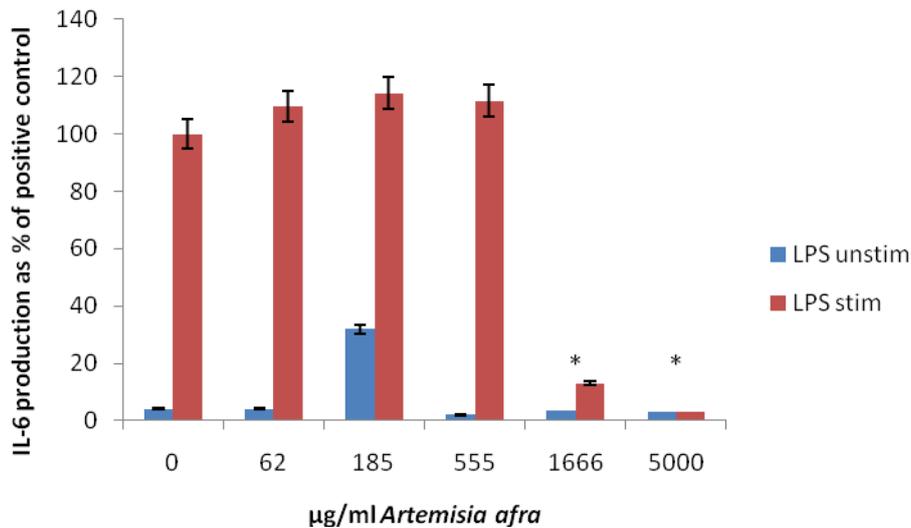### 4.3.2 The anti-inflammatory effect of *Artemisia afra*

Figure 4.2:   The inflammatory activity of *Artemisia afra*. Data represents the mean and standard deviation of triplicate assays (n=9). Results are expressed as a % IL-6 secreted by whole blood cultures in comparison to the 0 μg/ml *Artemisia afra* positive control. Significant changes (P<0.001) are indicated (*) at the 1 666 and 5 000 μg/ml concentrations.

IL-6 forms a vital mediator in fever and in the acute phase response of immune activation. It is mainly secreted by macrophages in response to microbial pathogens (Mayor, 2000). Results obtained from all the donors were similar and indicate that *Artemisia afra* has an effect on the stimulated whole blood cultures. At a high concentration (1 666 μg/ml) *Artemisia afra* significantly decreased (P<0.01) the amount of IL-6 produced (16 % compared to the control). This indicates that *Artemisia afra* could act as an anti-inflammatory agent.

Traditional uses of *Artemisia afra* include colds, influenza, mumps and wounds (Kramer, 2006, van Wyk and Wink, 2004). The decrease in IL-6 production induced *by Artemisia afra*

would be beneficial in these conditions as it could bring about symptomatic relief from fever caused by infection and also reduce the amount of inflammation at wound sites. Other traditional uses include joint pain and rheumatism (van Wyk and Wink, 2004). Research has indicated that IL-6 levels are increased in rheumatoid arthritis and mechanisms of IL-6-target blockade (such as those demonstrated by *Artemisia afra*) are currently being research as a potential treatment (Nishimoto and Norihiro, 2006). Increased IL-6 levels have been associated with an increased risk of intervertebral disc disease (IDD) commonly characterized by sciatica (Noponen-Hietala et al., 2005). *Artemsia afra* is traditionally used as a topical application for back pain and neuralgia (Kramer, 2006). These results indicate that it may have potential in the treatment of these conditions.

As *Artemisia afra* is considered a traditional "cure-all", it is used for a wide variety of complaints including diabetes (Mukinda, 2007). Increased IL-6 levels have also associated with obesity, non-insulin dependent diabetes mellitus (Herder et al., 2007) and the metastasis of cancer (Smith, 2001). In 2004, Tackey et al. described elevated IL-6 levels as being a key factor in the immunopathology of systemic lupus erythematosus (SLE) and may be directly responsible for tissue damage. Through inhibiting IL-6 expression, *Artemisia afra* may also prove to be beneficial in treating these conditions.

4.3.3 The effects of *Artemisia afra* on humoral immunity

IL-10 cytokine synthesis by whole blood cultures upon stimulation with PHA was used as a biomarker to monitor the humoral immune pathway.
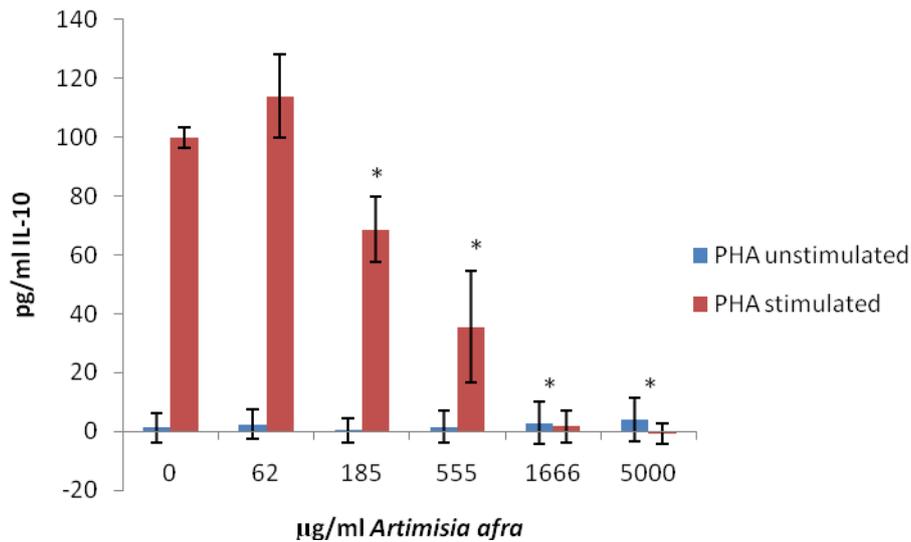
Figure 4.3: The effects of *Artemisia afra* on humoral immunity. Data represents the mean and standard deviation of triplicate assays (n=9). Results are expressed as a % IL-10 secreted by whole blood cultures in comparison to the 0 μg/ml *Artemisia afra* control. Significant changes ($P<0.001$) are indicated (*).

IL-10 acts mainly as an anti-inflammatory cytokine, inhibiting the Th1 pathway. Due to Th2 activation by IL-10, B cells and antibody production are activated. This plays an important role in acquired immunity and humoral responses to extracellular bacteria and parasites (Kumar and Clark, 1999; Mayor, 2000). Results show that *Artemisia afra* significantly reduces ($P<0.001$) the amounts of IL-10 expressed (Figure 4.3). This supression of the Th2 pathway may be useful in the treatment of asthma, reducing the activation of granulocytes, coinciding with its traditional use in asthma and other allergic conditions.

Although various digestive complaints are traditionally treated with *Artemisia afra*, these complaints are normally limited to conditions such as gastritis, flatulence, dyspepsia, *et cetera* (van Wyk and Wink, 2004) and not conditions such as food poisoning caused by *Chlostriduium species, Escherichia coli*, *Vibrio cholera* and other extracellular bacteria.

Although this study did not investigate the anti-microbial properties of *Artemisia afra* against these species, results produced still continue to coincide with traditional herbal folk-lore.

Artemisia species derive their common name (wormwood) from their traditional value in eliminating intestinal worms, or helminths. Basophils are recruited to the infection site from the circulation, limiting parasitic spread and aiding in pathogenic excretion from the host organism, (hence the "anthelmintic" action traditionally ascribed to *Artemisia afra*). Although basophils are incorporated via the Th2 pathway, their functions are regulated by cytokines not included in this study (IL-4, IL-5, IL-9 and IL-13) (Mitre and Nutman, 2006 and Paul, 2003).

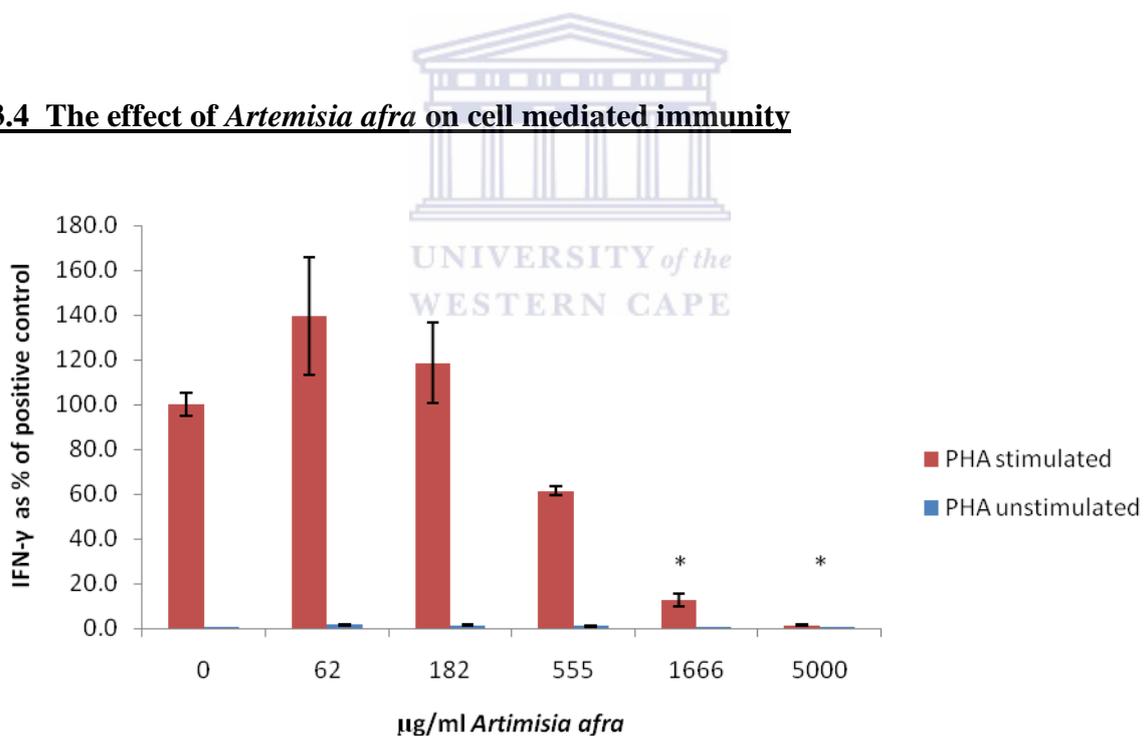### 4.3.4 The effect of *Artemisia afra* on cell mediated immunity

Figure 4.4 The effects of *Artemisia afra* on cellular immunity. Data represents the mean and standard deviation of triplicate assays (n=9). Results are expressed as a % IFN-γ secreted by whole blood cultures in comparison to the 0 μg/ml *Artemisia afra* control. Significant changes ($P<0.001$) are indicated (*).

IFN-γ plays a regulatory role in both innate and acquired immunity. By encouraging Th1 pathways, it results in increased macrophage activity and increased anti-viral activity via NK cell activity (Mayor, 2000). Because of this increase in cell-mediated immunity, it plays a key role in host defences against intracellular bacteria and viruses.

Results indicate a significant decrease in cytokine production (P<0.001) at the 1 666 µg/ml concentration (32.7 % compared to the control). *Artemisia afra* is commonly used for various viral infections such as colds, influenza and mumps, although all assays performed indicate a decrease in anti-viral activity. Malaria is caused by *Plasmodium malariae, Plasmodium vivax, Plasmodium ovale* or *Plasmodium falciparum*. It is endemic in Africa and is one of the most common conditions traditionally treated using *Artemisia afra* (Beers, 2006; Mukinda, 2007). The data obtained from these assays shows that IFN-γ was not significanly increased, as would normally be necessary in cases of intracellular parasitic invasion.

The study did not, however, investigate for any direct anti-microbial action against *Plasmodium species* nor any viral pathogens and it may possibly have an effect on the microbe itself. As confirmed under the inflammatory section of this study, using *Artemisia afra* may provide symptomatic benefit to those suffering from malaria and viral infections by acting as an anti-pyretic and decreasing fever. Fever is one of the classical symptoms of numerous infections, including infuenza and malaria (Beers, 2006).

Scientific literature focuses mainly on a different species of *Artemisia, Artemsia annua* (commonly known as Chinese wormwood or sweet wormwood) as having anti-malarial effects. The main pharmacological active compound in this species has been found to be artemisinin, a sesquiterpene lactone. Although *Artemsia afra* does contain sesquiterpene lactones, it does not contain artemisinin (van Wyk and Wink, 2004).

4.4 Conclusion

Artemisia afra is one of South Africa's most common herbs traditionally used as a medicine to treat a wide variety of complaints ranging from bacterial, viral and parasitic infections. Results show that *Artemisia afra* decreases the production of IL-10. This supression of the Th2 pathway may be useful in the treatment of asthma, reducing the activation of granulocytes, coinciding with its traditional use in asthma and other allergic conditions.

Results are also cohesive with traditional uses of the plant regarding inflammatory pathways. Data shows that *Artemisia afra* acts as an inflammation inhibitor. At higher concentrations, the amount of IL-6 is decreased. This demonstrates a potential ability to reduce the effects of auto-immune conditions such as RA and SLE. Conditions such as arteriosclerosis, rheumatic pains, sciatica, neoplastic metastasis and non-insulin dependant diabetes mellitus have all been associated with abnormally high levels of IL-6. *Artemisia afra* could play a promising role in the treatment and prevention of these diseases.   Fever is associated with numerous bacterial, viral, and parasitic infections, such as influenza, colds, mumps and malaria (Holmes, 2007). In these conditions, symptomatic relief could be brought about by using *Artemisia afra* to decrease the fever. Further studies will be needed to better explore these avenues of immunoregulation, as well as the antimicrobial effects of *Artemisia afra* on parasitic and viral pathogens and the long term effects of the herb on the immune and other physiological systems.

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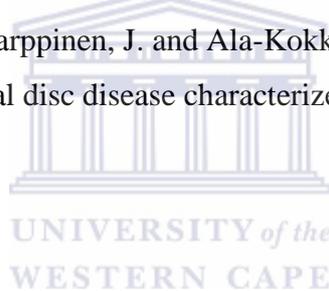
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Chapter 5: Summary, Conclusion and Recommendations

5.1 Summary

Cytokines play an important role in inducing and balancing immune defence mechanisms. IL-6 is responsible for regulating inflammation. It induces initial acute phase inflammation via innate processes. Via Th1 pathways, cellular immune processes result in NK cells up regulating IFN-γ production, vital to eliminating viral and intracellular bacterial infections. Th2 pathway activation results in T regulatory cells producing IL-10. This induces B cell proliferation and antibody production. These humoral responses are vital in eliminating extracellular and parasitic infection. It also plays a key role in hypersensitivity reactions against allergens (Clark, 2009; Mayor, 2000 and Paul, 2003).

Because of these key roles, these three cytokines were used as biomarkers in an *in vitro* study examining the immune regulating effects of the indigenous herb, *Artemisia afra.* IL-6 was used as a biomarker to indicate inflammatory activity. IFN-γ was used to indicate cellular immune activity, while IL-10 was used to indicate humoral activity. Herbal forms of medication are becoming increasingly popular within the general population, and medical practitioners are not well-equipped to address this growing popularity. Unpleasant side effects and potentially harmful adverse effects become common when the herb is taken incorrectly, or when it is taken together with certain pharmaceutical medications (Rai, 2005).

Limited literature exists regarding the biological and immune activities of herbs, and even less so regarding local herbs. Past studies involving indigenous herbs have focused on pharmacokinetic effects using HPLC and GC-MS. Antimicrobial studies have also been done on numerous South African species. No previous studies have been done on *Artemisia afra*, examining its effects on the immune system, despite its wide range of traditional indications ranging from various pathogenic infections to inflammatory and auto-immune

conditions. A quick and effective method of screening indigenous herbs for immune regulating effects is essential. The aim of this study was to determine if immunomodulating activities of herbs could be effectively validated using *in vitro* methods, and to determine the effects of *Artemisia afra* on inflammation and immune regulation.

Results were consistent with traditional uses of *Artemisia afra* such as fever, asthma and various conditions requiring a decrease in the amount of IL-6 produced such as sciatica, diabetes mellitus and rheumatoid arthritis. Results also coincided with traditional uses involving bacterial, viral and parasitic infections such as sore throats, influenza and malaria, where decreasing fever would be beneficial and bring about symptomatic relief.

5.2 Conclusion

Herbal medications can be effectively screened for immune regulating activity *in vitro* using whole blood cultures. *Artemisia afra* is cytotoxic at high concentrations. It appears to be beneficial in some conditions traditionally treated with the herb, such as diabetes and joint pain. It does not, however, appear to directly stimulate the immune system to fight off bacterial, viral and parasitic infections.

It demonstrates an inhibitory effect on the inflammatory process, down-regulating IL-6 production at higher concentrations. This could be potentially useful in treating auto-immune conditions such as rheumatoid arthritis and systemic lupus erythematosus and other conditions such as diabetes mellitus, cancer metastases and arteriosclerosis, where IL-6 levels are excessively high. In terms of cellular and humoral immune processes, results indicate that the herb acts mainly as an immune suppressant, decreasing the amounts of IL-10 and

IFN-γ produced. This coincides with previous studies done on other *Artemisia* species. It may also be potentially valuable in the treatment of certain allergies.

5.3 Recommendations

Further investigation is needed to help validate the findings produced by this study, including that of *in vivo* studies and more extensive studies investigating the anti-microbial effects of *Artemisia afra* against pathogens such as *Plasmodium malariae, Influenza* species and various helminths. Similar studies should also be done evaluating other indigenous herbs. When preparing the herb sample, extracts closer to the traditional dosage form and concentration represent the ideal. Water extractions are more often used. Although tinctures are traditionally used, these are normally made using a low-medium percentage of alcohol.

The general public and practitioners of Western medicine need to be more educated regarding the correct use and physiological effects of herbs. This will lead to a better working knowledge of herbs, enabling them to be used more effectively and reducing the changes of undesired adverse effects. Scientists and traditional herbal practitioners should regularly communicate in terms of traditional uses and modern findings (such as cytotoxic effects at certain concentrations) as both populations have both much to learn and much to offer the other.