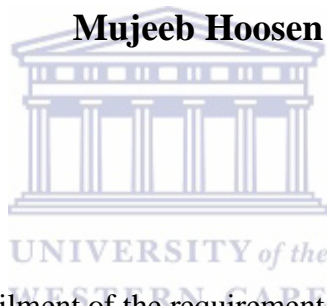


**The use of whole blood cell cultures as a model for assessing
the effects of Septilin™ on the immune system.**

By

Mujeeb Hoosen



Thesis submitted in partial fulfilment of the requirement for the degree of Magister Scientiae
(MSc) at the University of the Western Cape, South Africa.

August 2017

Supervisor: Professor E. J Pool

Abstract

The use of whole blood cell cultures as a model for assessing the effects of Septilin™ on the immune system.

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Key words: *Artemisia afra*, cell mediated immunity, cytokines, herbal medicinal products, human whole blood culture, humoral immunity, inflammation, RAW 264.7 macrophages, *Aspalathus linearis* (rooibos), Septilin™

In the past three decades there has been a huge increase in the use of herbal medicine globally. The active principles of these herbal medicines are mostly unknown with supportive evidence for safety and efficacy very rare. Septilin™ is a phytopharmaceutical formulation which is recommended for the treatment and management of various infections. It has been claimed to have immunomodulatory actions that potentiates the body's immune response. The immunomodulatory activity of Septilin™ has not been well investigated via appropriate *in vitro* models. Therefore this study was undertaken to investigate the *in vitro* effects of Septilin™ on biomarkers of specific immune pathways by using WBC. Stimulated and unstimulated WBC were incubated with the product. Enzyme linked immunosorbent assays were used to screen for IL-6, IL-10, and IFN γ as biomarkers for inflammation, humoral immunity, and cell mediated immunity, respectively. Results show that the presence of Septilin™ in LPS stimulated WBC has no effect on the release of IL-6 and IFN γ production but stimulated IL-10 production. Septilin™ in unstimulated WBC has no effect on the release of IL-10 and IFN γ production but stimulatory effects on IL-6 production. This study also assessed the effects of *Artemisia afra*, *Aspalathus linearis* (rooibos), and Septilin™ on inflammatory biomarkers namely, IL-6 and nitric oxide (NO)

using RAW 264.7 cells, a murine macrophage cell line. The results of this study indicate that *Artemisia afra* has anti-inflammatory effects while *Aspalathus linearis* (rooibos) up regulated the immune system. The study also shows that Septilin™ has no immunomodulatory effects on RAW 264.7 cells.



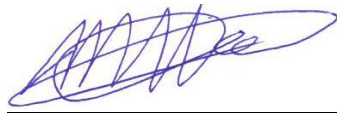
Declaration

I, Mujeeb Hoosen, declare that this thesis entitled “The use of whole blood cell cultures as a model for assessing the effects of Septilin™ on the immune system” is my work and has not been submitted before for any degree or examination in any other university, and that all the sources of my information have been indicated and acknowledged by complete references.

Mujeeb Hoosen

August 2017

Signed



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

I thank my Creator for the countless blessings in my life, I am truly humbled to be part of a profession that contributes to the wellbeing of mankind.

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Contents

Abstract.....	ii
Declaration.....	iv
Acknowledgements.....	v
List of abbreviations.....	x
List of tables.....	xii
List of figures.....	xiii
Chapter 1	1
Introduction.....	1
1.1 The increased use of herbal medicine.....	1
1.2 Herbal Medicine in Africa.....	2
1.3 Herbal medicine and immunology.....	3
1.4 Classification of Immunomodulators.....	4
1.5 Immunomodulation in herbal medicine.....	5
1.6 Immunomodulatory constituents in herbal medicine.....	8
1.7 <i>In vitro</i> models used for testing immunomodulatory HMPs.....	9
1.7.1 <i>In vitro</i> whole blood culture model.....	10
1.7.2 <i>In vitro</i> RAW264 macrophage model.....	12
1.8 WHO health strategy for traditional medicine 2014-2023.....	14
1.9 The Medicines Control Council (MCC) regulations of HMPs in SA.....	15
1.10 References.....	16
Chapter 2	23
An overview of <i>Aspalathus linearis</i> (rooibos), <i>Artemisia afra</i> and Septilin™.....	23
2.1 An overview of <i>Aspalathus linearis</i> (rooibos) tea.....	23

2.1.1 The chemical composition of <i>Aspalathus linearis</i> (rooibos) tea.....	23
2.1.2 The medicinal value of <i>Aspalathus linearis</i> (rooibos) tea.....	24
2.1.3 Antioxidant studies on <i>Aspalathus linearis</i> (rooibos) tea.....	25
2.1.4 Anti-inflammatory studies on <i>Aspalathus linearis</i> (rooibos) tea.....	27
2.2 An overview of <i>Artemisia afra</i>	30
2.2.1 The chemical composition of <i>Artemisia afra</i>	30
2.2.2 The medicinal value of <i>Artemisia afra</i>	31
2.2.3 Antioxidant studies on <i>Artemisia afra</i>	31
2.2.4 Anti-inflammatory studies on <i>Artemisia afra</i>	32
2.3 The chemical composition of Septilin™.....	35
2.4 The medicinal value of Septilin™.....	35
2.4.1 The herbal ingredients of Septilin™.....	35
2.4.1.1 The immunological activity of <i>Commiphora mukul</i>	36
2.4.1.2 The immunological activity Maharasnadi quath (herbal preparation).....	37
2.4.1.3 The immunological activity of <i>Tinospora cordifolia</i>	38
2.4.1.4 The immunological activity of <i>Rubies cordifolia</i>	40
2.4.1.5 The immunological activity of <i>Emblica officinalis</i>	41
2.4.1.6 The immunological activity of <i>Moringa pterygosperma</i>	42
2.4.1.7 The immunological activity of <i>Glycyrrhiza glabra</i>	43
2.4.1.8 The immunological activity Shankh bhasma.....	44
2.5 Immunomodulatory studies on Septilin™.....	45
2.6 Concluding remarks.....	49
2.7 References.....	51
Chapter 3	65
An <i>in vitro</i> study to elucidate the effects of <i>Artemisia afra</i> , <i>Aspalathus linearis</i> (rooibos) and Septilin™ on immune pathways.....	65
3.1 Abstract.....	65

3.2 Introduction.....	67
3.3 Materials and methods.....	70
3.3.1 Sample preparation of <i>Artemisia afra</i> , <i>Aspalathus linearis</i> (rooibos) and Septilin™.....	70
3.3.2 Preparation of RAW 264.7 Cell culture for <i>Artemisia afra</i> , <i>Aspalathus linearis</i> (rooibos) and Septilin™.....	71
3.3.3 Blood collection (preparation of WBC for Septilin™).....	72
3.3.3.1 The effects of Septilin™ on unstimulated blood (WBC).....	72
3.3.3.2 The effects of Septilin™ on LPS stimulated blood (WBC).....	73
3.3.3.3 The effects of Septilin™ on PHA stimulated blood (WBC).....	73
3.3.4 Metabolic activity and cytotoxicity (WST-1) of <i>Artemisia afra</i> , <i>Aspalathus linearis</i> (rooibos) and Septilin™ in RAW 264.7 cells.....	74
3.3.5 Measurement of nitrite formation of <i>Artemisia afra</i> , <i>Aspalathus linearis</i> (rooibos) and Septilin™ in RAW 264.7 cells.....	74
3.3.6 Cytokine analysis (IL-6 ELISA) of <i>Artemisia afra</i> , <i>Aspalathus linearis</i> (rooibos) and Septilin™ treated RAW264.7 macrophage culture supernatant.....	75
3.3.7 Cytokine analysis of Septilin™ (IL-6, IL 10 and IFN γ ELISAs) for WBC.....	76
3.3.8 Statistical analysis of data.....	77
3.4 Results and discussion.....	77
3.4.1 The effect of <i>Artemisia afra</i> , <i>Aspalathus linearis</i> (rooibos) and Septilin™ on the metabolic activity of unstimulated and LPS stimulated RAW 264.7 cells.....	77
3.4.2 The effects of <i>Artemisia afra</i> , <i>Aspalathus linearis</i> (rooibos) and Septilin™ on NO production in unstimulated and LPS stimulated RAW 264.7 cells.....	82
3.4.3 The effects of <i>Artemisia afra</i> , <i>Aspalathus linearis</i> (rooibos) and Septilin™ on IL-6 production in unstimulated and LPS stimulated RAW 264.7 cells.....	88
3.4.4 Effects of Septilin™ on IL-6 production in WBC.....	94
3.4.2 Effects of Septilin™ on IL-10 production in WBC.....	96
3.4.3 Effects of Septilin™ on IFN γ production in WBC.....	98
4. Concluding remarks.....	100
4.6 References.....	102

Chapter 4	113
Summary, concluding remarks and recommendations.....	113
4.1 Summary.....	113
4.2 Concluding remarks.....	114
4.3 Recommendations.....	115
Addendum 1: Chemical analysis of the <i>Artemisia Afra</i> extract sample - Central Analytical Facilities at Stellenbosch University	



List of abbreviations

ATM	African traditional medicine
C&TM	Complementary and traditional medicine
COX-2	Cyclooxygenase 2
DAS	Double antigen sandwich
ELISA	Enzyme linked immune-sorbent assay
GDP	Good dispensing practice
GLP	Good laboratory practice
GMP	Good manufacturing practice
HIV	Human immunodeficiency virus
HMPs	Herbal medicinal products
HRP	Horseradish peroxidase
IFN γ □	Interferon-gamma
Ig	Immunoglobulin
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6



IL-10	Interleukin-10
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MCC	Medicines control council
N	Mean
NF-kB	nuclear factor-kB
NO	Nitrous oxide
PHA	Phytohaemagglutinin
SA	South Africa
SD	Standard deviation
TB	Tuberculosis
TM	Traditional medicine
TNF	Tumour necrosis factor
WBC	Whole blood culture
WHA	World health assembly
WHO	World health organization



List of Tables

Table 1 Risk level, type of claim and evidence required for HMPs.....	16
Table 2.1 Studies on the effects of <i>Aspalathus linearis</i> (rooibos) on cytokine and NO activity.....	29
Table 2.2 Studies on the effects of <i>Artemisia</i> species on cytokine and NO activity.....	34
Table 2.3 Studies on the effects of <i>Commiphora mukul</i> on cytokine and NO activity.....	37
Table 2.4 Studies on the effects of <i>Tinospora cordifolia</i> on cytokine and NO activity.....	40
Table 2.5 Studies on the effects of <i>Embllica officinalis</i> on cytokine and NO activity.....	42
Table 2.6 Studies on the effects of <i>Moringa pterygosperma</i> on cytokine activity.....	43
Table 2.7 Studies on the effects of <i>Glycyrrhiza glabra</i> on cytokine and NO activity.....	44
Table 2.8 Studies on the effects of Septilin™ on cytokine and NO activity.....	49



List of Figures

Figure 3.1: Cell metabolic activity of unstimulated RAW 264.7 cells exposed to various concentrations of <i>Artemisia afra</i> , <i>Aspalathus linearis</i> (rooibos) and Septilin™.....	81
Figure 3.2: Cell metabolic activity of LPS stimulated RAW 264.7 cells exposed to various concentrations of <i>Artemisia afra</i> , <i>Aspalathus linearis</i> (rooibos) and Septilin™.....	81
Figure 3.3: Standard curve for NO assay.....	87
Figure 3.4: NO production in unstimulated RAW 264.7 cells exposed to various concentrations of <i>Artemisia afra</i> , <i>Aspalathus linearis</i> (rooibos) and Septilin™.....	87
Figure 3.5: NO production in LPS stimulated RAW 264.7 cells exposed to various concentrations of <i>Artemisia afra</i> , <i>Aspalathus linearis</i> (rooibos) and Septilin™.....	88
Figure 3.6: Standard curve for IL-6 ELISA.....	92
Figure 3.7: IL-6 production in unstimulated RAW 264.7 cells exposed to various concentrations of <i>Artemisia afra</i> , <i>Aspalathus linearis</i> (rooibos) and Septilin™.....	92
Figure 3.8: IL-6 production in LPS stimulated RAW 264.7 cells exposed to various concentrations of <i>Artemisia afra</i> , <i>Aspalathus linearis</i> (rooibos) and Septilin™.....	93
Figure 3.9: Standard curve for IL-6 ELISA.....	95
Figure 3.10: Effects of Septilin™ on IL-6 production.....	96
Figure 3.11: Standard curve for IL-10 ELISA.....	97
Figure 3.12: Effects of Septilin™ on IL-10 production.....	98
Figure 3.13: Standard curve for IFN γ ELISA.....	99
Figure 3.14: Effects of Septilin™ on IFN γ production.....	100

Chapter 1

Introduction

1.1 The increased use of herbal medicine

In the past three decades there has been a huge increase in the use of herbal medicine (Mukherjee and Houghton, 2009). Herbal medicinal products (HMPs) are defined as plants, parts of plants or extracts from plants that are used in health care or in combating disease (Mukherjee and Houghton, 2009). The World Health Organization (WHO) states that herbal medicine has an important role to play in the healthcare of many developing countries. Worldwide the use of herbal medicine has increased due to therapeutic and economic importance. Herbal medicine plays an important role in many forms of complementary and traditional medicine (C&TM) systems such as African traditional medicine (ATM), Unani-Tibb, traditional Chinese medicine, amongst others which are practiced and utilized by millions globally. It is estimated that up to 80% of Africans rely on C&TM for their healthcare needs (Mahlangeni *et al.*, 2014). The use of plant immunostimulants has a long history in these traditional medical systems (Tan and Vanitha, 2004). The use of botanical products is expected to increase exponentially in the United States where revenue of medicinal herbs or herbal extracts is predicted to exceed \$4 billion annually (Chang, 2000). HMPs research is an area of rapid growth in both human and veterinary medicine (Hart, 2005). The identification of suitable immunomodulatory preparations or drugs from natural sources to prevent and treat immunological complications are increasing in popularity (Varma *et al.*, 2011). Herbal immunomodulators have gained popularity amongst the public and researchers due to the emergence of drug resistant strains of micro-organisms and high costs of synthetic drugs (Mahima *et al.*, 2012). Worldwide, inflammatory and immune-related illnesses have been treated with herbal formulas for centuries (Burns *et al.*, 2010)

1.2 Herbal Medicine in Africa

In Africa, plants have been used for medicinal purposes for many centuries. Many of these African medicinal plants are assumed to be safe yet they could potentially be harmful (Adewunmi and Ojewole, 2004). Recent findings have shown that up to 25% of the world's plants are found south of Saharan Africa (van Wyk, 2008). In 2005 there were 5400 African medicinal plants already identified, with over 16,300 medicinal uses. Several African medicinal plants are used for immune related conditions. *Aspalathus linearis* (rooibos) and *Artemisia afra* are two of several immunomodulatory HMPs used extensively in South Africa as well as in other African regions (van Wyk, 2008). Septilin™ is a widely used health supplement claimed to strengthen immunity (Khanna & Sharma, 2003; Daswani & Yegnanarayan, 2002). Septilin™ is available as an over the counter herbal medication.

Herbal medicine has been standardized in India and China (Mahlangeni *et al.*, 2014). However, in Africa this is not the case despite its rich herbal medicinal traditions and wide range of medicinal plants. Most of the documented text on African traditional medicine (ATM) deals with medicinal plants and associated uses, while ignoring pharmacological and chemical studies (Mahlangeni *et al.*, 2014). Most of the western world views African traditional medicine as mysterious due to the lack of scientific evidence regarding safety and efficacy. Herbal medicine, also known as phytomedicine, displays distinctive characteristics that are often not well understood. The bio-activities are mostly unknown, with supportive evidence for safety and efficacy very rare. Since these herbal medicines are easily accessible over the counter and at times cheaper than conventional drugs, many patients use self-prescribed herbal medicine without revealing this to their healthcare practitioners. Regulatory studies are crucial to provide data on the toxicology, pharmacodynamics and pharmacokinetics of these herbal medicines (Adewunmi and Ojewole, 2004). There

is a growing trend worldwide to verify the efficacy of these HMPs and to establish user friendly data bases for easy reference to botanical and medical information on herbal ingredients. Commercialization of herbal medicine has led to the rise of many private entrepreneurs preparing and packaging HMPs. The marketing of these HMPs has become a lucrative business. Confirmation on the quality, safety and efficacy of these HMPs are of utmost importance to reduce potential health risks to consumers (Ndhlala *et al.*, 2012). The effective data collection methods on Chinese herbal medicine have led to the National Institutes of Health in United States recognition of Chinese medicine as an effective form of C&TM (Malangu, 2007). There is a definite need for Africa to follow suite (Oladosu *et al.*, 2012). The WHO reports that in South Africa 75% of Human immunodeficiency virus (HIV) infected patients use immunomodulatory HMPs as treatment (Malangu, 2007). The integrative use of immunomodulatory HMPs alongside conventional medication is increasing in popularity in Africa and around the world. More commonly in HIV infected patients, up to 30% in Uganda, 70% in South Africa and Zimbabwe and up to 70% of US African Americans are reported to use some form of traditional herbal medicine alongside conventional prescriptions (Gwaza *et al.*, 2013). The effectiveness and safety of many African traditional medicines (ATM) has yet to be established scientifically (Mills *et al.*, 2005).

1.3 Herbal medicine and immunology

The number of researchers publishing general C&TM articles have increased rapidly with more than 1700 articles cited in PubMed using “complementary medicine” as a keyword in 2007, compared to only 355 in 1990. There has been a similar increase in number of articles relating to the effect of C&TM on the immune system with the number of articles published annually increasing 3 fold since 1990 (Mainardi *et al.*, 2009). Herbal medicine with anti-inflammatory, antioxidant, antimicrobial,

immunomodulatory and/or analgesic properties are used therapeutically to treat acute infections and inflammatory conditions, particularly in humans (Hart, 2005). Various herbal medicines have been shown to exert anti-inflammatory and/or antioxidant effects in human and animal models based on several studies. The bioactive constituents found in herbal medicine, like plant phenols, vitamins, carotenoids, phytoestrogens and terpenoids have been shown to exert both anti-inflammatory and antioxidant activity. Modulation of various functions of inflammatory cells can be regulated by herbal constituents which can affect the immune system directly or indirectly (Iwalewa *et al.*, 2007). Recently there has been an exponential growth in the field of herbal medicine leading to its popularity in both developing and developed countries. Immunomodulatory medicinal plants can provide an alternative to conventional medicine for a variety of diseases (Ganju *et al.*, 2003). Herbal immunomodulators are reported to be valuable for regulating inflammatory pathways. *In vitro* and *in vivo* study observations have shown possible suppression and enhancement of immune functions (Varma *et al.*, 2011). A few immunomodulatory studies are available whilst most HMPs effects are unknown (Mahima *et al.*, 2012).

1.4 Classification of Immunomodulators

The term immunomodulator refers to medicines that modify the actions of the immune system by influencing the regulation of messenger molecules like cytokines, nitric oxide, hormones, neurotransmitters, and other peptides (Burns *et al.*, 2010). Immunomodulators are biological or synthetic substances that may stimulate, suppress or modulate various components of the immune system including both adaptive and innate immunity. Immunoadjuvants specifically enhances the efficacy of vaccines and therefore are considered to be the true modulators of the immune response. It is proposed that they act as selectors between cellular and humoral helper T1 (Th1) and helper T2 cells (Th2). Immunoadjuvants are believed to also influence

immunoprotective and immunodestructive activity. Immunoglobulin E (IgE) versus IgG type immune responses can be determined by immunoadjuvants. This can prove to be challenging to vaccine manufacturers (Kumar *et al.*, 2012). Immunostimulants enhances the overall immune response against pathogens. This occurs via humoral and cellular immune responses, by enhancing cytokine production or stimulating B- or T-lymphocytes (Tan and Vanitha, 2004). Immunostimulants are expected to offer prophylactic effects in healthy individuals by potentiating the basic level of the immune response. In those who have an immune impairment, immunostimulants are administered as therapeutic agents for immunity (Kumar *et al.*, 2012). Immunosuppressants act by suppressing the immune response (Mahima *et al.*, 2012). These drugs are commonly used to treat different types of organ transplant rejection and autoimmune diseases (Kumar *et al.*, 2012). From a therapeutic perspective immunomodulation refers to the alteration of the immune response to a desired level which is most beneficial to the host (Mahima *et al.*, 2012).

1.5 Immunomodulation in herbal medicine

The general public makes use of herbal immunomodulators for various reasons based on *in vivo* claims which includes enhancement of general well being or/and for prophylactic purpose. These HMP's may enhance or suppress immune function which could benefit or harm patients (Wilasrusmee *et al.*, 2002). Botanical immunomodulators may alter immune function and offer clinically relevant therapeutics. This necessitates research on the effects of these formulations to provide insights on safety and efficacy. Burns *et al.*, reviewed 385 polyherbal immunomodulatory studies and noted that many herbal mixtures have effects on one or more cytokines. The most popular cytokines assessed were IL-4, IL-6, IL-10, TNF and IFN γ (Burns *et al.*, 2010). An *in vitro* immunomodulatory study compared 10 commonly used HMP's which included; green tea (*Camellia sinensis*), garlic (*Allium*

sativum), dong quai (*Angelica sinensis*), Chinese ginseng (*Panax ginseng*), ephedra (*Ephedra sinica*), milk thistle (*Silybum marianum*), ginger (*Zingiber officinale*), Licorice (*Glycyrrhiza glabra*), Echinacea (*Echinacea purpurea*), and St. John's wort (*Hypericum perforatum*). This study employed the lymphocyte proliferation test using concanavalin A (Con A) and mixed lymphocyte culture (MLC). The results showed that dong quai, ginseng, milk thistle and St. John's wort had a significant up regulation effect ($p < 0.05$) on mitogen stimulation whilst green tea and ginger displayed immunosuppression. Garlic, ephedra, licorice, and Echinacea showed insignificant effects on mitogen-stimulated lymphocyte proliferation which was surprising in light of various immunomodulatory results reported in previous literature. Echinacea is marketed as an immune stimulant and previous studies indicated to its stimulatory effects on IL-1 and IL-6 production by macrophages which could mean that this herb acts on innate immunity and not adaptive as tested in this study (Wilasrusmee *et al.*, 2002).

Artemisia afra and *Aspalathus linearis* (rooibos) are 2 of several South African HMPs used traditionally for infections. These HMPs are claimed to have immunomodulatory effects *in vivo* (van Wyk, 2011). One study showed that artemisinin extracted from *Artemisia annua* possessed immunosuppressive activity by inhibiting calmodulin, the inflammatory and immune mediator. An immunomodulatory study using a whole blood cell culture model showed that *Artemisia afra* significantly decreased ($P < 0.01$) the amount of IL-6 produced (16 % compared to the control) at a high concentration (1666 $\mu\text{g/ml}$) whilst also significantly reducing ($P < 0.001$) the amounts of IL-10 and IFN γ ($P < 0.001$) at the concentration of 1666 $\mu\text{g/ml}$ (32.7 % compared to the control) (Kriel and Pool, 2010). Due to the global popularity of *Aspalathus linearis* (rooibos), since 1979 there has been several immunomodulatory *in vitro*, *in vivo* (animal and human model) and *ex*

in vivo studies conducted (Joubert *et al.*, 2008). The *in vitro* studies employed subcellular fractions and cell culture. One study on the water extract of *Aspalathus linearis* (rooibos) in murine splenocytes using anti-ovalbumin (OVA) and sheep red blood cells showed an increase in antibody production with no effect seen in lipopolysaccharide-stimulated (LPS) spleen cells. *Aspalathus linearis* (rooibos) stimulated interleukin (IL)-2 in splenocytes primed with OVA and CD3, whilst down regulating IL-4 in OVA primed cells (Joubert *et al.*, 2008). Similar results were seen *in vivo*, in Wistar rats given oral doses of *Aspalathus linearis* (rooibos) water extract. These cyclosporine treated rats displayed restoration in OVA-induced antibody production however no difference was seen between OVA-stimulated and control rats (Joubert *et al.*, 2008).

Inflammatory and immune-related conditions have a long history of being treated with polyherbal immunomodulators. Traditional herbal pharmacotherapy often combines immunomodulatory plants producing complex phytochemical mixtures which could potentially influence numerous molecular pathways. The sum effects of these polyherbal formulations may differ from the individual plants (Burns *et al.*, 2010). An *in vitro* herbal formulation comparison study on Abana™, Geriforte™, Septilin™, Triphala™, Chyavanaprasha™ and Mentat™ reported decreased dose dependant NO scavenging activity of all HMPs tested using sodium nitroprusside as a NO donor (Jagetia *et al.*, 2004). Septilin™, a proprietary polyherbal formulation, has been reported to have immunomodulatory, antibacterial and wound healing properties. Septilin™ is a widely used health supplement claimed to strengthen immunity and to be effective against Gram-positive and Gram-negative infections (Khanna and Sharma, 2003; Daswani and Yegnanarayan, 2002). Septilin™ has shown some immunomodulatory potency in animal models (mice and rats) by improving the phagocytic index and reducing the risk of bacteria induced sepsis (Varma *et al.*,

2011). Several immunomodulatory HMPs has been studied on mouse, chicken and human cell lines (Mahima *et al.*, 2012). Cytokines are widely researched for many inflammatory and immune related diseases (refer to chapter 2). The majority of the investigations of herbal medicine and cytokine activity have been conducted via *in vitro* assays (Burns *et al.*, 2010).

1.6 Immunomodulatory constituents in herbal medicine

Immunomodulatory activities of botanicals on animal immune systems have been largely attributed to plant secondary metabolites. For example, Ginseng alongside its steroidal saponine has displayed immune stimulating properties on cytokine production namely; IL-2, IL-6 TNF and IFN γ synthesis and an increase in macrophage activation and lymphocyte activity (Mahima *et al.*, 2012). Saponins are known to stimulate cell mediated immunity and enhance antibody production (Mahima *et al.*, 2012). Saponins have been reported to up regulate cytokine (interleukins and interferon) production. Plant polysaccharides of medicinal plants like *Astragalus* has been reported to enhance immunoglobulin production and macrophage activation (Mahima *et al.*, 2012). Numerous glycosides have been recognized to exert immunomodulatory effects. Three new sesquiterpene glycosides namely dendroside A and dendronobilosides A and B have been isolated from the stems of the Chinese traditional medicinal plant called dendrobium. An *in vitro* study on murine T and B lymphocytes showed that dendroside A and a mixture of dendronobilosides A and B stimulated proliferation. Dendronobiloside B as a single compound showed inhibitory activity (Kumar *et al.*, 2012). The polyphenol content in green tea has been reported to provide potential benefit in inhibiting IL-1B induced catabolic effects in osteoarthritis chondrocytes (Huang *et al.*, 2008). Polyphenols in green tea also contributes to potent anti-oxidant and anti-inflammatory effects by decreasing IL-2 secretion in animal models (Huang *et al.*, 2008). The potent

antioxidant action of *Aspalathus linearis* (rooibos) tea has been attributed to its rich and unique polyphenol content. Flavonoids (luteolin and quercetin) in *Aspalathus linearis* (rooibos tea) showed inhibition of pro-inflammatory cytokines, IL-6 and TNF- α using a LPS-stimulated macrophage model (Ajuwon *et al.*, 2014). Luteolin, a major flavanoid of *Artemisia afra* has been reported to exert anti-oxidant, anti-inflammatory, anti-allergenic, anti-carcinogenic, and immune-modulating effects. Seven different compounds were isolated from the bio-active fraction of *Tinospora cordifolia*. These bio-actives belong to different groups of chemicals such as alkaloids, phenylpropanoids and sesquiterpenes. Two of these (cordifolioside A and syringin) were reported to have immunomodulatory effects (Sharma *et al.*, 2012). The potent anti-oxidant properties of Amla (*Emblica officinalis*) were due to the presence of hydrolysable tannins emblicanin A, emblicanin B, puningluconin, and ped-unclagin. Unique sesquiterpenoids from the roots of *Phyllanthus emblica* have showed cytotoxic properties *in vitro* (Sai Ram *et al.*, 2002). Guggulsterone (plant sterol), the bioactive constituent of the resinous sap from the guggul tree (*Commiphora mukul*) has been shown to strongly suppress inflammatory gene expression by inhibiting NF- κ B signaling via the blocking of I κ B degradation in LPS induced inflammation of human middle ear epithelial cells (Song *et al.*, 2010). Piperine, an active alkaloid extracted from *Piper longum* has been reported to possess anti-oxidative potency in both *in vitro* and *in vivo* studies. Isolated diterpenes (andrographiside and andrographolide) from the plant *Andrographis paniculata* proved to be beneficial against tumourigenesis by exerting anti-lipoperoxidative action and carcinogen detoxification action (Balachandran and Govindarajan, 2005).

1.7 *In vitro* models used for testing immunomodulatory HMPs

Herbal medicines are extremely complex in nature. The use of highly sensitive and miniaturized assays is therefore essential alongside innovative technologies like

immunochemical and enzymatic methods to elucidate the mode of action of these products. These techniques are used directly for a wide range of mechanism-based and cellular assays. A variety of *in vitro* bio-assays have been utilised to determine mechanisms of action, pharmacological actions, efficacy and safety, leading to more effective products in healthcare (Mukherjee and Houghton, 2009). In depth study of the effect of herbal medicine on the immune system requires the use of both *in vitro* and *in vivo* experimentation. *In vitro* models have proved to be useful in evaluating the immunomodulatory effects of herbal constituents (Silliman and Wang, 2006). Pro-inflammatory cytokines like tumor necrosis factor alpha (TNF α), IL-1, IL-6, IL-8 and nitric oxide (NO) have been implicated in numerous immuno-pathological conditions (Varma *et al.*, 2011). The majority of *in vitro* experiments in herbal medicine make use of aqueous extractions as opposed to ethanolic extractions with varying concentrations of the wet or dried plants ranging between 0.3 μ g/ml to 1g/ml. Popular cell types used are splenocytes, T cells, monocytes and macrophages and the most common stimulants utilized for immune pathway activations are Con A, phytohemagglutinin (PHA) and LPS. *In vitro* cell culture incubation times varied from 6 hours to 4 weeks (Burns *et al.*, 2010).

1.7.1 *In vitro* whole blood culture model

Stimulated monocytes produce IL-6, TNF α , and IL-1, which promotes inflammation, T-cell growth and differentiation and the release of the anti-inflammatory cytokine IL-10. Monocyte cytokine production can be assessed *in vitro* by stimulating isolated monocytes but this method is time consuming, expensive and involves numerous steps which could compromise cell viability, stimulation and function (Damsgaard *et al.*, 2009). The use of peripheral blood is a far simpler and an inexpensive procedure. Whole blood cultures contain all the physiological requirements for cells to function naturally and therefore is ideal as a reference for immune activity. Other advantages

of using whole blood cultures are that samples can be used immediately and only small volumes are needed. Cytokine production based on the whole blood culture model is considered to be a reliable method as it has good reproducibility (Damsgaard *et al.*, 2009). This system provides better outcomes over culture of single cell types because it creates a natural environment with physiological concentrations of factors influencing immune cell function which allows analysis of the effects of various experimental substances on inflammatory leucocytes. In other models the pattern of cytokine release may be altered by experimental procedures during cell isolation (Schmid *et al.*, 2009). *In vitro* assays using whole blood cells are ideal as they resemble the *in vivo* environment (Pool and Bouic, 2001).

An *in vitro* study on the effects of triterpene saponins from the *Astragalus* species on cytokine release by human whole blood cultures included IL-1, IL-8, IL-2, IL-4 and IFN γ (Yesilada *et al.*, 2005). Lipopolysaccharide (LPS) and phytohaemagglutinin (PHA) stimulation were used. Triterpene glycosides were screened, isolated and chemically defined from various Turkish *Astragalus* species: *Astragalus brachypterus*, *Astragalus cephalotes*, *Astragalus microcephalus*, and *Astragalus trojanus*. The results showed that all triterpene saponins tested induced IL-2 stimulation between 35.9% and 139.6%, *Astragalus oleifolius* (141.2%) producing the highest score. This study noted that the mechanism of action for the anticancer and immunomodulatory effects of the *Astragalus* species (Yesilada *et al.*, 2005). An *in vitro* study on the aqueous extracts of *Cimicifuga racemosa* (black cohosh) and its phenolcarboxylic constituents was conducted on proinflammatory cytokines (IL-6, TNF α , IFN γ , and the chemokine IL-8) using LPS-stimulated human whole blood (Schmid *et al.*, 2009). The root extract of *Cimicifuga racemosa* (3mg/ml and 6mg/ml) reduced LPS-induced release of IL-6 and TNF α in a dose and time-dependent manner and inhibited the release of IFN γ in whole blood culture supernatant whilst

stimulating IL-8. These findings may explain the anti-inflammatory mechanism of action of *Cimicifuga racemosa* when used for rheumatism and other inflammatory diseases (Schmid *et al.*, 2009). The anti-inflammatory effects of a polyherbal Tibetan preparation (PADMA-28), was undertaken in LPS-stimulated human whole blood. The cytokines evaluated included IL-1, IL-6, IL-8, IL-10 and TNF α . The aqueous extract of PADMA-28 inhibited the production of the inflammatory cytokines IL-1, IL-6, IL-8 and TNF α whilst decreasing the anti-inflammatory cytokine, IL-10. These findings suggests possible clinical efficacy in autoimmune and inflammatory conditions (Barak *et al.*, 2004).

1.7.2 *In vitro* RAW264 macrophage model

Inflammatory mediators includes pro-inflammatory cytokines like TNF α , IL-1, IL-6, IL-8, NO, prostaglandins amongst others (Varma *et al.*, 2011). NO is a product of three enzymes which include inducible nitric oxide synthase (iNOS) from the amino acid L-arginine. The enzymatic activity of iNOS in diverse cell types contributes to the overproduction of NO which is responsible for inflammation in several pathophysiological conditions like cancer, rheumatoid arthritis, diabetes and liver cirrhosis amongst others. Inhibition of NO has become the main focus area in the field of anti-inflammatory research (Konkimalla *et al.*, 2008). Macrophages and monocytes play a crucial role in innate and adaptive immunity. Macrophages affect various immune responses when encountering invading pathogens. The versatile role of macrophages includes antigen recognition, capture, clearance and transport of foreign products. Macrophages stimulated by LPS and microbes elicit the release of various proteins like iNOS which leads to the production of NO (Bisht *et al.*, 2009). The LPS stimulated cell system has become a popular tool in the discovery of new anti-inflammatory drugs (Varma *et al.*, 2011). The use of RAW 264.7 mouse

macrophage cell lines is a well-established model to determine NO production (Konkimalla *et al.*, 2008).

The anti-inflammatory and anti-oxidant effect of the lyophilized aqueous extract of a Mexican herbal medicine (*Calea urticifolia*) was assessed on LPS stimulated RAW 264.7 macrophages (Torres-Rodriguez, *et al.*, 2016). This study also aimed to provide evidence on the presence of phenolic compounds in this product. The results showed anti-inflammatory effects of *Calea urticifolia* via suppression of the iNOS/NO pathway through inhibition of nucleus translocation of NF- κ B p65 and p50 sub-units. *Calea urticifolia* caused a significant ($P < 0.05$) inhibition of ROS production in a dose-dependent manner in LPS-stimulated macrophages. Inflammatory markers (GCSF, MCSF, IFN γ , IL-1a, IL-1B, IL-4, IL-6, IL-9, IL-12, IL-17, MIG, I-TAC, SDF-1, MCP-1, MIP-1 α , MIP-1 γ , TECK, Fractalkine, Lymphotactin, KC, LIX, CXCL1, s TNF RI, s TNF RII) expression were suppressed (34.5 to 88.3%). The phenolic compounds that were found in *Calea urticifolia* were caffeoylquinic acid derivatives and flavonoid glycosides which could account for its anti-inflammatory and anti-oxidant effect (Torres-Rodriguez *et al.*, 2016). St. John's wort (*Hypericum perforatum*) is a well known herbal supplement prescribed for mild to moderate depression (Huang, *et al.*, 2011). Several previous studies reported synergistic anti-inflammatory actions due to the presence of 4 compounds. Other species could possess similar or additional compounds which contribute to its anti-inflammatory effects. An *in vitro* study on the anti-inflammatory constituents of the extracts in several species of *Hypericum* using RAW 264.7 mouse macrophages reported that *Hypericum perforatum* and *Hypericum gentianoides* possessed the strongest overall anti-inflammatory efficacy (inhibitory effects on PGE₂, NO, TNF α and IL-1 β). Pseudohypericin was reported to be the main constituent which contributed to the anti-inflammatory effects in both species however a combination of different

constituents were found to be present in both *Hypericum perforatum* and *Hypericum gentianoides* (Huang *et al.*, 2011). An *in vitro* study on the isolated compounds (artemisolid, 3-methoxytanaparholide, deacetyllaurenobiolide, moxartenolide and arteminolides B and D) of *Artemisia sylvatica* was conducted in RAW 264.7 murine macrophages. These compounds were obtained from the methanol extract of the aerial parts of *Artemisia sylvatica*. All 6 compounds displayed inhibitory activity on the LPS-induced NF- κ B activation, NO and TNF α production (Jin *et al.*, 2004).

1.8 WHO health strategy for traditional medicine 2014-2023

The WHO health strategy for traditional medicine 2014-2023 was formulated in response to the world health assembly (WHA) resolution on traditional medicine (WHA62.13) (1) which emphasized the need to address current challenges that concerns the use of traditional medicine globally. C&TM is recognized as an essential component of healthcare in most countries which is often underestimated. C&TM services and products are increasing in demand. Therefore many countries have realized the need to ensure safety, quality and efficacy for the wellbeing of patients. The regulatory measures for HMPs are of primary importance to ensure the viable practice of C&TM worldwide. This can be achieved by implementing these strategic objectives:

- building the database C&TM services and products
- formulating national policies on C&TM services and products
- to ensure safety, quality and effectiveness through regulation
- integrating C&TM services and self-health care into national health systems on a global level (WHO Traditional Medicine Strategy, 2013).

1.9 The Medicines Control Council (MCC) regulations of HMPs in SA

The Medicines and Related Substances Act (Act 101 of 1965) has led to the establishment of the MCC in South Africa. The MCC consists of eleven technical expert committees, C&TM is the focus area of two of eleven committees. This is crucial to the regulation of C&TM medicines in SA (Gqaleni *et al.*, 2007). The MCC recognizes that HMPs are used and sold extensively in SA and therefore has implemented regulatory guidelines to ensure the safety, efficacy and quality of these HMPs. This aims to ensure good manufacturing practice (GMP), good laboratory practice (GLP) and good dispensing practice (GDP) as well as good regulatory practice in relation to HMPs. This would promote these HMPs rendering them acceptable in terms of quality, safety and efficacy. HMPs manufacturers can make high and low risk claims and proof of absolute efficacy can be challenging. These claims of efficacy should be supported by current scientific findings and must include relevant *in vitro*, *in vivo* and clinical trial studies (MCC Complementary medicines-quality, safety and efficacy, 2013).

Septilin™ is a phytopharmaceutical formulation which is recommended for the treatment and management of various infections. It is claimed to have immunomodulatory action. HMPs manufactured in one region is often used elsewhere. This HMP is manufactured in India and used by many C&TM practitioners in SA. Septilin™ is also available as an over the counter medication in health stores and pharmacies. Septilin™ falls within the high risk level category due to the claims of efficacy made by the manufacturer and therefore requires continuous scientific validation via relevant *in vitro*, *in vivo* and clinical trial studies (refer to Table 1).

Table 1: Risk level, type of claim and evidence required for HMPs

Risk level	Type of claim	Evidence required to support claim
High	Treats/cures/manages any disease/disorder. <ul style="list-style-type: none"> • Prevention of any disease or disorder. • Reduction of risk of a disease/disorder. • Aids/assists in the management of a named symptom/disease/disorder. • Relief of symptoms of a named disease or disorder • Treatment of proven vitamin or mineral deficiency disease/s. 	Clinical data to be evaluated and two of the following four sources that demonstrates adequate support for the indications claimed: <ol style="list-style-type: none"> 1. Recognized pharmacopoeia. 2. Recognized monograph. 3. Three independent written histories of use in the classical or traditional medical literature, or 4. Citations from other <i>in vivo</i>, <i>in vitro</i> studies, case reports or others.
Low	General health enhancement without any reference to specific diseases or conditions <ul style="list-style-type: none"> • Health maintenance, including nutritional support. • Relief of minor symptoms (not related to a disease or disorder) 	Clinical data to be evaluated and/or two of the following four sources that demonstrates adequate support for the indications claimed: <ol style="list-style-type: none"> 1. Recognized pharmacopoeia. 2. Recognized monograph. 3. Three independent written histories of use in the classical or traditional medical literature, or 4. Citations from other <i>in vivo</i>, <i>in vitro</i> studies, case reports or others.

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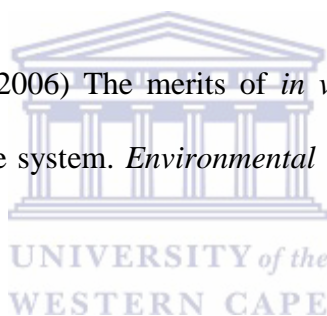
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Chapter 2

An overview of *Aspalathus linearis* (rooibos), *Artemisia afra* and Septilin™

2.1 An overview of *Aspalathus linearis* (rooibos) tea

Aspalathus linearis (rooibos) tea is a commercialized popular health drink from South Africa (SA). It is most commonly used as a beverage and in cosmetic products (van Wyk, 2008). It is a 1.5 meter high shrub consisting of bright green needle-shaped leaves and pea-shaped yellow flowers (Erickson, 2003). *Aspalathus linearis* (rooibos) tea is considered to be one of the 'big five' phytomedicines in SA due to its popularity as an herbal health drink. A traditional beverage consumed by the Khoi people, *Aspalathus linearis* (rooibos) originates from the Cederberg area in the Western Cape (George *et al.*, 2001). *Aspalathus linearis* (rooibos) tea also known as Redbush tea has several health benefits which includes antioxidant effects (Nel *et al.*, 2007). Due to its low tannin content, zero caffeine and potent antioxidant properties, *Aspalathus linearis* (rooibos) has been gaining popularity globally as much more than a health beverage and accepted as a nutraceutical. The health-promoting benefits of *Aspalathus linearis* (rooibos) have been confirmed in several *in vitro* and *in vivo* studies (Mahomoodally, 2013). There are consistent reports on the safety of *Aspalathus linearis* (rooibos) (Smith and Swart, 2016).

2.1.1 The chemical composition of *Aspalathus linearis* (rooibos) tea

Aspalathus linearis (rooibos) is known to contain various flavonoids which include flavonols, flavones and dihydrochalcones. Aspalathin, a C-C linked dihydrochalcone glucoside is considered to be the main flavonoid in *Aspalathus linearis* (rooibos) (Joubert *et al.*, 2008). Other known flavonoids isolated from *Aspalathus linearis* (rooibos) are; aspalalinin, nothofagin, orientin, iso-orientin, isovitexin, dihydro-orientin, dihydro-iso-orientin, hemiphlorin, quercetin, quercetin-3-robinobioside,

hyperoside, isoquercitrin and rutin amongst others (Shimamura *et al.*, 2006; Joubert *et al.*, 2008). *Aspalathus linearis* (rooibos) also contains non-flavonoid components such as lignans (vladinol E, secoisolariciresinol, secoisolariciresinol glucoside) and phenolic acids (caffeic acid, ferulic acid, p-coumaric acid, p-hydroxybenzoic acid, vanillic acid, protocatechuic acid) (Shimamura *et al.*, 2006; Joubert *et al.*, 2008; Joubert and De Beer, 2011).

2.1.2 The medicinal value of *Aspalathus linearis* (rooibos) tea

The absence of alkaloids and its low tannin content accounts for the reason why many consider *Aspalathus linearis* (rooibos) tea to be harmless. In 1968 *Aspalathus linearis* (rooibos) was recognized as an anti-colic agent as it relieved vomiting and chronic restlessness in babies. The anecdotal uses suggest that *Aspalathus linearis* (rooibos) exerts anti-allergic, appetite stimulatory and sedative effects (Joubert *et al.*, 2008). Several compounds in *Aspalathus linearis* (rooibos) tea have been recognized to have antioxidant activity namely; aspalathin, iso-orientin, orientin, rutin, isovitexin, vitexin, isoquercitrin, hyperoside, quercetin, luteolin and chrysoeryol. *Aspalathus linearis* (rooibos) revealed low anti-microbial activity when compared to other herbal tea infusions. Aspalathin, isoorientin, orientin and rutin are the compounds that are suggested to contribute to the anti-microbial activity of *Aspalathus linearis* (rooibos) (Almajano *et al.*, 2008). In one *in vitro* study the anti-mutagenic effects of *Aspalathus linearis* (rooibos) were attributed to the flavonoid compounds aspalathin, nothofagin, luteolin and chrysoeriol. Various studies have confirmed the anti-mutagenic, antioxidant and dermatological benefits of *Aspalathus linearis* (rooibos) extracts (Snijman *et al.*, 2007). Aspalathin found in certain *Aspalathus linearis* (rooibos) extracts has been suggested to have anti-diabetic effects. Others claim that aspalathin can be used as a medicament for the treatment of neurological and psychiatric conditions of the central nervous system. Studies support the potential

healing role of *Aspalathus linearis* (rooibos) in skin conditions (Joubert and de Beer, 2011). A comparative study on the chemo protective properties of various herbal teas in rat models revealed that *Aspalathus linearis* (rooibos) significantly ($p < 0.05$) inhibits cancer promotion induction of fumonisin B1 in rat liver (Marnewick *et al.*, 2009).

2.1.3 Antioxidant studies on *Aspalathus linearis* (rooibos) tea

An antioxidant is a substance that can trap free radicals before oxidative damage occurs. Antioxidants such as flavanoids, polyphenol and phenolic acids are able to scavenge free radicals thereby preventing oxidative cellular damage (Joubert *et al.*, 2008). The link between oxidative stress and inflammation is well known especially with regards to stress related chronic diseases and accelerated aging (Smith and Swart, 2016). Two *in vitro* studies investigated the free radical scavenging activity of *Aspalathus linearis* (rooibos) and its effect on reactive oxygen species (ROS), catalase (CAT), and superoxide dismutase (SOD) (Waisundara and Hoon, 2015). The oxygen radical absorbance capacity (ORAC) assay was used to quantify the antioxidant capacity of *Aspalathus linearis* (rooibos) in a cancer and diabetic model using human umbilical vein endothelial cells (HUVECs) and HeLa cells. The results showed statistically significant decreases ($p < 0.05$) in the fluorescence intensities as compared to the control. This study noted that even though antioxidant effects of *Aspalathus linearis* (rooibos) were observed caution should be practised for *in vivo* application as previous studies have reported on pro-oxidant effects of the phenolic compound in *Aspalathus linearis* (rooibos) (Waisundara and Hoon, 2015). A controlled clinical trial based on a 12 week pre-measurement and post-measurement single group intervention design was conducted to assess the effects of *Aspalathus linearis* (rooibos) on oxidative stress and biochemical parameters in adults at risk for cardiovascular disease. Total polyphenols, lipid peroxidation (conjugated dienes –

CDs, thiobarbituric acid reactive substances – TBARS), redox status (total glutathione – tGSH, ratio of reduced to oxidized glutathione – GSH:GSSG), lipid profile (total cholesterol, low density lipoprotein – LDL and high density lipoprotein – HDL cholesterol and triacylglycerol levels) as well as liver and kidney function were included (Marnewick *et al.*, 2011). This study reported a decreased lipid profile (decreased HDL and saturated fats) and redox status in the participants however there were no significant changes to the non-specific marker of generalized inflammation, C-reactive protein, after the 6 week consumption of *Aspalathus linearis* (rooibos) (Marnewick *et al.*, 2011). An *in vivo* study used *Caenorhabditis elegans* as a model organism to assess the effect of *Aspalathus linearis* (rooibos) extracts against oxidative stress (Chen *et al.*, 2013). This study employed juglone, a generator of ROS, known to cause damage to cells and organisms. Age-synchronized *Caenorhabditis elegans* were treated with green *Aspalathus linearis* (rooibos) extract (100µg/ml), red *Aspalathus linearis* (rooibos) extract (100µg/ml) or aspalathin (0, 10, 20 and 50µM). After exposure to acute oxidative damage, the surviving organisms were examined and scored. *C. elegans* treated with *Aspalathus linearis* (rooibos) extract displayed an extended lifespan. Green *Aspalathus linearis* (rooibos) exhibited a more potent antioxidant effect than red *Aspalathus linearis* (rooibos), most likely due to its higher aspalathin content. Quantitative real-time PCR results demonstrated that aspalathin reduced endogenous intracellular level of ROS by targeting stress and ageing related genes. This study suggests that the antioxidant effect of *Aspalathus linearis* (rooibos) could be mediated by regulation of the DAF-16/FOXO insulin-like signalling pathway (Chen *et al.*, 2013). However, Joubert *et al.*, reported on both antioxidant and/or pro-oxidant activities of the aqueous extracts and crude polyphenolic fractions of unfermented and fermented *Aspalathus linearis* (rooibos) (Joubert *et al.*, 2005).

2.1.4 Anti-inflammatory studies on *Aspalathus linearis* (rooibos) tea

Active oxygen and free radicals can induce inflammation. Vitamin C and E, flavonoids and enzymes such as catalase, glutathione peroxidase (GPx), and serum superoxide dismutase (SOD) are known antioxidants (Baba *et al.*, 2009). An *in vivo* study investigated the anti-inflammatory effects of *Aspalathus linearis* (rooibos) tea using a rat colitis model. SOD levels were determined as an indicator of antioxidant activity whilst urine 8-hydroxy-2'-deoxyguanosine (8-OHdG) concentrations were indicative of DNA damage. Dextran sodium sulfate (DSS) was used to induce colitis in rodents. Clinical symptoms, hemoglobin, serum iron, (8-OHdG) concentration and SOD levels were compared between the *Aspalathus linearis* (rooibos) and control groups. Levels of SOD of the *Aspalathus linearis* (rooibos) group were significantly increased ($P < 0.05$) compared with the controls whilst urine 8-OHdG levels were significantly decreased ($P < 0.05$) in the *Aspalathus linearis* (rooibos) group compared with the controls. The antioxidant activity of *Aspalathus linearis* (rooibos) tea were proposed as the mechanism preventing DNA damage and inflammation *in vivo* (Baba *et al.*, 2009). *Aspalathus linearis* (rooibos) was included in an anti-inflammatory study using LPS-stimulated macrophage model. Several herbs and spices and their components were included in this study. This study reported a 25% reduction in IL-6 secretion in samples incubated with *Aspalathus linearis* (rooibos) (0.5mg/ml) and a significant reduction in IL-10 as well. Western blot analysis showed an increased expression of COX-2 (more than 25%) by the extracts of *Aspalathus linearis* (rooibos) tea (0.5mg/ml) (Mueller *et al.*, 2010). A study on the effect of *Aspalathus linearis* (rooibos) on rat adrenal cytokine expression showed that *Aspalathus linearis* (rooibos) decreased IL-6 synthesis and increased IL-10 production in cortical tissue (Swart *et al.*, 2013). Inconsistent results were seen when *in vitro* and *in vivo* studies were compared for herbal products (Spelman *et al.*, 2006). An *in vitro* and *in vivo*

study assessed the effects of the two main actives dihy-drochalcones (aspalathin and nothofagin) of *Aspalathus linearis* (rooibos) on high glucose-induced inflammation using human umbilical vein endothelial cells (HUVECs) and mice. Aspalathin and nothofagin inhibited high glucose-mediated vascular hyperpermeability, adhesion of monocytes toward HUVECs and expression of CAMs in a dose dependant manner whilst significantly suppressing ROS formation ($P < 0.05$) and NF- κ B activation ($P < 0.05$) (Ku *et al.*, 2015). An *in vivo* (male wistar rats) study on the ameliorative effect of *Aspalathus linearis* (rooibos) in LPS induced liver injury was undertaken (Ajuwon *et al.*, 2014). This study monitored hepatic levels of pro-inflammatory cytokines, IL-1 β , IL-6 and TNF- α . Results showed that the *Aspalathus linearis* (rooibos) extract significantly ($p < 0.05$) decreased the LPS-induced elevation in TNF- α and IL-6 when compared to the controls. IL-1 β and IL-10 levels remain similar across all 4 groups (Ajuwon *et al.*, 2014). Persson *et al.*, investigated the effects of *Aspalathus linearis* (rooibos), green and black tea on angiotensin-converting enzyme activity (ACE) and nitric oxide production in human endothelial cells. This *in vitro* study showed dose dependant inhibition of ACE activity by green tea and black tea only and that green tea, black tea and *Aspalathus linearis* (rooibos) tea showed a dose dependant increase in NO concentration in the same cells (Persson *et al.*, 2006). A follow-up randomized three-phase crossover design on healthy volunteers was undertaken to determine the effects of *Aspalathus linearis* (rooibos), green and black tea on angiotensin-converting enzyme and nitric oxide *in vivo*. After administering 400 ml of green, black or *Aspalathus linearis* (rooibos) tea, ACE activity was significantly inhibited with *Aspalathus linearis* (rooibos) tea after 30 min ($P < 0.01$) and after 60 min ($P < 0.05$) whilst no significant inhibition was seen with the green or black tea. No significant effect on NO concentration after oral intake of any of the teas were observed (Persson *et al.*, 2006). According to Persson *et al.*, the

conflicting findings between the *in vitro* and *in vivo* studies may be due to differences in the content of the flavonoids or/and the metabolism of the components in the different teas. It is known that there are differences in the pharmacokinetics/metabolism between various flavanoids (Persson *et al.*, 2010). Many studies reports on the anti-inflammatory effects of *Aspalathus linearis* (rooibos). However there are a few studies which reports on its pro-inflammatory effects as well (Smith and Swart, 2016).

Table 2.1 Studies on the effects of *Aspalathus linearis* (rooibos) on cytokine and NO activity.

Preparation	Dose	Model	Cytokine and/or NO effect	Reference
1.63g leaves/100ml water, boiled for 15min and freeze-dried	1-1000µg/ml	<i>in vitro</i> , murine splenocytes	Increased IL-2 Inhibition of IL4 (OVA-primed murine splenocytes)	Kunishiro <i>et al.</i> , 2001
1.63g leaves/100ml water, boiled for 15min	4ml/day extract for 3 weeks	<i>ex vitro</i> , splenocytes of female mice	Increased IL-2 (OVA-induced)	Kunishiro <i>et al.</i> , 2001
5g/100 ml freshly boiled phosphate-buffered saline, steeped for 10 min	0-730µg/ml	<i>in vitro</i> , cultured human umbilical veins endothelial cells	increased NO production	Persson <i>et al.</i> , 2006
17.5g/1000ml boiled for 15mins	100µg/ml <i>in vitro</i> 0.56g/l and 0.16g/l solutions <i>in vivo</i>	<i>in vitro</i> , murine splenocytes <i>in vivo</i> , mice	Increased IL-10 (OVA-induced) Decreased IL-2, IL-4 IFN γ	Ichiyama <i>et al.</i> , 2007
25g tea bags/1000ml Seeped in boiled water, frozen	0-250µg/ml	<i>in vitro</i> , whole blood culture	Increased IL-6, IL-10, and IFN γ (unstimulated) Increased IL-6, decreased IL-10 and No effect on IFN γ (LPS/PHA stimulated)	Hendricks and Pool, 2010
10g tea in 400ml fresh-boiled water for 5 min	400ml per week for 4 weeks	<i>in vivo</i> , randomized three-phase crossover design (human)	No effect on NO	Persson <i>et al.</i> , 2010
100mg/ml DMSO powder leaf extract	0.5mg/ml	<i>in vitro</i> , RAW 264.7 macrophages	Decreased IL-6 and IL-10 (LPS-stimulated) No effect on TNF α	Mueller <i>et al.</i> , 2010
15g leaves /250ml (human) 160mg dried extract/ml (rat)	90g <i>Aspalathus linearis</i> (rooibos)leaves/subject daily 0.25g <i>Aspalathus linearis</i> (rooibos)leaves/rat daily	<i>in vivo</i> , humans and rats	Increased IL-10 Inhibition of IL-6	Swart <i>et al.</i> , 2013
2g/100 ml, seeped for 30mins	2%, w/v	<i>in vivo</i> , rats	Decreased TNF α and IL-6 No effect on IL-1 β and IL-10	Ajuwon <i>et al.</i> , 2014
Aspalathin and Nothofagin, <i>Aspalathus linearis</i> (rooibos) compounds	10uM-30uM 8.7µg- 27.1µg (per mouse)	<i>in vitro</i> , human umbilical vein endothelial cells <i>in vivo</i> , mice	Decreased TNF α and IL-6	Lee and Bae, 2015
30g plant (rooibos) material with 300mL chloroform for 8h using a glass soxhlet in 300ml methanol.	twice daily by gavage (250µl), with a gavage dosage extracted from 0.25g rooibos leaves	<i>in vivo</i> , rats	Decrease IL-6 Increased IL-10	Smith and Swart, 2016

2.2 An overview of *Artemisia afra*

In South Africa, *Artemisia afra* remains one of the most popular herbal medicines used for a variety of illness conditions. Despite its popularity there is limited research on this species (Liu *et al.*, 2009). It is estimated that up to 70% of the South African population use medicinal plants such as *Artemisia afra* for healing purposes (Mjiqiza *et al.*, 2013). *Artemisia afra* grows up to 2 m tall and is a perennial woody shrub with a leafy ridged stem. *Artemisia afra* is found in the mountainous regions of Uganda, Tanzania, Kenya and Ethiopia. It is widely distributed in SA, Namibia and Zimbabwe. Known in Xhosa as “Umhlonyane”, in the Zulu language as “Mhlonyane”, in Sotho as “Lanyana” and in the Tswana language as “Lengana”. *Artemisia*'s common name in English is “African wormwood” and in Afrikaans it is well known as “Wilde als” (Liu *et al.*, 2009).

2.2.1 The chemical composition of *Artemisia afra*

Artemisia afra contains acetylenes, coumarins (scopoletin), flavonoids (methyl ethers of luteolin), terpenoids and volatile oil (Mukinda *et al.*, 2010). Common volatile oils found in *Artemisia afra* are camphor, borneol, 1.8-cineole (eucalyptol), alpha-thujone, beta-thujone and sesquiterpenoids (chrysanthenyl acetate and devannone) (van Wyk and Gericke, 2005 and van Wyk, 2008). There are several flavonoids found in *Artemisia afra* which includes tamarixetin, apigenin, genkwanin, chrysoeriol, kaempferol, and acacetin (Mjiqiza *et al.*, 2013). Other known constituents (non-volatile) contained in *Artemisia afra* include triterpenes (friedelin and amyrin), alkanes (ceryl cerotate and N-nonacosane) and polyacetylenes (van Wyk and Gericke, 2005 and van Wyk, 2008).

2.2.2 The medicinal value of *Artemisia afra*

In South Africa, *Artemisia afra* is commonly used for colds, coughs, diabetes mellitus, heartburn, bronchitis, asthma and for many other illness conditions (Mukinda *et al.*, 2010). Traditional medicinal preparations of *Artemisia afra* include molasses, decoctions, infusions and alcohol extracts (Burits *et al.*, 2001). Flavonoids are considered to be antioxidant agents that provide health promoting benefits which includes anticancer, anti-viral, anti-osteoporotic, anti-inflammatory, anti-allergic and antimicrobial activities (Mukinda *et al.*, 2010). A study tested the folklore remedy of *Artemisia afra* for stomach pains and intestinal cramps. *Artemisia afra* has been proven to have anti-convulsive and sedative effects which are dose dependant. The presence of flavonoids is suggested to influence CNS activity (Liu *et al.*, 2009). A study on the effects of *Artemisia afra* on U937 and HeLa cells lines showed that *Artemisia afra* has potential anticancer activity (Spies *et al.*, 2013).

2.2.3 Antioxidant studies on *Artemisia afra*

The traditional medicinal use of *Artemisia afra* for heart related conditions was tested in animal models. Cardio-protective effects were seen in rats (isoproterenol induced myocardial injury) pre-treated with the aqueous extracts of *Artemisia afra* (Sunmonu and Afolayan, 2010). There were significant decreases in serum marker enzymes namely lactate dehydrogenase (LDH), aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP). Isoproterenol treated rats exhibited decreased myocardial levels of glutathione reductase (GR), glutathione peroxides (GPx), superoxide dismutase (SOD) and glutathione (GSH) whilst pre-treated (aqueous extracts of *Artemisia afra*) rats exhibited near normal levels. GSH is a well known antioxidant which plays an important role as an intracellular radical scavenger. Higher levels of GSH are directly linked to an increased antioxidant status. Likewise decrease levels of GR, GPx and SOD are linked to pro-oxidant

activities (Sunmonu and Afolayan, 2010). In a similar study conducted on the pancreas of Streptozotocin-induced diabetic Wistar rats, antioxidant enzyme activities of GPx, GR, and SOD increased significantly after treatment with the extract of *Artemisia afra*. This study reported that the extract of *Artemisia afra* was effective as a corrective for hyperglycaemia and in alleviating pancreas oxidative stress in streptozotocin-induced diabetic rats (Sunmonu and Afolayan, 2014). A comparative *in vitro* study was conducted on the essential oils of *Artemisia afra*, *Artemisia abyssinica* and *Juniperus procera* to assess the antioxidant potential of these medicinal plants. This study reported that the oil of *Artemisia afra* was an effective hydroxyl radical scavenging agent based on the results of the deoxyribose degradation assay (Burits *et al.*, 2001). The ethanolic extract of *Artemisia afra* leaves displayed significant spasmolytic properties in isolated mouse duodenum and guinea pig ileum. *Artemisia* oil showed antioxidant potential in the bioassay for non-enzymatic lipid peroxidation in liposomes. The radical scavenging effects of the oil were attributed to the presence of sesquiterpene and chamazulene (Liu *et al.*, 2009). A comparative *in vivo* study on four plant extracts with antioxidant activity was conducted in broiler chickens. Toltrazuril, an anti-parasitic veterinary drug was used as the positive control. This study reported that the numerous antioxidant compounds in *Artemisia afra* contributed to its anti-coccidial activity (Naidoo *et al.*, 2008).

2.2.4 Anti-inflammatory studies on *Artemisia afra*

A tuberculosis experimental animal model study looked at the efficacy of *Artemisia afra* against *Mycobacterium aurum* and *Mycobacterium tuberculosis*. Results showed that in tuberculosis infected mice treated with aqueous *Artemisia* extracts, regulation of pulmonary inflammation was evident. This study concluded that *Artemisia* does have *in vivo* anti-myobacterial activity and modulatory effects on pulmonary inflammation in the early stages of the infection (Ntutela *et al.*, 2009). A comparative

in vitro study assessed the anti-inflammatory activity of *Artemisia afra* and *Sutherlandia frutescens* using a whole blood culture model. The results showed that *Artemisia afra* significantly ($P < 0.001$) decreased IL-6 production in LPS stimulated whole blood cells. Results also showed that *Artemisia afra* was cytotoxic at highest concentration tested ($5000\mu\text{g/ml}$) (Kisten, 2011). A comparative study was conducted on the phosphodiesterase 4 inhibitory activity of 3 indigenous African plant medicines namely; *Artemisia afra*, *Leonotis leonorus* and *Mentha longifolia*. Results showed that hydrolysed and unhydrolysed aqueous extracts of *Artemisia afra* inhibited phosphodiesterase 4 activity in a dose dependant manner ($25\text{-}2000\mu\text{g/ml}$) (Mulubwe and Syce, 2007). The genus *Artemisia* comprises of approximately 500 species, found throughout the world. Differences in the chemical profiles of the volatile constituents of *Artemisia* species is due to geographical locations and genetic differences within populations. Plant parts used, variations in sample preparation and extraction methods contributes significantly to findings amongst the same and various species of *Artemisia* (Liu *et al.*, 2009). In South Africa, *Artemisia afra* is used extensively as an anti-inflammatory agent yet there are very few studies available on its effects on cytokine and NO activity.

Table 2.2 Studies on the effects of *Artemisia* species on cytokine and NO activity.

Preparation	Dose	Model	Cytokine and/or NO effect	Reference
Methanol extract of the aerial parts (<i>Artemisia sylvatica</i>)	1.46-6.16uM (IC50 values)	<i>in vitro</i> , RAW 264.7 macrophages	Decreased NO production	Jin <i>et al.</i> , 2004
200g of <i>Artemisia capillaries</i> was ground and extracted with boiling water for 4 h	0-500µg/ml	<i>in vitro</i> , cell culture, RINm5F (RIN) cells	Decrease IFN γ IL-1 β and NO	Kim <i>et al.</i> , 2007
A 20% (w/v) extract was prepared using 94.4% ethanol (<i>Artemisia afra</i>)	0-5000µg/ml	<i>in vitro</i> , whole blood culture	Decreased IL-6, IL-10 and IFN γ production	Kriel and Pool, 2010
Essential oils from the leaves of <i>Artemisia fukudo</i> were obtained by the hydrodistillation of separated 1kg of fresh leaves for 4 h	12.5 -50µg/ml	<i>in vitro</i> , RAW 264.7 macrophages	Inhibition of IL-6, IL-1 β , TNF α and NO	Yoon <i>et al.</i> , 2010
Powdered aerial parts (<i>Artemisia absinthium</i>) (500g) were macerated with distilled water (5 L) at room temperature for 12h, and then boiled for 1h.	Aqueous extract 50, 100 or 200mg/kg body weight/day was administered orally	<i>in vivo</i> , mice model	Decrease TNF α and IL-1	Amata <i>et al.</i> , 2010
20g of the pulverized plant (flowering aerial parts of <i>Artemisia herba alba</i> were macerated for 24 hours in methano-containing water (7:3).	5-50µg/ml	<i>ex vivo</i> , peripheral blood mononuclear cells isolated from Algerian patients (Adamantiades- Behçet's disease)	Decreased IL-12 and NO Increased IL-4	Messaoudene <i>et al.</i> , 2011
Dried stems and leaves (260g) of <i>Artemisia morrisonensis</i> were sliced into small pieces and extracted with 70% ethanol for four times and passed through filter paper.	Oral dose 20, 100, and 500mg/kg	<i>in vivo</i> , mice model	Decreased IL-6, TNF α and NO	Chou <i>et al.</i> , 2012
Aqueous, hydroalcoholic, (50% ethanol and 50% distilled water, v/v), methanol and hexane extracts (dried <i>Artemisia dracunculus</i>)	100mg/kg for 21 consecutive days	<i>in vivo</i> , mice model	Decreased IL-10, IL-17, IFN γ and NO	Froushani <i>et al.</i> , 2016

2.3 The chemical composition of Septilin™

A study on the evaluation of the phytochemicals present in Septilin™ drops was conducted using thin layer chromatography. This study reported the presence of sugars, tannins, alkaloids, saponins, flavonoids, proteins and glycosides (Pushpa *et al.*, 2014).

2.4 The medicinal value of Septilin™

According to the Therapeutic Index of the Himalayan herbal drug company, Septilin™ is a phytopharmaceutical formulation which is recommended for the treatment and management of various infections (Manal, 2014). It is claimed to have immunomodulatory action which potentiates the body's immune response. Septilin™ is considered to be a valuable adjuvant in infection management as it builds the defence mechanism of the body and ensures a faster recovery rate when co-prescribed with antibiotics. It is claimed to have stimulatory effects on the humoral immunity by increasing antibody forming cells. Septilin™ is indicated as an immunomodulator in upper respiratory tract infections, lower respiratory tract infections, allergic disorders of the upper respiratory tract, skin and soft tissue infections, ocular infections, bone and joint infections, urinary tract infections, for early recovery in postoperative conditions, to reduce recurrence in infection-prone individuals, as an adjuvant to anti-infective therapy and for those patients who are resistant to antibiotic therapy (Manal, 2014). The above mentioned claims have yet to be substantiated scientifically.

2.4.1 The herbal ingredients of Septilin™

Septilin™ contains the following herbal ingredients (452mg);

- Guggulu / Indian bedellium (*Balsamodendron mukul* / *Commiphora mukul*)
0.324gm

- Maharasnadi quath 130mg
- Guduchi / Gulancha tinospora (*Tinospora cordifolia*) 98mg
- Manjishtha / Indian madder (*Rubia cordifolia*) 64mg
- Amalaki / Indian gooseberry (*Emblica officinalis*) 32mg
- Shigru / Horse-radish tree (*Moringa pterygosperma*) 32mg
- Yashti-Madhu / Licorice (*Glycyrrhiza glabra*) 12mg
- Conch shell calx (Shankha / *Shankh bhasma*) 64mg (Deore *et al.*, 2014; Manal, 2014).

2.4.1.1 The immunological activity of *Commiphora mukul*

Guggulsterone is an anti-inflammatory phytochemical, a plant sterol derived from the oleogum resin from the Guggul tree known as *Commiphora mukul* which has been used for centuries to treat various diseases, including atherosclerosis, rheumatism, and obesity, and its biological activity and anti-inflammatory activities were first demonstrated in 1960 (Song *et al.*, 2010). Guggulsterone is known to suppress nuclear factor- κ B (NF- κ B) activation induced by inflammatory agents which has led to a considerable amount of interest on this phytochemical. An *in vitro* study on cultured human middle ear epithelial cells focused on the molecular mechanisms underlying the anti-inflammatory activities of guggulsterone in relationship to otitis media. The results showed that guggulsterone (2.5-50 μ M) has an inhibitory effect on TNF α expression and COX-2 production (LPS induced) which may be mediated through its inhibition of NF- κ B activation (Song *et al.*, 2010). Guggulsterone, the plant sterol, has been extensively used in Indian medicine to treat inflammatory disorders such as hyperlipidemia, obesity, and arthritis for many years. Pregnane

plant sterols cis-guggulsterone (E-GS) and trans-guggulsterone (Z-GS) are the active substances in *Commiphora mukul* (Mencarelli *et al.*, 2009). Guggulsterone has been shown to reduce colonic inflammation in two mice colitis models. Guggulsterone fed mice (15 and 30mg/kg) were shown to be protected against the development of signs and symptoms of colon inflammation (based on macroscopic and microscopic damage scores). The *in vitro* (mechanistic study) that employed CD4+ cells isolated from the intestinal lamina propria of these colitis induced mice demonstrated that guggulsterone can effectively regulate the function of effector T cells by modulating cell signalling activation pathway caused by CD3/CD28. Guggulsterone attenuated the generation of IL-2, IL-4 and IFN γ as well as T cell proliferation (*in vitro and in vivo*) in the above mentioned study (Mencarelli *et al.*, 2009).

Table 2.3 Studies on the effects of *Commiphora mukul* on cytokine and NO activity.

Preparation	Dose	Model	Cytokine and/or NO effect	Reference
Methanolic extract of the gum resin	13 μ g/ml	<i>in vitro</i> , RAW 264.7 macrophages	Decrease IL-12, IL-1B, TNF α , IFN γ and NO	Matsuda <i>et al.</i> , 2004
Guggulsterone	2.5-50 μ M	<i>in vitro</i> , cultured human middle ear epithelial cells	Inhibitory effect on TNF α	Song <i>et al.</i> , 2010
Guggulsterone in 100 mM DMSO, diluted in methylcellulose 1%, administrated intraperitoneally, final volume of 200 μ l/mouse	15 and 30mg/kg	<i>in vivo</i> mice colitis models <i>in vitro</i> , isolated CD4+ cells (intestinal lamina propria) from mice colitis model	Decrease IL-2, IL-4, IFN γ and TNF α Decrease IL-2, IL-4 and IFN γ secretion	Mencarelli <i>et al.</i> , 2010
Guggulsterone, isolated phytosterol (<i>Commiphora mukul</i>)	0-25 μ M	<i>in vitro</i> , RAW 264.7 macrophages	Decrease IL-1 β , TNF α and NO secretion	Zhang <i>et al.</i> , 2016

2.4.1.2 The immunological activity Maharasnadi quath

Today suitable preparations from natural sources are the centre of attention in the field for preventing immunological complications of various organs. Herbal constituents such as Maharasnadi quath of immunomodulatory drugs have been found

to be promising in managing inflammatory disorders. Maharasnadi quath have been reported to possess antibacterial, anti-inflammatory, antiexudative, and immunomodulation properties (Deore, 2014). Maharasnadi quath is one of the main components of Indian polyherbal formulations indicated as an analgesic, antiphlogistic and anti-pyretic properties. Maharasnadi quath has also been used for ages in the treatment of rheumatism and arthritis in traditional Indian medicine (Bhalerao *et al*, 2011). There are several studies on polyherbal immunomodulatory products containing Maharasnadi quath as a constituent. However there are no studies available that focus on Maharasnadi quath on its own.

2.4.1.3 The immunological activity of *Tinospora cordifolia*

Tinospora cordifolia is a vital component to many Indian polyherbal preparations. The many therapeutic properties exerted by the extracts of this plant includes its use as a general tonic, an anti-inflammatory, anti-arthritic, antimalarial, aphrodisiac, anti-allergic, antidiabetic, antihepatotoxic, antipyretic and nephro-protective (Sachdeva *et al.*, 2014). The immune activity of *Tinospora cordifolia* was assessed in one experimental animal study (*Leishmania donovani* infected BALB/c mice). Mice were fed the dried pure herb (100mg/kg body weight for 15 days daily), *Tinospora cordifolia* displayed stimulated differentiation of T cells into Th1 sub population and an up-regulation in the release of cytokines such as IFN γ and IL-2 by these cells were also noted (Sachdeva *et al.*, 2014). Seven immunomodulatory active compounds belonging to different classes have been isolated and characterized from the extracts of *Tinospora cordifolia*. These groups of compounds may interact synergistically (Sharma *et al.*, 2012). Increase in ROS is directly related to inflammation via intracellular signalling (Tiwaria *et al.*, 2014). The protective efficacy of the extracts of *Tinospora cordifolia*, were assessed in asthma induced mice. Protective efficacy against oxidative stress and inflammation during asthma,

based on modulation of glutathione homeostasis, as well as increased total anti-oxidant capacity of serum and decrease in lipid peroxidation were observed (Tiwaria *et al.*, 2014). The extract also showed increased levels of I κ B down-regulate translocation of NF- κ B to nucleus resulting in down-regulation of pro-inflammatory genes iNOS, COX-2 and ICAM-1, TNF α , IL-4, NO and IgE leading to prevention of inflammation in the mice models (Tiwaria *et al.*, 2014). On the other hand, one study that looked at 8 immune enhancing botanicals which included *Tinospora cordifolia* reported that the *in vitro* macrophage activation exhibited by extracts of these plants were due the presence of bacterial lipoproteins and lipopolysaccharides. Trace amounts of bacterial lipoproteins was shown to contribute significantly to the activity exhibited by the melanin fraction derived from these immunomodulatory herbal products. This study pointed out that careful consideration should be taken when monitoring the activity of “purified” plant constituents such as a polysaccharide or other large molecular weight plant constituent in order to rule out the influence of bacterial lipoproteins. Contaminating bacterial lipoproteins present at 10ng/ml (0.10% to 0.01% contaminants) within polysaccharide preparations contributes substantially to the activity of these botanicals when monitored at concentrations of 10 to 100 μ g/ml, *in vitro* (Pugh *et al.*, 2008).

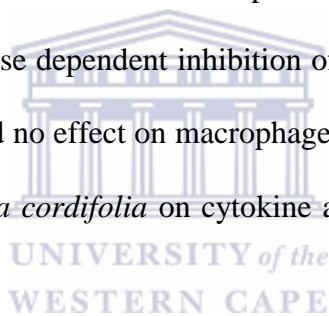
Table 2.4 Studies on the effects of *Tinospora cordifolia* on cytokine and NO activity.

Preparation	Dose	Model	Cytokine and/or NO effect	Reference
Isolated polysaccharide, 0.1% yield of the total dry material (powdered) extraction as a puffy solid that dissolved in water	0-100µg/ml	<i>in vitro</i> , human lymphocytes	Increased IL-1B, IL-6 IL-12, IL-18, IFN γ and TNF-B No effect on IL-2, IL-4, IL-10, IFN- α , TNF α and NO.	Raveendran Nair <i>et al.</i> , 2004
Isolated polysaccharide (methanol extract) from dried powdered stem	2.5, 5.0, 12.5, 25.0mg/kg body weight, intraperitoneally	<i>in vivo</i> , mice model	Increase IL-1B, IL-6 and IFN γ Decrease TNF α and IL-10 Increase NO	Desai <i>et al.</i> , 2007
Aqueous extract from fine dried stem powder (60gm) soaked in 600ml of water.	25-625µg/ml	<i>in vitro</i> , B16F10 mouse melanoma cells.	Increased IL-6 and NO production	Upadhyaya <i>et al.</i> , 2011
Protein quantitation of the dry stem powder(capsule), dried powdered plant and aqueous extract (20% w/v)	0-10µg/ml	<i>in vitro</i> , RAW 264.7 macrophages	Increase NO secretion	Aranha <i>et al.</i> , 2012
Ethyl acetate, water fractions and hot water extract of air dried powdered stem	0.1- 2.5µg/ml	<i>in vitro</i> , human isolated PMNs	Increase NO secretion	Sharma <i>et al.</i> , 2012
Pure dried herb (tablet form) dissolved in distilled water	100 mg/kg (daily) for 15 days	<i>in vivo</i> , mice model	Decrease IL-10 and IL-4 Increase IL-2 and IFN γ	Sachdeva <i>et al.</i> , 2014
Powdered stem (20g) ethanol extract (100ml, 50%)	Oral dose of 100 mg/kg from days 15 to 23	<i>in vivo</i> , asthma mice model	Decreased IL-4 and TNF α and increased IFN γ Decreased NO secretion	Tiwaria <i>et al.</i> , 2014

2.4.1.4 The immunological activity of *Rubia cordifolia*

Rubia cordifolia is a perennial, herbaceous climbing plant known for its traditional therapeutic uses in Indian medicine for the treatment of various immune-related diseases (Kannan, *et al.*, 2009). Free radical-induced oxidative stress has been shown to impair the cellular and humoral components of the immunity. Chronic inflammation also contributes to free radical formation (Joharapurkar *et al.*, 2003). The aqueous extract of *Rubia cordifolia* showed anti-inflammatory activity in rats with carrageenan paw oedema at a dose of 10 and 20ml/kg which was comparable to that of phenylbutazone (100mg/kg) (Joharapurkar *et al.*, 2003). The administration of the ethanol extracts of *Rubia cordifolia* (100mg/kg, body weight orally for 21 days)

in cyclophosphamide induced immunosuppressed Albino rats significantly increased ($P < 0.05$) total white blood and red blood cell count indicating stimulation of the haemopoetic system (Kannan *et al.*, 2009). An *in vivo* evaluation study was conducted on the antioxidant effects of the ethanol extracts of *Rubia cordifolia* in ethanol-induced immuno-suppressed rats (Joharapurkar *et al.*, 2003). The treatment group (100mg/kg of herbal extract) showed significantly decreased ($P < 0.05$) oxidative stress parameters when compared to the control group (Joharapurkar *et al.*, 2003). A comparative study was conducted on the anti-inflammatory compounds from *Ventilago madraspatana*, *Rubia cordifolia* and *Lantana camara* (Ghosh *et al.*, 2010). The NO scavenging activity of these plants were determined on LPS/IFN γ activated murine peritoneal macrophage cultures and Western blot analysis evaluated iNOS and COX-2 expression. The isolated compound, 1-hydroxytectoquinone from *Rubia cordifolia* displayed dose dependent inhibition of NO via suppression of iNOS protein. *Rubia cordifolia* had no effect on macrophage viability (Ghosh *et al.*, 2010). Studies on the effect of *Rubia cordifolia* on cytokine and NO activity are extremely rare.



2.4.1.5 The immunological activity of *Emblica officinalis*

Emblica officinalis belongs to the genus *Emblica* (Family *Euphorbiaceae*) well known in Indian medicine as amla or as the Indian gooseberry. The fruits of this species have been reported to have anti-inflammatory effects. However there are very few *in vitro* or *in vivo* studies to support this (Nicolis *et al.*, 2008). An *in vitro* study on the anti-inflammatory effects of the whole plant ethanolic extracts of *Emblica officinalis* was conducted on I β 3-1 cystic fibrosis airway bronchial epithelial cells. IL-6 and the PAO1-dependent expression of the neutrophil chemokines, IL-8, GRO-a, GRO-y and of the adhesion molecule ICAM-1 were strongly inhibited by *Emblica officinalis* (Nicolis *et al.*, 2008). Another *in vitro* study looked at the antioxidant and

immunomodulating properties of *Emblica officinalis* on chromium (immunosuppressive agent) treated lymphocytes. This study reported that the fruit extracts of *Emblica officinalis* (10µg/ml-1mg/ml) significantly inhibited chromium-induced free radical production in lymphocytes (Sai Ram *et al.*, 2002). An *in vitro* study was performed on the synergistic anti-*Staphylococcus aureus* activity of amoxicillin in combination with the stamen extracts of *Emblica officinalis*. This study noted increased efficacy of amoxicillin when combined with *Emblica officinalis* (Mandal *et al.*, 2010).

Table 2.5 Studies on the effects of *Emblica officinalis* on cytokine and NO activity

Preparation	Dose	Model	Cytokine affected	Reference
Ethyl acetate extract (polyphenol-rich fraction)	40 or 10mg/kg body weight per day for 100 days	<i>in vivo</i> , rat model	Decrease NO secretion	Yokozawa <i>et al.</i> , 2006
Dried fruit ethanolic extracts (9.33% yield)	500µg/ml of the herbal extract for 24 hrs	<i>in vitro</i> , IB3-1 cystic fibrosis airway bronchial epithelial cells	Decrease IL-6 and IL-8	Nicolis <i>et al.</i> , 2008
Extraction of the powder (2g) was carried out with 200 ml of 90% ethanol in Soxhlet apparatus for 5h.	10µg/ml-mg/ml	<i>in vitro</i> , isolated splenocytes from Sprague-Dawley rats	Decreased IL-2 and IFN γ	Sai Ram <i>et al.</i> , 2012

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2.4.1.6 The immunological activity of *Moringa pterygosperma*

Moringa oleifera also known *Moringa pterygosperma* belongs to the *Moringaceae* family. This is a popular Indian medicinal herb known commonly as drumstick tree, sajiwan or sajna. *Moringa oleifera* has been reported to have antipyretic, anti-inflammatory, anti-ulcer, antioxidant, renal and hepatoprotective activities amongst others (Sharma and Paliwal, 2014). An *in vivo* animal study on the antioxidant potential of *Moringa oleifera* pods and its isolated saponin was conducted in mice treated with renal carcinogens and with the hydroethanolic extract of the herb (200 and 400mg/kg body weight). The results showed significant ($p < 0.001$) suppressed renal oxidative stress and toxicity in the treatment group when compared to the

control group (Sharma and Paliwal, 2014). A study on the anti-inflammatory effects of the aqueous extract of the root of *Moringa oleifera* was conducted on rats using indomethacin (10mg/kg) as standard drug. Inhibition in the development of oedema was observed in the *Moringa oleifera* treatment group (dose of 750mg/kg) (Ndiaye *et al.* 2002). Studies on the effect of *Moringa* species on NO activity are extremely rare.

Table 2.6 Studies on the effects of *Moringa pterygosperma* on cytokine activity.

Preparation	Dose	Model	Cytokine affected	Reference
Coarse powder (500g) of the dried seeds was defatted using petroleum ether (60°C-80°C) and then extracted using 95 % ethanol (2L) in a Soxhlet extractor at 55 C for 6 hours	100mg/kg orally	<i>in vivo</i> , guinea pig model	Decrease IL-6, IL-4 and TNF α	Mahajan <i>et al.</i> , 2009
Extraction of the powder (2g) was carried out with 200 ml of 90% ethanol in Soxhlet apparatus for 5h.	10 μ g/ml-mg/ml	<i>in vitro</i> , isolated splenocytes from Sprague-Dawley rats	Decreased IL-2 and IFN γ	Sai Ram <i>et al.</i> , 2012

2.4.1.7 The immunological activity of *Glycyrrhiza glabra*

Glycyrrhiza glabra Linn. (Leguminosae), also known as licorice has been used for ages for medicinal purposes. The roots and stolons of licorice are considered to contain its medicinal active constituents (Asha *et al.*, 2013). Numerous triterpenes such as glycyrrhizin and glycyrrhetic acid, alongside flavones, isoflavones, chalcones and numerous related compounds are suggested to contribute to the many therapeutic properties ascribed to *Glycyrrhiza glabra*. These include anti-inflammatory, antioxidant, antimicrobial and immunomodulatory properties (Siracusa *et al.*, 2011). The immune and antioxidant activities of *Glycyrrhiza glabra* were investigated in four groups of growing Kunming mice on a high fat diet. Mice that were fed a high fat diet had reduced levels of antioxidant enzyme activity (SOD, CAT, GSH-Px and total antioxidant capacity). Significant (P<0.01) dose-dependent increased

antioxidant enzyme activity (SOD, CAT, GSH-Px and total antioxidant capacity) were observed in the treatment group (Hong *et al.*, 2009).

Table 2.7 Studies on the effects of *Glycyrrhiza glabra* on cytokine and NO activity.

Preparation	Dose	Model	Cytokine affected	Reference
Dried roots (2 kg) from <i>Glycyrrhiza glabra</i> grinded into fine powder in liquid nitrogen, with a mortar and pestle. Afterwards, two consecutive extraction with 4L of boiling distilled water for 2.5 h	50 µM for 24 hours.	<i>in vitro</i> , human myelomonocytic leukaemia cells	Decreased IFN γ and NO secretion	Franceschelli <i>et al.</i> , 2011
Dried roots were extracted thrice using acetone in 1:4 proportions at room temperature. The resultant liquid extract obtained after every extraction was mixed and filtered under vacuum at temperature 55°C	1-40µg/ml	<i>in vitro</i> , J774A.1 murine macrophages.	Inhibition of IL-6, IL-1 and NO	Thiyagarajan <i>et al.</i> , 2011
Glycyrrhizin (93%) was extracted from the dried roots of licorice	10, 20 and 30 mg/kg orally for 20 days once a day	<i>in vivo</i> , allergic rhinitis mice model	Decrease IL-4, IL-5, IL-6, and TNF α Increase IL-2, IL-12 and NO secretion	Li and Zhou, 2011

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2.4.1.8 The anecdotal claims of Shankh bhasma

Conch refers to the empty shell of *Turbinella rapa/Xanachus pyrum*, a marine gastropod. The shell is well known in Indian medicine as Shankh bhasma and recognized as a source of calcium salts (Gopal *et al.*, 2008). Shankh bhasma is obtained by incinerating the conch shell and indicated for the treatment of dyspepsia, digestive impairment, malabsorption syndrome, enlargement of liver-hepatomegaly, hyperacidity and duodenal ulcer hyperpyrexia. The Conch shell ash is used both internally and externally to treat various illnesses including: ophthalmic and other ocular infections, earache, ulcers, dyspepsia, gonorrhoea, colic, dysentery, jaundice, tympanitis and flatulence (Gopal *et al.*, 2008).

2.5 Immunomodulatory studies on Septilin™

An animal study was undertaken by Sharma and Ray in 1997 to investigate the effect of Septilin™ as an immunomodulator in immunosuppressed mice (primary and secondary immunization with sheep red blood cells) specifically focused on humoral (antibody titre of IgM and IgG) and cellular immunity (footpad thickness as an indicator of localised delayed hypersensitivity). The oral administration of Septilin™ (500mg/kg) alone or alongside the immuno-suppressive drug (prednisolone 4mg/kg) showed stimulation of both primary and secondary immune response (Sharma and Ray, 1997). The Septilin™ treated group demonstrated a significant increase in cell mediated immunity ($p < 0.01$) whilst the Septilin™ with prednisolone treated group demonstrated an insignificant difference in cell mediated immunity ($p > 0.05$). Results of secondary antibody titre showed significant increase ($p < 0.001$) in both IgM and IgG concentrations in the Septilin™ treated group. This suggests significant potentiating action of Septilin™ on humoral immunity (Sharma and Ray, 1997).

Immunomodulatory studies on male albino rats and mice were conducted to assess various arms of the immune response to Septilin™ at varying doses (1-3g/kg) (Daswani and Yegnanarayan, 2002). The parameters monitored included that of weight gain, resistance against *E. coli* sepsis, haemo-gram, phagocytic activity of polymorphonuclear (PMN) cells and reticulo-endothelial system, delayed hypersensitivity to oxazolone and the plaque forming cell response of splenic lymphocytes to sheep erythrocytes. The results showed that high doses of Septilin™ reduced phagocytic activity of the PMN cells/reticulo-endothelial system whilst both high and low doses increased the percentage and absolute quantity of circulating neutrophils. These results showed stimulation of humoral immunity (B-lymphocyte function was determined by the plaque forming cell response of rat splenic lymphocytes to sheep erythrocytes) and suppression of cellular immunity (delayed

hypersensitivity to oxazolone) (Daswani and Yegnanarayan, 2002). The humoral stimulatory findings agreed with previous results of Sharma and Ray, 1997. This study reported dual effects of Septilin™, high doses showed suppressive effects on cellular immunity and low doses yielded greater stimulatory effects on humoral immunity (Daswani and Yegnanarayan, 2002; Varma *et al.*, 2011). In a similar animal model study conducted by the Pharmacology Department of the University College of Medical Sciences in Dehli in India, researchers looked at the anti-inflammatory and analgesic effects of Septilin™ (Khanna and Sharma, 2003). Acute, sub-acute and chronic models of inflammation were used to assess inflammatory activity based on four study groups consisting of a control, a Septilin™ treated group (500mg/kg), a prednisolone treatment group (4mg/kg) and a combination treatment group of Septilin™ (500mg/kg) and prednisolone (4mg/kg). The results confirmed consistent anti-inflammatory effects of Septilin™ when compared to the reference drug, prednisolone (Khanna and Sharma, 2003). The acute and sub-acute models demonstrated that Septilin™ was less effective as an anti-inflammatory than prednisolone. Septilin™ as an adjuvant over a long period proved to be superior to prednisolone. Prednisolone demonstrated potent anti-inflammatory effects but suppressed host immune response over long term whilst Septilin™ potentiated non-specific defence mechanisms (Khanna and Sharma, 2003).

Studies on the effects of Septilin™ on irradiated mice, with focus on mortality and symptoms of radiation sickness, reported significant differences between the treated group and the control (Jagetia and Balinga, 2004). Pre-treatment of mice to the herbal product 4 days before exposure to radiation compared to the untreated group resulted in a dose-dependent reduction in animal mortality up to 100mg/kg, higher dosages led to decreased survival rates. The group pre-treated with Septilin™ were protected against radiation sickness and death due to gastrointestinal complications when

compared to the untreated group. This study considered Septilin™ to be an effective radio protective agent at specific doses of 40, 60, 80 and 100mg/kg (Jagetia and Balinga, 2004). Another study noted an increase in phagocytosis, leukocyte counts, percentage of polymorphs in blood, proliferation of bone marrow cells and protection against myelosuppression and leukopenia in cyclophosphamide induced mice (Kumar *et al*, 1997).

In a review on the cytokine expression by herbal immunomodulators, Spelman *et al*, suggests that the inconsistencies noted in previous studies on the effects of several herbal products on cytokine expressions (immune activity) is due to a biphasic dose response. The biphasic effect explains that exogenous and endogenous compounds may have opposing, dose-dependent physiological effects. Paradoxical responses in cytokine activity by herbal products in both *in vitro* and *in vivo* studies is not an uncommon finding (Spelman *et al*, 2006). Contradictions in results of Septilin™ and its ingredients (single herbs) in similar models in the above mentioned studies may be due a biphasic dose response. Divergent models, dosages, duration of exposure (incubation time), and method of administration of herbal products are factors which also contributes to variation in cytokine expression.

Bhattacharya and Deepa, 2011, conducted three clinical trials on the efficacy of Septilin™ (tablets and syrup) on children and infants with upper respiratory tract infections (RTIs) (Bhattacharya and Deepa, 2011). Clinical trial one assessed hemoglobin, total and differential white cell counts, and throat swab for culture and sensitivity in patients diagnosed with upper respiratory tract infections. Younger patients were given 1 tablet twice a day whilst older children were given 1 tablet three times a day for 12 weeks. Results showed that 80% patients showed no recurrence of infection and 20% patients showed a poor response in relation to disappearance of previous documented signs and symptoms (Bhattacharya and Deepa, 2011).

Clinical trial two assessed routine blood counts, throat cultures, and chest x-ray in infants and children (9 months to 5 years old) with upper RTIs. Infants were given one teaspoon of Septilin™ whilst children were given 2 teaspoons a day for 12-16 weeks. Significant decreases in symptoms were noted in most patients, good appetite with concomitant weight gain was seen in all patients (Bhattacharya and Deepa, 2011). Clinical trial three included children (6 months to 5 years) with repeated attacks of acute tonsillopharyngitis. Dosages of half a tablet to one tablet 2-4 times per day were administered according to age for 6-7 weeks after antibiotic therapy (7 days). These patients were followed up after 6-9 months and results showed a reduction of recurrent infections in 80% of patients compared to previous history of recurrent infection without the use of Septilin™ as an intervention (Bhattacharya and Deepa, 2011). This clinical trial does not clearly explain the impact that antibiotic therapy could have on the results reported. This study also did not include a control group. However the results could be valuable for future follow up clinical trials.

Shetty *et al.*, conducted a double blinded randomized controlled clinical trial on 96 healthy patients with chronic periodontitis following scaling and root planning. Group 1 was administered Septilin™ tablets twice daily for 2 weeks while group 2 was given probiotic tablets twice daily for 2 weeks, and group 3 was not given any intervention. Gingival index, gingival bleeding index, pocket depth and IL-6 levels in gingival crevicular fluid and saliva were assessed. Results showed statistically significant reduction ($p < 0.001$) in clinical parameters and IL-6 levels in group one (Septilin™) when compared to group 2 and 3 (Shetty *et al.*, 2015).

Table 2.8 Studies on the effects of Septilin™ on cytokine and NO activity

Preparation	Dose	Model	Cytokine affected	Reference
100grams of Septilin™ powder in 50% ethanol (11) at 50 to 60°C in a Soxhlet apparatus for 120 h. The cooled hydroalcoholic liquid extracts were concentrated by evaporating its liquid contents	0-1000µg/ml	<i>in vitro</i> , sodium nitroprusside treated	Inhibition of NO secretion	Jagetia <i>et al.</i> , 2004
Tablet form as commercially available	1 tablet twice a day for two weeks	<i>in vivo</i> , human chronic periodontal disease model	Reduction in TNFα	Bhattacharya and Deepa, 2011
Liquid preparation of Septilin™ was received from Himalaya Drug Company,	2.5-5% Septilin™ incubation for 24hrs	<i>in vitro</i> , RAW 264.7 macrophages	Decrease IL-6, IL-8, TNFα and NO secretion	Varma <i>et al.</i> , 2011
Liquid preparation of Septilin™ was received from the Himalaya Drug Company	Septilin™ injected intraperitoneally (100 mg/kg b.wt.) for five consecutive days	<i>in vivo</i> , radiation induced rat model	Decreased TNFα and NO secretion	Mansour <i>et al.</i> , 2014
Tablet form as commercially available	Septilin™ tablets twice daily for 2 weeks	<i>in vivo</i> , human double blinded randomized controlled clinical trial	Decrease IL-6	Shetty <i>et al.</i> , 2015



Septilin™ has shown some immunomodulatory potency in a few animal and human models, however further studies are needed as the cellular signalling mechanism underlying the effects on inflammation remains unclear. The effects of Septilin™ on inflammation have yet to be established via suitable *in vitro* models.

2.6 Concluding remarks

Scientific literature confirms that many of the medicinal effects of botanicals are due to cytokine modulation. Many immunomodulatory HMPs are polyherbal formulations (Spelman *et al.*, 2006). These plant based medicines are extremely complex in nature due to the presence of numerous active constituents that may interact synergistically. These compounds may target several pharmacological sites that may potentiate therapeutic benefits (Burns *et al.*, 2010). The anti-inflammatory

effects of several herbal medicines have been reported previously. Most of these plant products are antioxidant in nature, the ability to scavenge oxygen radicals is directly linked to de-activation of pro-inflammatory genes. Herbal antioxidant effects have been widely studied in the context of inflammation (Gertsch *et al.*, 2010). Most immune related studies of herbal medicines are conducted *in vitro* on cytokines such as IL-6, IL-10, IFN γ amongst others. Divergent experimental *in vitro* study designs have been reported to be the reason for variations in results of cytokine effects which at times could exist in the same type of study and methods followed. Often *in vitro* or/and *in vivo* animal models uses unrealistic dosages of plant materials to support various anecdotal claims (Spelman *et al.*, 2006).

The effects of *Aspalathus linearis* (rooibos) on inflammation has been researched in a few *in vitro* and *in vivo* studies. Its effects on several cytokines has been observed with various reports differing in results. Numerous factors contribute to these variations as mentioned before. There are several reports on the antioxidant and anti-inflammatory effects on *Aspalathus linearis* (rooibos) however there are a few reports on its pro-oxidant and pro-inflammatory effects. There may be a relation between the type of preparation used (solvent or aqueous) and inflammation activity.

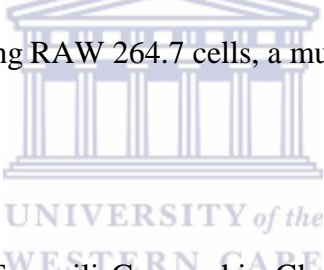
There are several studies on various species of *Artemisia* worldwide. Previous studies on herbal products have shown that the quantity and presence of active constituents vary in different species of medicinal plants which accounts for varying pharmacological effects. *Artemisia afra* is widely used in S.A for inflammatory conditions. Studies on the effects *Artemisia afra* on inflammation with reference to NO and IL-6 are few.

The anti-inflammatory effects of Septilin™ has been reported in a few *in vivo* (animal and human) studies. Spelman *et al.*, reports that the dosage of 400-500mg/kg

(Septilin™) used in previous animal models are unrealistic and cannot be extrapolated for human use as it represents a dosage that is far too high when compared to its anecdotal use. Several previous *in vitro* studies on Septilin™ also used large dosages which could account for the significant effects reported. Most *in vitro* studies on Septilin™ obtained samples of the product from the Himalaya drug company in a liquid preparation. Studies on the effects of Septilin™ on inflammation using whole blood cultures and macrophages are rare. Septilin™ has very few citations on its immunomodulatory action *in vitro*.

In light of the above, this study was undertaken to investigate the *in vitro* effects of Septilin™ on biomarkers (IL-6, IL-10 and IFN γ) of specific immune pathways by using human whole blood culture assays. This study also assessed the effects of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ on inflammatory biomarkers (IL-6 and NO) using RAW 264.7 cells, a murine macrophage cell line.

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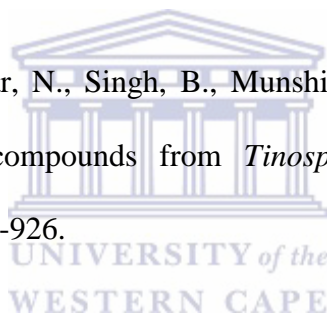
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Chapter 3

An *in vitro* study to elucidate the effects of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ on immune pathways

3.1 Abstract

Septilin™, a commonly used herbal medicinal product (HMP) in South Africa (SA) has been reported to have immunomodulatory properties. *Aspalathus linearis* (rooibos) is a commercialised South African tea recognised for its phytopharmaceutical potential. *Artemisia afra* (Wilde als) is a well known herbal medicine used for various inflammatory conditions. This study assessed the effects of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ on inflammatory biomarkers using RAW 264.7 cells, a murine macrophage cell line. This study also assessed the effects of Septilin™ on biomarkers of specific immune pathways by using whole blood culture assays (WBC). RAW 264.7 cells and lipopolysaccharide (LPS) activated RAW 264.7 cells were treated with various concentrations of the above mentioned samples after which the culture supernatants were assayed for specific inflammatory biomarkers namely, IL-6 and nitric oxide (NO). Stimulated and unstimulated WBC were incubated with Septilin™. Enzyme linked immunosorbent assays were used to screen for IL-6, IL-10, and IFN γ as biomarkers for inflammation, humoral immunity, and cell mediated immunity, respectively. *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ were shown to be non-cytotoxic on unstimulated RAW 264.7 cells across all concentrations tested (31-1000 μ g/ml). Addition of *Aspalathus linearis* (rooibos) to unstimulated RAW 264.7 cells significantly up regulated (P<0.001) NO and IL-6 production at concentrations of 500 μ g/ml and 1000 μ g/ml when compared to the control, whilst Septilin™ and *Artemisia afra* had no effect. *Artemisia afra* and *Aspalathus linearis* (rooibos) were shown to be non-cytotoxic on stimulated RAW 264.7 cells across all concentrations

tested (31-1000µg/ml). However, Septilin™ significantly (P<0.001) decreased metabolic activity at the highest concentration tested (1000µg/ml). Addition of *Artemisia afra* to stimulated RAW 264.7 cells significantly down regulated (P<0.001) NO and IL-6 production when compared to the control. *Aspalathus linearis* (rooibos) and Septilin™ samples had no effect on the synthesis of NO and IL-6 in stimulated RAW 264.7 cells when compared to the controls. WBC results show that Septilin™ had no effect on the release of IL-6 production by LPS stimulated WBC across all concentrations tested. However, Septilin™ induced significantly higher levels (P<0.001) of IL-6 release in unstimulated WBC across all concentrations between 0µg/ml-258µg/ml. Septilin™ had no effect on the release of IL-10 release in unstimulated WBC across all concentrations. However the presence of Septilin™ in phytohaemagglutinin (PHA) stimulated WBC induced significantly higher release (P<0.01) of IL-10 at concentrations between 64.5µg/ml-258µg/ml when compared to the control. The presence of Septilin™ in unstimulated WBC had no effect on the release of IFN γ production across all concentrations. The presence of Septilin™ in PHA stimulated WBC release of IFN γ is inconclusive. The results of this study indicate that *Artemisia afra* has anti-inflammatory effects while *Aspalathus linearis* (rooibos) up regulated the immune system. The study also shows that Septilin™ had no effects on RAW 264.7 cells whilst the *in vitro* study on WBC indicates that Septilin™ had immunomodulatory effects.

3.2. Introduction

Some herbal immunomodulatory preparations have been observed to exert anti-inflammatory effects and modulate both humoral and cellular functions (Varma, *et al.*, 2011). These formulas may modify the actions of the immune system by influencing the regulation of messenger molecules like cytokines, nitric oxide, hormones, neurotransmitters, and other peptides. Herbal formulas are often prescribed for inflammatory and immune-related illnesses (Burns *et al.*, 2010).

Immunity refers to the protection against pathogenic microbes. Innate and adaptive immunity are the two types of reactions of the immune system. The reactions of cells within innate and adaptive immunity are involved in the process of inflammation (Kumar *et al.*, 2007). Inflammation is a highly complex process that has a domino-like effect on the initiation and development of a wide range of systemic diseases (Shahabi *et al.*, 2014). Polypeptide products of numerous cell types, known as cytokines, are mediators of inflammatory and immune responses (Kumar *et al.*, 2007). Influencing the cytokine activity shows promise as treatment for many illness conditions (Burns *et al.*, 2010). It is clear that the cytokine IL-6 is involved in the systemic changes associated with inflammation and infection (Medeiros *et al.*, 2009). Various cells like fibroblasts, cells of the monocyte-macrophage lineage, and endothelial cells can secrete IL-6, which is also known as an alarm hormone due to its release when tissues are damaged, which in turn induces the synthesis of proteins in the liver (acute-phase proteins) that protect the host against inflammatory reactions. IL-6 is known for its pro-inflammatory actions which make it ideal as a biomarker for inflammation. The availability of bioassays which are sensitive for IL-6 allows for the determination of this cytokine in clinical samples (Hack *et al.*, 1989; Jones *et al.*, 2001; Medeiros *et al.*, 2009). Biomarkers can be employed as an indicator to objectively measure and analyse biological, pathological or pharmacological

responses to a therapeutic intervention. The pharmaceutical potency of an agent and the mechanism of its actions can be measured by the presence of biomarkers (Kisten and Pool, 2010). Lipopolysaccharide (LPS), pathogens, toxins, drugs and oxidants are all known to be potent stimulators of IL-6 (Shahabi *et al.*, 2014). LPS, a bacterial antigen, is a potent activator of a wide range of signalling pathways particularly pathways of inflammation. Inflammatory mediators includes pro-inflammatory cytokines like TNF α , IL-1, IL-6, IL-8 and also NO and prostaglandins amongst others. NO is a metabolite produced by enzymes, which include inducible nitric oxide synthase (iNOS). The enzymatic activity of iNOS in diverse cell types contributes to the overproduction of NO which is responsible for inflammation in several pathophysiological conditions like cancer, rheumatoid arthritis, diabetes and liver cirrhosis amongst others (Erickson, 2003). An unstable molecule that has lost an electron is referred to as a free radical. Free radicals can oxidise DNA, nucleic acid, proteins or lipids which contributes to degenerative illnesses like cardiovascular diseases and cancers (Erickson, 2003). The production of reactive oxygen species is mechanistically linked to inflammation (Ku *et al.*, 2015). The excessive production of NO and its oxidation product, peroxynitrite has been implicated in several inflammatory conditions. Inhibition of NO has become the main focus area in the field of anti-inflammatory research (Konkimalla *et al.*, 2008). Macrophages and monocytes play a crucial role in innate and adaptive immunity. Macrophages affect various immune responses when encountering invading pathogens. The versatile role of macrophages includes antigen recognition, capture, clearance and transport of foreign products. Macrophages stimulated by LPS and microbes elicit the release of various proteins like iNOS which leads to the production of NO (Bisht *et al.*, 2009).

The use of RAW 264.7 mouse macrophage cell lines is a well-established model to determine NO production (Konkimalla *et al.*, 2008). The LPS stimulated cell system

has become popular in the area of new anti-inflammatory drug discovery (Varma *et al.*, 2011).

Adaptive immunity consists of two pathways namely the humoral and cell mediated immunity (Kumar *et al.*, 2007). Humoral immunity promotes natural resistance and host defence against certain infections. B cells are lymphocytes that contribute significantly to humoral immunity by producing antibodies against antigens (Kisten and Pool, 2010). These antibodies contribute to the destruction of extracellular pathogens and prevent the spread of intracellular infections (Murphy, 2012). The cytokine IL-10 originates from T helper type 2 (Th2) cells. IL-10 plays an essential role in B cell lymphocyte maturation, as it is required for B cell survival, differentiation and isotype switching. IL-10 production is one of the main mechanisms contributing to the function of B lymphocytes (Mion *et al.*, 2014). IL-10 is an essential regulator of defence mechanisms involved in combating intestinal parasites, neutralising bacterial toxins and in local mucosa defence (Hendricks and Pool, 2010). IL-10 is an important immunoregulator during infections caused by viruses, bacteria, fungi and protozoa (Couper *et al.*, 2008). The production of IL-10 by whole blood cells can be used as a biomarker for humoral immunity (Kisten and Pool, 2010).

Cell mediated immunity consists mainly of T cell-mediated cytotoxicity and the activation of macrophages by effector T cells. Th1 is specifically responsible for synthesizing the cytokine IFN γ . The production of IFN γ continues when antigens are recognised on target cells. Reinforcement of this signal leads to further differentiation of more Th1 cells (Murphy, 2012). IFN γ is a complex modulatory cytokine that plays a crucial role in immune defence during microbial infections. IFN γ is a powerful mediator of multiple immune pathways in the inflammatory process and in the presence of bacterial infections. IFN γ is known to activate the microbicidal ability of

macrophages (Too *et al.*, 2014). The cytokine IFN γ has a wide range of properties which include that of an anti-fibrotic, anti-infective, anti-proliferative, and an immunomodulatory. IFN γ affects fibroblast proliferation, collagen-matrix deposition, and collagen synthesis all of which has been observed *in vitro* and in rodent models (Raghu, *et al.*, 2004). The production of IFN γ by whole blood cells can be used as a biomarker for cellular immunity (Kisten and Pool, 2010).

This study investigated the immunomodulatory effects of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ on RAW 264.7 cells using cytotoxicity and inflammatory biomarkers as end points. The inflammatory biomarkers used in this study were IL-6 and NO. This study also investigated the *in vitro* effects of Septilin™ using whole blood cultures. Biomarkers of specific immune pathways (IL-6, IL-10 and IFN γ) were then monitored to determine the effect of Septilin™ on these pathways.

3.3 Materials and methods

3.3.1 Sample preparation of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™

A 20% (w/v) plant extract of *Artemisia afra* was prepared using 94.4% ethanol (Parceval (Pty) Ltd pharmaceuticals, South Africa). The aerial parts of the *Artemisia afra* plant were milled (sieve size 2-3mm) and mixed with 94.4% ethanol (20g *Artemisia afra*: 100ml ethanol). The milled leaves were separated from the remaining tincture. The tincture was sterilised by filtration using a 0.50 μ m sterile filter and stored at 4°C.

Chemical analysis of the *Artemisia Afra* extract sample was conducted by Central Analytical Facilities at Stellenbosch University (see addendum 1)

Aspalathus linearis (rooibos) in a tea bag form (net weight: 25g; manufacture date 02/06/2013; expiry date 01/06/2014) was seeped in 500ml of boiling water. The sample was allowed to cool to room temperature. The sample was sterilised by filtration using a 0.50µm sterile filter. Aliquots of the extract 1ml/vial were stored at -80°C.

Septilin™ (net weight: 452mg; batch nr: E281004; manufacture date: 10/08/2011; expiry date: 04/2014) in tablet form was crushed by means of a sonicator then diluted in 35ml of distilled water. The sample was incubated on a shaker for 1 hour at ambient temperature. The sample was then centrifuged at 40 000rpm for 10mins. After that it was sterile filtered using 0.50µm sterilized filters and stored in 1ml aliquots at -80°C.

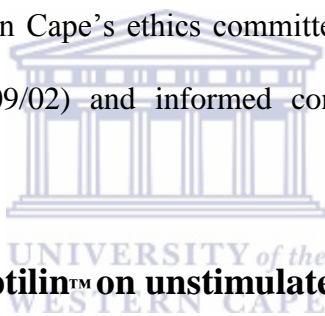
3.3.2 Preparation of RAW 264.7 Cell culture for *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™

Cultures were prepared under sterile conditions. Mouse macrophage RAW 264.7 cell line (ATCC-TB-71) was cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat inactivated foetal bovine serum (FBS), 1% antibiotic/antimycotic (Sigma, Germany), 0.05% gentamycin (Sigma, Germany), and 1% Glutamax™, at 37°C and 5% CO₂. The cells were cultured in 96 well plates (Nunc maxisorp, Nunc™, Denmark) at a density of 5x10⁵ cells/ml till they were almost confluent. At this stage the following solutions were prepared: control medium for unstimulated cultures consisted of normal culture medium only, while stimulation medium for cultures was supplemented with 1µg/ml lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4 (Sigma, Germany). Extracts of the various products (*Aspalathus linearis*, *Artemisia afra* and Septilin™) were diluted in normal medium to give a concentration range from 0-2000µg/ml. At confluence half the plate received normal medium (unstimulated cultures), while the other half of the plate received LPS

containing medium (stimulated cultures) at 100µl/well. This was followed by the addition of a further 100µl/well of the medium containing various extract concentrations. Final concentration ranges of the extracts were between 0-1000µg/ml. After overnight incubation at 37°C and 5% CO₂, culture supernatants were collected for NO and IL-6 assays. The cells on a plate were used for cell viability assays.

3.3.3 Blood collection (preparation of WBC for Septilin™)

The blood was collected at the University of the Western Cape (UWC) campus clinic. Blood from 4 healthy male volunteers, not on any medication, was used. Samples of blood were collected by venipuncture directly into heparinised vacuum tubes. The blood was stored at room temperature and used within 2 hours of collection. Whole blood cell cultures were prepared under sterile conditions in a laminar flow cabinet. The University of the Western Cape's ethics committee approved this study (Ethics number: ScRIRC2010/exco/09/02) and informed consent was obtained from all participants.



3.3.3.1 The effects of Septilin™ on unstimulated blood (WBC)

Cultures were prepared under sterile conditions. The following media were prepared for the cultures: Medium for unstimulated cultures was RPMI 1640 supplemented with 1% antibiotic/antimycotic (Sigma, Germany), 0.05% gentamycin (Sigma, Germany), and 1% glutamax (Sigma, Germany). Blood was diluted to a final concentration of 20% in medium. Aqueous extracts of the herbal product (Septilin™) were diluted in normal medium to give a concentration range from 0-2000µg/ml. Three replicates of the diluted extracts were transferred to a 96 well plate at 100µl/well. This was followed by the addition of 100µl/well of diluted blood in normal medium to each of the diluted extract replicates. After overnight incubation at

37°C and 5% CO₂, culture supernatants were collected for IL-6, IFN γ and IL-10 assays.

3.3.3.2 The effects of Septilin™ on LPS stimulated blood (WBC)

Cultures were prepared under sterile conditions. The following media were prepared for the cultures: Medium for LPS stimulated cultures was RPMI1640 supplemented with 1% antibiotic/antimycotic (Sigma, Germany), 0.05% gentamycin (Sigma, Germany), and 1% glutamax (Sigma, Germany) and 1 μ g/ml LPS. Blood was diluted to a final concentration of 20% in LPS enriched medium. Extracts of the herbal product (Septilin™) were diluted in normal medium to give a concentration range from 0-2000 μ g/ml. Three replicates of the diluted extracts were transferred to a 96 well plate at 100 μ l/well. This was followed by the addition of 100 μ l/well of diluted blood in LPS stimulated medium to each of the diluted extract replicates. After overnight incubation at 37°C and 5% CO₂, culture supernatants were collected for IL-6 assays.

3.3.3.3 The effects of Septilin™ on PHA stimulated blood (WBC)

Cultures were prepared under sterile conditions. The following media were prepared for the cultures: Medium for phytohemagglutinin (PHA) stimulated cultures was RPMI 1640 supplemented with 1% antibiotic/antimycotic (Sigma, Germany), 0.05% gentamycin (Sigma, Germany), and 1% glutamax (Sigma, Germany) containing 16 μ g/ml PHA (Sigma, Germany). Blood was diluted to a final concentration of 20% in PHA enriched medium. Extracts of the herbal product (Septilin™) (net weight: 452 mg in 35ml of distilled water) were diluted in normal medium to give a concentration range from 0-2000 μ g/ml. Three replicates of the diluted extracts were transferred to a 96 well plate at 100 μ l/well. This was followed by the addition of 100 μ l/well of diluted blood in PHA stimulated medium to each of the diluted extract replicates.

After overnight incubation at 37°C and 5% CO₂, culture supernatants were collected for IFN γ and IL-10 assays.

3.3.4. Metabolic activity and cytotoxicity (WST-1) of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ in RAW 264.7 cells

The cell metabolic activity and cytotoxicity of RAW 264.7 cells were evaluated using the WST-1 Cell Proliferation Reagent (Roche, Almere, the Netherlands). The WST-1 conversion assay is based on the mitochondrial integrity of whole cells which allows them to metabolise the stable tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) to a soluble violet formazan product. The metabolic conversion of WST-1 by the cells exposed to various concentrations of the above extracts was assessed in a 96-well microtitre plate. The assay was conducted according to the manufacturer's specifications. Briefly, cells were exposed to various concentrations (0-1000 μ g/ml) of the above extracts for 24 hours and subsequently incubated with WST-1 reagents for 1 hour. Absorbance was measured using a SpectraMax® spectrophotometer at a wavelength of 450nm. The absorbance of the extracts in the culture medium, measured in the absence of cells, was subtracted from the total absorbance of the extract treated cells.

3.3.5 Measurement of nitrite formation of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ in RAW 264.7 cells

Nitrite production was determined in the supernatant of the media by Griess reaction. The reagents for the Griess assay were purchased from Sigma (USA). After the 24 hour incubation of the test cells, cell culture supernatant (100 μ l/well) was added to a solution of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylene diaminedihydrochloride in 5% H₃PO₄), incubated at ambient temperature for 15 minutes to form a purple azodye. The absorption reading at 540nm was determined

using a SpectraMax® spectrophotometer. Excel was used to generate a standard curve.

3.3.6 Cytokine analysis (IL-6 ELISA) of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ treated RAW 264.7 macrophage culture supernatants

The release of the inflammatory biomarker IL-6 was measured in the supernatant of the RAW 264.7 cells after exposure to various concentrations of the plant extracts and Septilin™ for 24 hours at 37°C in a humidified atmosphere of 5% CO₂. The effects of the extracts on IL-6 released from the RAW 264.7 cells were assessed using the Mouse Cytokine IL-6 ELISA kit (E-Bioscience kit, Biocom biotech).

Cytokine analysis was performed according to the manufacturer's instructions. Nunc maxisorp (Nunc™, Denmark) plates were used for the assays. Briefly, 96 well plates were coated with primary antibody against the IL-6 and incubated overnight at -4°C. After incubation, the plates were washed with phosphate buffered saline containing 0.05% Tween-20. Non-specific binding sites were then blocked with assay diluent for 1 hour at ambient temperature after which the wells received either recombinant mouse IL-6 standards or sample. The plate was sealed and incubated for 2 hours at ambient temperature on a shaker. After incubation the wells were washed. The wells then received Biotin-conjugated antibody against IL-6. The plate was incubated for 1 hour at ambient temperature on a shaker followed by washing as before. The wells then received avidin horseradish peroxidase (Avidin-HRP conjugate). The plate was incubated for 30 minutes at ambient temperature on a shaker followed by washing. After the last wash, the bound peroxidase was monitored by addition of Tetramethylbenzidine substrate (Sigma) solution to each well, after which the plate was incubated for approximately 15 minutes. The reaction was stopped by adding 50µl of 2M H₂SO₄ to each well. The absorbance was read at 450nm on an ELISA

plate reader. Excel was used to generate a standard curve for each ELISA plate. This was then used to determine the cytokine concentrations of the culture supernatants.

3.3.7 Cytokine analysis of Septilin™ (IL-6, IL 10 and IFN γ ELISAs) for WBC

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

3.3.8 Statistical analysis of data

RAW 264.7 cells and WBC (Septilin™) experiments were performed in triplicate. The data of the above mentioned assays (IL-6, IL-10 and IFN γ) for WBC (Septilin™) were not compared to each other. The unstimulated WBC versus the stimulated WBC for each biomarker (IL-6, IL-10 and IFN γ) were compared. All data was captured on excel spreadsheets and were expressed as mean \pm standard deviation (SD). The statistical significance of data was analysed via one-way analysis of variance and regression analysis (ANOVA).

3.4 Results and discussion

3.4.1 The effect of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ on the metabolic activity of unstimulated and LPS stimulated RAW 264.7 cells

Cellular proliferation refers to an increase in the number of cells due to cell growth and cell division which result in the increase in subcellular organelles like mitochondria. In all living organisms, tissue growth is dependent on a balance between cell proliferation and cell death. Many drugs affect particular stages of the cell cycle. Cell injury and cell death is a consequence of specific interferences with cell metabolism. Abnormal cell proliferation is the underlying factor to many pathological conditions. Alterations to the cell cycle and cell proliferation plays an important role in immunity (Underwood, 2004).

The results on metabolic activity of unstimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ were evaluated using the WST-1 Cell Proliferation Reagent (Figure 3.1). *Artemisia afra* induced a significant increase in metabolic activity ($P < 0.001$) at a concentration of 500 μ g/ml whilst *Aspalathus linearis* (rooibos) induced significant increases in metabolic activity ($P < 0.001$) across the concentrations of 31-250 and 1000 μ g/ml in

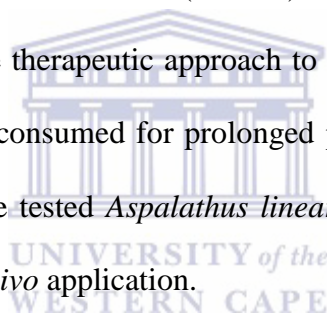
unstimulated RAW 264.7 cells. Septilin™ induced significant increases in metabolic activity ($P < 0.001$) across the concentrations of 63-1000 µg/ml in unstimulated RAW 264.7 cells. These findings suggest that *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ are non-cytotoxic at the above mentioned concentrations in unstimulated RAW 264.7 cell activity.

Furthermore, a study on the toxicity of *Artemisia afra* by Mukinda and Syce (2007), reported that the extract of *Artemisia afra* is non-toxic in acute doses. In a similar study on scoparone, a major constituent of *Artemisia capillaris*, no cytotoxic effects were reported in unstimulated macrophage cells (Jang *et al.*, 2005). Additionally the inhibition potential of the extract of *Artemisia capillaris* on cytokine-induced nitric oxide formation and cytotoxicity on RINm5F cells reported no significant difference in cell viability in the absence of a stimulus even at the highest concentrations (Kim *et al.*, 2007). Possible differences that exist amongst studies could be due to differences in activities of these two species of *Artemisia*.

These current findings with regards to *Aspalathus linearis* (rooibos) agree with Hendricks and Pool (2010), who has also found no cytotoxic effects at all concentration tested *in vitro* however, using whole blood cell cultures. *Aspalathus linearis* (rooibos) has gained popularity globally as an accepted nutraceutical. The health-promoting benefits of *Aspalathus linearis* (rooibos) have been confirmed in several *in vitro* and *in vivo* studies (Mahomoodally, 2013; Smith and Swart, 2016). These findings also agree with previously mentioned studies which refer to the safety of Septilin™ as it is widely used as a health supplement (Khanna & Sharma, 2003; Daswani & Yegnanarayan, 2002). A non-randomized non-placebo controlled pilot study using Septilin™ in chronic periodontitis reported no adverse effects in patients indicating to its relative safety (Deore *et al.*, 2014).

The results of the cell metabolic activity of LPS stimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ were evaluated using the WST-1 Cell Proliferation Reagent (Figure 3.2). Stimulated RAW 264.7 (Figure 3.2) compared to unstimulated RAW 264.7 (Figure 3.1) shows that the cell metabolic activity of RAW 264.7 cells exposed to LPS (control) increased cell proliferation. LPS is a well known mitogen (a substance which induces cell mitosis) (Murphy, 2012). *Artemisia afra* induced significant increases in metabolic activity ($P < 0.001$) across concentrations of 63-500 µg/ml in LPS stimulated RAW 264.7 cells. These findings agree with the previously mentioned study on *Artemisia capillaris* which reported no cytotoxic effects on stimulated macrophage cells (Jang *et al.*, 2005). In the previously mentioned study on the extract of *Artemisia capillaris* on RINm5F cells it was reported that the extract restored the cell proliferation potential proportional to its concentration (Kim *et al.*, 2007). These findings may differ to due the use of different cell lines in both studies. Septilin™ significantly decreased metabolic activity ($P < 0.001$) at the highest concentration tested (1000 µg/ml) in LPS stimulated RAW 264.7 cells. These findings are contrary to the results in unstimulated RAW 264.7 (Figure 3.1). Septilin™ may be cytotoxic at high doses however further investigation would be needed. This could indicate to the importance of dosage optimisation when prescribing this medication for infectious conditions. Septilin™ is indicated for acute infectious conditions. Wiesner and Knoss reports that globally, most patients believe that HMPs are safe which improves compliance (Wiesner and Knoss, 2014). The misconception regarding the safety of HMPs may cause patients to misuse these medicines. This is the first study to note decreased metabolic activity at the above mentioned dose (1000 µg/ml) in LPS stimulated RAW 264.7 cells. No toxicological studies have been done on this herbal preparation and the current literature lacks sufficient evidence regarding

its safety and toxicity therefore further studies are recommended. *Aspalathus linearis* (rooibos) had no effects on the metabolic activity of LPS stimulated RAW 264.7 cells at all concentration tested indicating that this herbal extract is non-toxic even at high concentrations. The popular use of *Aspalathus linearis* (rooibos) over time has contributed to the assumption of its relative safety (Joubert, *et al.*, 2008). Many studies have looked at aspects of safety and toxicity of *Aspalathus linearis* (rooibos) however no toxicological studies have been done as yet. The minor component of *Aspalathus linearis* (rooibos), quercetin is suggested to be implicated in its mutagenic effects. However these effects were seen in concentration of 220-230 times more than that of the normal tea drinking quantities (Joubert, *et al.*, 2008). The present study provides *in vitro* evidence suggesting that the product is non-toxic. A limitation to this study was that *Aspalathus linearis* (rooibos) was introduced to cells after stimulation which mimics the therapeutic approach to infection. *Aspalathus linearis* (rooibos) is most commonly consumed for prolonged periods as a daily beverage or health drink therefore to have tested *Aspalathus linearis* (rooibos) as a preventative would add more value for *in vivo* application.



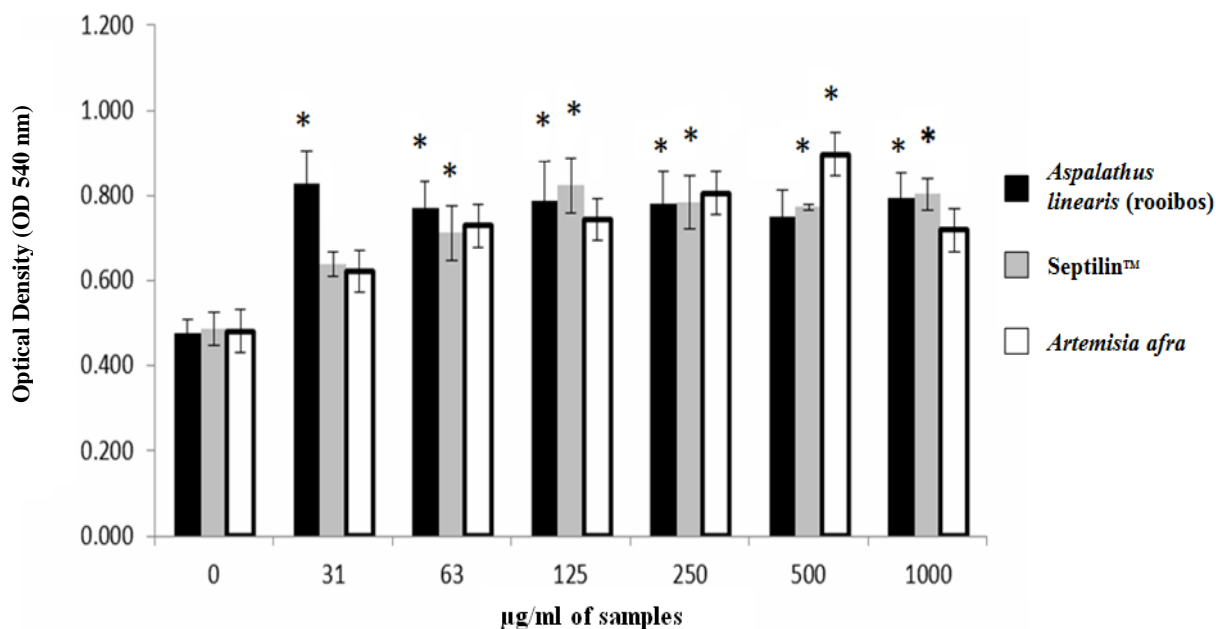


Figure 3.1. Cell metabolic activity of unstimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™. The statistical significant ($P < 0.001$) difference compared to the control is designated by an asterisk (*).

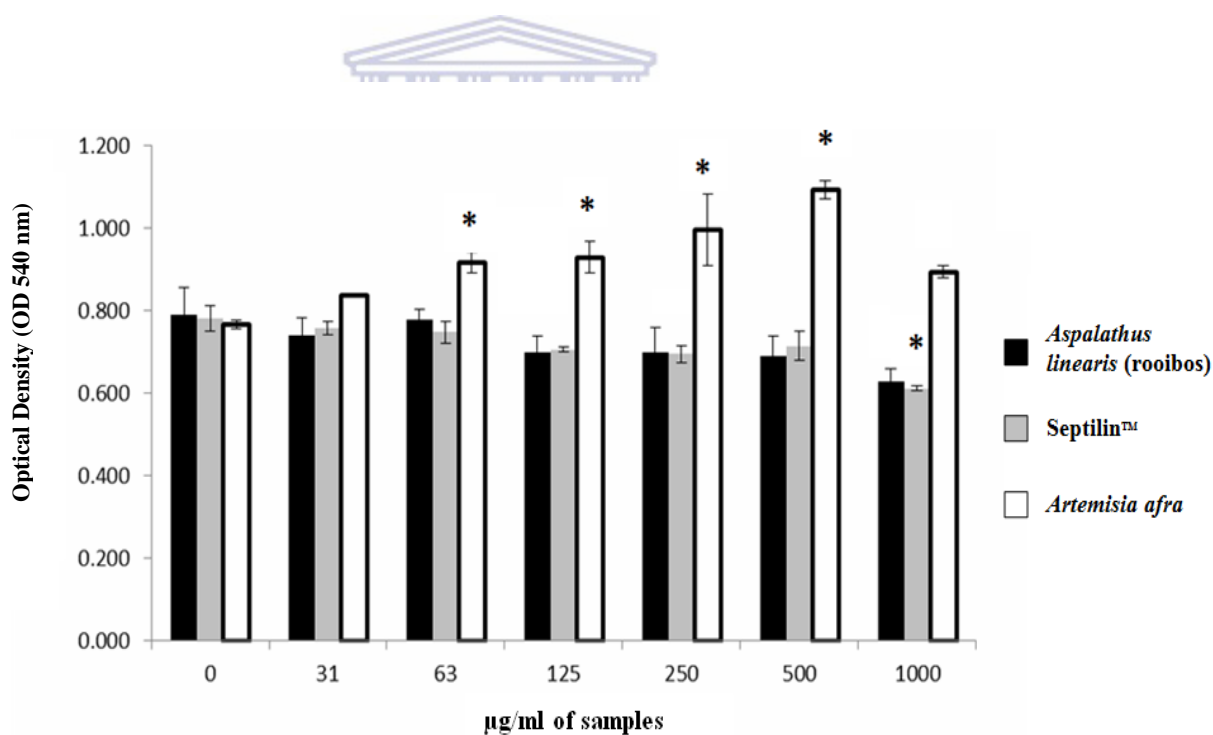


Figure 3.2. Cell metabolic activity of LPS stimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™. The statistical significant ($P < 0.001$) differences compared to the control is designated by an asterisk (*).

3.4.2 The effects of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ on NO production in unstimulated and LPS stimulated RAW 264.7 cells

The overproduction of NO is responsible for inflammation in several pathophysiological conditions like cancer, rheumatoid arthritis, diabetes, liver cirrhosis and septic shock. Inhibition of NO has become the main focus area in the field of anti-inflammatory research (Konkimalla *et al.*, 2008). Herbal medicines may be valuable in the modulation of NO. iNOS is a popular investigated enzyme system utilised for *in vitro*, *ex vivo*, *in vivo*, animal, or human research on HMPs. Research on herbal medicines in whole, standardized or extract forms are frequently investigated with regards to nitric oxide activity (Bouchard *et al.*, 2012).

The standard curve for the NO assay is shown in Figure 3.3. The standard curve was used to calculate the concentrations of NO in samples. The standard curve displays a good correlation ($R^2 = 0.9995$) between the absorbance and NO concentration. Nitrite production, a marker of NO synthesis, was determined in the supernatant of unstimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ (Figure 3.4). There were no significant differences on NO secretion in unstimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*.

Aspalathus linearis (rooibos) significantly increased ($P < 0.001$) NO production at concentrations of 500 µg/ml and 1000 µg/ml in unstimulated RAW 264.7 cells. This suggests that *Aspalathus linearis* (rooibos) possess pro-oxidant potential at these concentrations in absence of a stimulus. These findings are contrary to several studies reporting on the antioxidant effects of *Aspalathus linearis* (rooibos) *in vitro* and *in vivo* (Nel *et al.*, 2007; Snijman *et al.*, 2007; Joubert *et al.*, 2008; Baba *et al.*, 2009; Marnewick *et al.*, 2011; Chen *et al.*, 2013; Mahomoodally, 2013; Ku *et al.*, 2015; Waisundara and Hoon, 2015; Smith and Swart, 2016). However these findings agrees

with Persson *et al.*, who reported increased NO production of *Aspalathus linearis* (rooibos) *in vitro* on cultured human umbilical veins endothelial cells at doses of 0-730 µg/ml (Persson *et al.*, 2006). In a follow up *in vivo* study, Persson *et al.*, reported no effect on NO activity in human subjects who consumed 400ml of *Aspalathus linearis* (rooibos) per week for 4 weeks in a randomized three-phase crossover design. Differences between the *in vitro* and *in vivo* studies may be due to differences in the content of the flavonoids or/and the metabolism of the components in the different teas as well as the use of different models (Persson *et al.*, 2010). Waisundara and Hoon reported on the antioxidant effects of *Aspalathus linearis* (rooibos) but cautioned against the *in vivo* application of these findings due to the pro-oxidant reports of *Aspalathus linearis* (rooibos) in other studies (Waisundara and Hoon, 2015). *Aspalathus linearis* (rooibos) is mainly consumed as a health promoting beverage as mentioned in previous studies. Its pro-oxidant potential should be considered especially in chronic inflammatory conditions. NO stimulation is responsible for cellular and tissue damage which contributes to numerous inflammatory conditions affecting different organs (Varma *et al.*, 2011).

In this study, Septilin™ had no effect on NO secretion in unstimulated RAW 264.7. Septilin™ had no significant anti-inflammatory effects (NO inhibition) in unstimulated RAW 264.7 cells (Varma *et al.*, 2011). This is the second known study which followed a similar model to that of Varma *et al.*, 2011 by assessing anti-inflammatory effects (NO inhibition) of Septilin™ and hence its importance since this herbal preparation is widely used as an anti-inflammatory agent.

The NO production was also determined in the supernatant of LPS stimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ (Figure 3.5). *Artemisia afra* significantly decreased NO production (P<0.001) at all concentrations tested (31,25-1000µg/ml) in LPS

stimulated RAW 264.7 cells. These findings agree with the study of Jang *et al.*, (2005) which reported on significant ($P < 0.01$) inhibition of NO production of *Artemisia capillaris* in LPS stimulated macrophage cells. The previously mentioned study on the extract of *Artemisia capillaris* on RINm5F cells reported potent dose dependant inhibition of NO (Kim *et al.*, 2007). Three *in vitro* studies on *Artemisia* species reported on the inhibition/reduction of NO secretion in macrophages which suggest the anti-inflammatory potential of *Artemisia* species (Kim *et al.*, 2006; Yoon *et al.*, 2010; Froushani *et al.*, 2016). The results support the anecdotal uses of *Artemisia* species for inflammatory conditions.

An indepth study of the effect of herbal medicine on the immune system requires the use of both *in vitro* and *in vivo* experimentation. *In vitro* models are valuable in evaluating the immunomodulatory effects of herbal constituents (Silliman and Wang, 2006). *Aspalathus linearis* (rooibos) and Septilin™ showed no effect on NO activity on stimulated RAW 264.7 cells. These findings are contrary to that of Varma *et al.*, (2011) who reported significant inhibition ($P < 0.001$) of NO in LPS stimulated macrophages by Septilin™. The findings of Varma *et al.*, (2011) were tested at concentrations of 2.5% and 5% of Septilin™ which are 25 to 50 fold higher than the concentrations of Septilin™ (31-1000µg/ml) used in this study. Such high concentrations of the herbal product could be unrealistic and problematic if these concentrations were to be extrapolated for *in vivo* application. Mansour *et al.*, reported on the reduction of NO secretion in an *in vivo*, radiation induced rat model. In this study liquid preparation of Septilin™ was injected intraperitoneally (100 mg/kg b.wt.) for five consecutive days (Mansour *et al.*, 2014). Sharma and Ray, 1997 conducted a study using an oral dose of 500mg/kg of Septilin™ in rodents which is equivalent to an intake of 25-50g in humans. These dosages are clearly too high

which is a common problem found in *in vitro* and *in vivo* studies on herbal medicines (Gertsch *et al.*, 2010).

Pre-clinical evaluation of HMPs should begin with *in-vitro* models, by testing cytotoxicity, mutagenicity and acute and sub-chronic safety. These safety studies should be followed by *in-vivo* models at appropriate doses of the HMP's according to internationally accepted standards. Extrapolating doses of the HMPs for *in vivo* application proves to be challenging. Gericke (2011), states that dose-finding studies before formal animal studies are crucial in the preliminary phase to establish efficacy of HMPs

In a comparative study by Jagetia *et al.*, (2004) on the nitric oxide (NO) scavenging activities of traditional polyherbal drugs, Septilin™ was tested at the same concentrations (31-1000µg/ml) as this current study. It was reported that Septilin™ inhibited the production of NO in a dose dependent manner up to 125 µg/ml (69.66%) which was followed by a gradual increase of NO production thereafter at the higher doses. The results of Jagetia *et al.*, (2004) showed far less efficacy of NO inhibition by Septilin™ to that of Varma *et al.*, (2011). This could be due to the differences in the concentrations tested.

Another contributing factor to differences in findings of these two studies could be attributed to variations that exist in different batches of HMPs. The chemical composition of HMPs differ depending on various factors which includes the botanical species, the anatomical part of the plant used, storage methods, sun, humidity, type of soil, time of harvest, geographic location amongst others. Batch to batch variations can be found within the same manufacturing company which can result in significant variations in pharmacological activities influenced by pharmacodynamic and/or pharmacokinetic factors (Firenzuoli and Gori, 2007).

Several *in vitro* and *in vivo* studies on the individual ingredients of Septilin™ were conducted on various models with varying effects on NO activity (refer to chapter 2). *Commiphora mukul* (Matsuda *et al.*, 2004; Zhang *et al.*, 2016), *Rubia cordifolia* (Ghosh *et al.*, 2010), *Emblica officinalis* (Yokozawa *et al.*, 2006), *Moringa pterygosperma* (Yokozawa *et al.*, 2006) decreased NO secretion. Most studies of *Tinospora cordifolia* (Desai *et al.*, 2007; Upadhyaya *et al.*, 2011; Aranha *et al.*, 2012; Sharma *et al.*, 2012) reported increased NO production. *Glycyrrhiza glabra* studies reported either increased NO production (Li and Zhou, 2012) or decreased NO production (Franceschelli *et al.*, 2011; Thiyagarajan *et al.*, 2011). Many studies on the molecular modes of activities of individual herbs have little relevance to its practical application as most herbal medicines are formulations (combinations of several herbs) (Burns *et al.*, 2009). These formulas introduce extremely complex mixtures of compounds that may act synergistically to produce therapeutic effects. The overall effect of the formulation may be different to the sum of the individual effects of each herb which makes the study on herbal medicines extremely challenging due to its complex chemistry (Burns *et al.*, 2009). The current results are contrary to several *in vitro* and *in vivo* reporting on the antioxidants and/or anti-inflammatory effects of *Aspalathus linearis* (rooibos) previously mentioned. Most of these *in vitro* studies used the ethanolic extract of *Aspalathus linearis* (rooibos) which may account for differences in findings. However in a previous study, Joubert *et al.*, (2005) reported on the pro-oxidant activity of the aqueous extracts of *Aspalathus linearis* (rooibos).

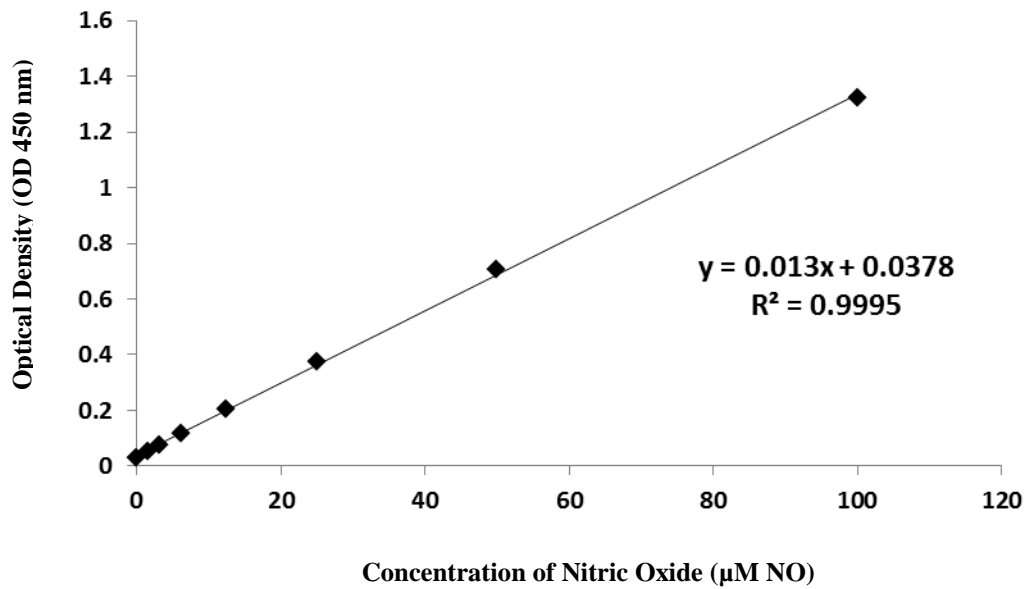


Figure 3.3. Standard curve for NO assay. This standard curve shows a good correlation ($R^2 = 0.9995$) between absorbance readings and NO concentration.

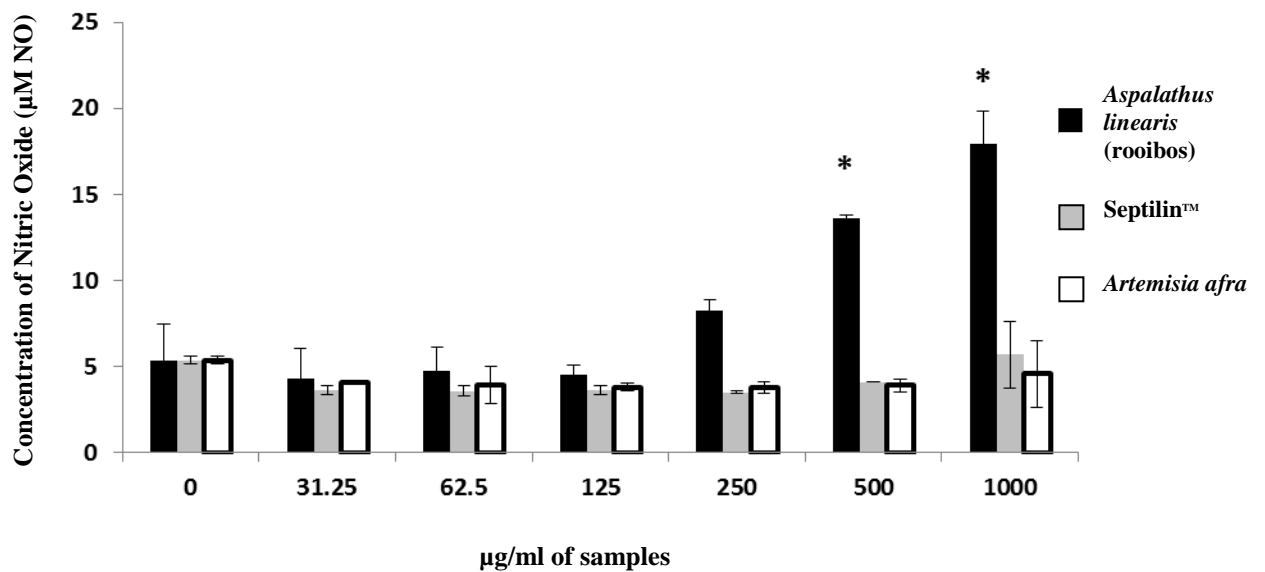


Figure 3.4. NO production in unstimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™. The statistical significant ($P < 0.001$) differences designated by an asterisk (*).

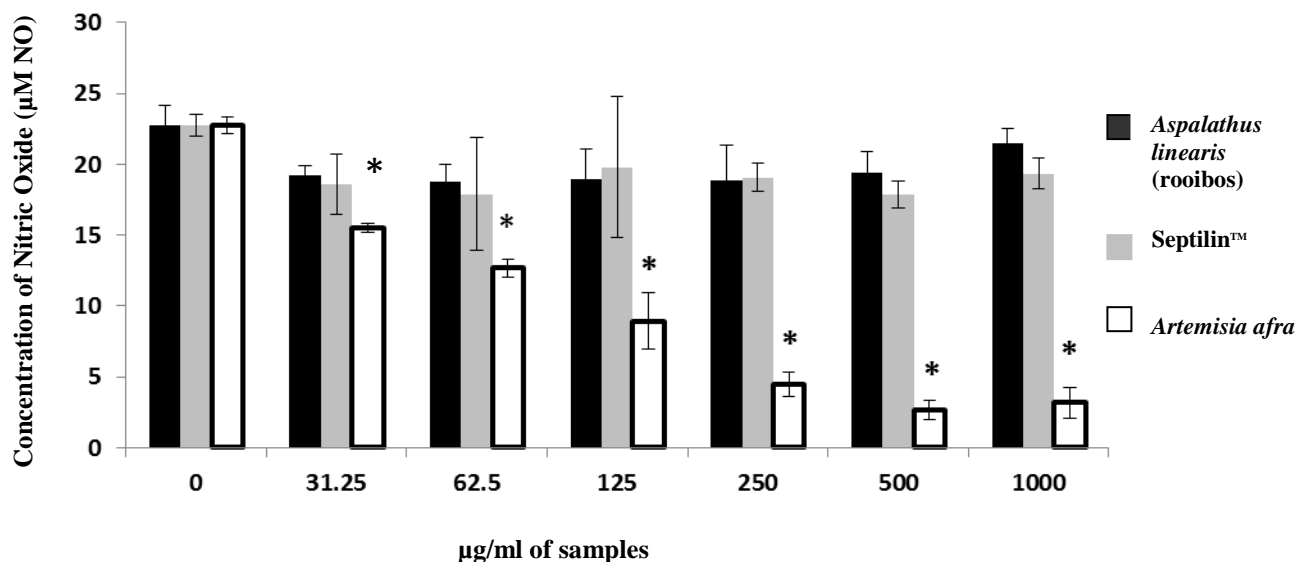


Figure 3.5. NO production in LPS stimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™. The statistical significant ($P < 0.001$) difference compared to the control is designated by an asterisk (*).

3.4.3 The effects of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ on IL-6 production in unstimulated and LPS stimulated RAW 264.7 cells

The cytokine, interleukin-6 (IL-6) is involved in the systemic changes associated with inflammation and infection (Kumar *et al.*, 2007). IL-6 concentrations were determined using a DAS-ELISA. The standard curve for the IL-6 ELISA is shown in Figure 3.6. The standard curve was used to calculate the concentrations of IL-6 in samples. The standard curve displays a good correlation ($R^2 = 0.9991$) between the absorbance and IL-6 concentration. IL-6 was used as a biomarker to determine the inflammatory response of LPS on unstimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ (Figure 3.7).

Artemisia afra had no effect on IL-6 production in unstimulated RAW 264.7 cells.

These findings correspond to the previously mentioned results for Figure 3.4 which

shows no effect of *Artemisia afra* on NO production in unstimulated RAW 264.7 cells.

Aspalathus linearis (rooibos) significantly increased ($P < 0.001$) IL-6 production at concentrations of 500 μ g/ml and 1000 μ g/ml in unstimulated RAW 264.7 cells. These findings are contrary to most of the previous studies which reports on the anti-inflammatory properties of *Aspalathus linearis* (rooibos) *in vitro* and *in vivo* (Nel *et al.*, 2007; Snijman *et al.*, 2007; Joubert *et al.*, 2008; Baba *et al.*, 2009; Marnewick *et al.*, 2011; Chen *et al.*, 2013; Mahomoodally, 2013; Ku *et al.*, 2015; Waisundara and Hoon, 2015; Smith and Swart, 2016). Most of these *in vitro* studies were conducted using similar concentrations of *Aspalathus linearis* (rooibos) (0-1000 μ g/ml) as this study however within different models which may account for variations in findings. Mueller *et al.*, conducted a similar study on *Aspalathus linearis* (rooibos) on RAW 264.7 macrophages. Results showed decreased IL-6 at concentrations of 500 μ g/ml (Mueller *et al.*, 2010). Studies on the pro-inflammatory effects of *Aspalathus linearis* (rooibos) are few (Smith and Swart, 2016). However these studies tested the aqueous extract of *Aspalathus linearis* (rooibos) whilst the majority of anti-inflammatory studies on *Aspalathus linearis* (rooibos) were conducted on the ethanolic extract. This maybe due to the presence of different bio-actives in aqueous extracts compared to ethanol extracts.

These current findings suggest the pro-inflammatory effects of *Aspalathus linearis* (rooibos) *in vitro* in absence of a stimulus which corresponds to the results in Figure 3.4, showing that *Aspalathus linearis* (rooibos) induced IL-6 production at concentrations of 500 μ g/ml and 1000 μ g/ml in unstimulated RAW 264.7 cells. Up regulation of IL-6 could potentially activate hepatocytes to produce acute phase proteins leading to complement activation allowing phagocytosis. Cellular responses to microbial pathogens could be improved by consuming *Aspalathus linearis*

(rooibos) tea (Hendricks and Pool, 2010). This suggests that the consumption *Aspalathus linearis* (rooibos) tea could potentially be used for prophylactic purposes. However, important consideration should be given to its possible pro-inflammatory action in midst of inflammation which could lead to or worsen tissue damage. IL-6 is well known to mediate the involvement of inflammatory cells in acute and chronic inflammation (Varma *et al.*, 2011). IL-6 is involved in the systemic changes associated with tissue damage, inflammation and infection (Hack *et al.*, 2014). In an *in vitro* whole blood culture study on unstimulated WBC, *Aspalathus linearis* (rooibos) also induced higher IL-6 secretion at concentrations between 7.8125 µg/ml - 250 µg/ml (Hendricks and Pool, 2010).

Septilin™ had no effects on unstimulated RAW 264.7 cells. These findings correspond to the previously mentioned results which reported that Septilin™ did not effect NO secretion in unstimulated RAW 264.7 cells (Figure 3.4).

IL-6 was used as a biomarker to determine the inflammatory response on LPS stimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ (Figure 3.8). *Artemisia afra* significantly decreased ($P < 0.001$) production of IL-6 by LPS stimulated RAW 264.7 cells in a concentration dependant manner (63-1000µg/ml). These results suggest the anti-inflammatory potential of *Artemisia afra* which also corresponds to the results in Figure 3.5 showing that *Artemisia afra* significantly decreased NO production of stimulated RAW 264.7 cells. These results supports the use of *Artemisia afra* as an anti-inflammatory agent for infectious conditions (Kim *et al.*, 2006; Yoon *et al.*, 2010; Froushani *et al.*, 2016). Several previous studies reported on the anti-infective properties of the active constituents of *Artemisia afra* which includes; camphene, 1,8-cineole, Artemisia ketone, camphor, borneol, terpineol, chrysanthenyl acetate, amyryn amongst others (Abad *et al.*, 2012; Liu *et al.*, 2009). These constituents amongst

several others were present in the *Artemisia afra* ethanolic extract tested in this study (see addendum 1) which may have contributed to the anti-inflammatory effects.

Aspalathus linearis (rooibos) did not induce significant changes in IL-6 secretion by stimulated RAW 264.7 cells. These findings are consistent with the results shown in Figure 3.5. (*Aspalathus linearis* did not induce significant changes in NO secretion) but inconsistent with the majority of previous studies which reported on the anti-inflammatory effects of *Aspalathus linearis* by inhibiting/reducing IL-6 secretion (Mueller *et al.*, 2010; Swart *et al.*, 2013; Lee and Bae, 2015; Smith and Swart, 2016).

In this study Septilin™ showed no effects in IL-6 secretion by stimulated RAW 264.7 cells. These findings are contrary to that of Varma *et al.*, 2011 and others who reported significant inhibition ($P < 0.001$) in IL-6 secretion in LPS stimulated macrophages by Septilin™ (Varma *et al.*, 2011). The anti-inflammatory effect of Septilin™ has been observed in previous studies (Kumar *et al.*, 1997; Sharma and Ray, 1997; Khanna and Sharma, 2003; Daswani and Yegnanarayan, 2002; Jagetia and Balinga, 2004; Varma *et al.*, 2011; Manal, 2014) which indicated that Septilin™ suppressed various inflammatory mediators like TNF α , IL-6 and IL-8 in LPS stimulated *in vitro* cell culture models. Studies also showed that Septilin™ inhibits iNOS gene expression, COX-2 enzyme activity and PDE4B gene expression. These are suggested to be the anti-inflammatory modes of action of this herbal product (Varma *et al.*, 2011). The current findings are contrary to the previously mentioned studies with regards to the anti-inflammatory effects of Septilin™. A possible reason for this could be due to the use of an aqueous preparation of Septilin™ in this study. A study by Raveendran Nair and Chanda, on the efficacy of medicinal plants against pathogenic bacterial strains reported greater effects by the ethanol extract of the samples than the aqueous extract (Raveendran Nair and Chanda, 2006). An anticancer *in vitro* study compared the effects of fifteen crude aqueous herbal extracts

to the ethanol herbal extracts against human cancer cell lines. This study reported that the aqueous herbal extracts decreased cell proliferation by more than 50% when compared to the ethanol herbal extracts. Another study also suggested that the ethanol extracts contained the herbal active constituents responsible for the significant results (Sun *et al.*, 2007). Further studies should include both ethanol and aqueous extracts of Septilin™, *Aspalathus linearis* (rooibos) and *Artemisia afra* within the same model.

The overall findings of this study suggests the anti-inflammatory effects of *Artemisia afra* and pro-inflammatory effects of *Aspalathus linearis* (rooibos) in RAW 264.7 cells. Septilin™ showed no effects in RAW 264.7 cells.

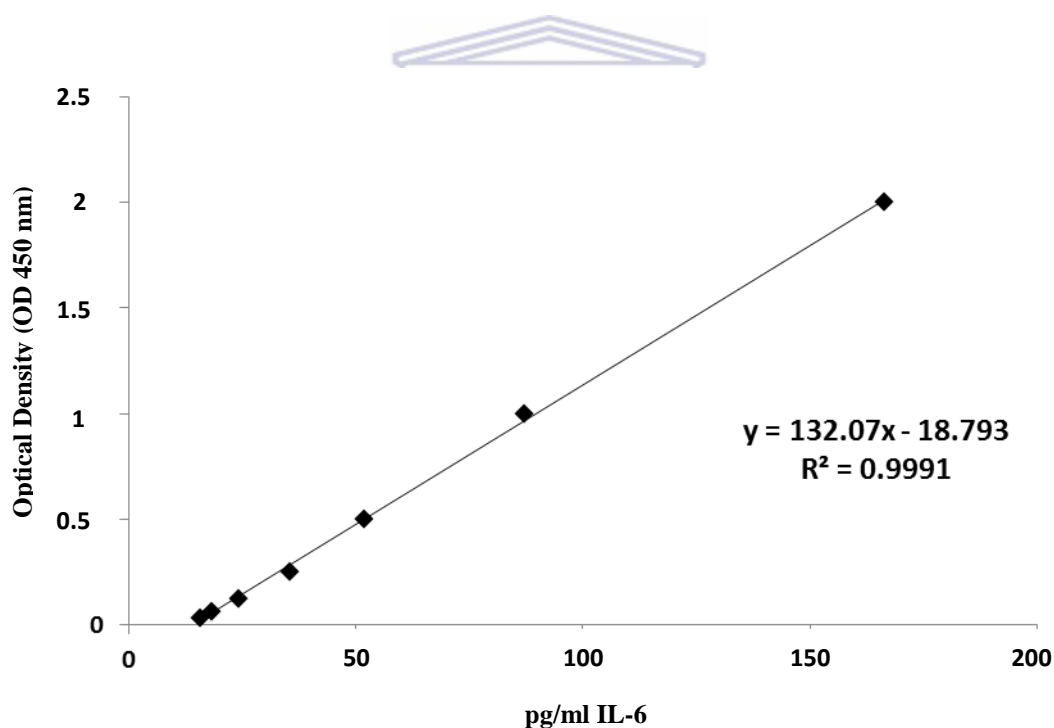


Figure 3.6. Standard curve for IL-6 ELISA. This standard curve shows a good correlation ($R^2 = 0.9991$) between absorbance readings and IL-6 concentration.

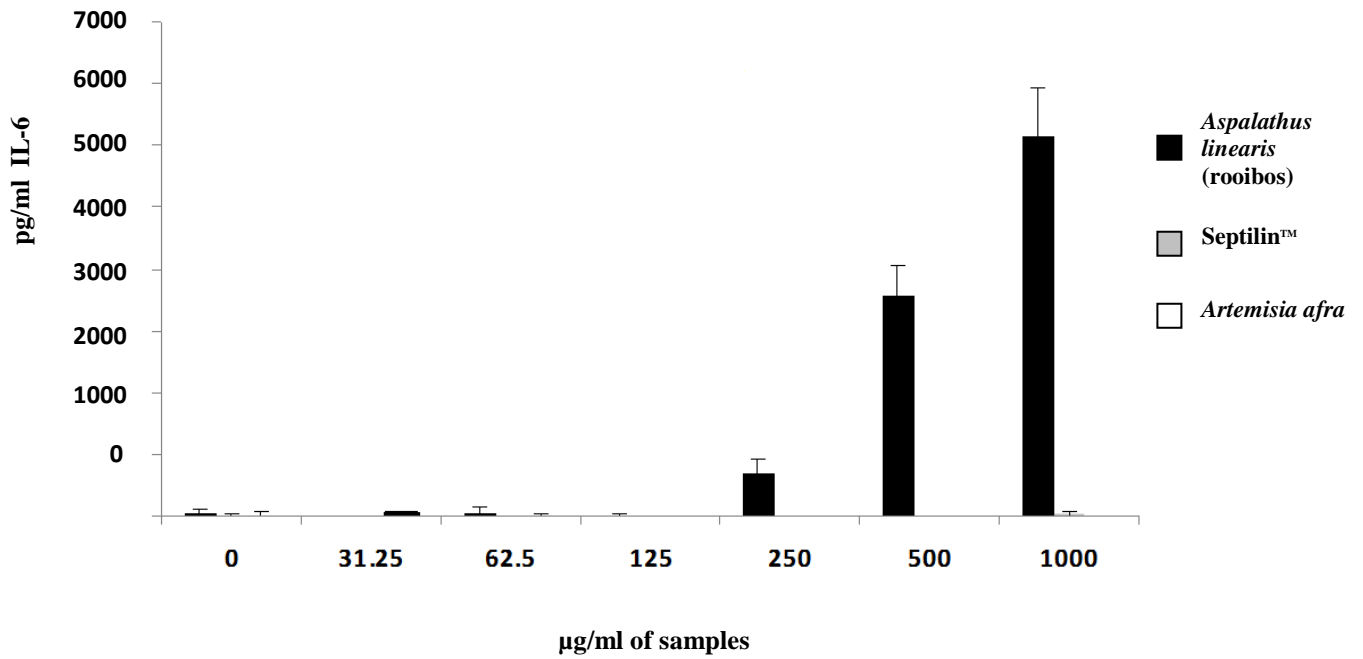


Figure 3.7. IL-6 production in unstimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™. The statistical significant ($P < 0.001$) difference designated by an asterisk (*).

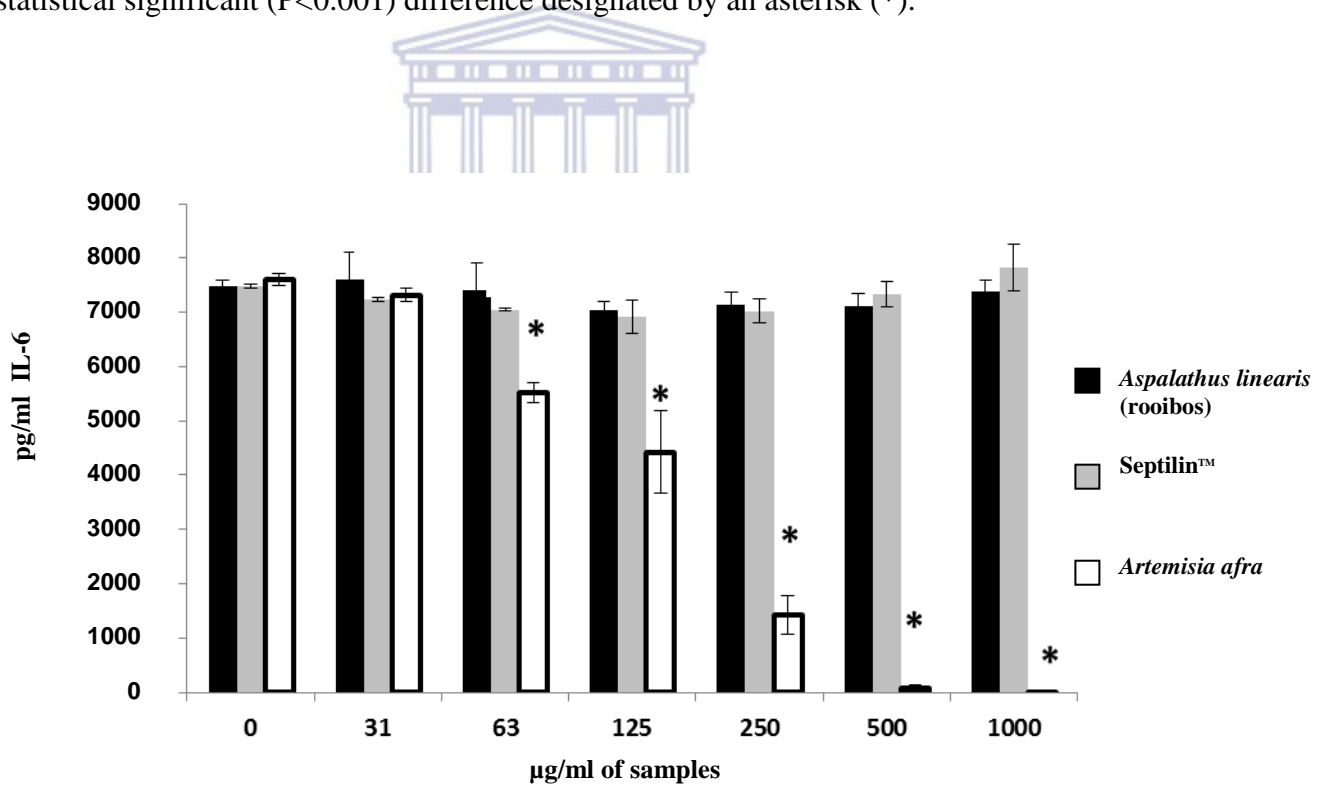


Figure 3.8. IL-6 production in LPS stimulated RAW 264.7 cells exposed to various concentrations of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™. The statistical significant ($P < 0.001$) difference compared to the control is designated by an asterisk (*)

3.4.4 Effects of Septilin™ on IL-6 production in WBC

IL-6 was used as a biomarker to determine the effects of Septilin™ on inflammatory activity in stimulated and unstimulated whole blood cultures (WBC). The standard curve for the IL-6 ELISA is shown in Figure 3.9. The standard curve was used to calculate the concentrations of IL-6 in samples. The standard curve displays a good correlation ($R^2 = 0.997$) between the absorbance and IL-6 concentration. Septilin™ has no effect on IL-6 production in LPS stimulated WBC (Figure 3.10). These findings are contrary to the majority of *in vitro* and *in vivo* studies previously mentioned which reported on the anti-inflammatory effects of Septilin™ via IL-6 inhibition/reduction. The biphasic effect (refer to chapter 2) which refers to the paradoxical responses in cytokine activity by herbal products in both *in vitro* and *in vivo* studies is not an uncommon finding (Spelman *et al*, 2006). Divergent models, dosages, duration of exposure and method of administration of Septilin™ are factors which may explain differences in cytokine expression. In this experimental design blood was first diluted in LPS enriched medium before Septilin™ was added. This mounted an immune response indicated by IL-6 release before the addition of Septilin™. Septilin™ had no effect on IL-6 release in stimulated WBC which suggests that Septilin™ may not be potent enough to serve as a therapeutic intervention during or after infection. However this does not rule out the possibility that Septilin™ may be effective as a preventative treatment. Septilin™ as with many similar herbal products are prescribed as daily health supplements used for preventative treatment. This *in vitro* experimental design only assessed Septilin™ as a therapeutic intervention and not as a preventative treatment.

Addition of Septilin™ to unstimulated WBC resulted in a significantly higher release of IL-6 across all concentrations (16.125µg/ml-258µg/ml) of Septilin™ when compared to the control. This suggests that Septilin™ has a stimulatory effect on IL-6

production in the absence of a stimulus. These findings differ from the previous data on RAW 264.7 cells which shows no effect of Septilin™ in both stimulated and unstimulated conditions. Activation of the immune system may be valuable in preventative treatment. On the other hand an overactive immune system is implicated in many pathologies including autoimmunity, chronic inflammatory diseases, systemic vasodilatation, carcinogenesis sepsis and the anaphylactic shock (Gertsch *et al.*, 2010). Residual bacterial endotoxins are known to be highly potent pro-inflammatory agents. A few molecules may induce cytokine expression. Plant extracts and herbal preparations have been reported to contain endotoxin contaminants (Gertsch *et al.*, 2010). The above is an important consideration especially in patients with chronic inflammatory conditions. IL-6 secretion in absence of a stimulus has been noted in previous studies on other herbal products. However, very few studies have reported this on Septilin™ using an *in vitro* whole blood culture model.

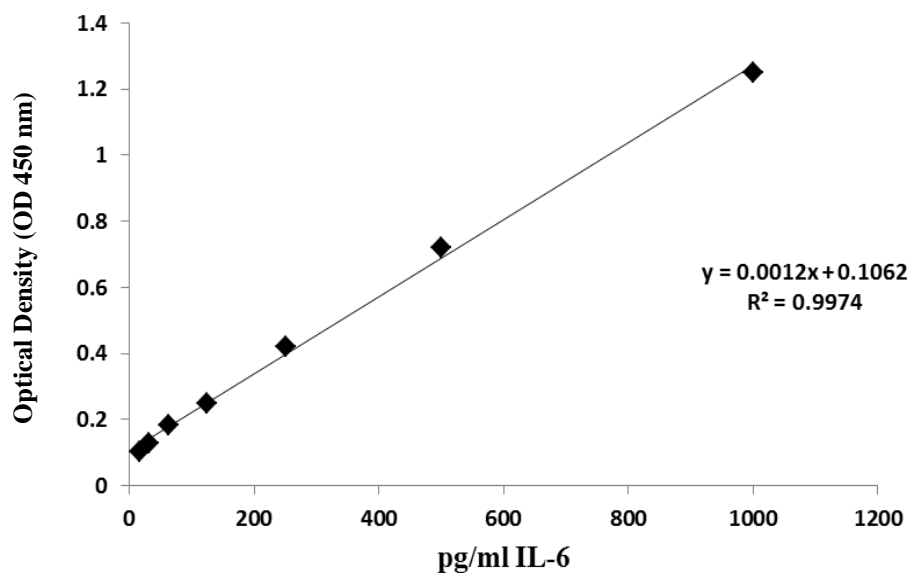


Figure 3.9. Standard curve for IL-6 ELISA. This standard curve shows a good correlation ($R^2 = 0.997$) between absorbance readings and IL-6 concentration.

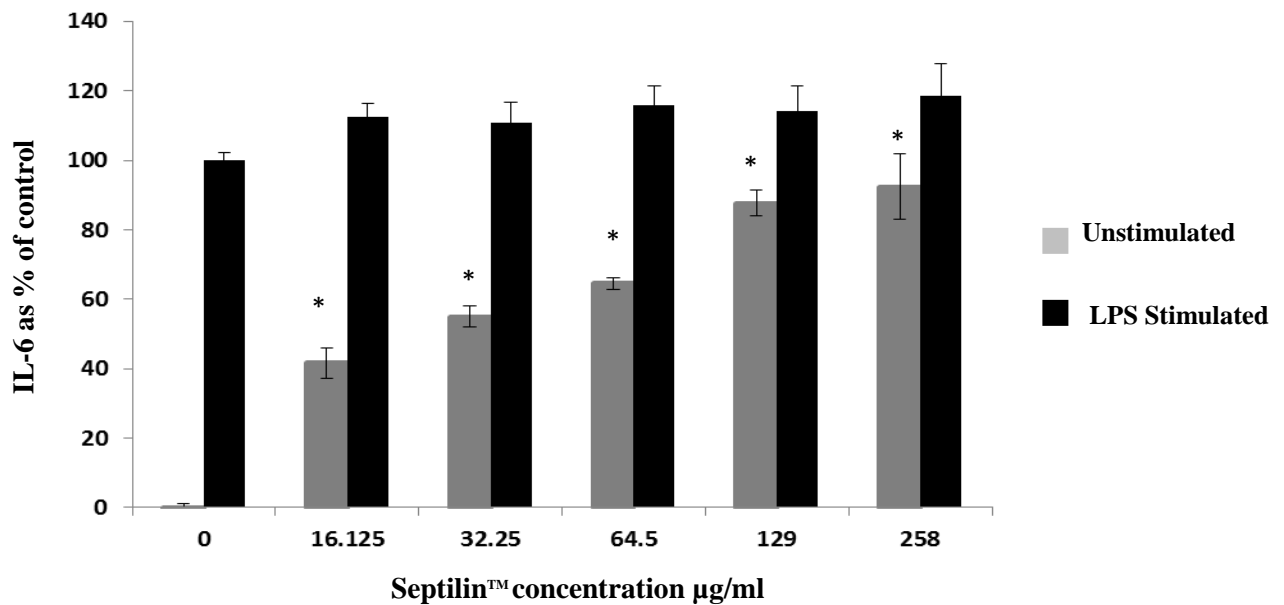


Figure 3.10. Effects of Septilin™ on IL-6 production. IL-6 was used as a biomarker to determine the inflammatory response on stimulated (LPS) and unstimulated WBC in the presence of Septilin™ across various concentrations. The statistical significant ($P<0.001$) difference compared to the control is designated by an asterisk (*)

3.4.2 Effects of Septilin™ on IL-10 production in WBC

IL-10 was used as a biomarker to determine the effect on humoral immunity of stimulated and unstimulated WBC in the presence of Septilin™. The standard curve for the IL-10 ELISA is shown in Figure 3.11. The standard curve was used to calculate the concentrations of IL-10 in samples. The standard curve displays a good correlation ($R^2=0.973$) between the absorbance and IL-10 concentration.

Septilin™ has no effect on the release of IL-10 release by unstimulated WBC (Figure 3.12). Addition of Septilin™ to PHA stimulated WBC resulted in a significantly ($P<0.01$) higher release of IL-10 between 64.5µg/ml-258µg/ml of Septilin™ when compared to the control.

B cells are known to play an important role in the immune system (Mion, *et al.*, 2014). IL-10 (Th2-type response) is a well known immunosuppressive and anti-inflammatory cytokine which counteracts the effects of IL-6. IL-10 inhibits IFN γ production and Th1 cells. Both exogenous infectious signals and endogenous immune mediators induces IL-10 secretion (Mion, *et al.*, 2014). The results obtained for this study suggests that Septilin™ may induce anti-inflammatory effects by means of IL-10 secretion. IL-10 is a known anti-inflammatory cytokine that acts on macrophages and may regulate the release of pro-inflammatory cytokines (Murphy, 2012). During infection Septilin™ may regulate the inflammatory process by means of increased IL-10 production. IL-10 activates the proliferation and differentiation of B cells, and upregulates Immunoglobulin (Ig) production. These findings agrees with results of Sharma and Ray (1997), who reported a significant increase ($p < 0.001$) in both IgM and IgG concentrations in the Septilin™ treated group (*in vivo* rodent model). Very few studies are available on the effects of Septilin™ on IL-10 production by WBC *in vitro*.

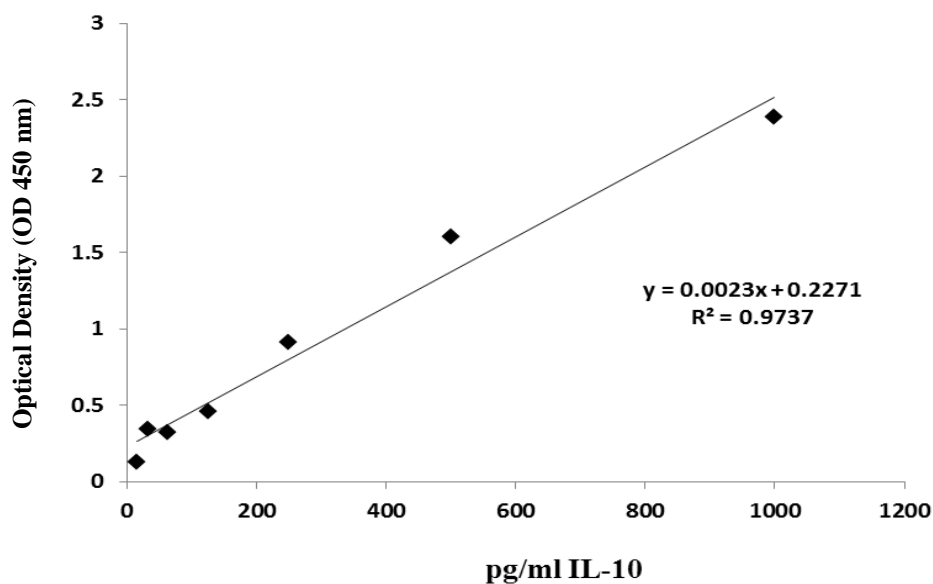
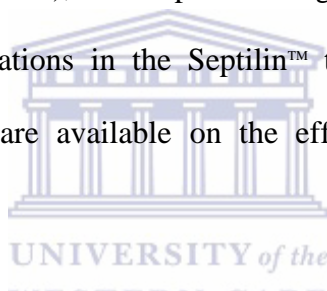


Figure 3.11. Standard curve for IL-10 ELISA. This standard curve shows a good correlation ($R^2 = 0.973$) between absorbance readings and IL-10 concentration.

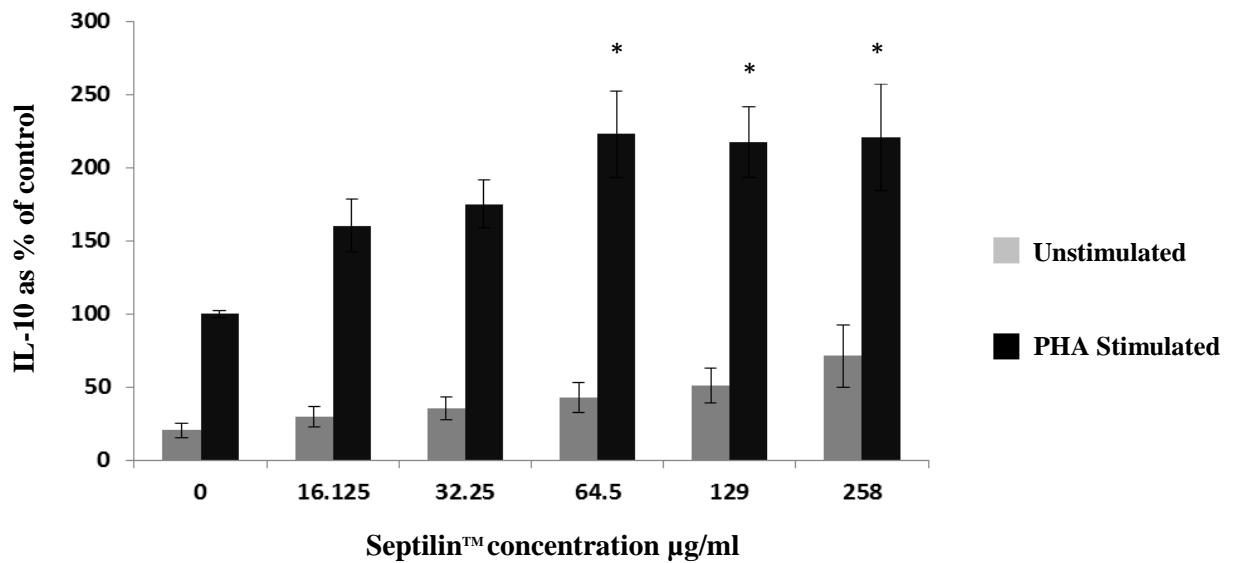


Figure 3.12. Effects of Septilin™ on IL-10 production. IL-10 was used as a biomarker to determine the humoral immune response on stimulated (PHA) and unstimulated WBC in the presence of Septilin™ across various concentrations. The statistical significant ($P < 0.01$) difference compared to the control is designated by an asterisk (*).



3.4.3 Effects of Septilin™ on IFN γ production in WBC

IFN γ was used as a biomarker to determine the effect of Septilin™ on cellular immunity of stimulated and unstimulated WBC. The standard curve for the IFN γ ELISA is shown in Figure 3.13. The standard curve was used to calculate the concentrations of IFN γ in samples. The standard curve displays a good correlation ($R^2 = 0.985$) between the absorbance and IFN γ concentration. Septilin™ has no effect on the release of IFN γ production by unstimulated WBC (Figure 3.14).

The effect of Septilin™ on IFN γ synthesis by PHA stimulated WBC was inconclusive. These findings are contrary to previous *in vitro* and *in vivo* (rodent models) findings which assessed the effects of Septilin™ on cell mediated immunity. These studies did not look at IFN γ as a marker of cellular immunity. IFN γ plays an important role in innate and adaptive immunity by increasing macrophage and anti-viral activity

through Nuclear Kappa (NK) cell activation. This ensures host defences against bacteria and viruses (cell mediated immunity) (Murphy, 2012). Septilin™ is commonly prescribed for colds, influenza and respiratory conditions. This current *in vitro* study indicates that Septilin™ may not be effective in these conditions.

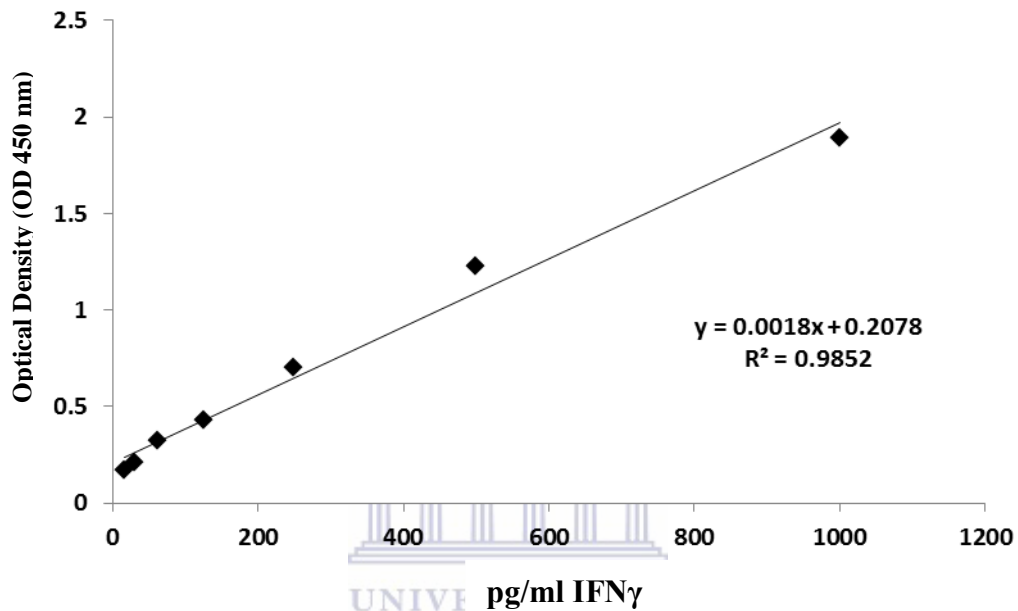


Figure 3.13. Standard curve for IFN γ ELISA. This standard curve shows a good correlation ($R^2 = 0.985$) between absorbance readings and IFN γ concentration.

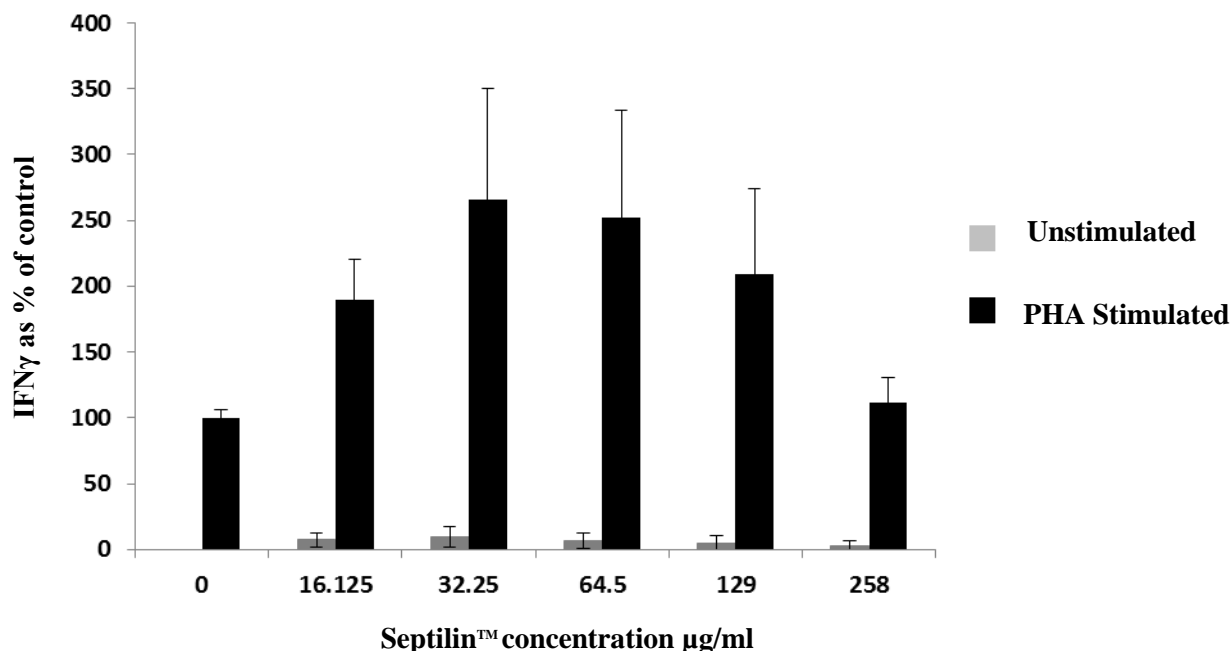


Figure 3.14. Effects of Septilin™ on IFN γ production. IFN γ was used as a biomarker to determine the cellular immune response on stimulated (PHA) and unstimulated WBC in the presence of Septilin™ across various concentrations.



4. Concluding remarks

This study assessed the effects of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ on inflammatory biomarkers using RAW 264.7 cells. LPS activated RAW 264.7 cells were treated with various concentrations of the above mentioned samples after which the culture supernatants were assayed for specific inflammatory biomarkers namely, IL-6 and nitric oxide (NO). Septilin™ significantly decreased metabolic activity ($P < 0.001$) of LPS stimulated RAW 264.7 cells at 1000 μ g/ml. This has not been reported before. These findings have to be confirmed in follow up studies to ensure patient safety when high doses are prescribed. Most patients consider HMPs to be safe and free of adverse effects which increase their use of these medicines.

The anti-inflammatory findings of *Artemisia afra* in this study agree with previous studies as well as with anecdotal uses. *Artemisia afra* may be effective in the treatment of arteriosclerosis, rheumatoid arthritis, sciatica, neoplastic metastasis and non-insulin dependant diabetes mellitus, all of which are marked by elevated IL-6 levels. The pro-inflammatory findings of *Aspalathus linearis* (rooibos) have been observed in a few previous studies however most studies reports otherwise (anti-inflammatory effects of rooibos). These differences in findings could be due to the extracts (aqueous vs ethanolic) used in these studies. Most reports on the anti-inflammatory effects of *Aspalathus linearis* (rooibos) were conducted using ethanolic extract of *Aspalathus linearis* (rooibos). On the other hand most of the reports on the pro-inflammatory effects of *Aspalathus linearis* (rooibos) were conducted on aqueous extracts.

This study is contrary to previous reports suggesting the IL-6 inhibitory effects of Septilin™ (Varma *et al.*, 2011; Shetty *et al.*, 2015). This could be due to the vast differences in concentrations of Septilin™ used in this study when compared to other studies as well as the use of an aqueous preparation of Septilin™. This *in vitro* study shows that Septilin™ was not effective as a therapeutic intervention during infection. However its possible preventative capabilities has not been explored in this study. Therefore further studies are recommended.

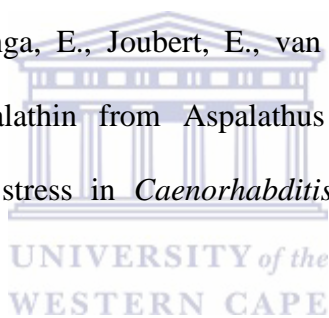
This current *in vitro* study on WBC indicates the pro-inflammatory effect (basal) of Septilin™ in the absence of a stimulus. Septilin™ could potentially activate the immune system and contribute to healing and tissue repair. This could activate hepatocytes to produce acute phase proteins leading to activation of complement and allows for the phagocytosis of pathogens. These findings could support the anecdotal use of Septilin™ for prophylactic purposes. Septilin™ is commonly prescribed as a preventative for colds and flu before the winter season. However chronic

inflammation is implicated in several pathological conditions. Basal pro-inflammatory effects are detrimental in patients with increased allostatic load. This basal pro-inflammatory effect may be due endotoxin contamination. HMPs have been reported to contain various contaminants and residues which could potentially be dangerous to the health of consumers. These could include naturally occurring radionuclides, toxic metals or bacteria (WHO guidelines for assessing quality of herbal medicines with reference to contaminants and residues, 2007). This study indicated an increase in IL-10 production by stimulated WBC exposed to Septilin™. This may contribute to stimulation of B-cells and synthesis of Ig which are consistent with previous *in vitro* and *in vivo* studies. Septilin™ was shown to have anti-inflammatory effects by means of increased IL-10 production which may be beneficial during infection. The effect of Septilin™ on IFN γ production on stimulated WBC were inconclusive. Septilin™ is a polyherbal formulation. Many of its herbal constituents have known immunomodulatory effects when tested as single ingredients. Possible herbal interactions should be investigated for future studies. This *in vitro* study indicates to the immunomodulatory effects of Septilin™ in WBC. This study also indicates that *Artemisia afra* has anti-inflammatory effects while *Aspalathus linearis* (rooibos) up regulated the immune system and Septilin™ showed no effects on RAW 264.7 cells.

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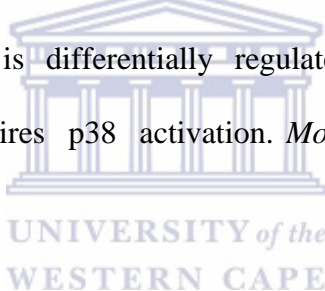
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Chapter 4

Summary, concluding remarks and recommendations

4.1 Summary

The increased popularity of the use of herbal medicinal products (HMP) is a global phenomena. HMPs include raw herbs, herbal preparations and herbal teas which are used to treat various illness conditions. Herbal immunomodulators can alter immune function and may be used as alternatives or as adjunct treatment to allopathic medicines that possess harmful adverse effects. Many of these herbal immunomodulators are prepared from combinations of medicinal plants which may stimulate, down regulate, inhibit or have no effect on immune pathways. These effects can be easily monitored and understood on isolated constituents and single herbal extracts. However, polyherbal formulations present a complex mixture of chemistry into a human system. Septilin™ a polyherbal HMP, has been reported to have immunomodulatory properties. Septilin™ is prescribed by many complementary and alternative medicine (C&TM) practitioners for inflammatory and infectious conditions. Septilin™ is also available as an over the counter medication in health stores and pharmacies. Most of the studies on Septilin™ are conducted by its manufacturing company with very few citations on its immunomodulatory action using suitable *in vitro* models. According to the Medicine Control Council (MCC) regulation on C&TM in South Africa (SA), Septilin™ falls within the high risk level category due to the claims of efficacy by the manufacturer and therefore requires continuous scientific validation via relevant *in vitro*, *in vivo* and clinical trial studies. Scientific data on Septilin™ and other HMPs are crucial to the safety of patients and to the regulation of the practice of C&TM in SA, Africa and the rest of the world.

This study was undertaken to assess the effects of Septilin™ on immune pathways (*in vitro*) by means of using whole blood cultures (WBC) and RAW 264.7 cells. Enzyme

linked immunosorbent assays were used to screen for IL-6, IL-10, and IFN γ as biomarkers for inflammation, humoral immunity, and cell mediated immunity, respectively. Results show that the presence of Septilin™ in LPS stimulated whole blood cultures (WBC) has no effect on the release of IL-6 and IFN γ production but stimulated IL-10 production. Septilin™ in unstimulated WBC has no effect on the release of IL-10 and IFN γ production but stimulatory effects on IL-6 production. These findings agree with previous *in vitro* studies on the effects of Septilin™ on IL-10 and IFN γ production. However, it disagrees with previous studies with regards to its anti-inflammatory action using IL-6 as a biomarker for inflammation.

This study also assessed the effects of *Artemisia afra*, *Aspalathus linearis* (rooibos) and Septilin™ on inflammatory biomarkers namely, IL-6 and nitric oxide (NO) using RAW 264.7 cells, a murine macrophage cell line. The results of this study indicate that *Artemisia afra* has anti-inflammatory effects while *Aspalathus linearis* (rooibos) up regulated the immune system. The study also shows that Septilin™ has no immunomodulatory effects on RAW 264.7 cells. These findings were inconsistent with previous *in vitro* studies on Septilin™.

4.2 Concluding remarks

The LPS stimulated cell system has become an accepted tool in the detection of new anti-inflammatory drugs. *In vitro* whole blood cell cultures and RAW 264.7 cells are effective instruments used for assessing the effect of HMPs on the immune system. The *in vitro* data produced from this study support the humoral stimulating effects of Septilin™ as reported by its manufacture and previous studies. Stimulation of humoral immunity may increase antibody production and improve host defence against bacterial and parasitic infections. Septilin™ had no effect in the release of IFN γ in PHA stimulated WBC. Septilin™ up regulated IL-6 production in LPS unstimulated

WBC and displayed no effect in LPS stimulated WBC. This is contrary to previous studies that reported anti-inflammatory effects. This study suggests that Septilin™ may have prophylactic benefits in conditions that requires up regulation of IL-6. This study also differed to previous studies in relation to the effects of Septilin™ on inflammatory biomarkers namely, IL-6 and nitric oxide (NO). One possible reason for this is the differences in dosages of the herbal product used in previous studies. Many of the previous *in vitro* studies used high to extremely high dosages of the herbal product. High doses used in previous studies may be cytotoxic when applied *in vivo*. Previous studies have reported on the vast differences between the efficacy of ethanol and aqueous herbal extract *in vitro*. Future studies conducted on these herbal medicines should include both ethanol and aqueous extracts.

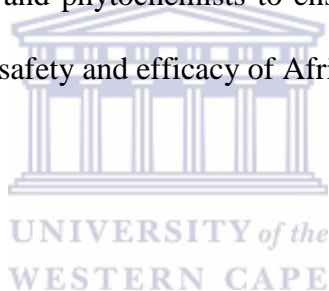
The results of this study indicate that *Artemisia* has anti-inflammatory effects which agree with all previous studies as well as anecdotal uses. *Aspalathus linearis* (rooibos) up regulated the immune system which agrees with a few previous studies whilst most studies reports on its anti-inflammatory effects. These current findings suggests that the aqueous extract of *Aspalathus linearis* (rooibos) may account for the its pro-inflammatory effects *in vitro*.

4.3 Recommendations

This is the first study to report on the immunomodulatory effect of Septilin™ using WBC and RAW 264.7 *in vitro* in one study. This is the first study that observed that high doses of Septilin™ decreased metabolic cellular activity of stimulated RAW 264.7 cells *in vitro*. Further studies on toxicity are recommended. This is an important factor to consider when prescribing this medication as incorrect/high dosages could have detrimental effects in patients. However further studies are needed to confirm these findings. This study also differed with previous studies with

regard to the anti-inflammatory activities of Septilin™. *In vitro* studies on HMPs should be standardised and regulated in terms of dosages used. Concentrations used *in vitro* should be calculated based on anecdotal dosages with the objective of extrapolating *in vitro* data for *in vivo* application. This could contribute to an efficient system of researching HMPs in Africa which could be cost effective and less time consuming. Future studies on these HMPs should consider that different batches of the product may produce varying results due to the complex chemistry of HMPs. Also, there are vast pharmacological differences between aqueous and ethanol extracts of HMPs therefore studies should include both forms. Divergent *in vitro* cell models produce varying results regarding cytokine and NO activity by HMPs.

Future research into African HMPs should include collaborations between C&TM practitioners, microbiologists and phytochemists to ensure better research outcomes. This is essential to ensure the safety and efficacy of African HMPs.



Central Analytical Facilities (Stellenbosch University)

Requested by: Edmund Pool

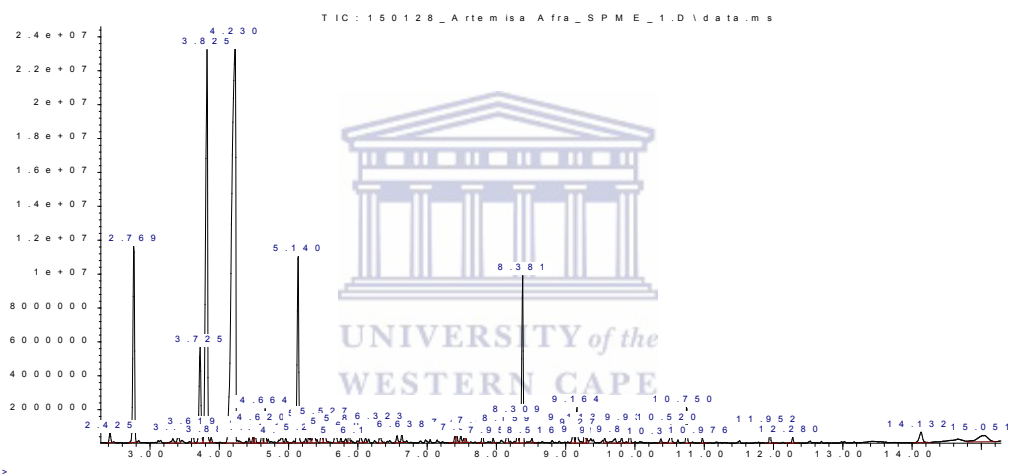
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Scope: GCMS Analysis of Artemisia Afra Extract Sample

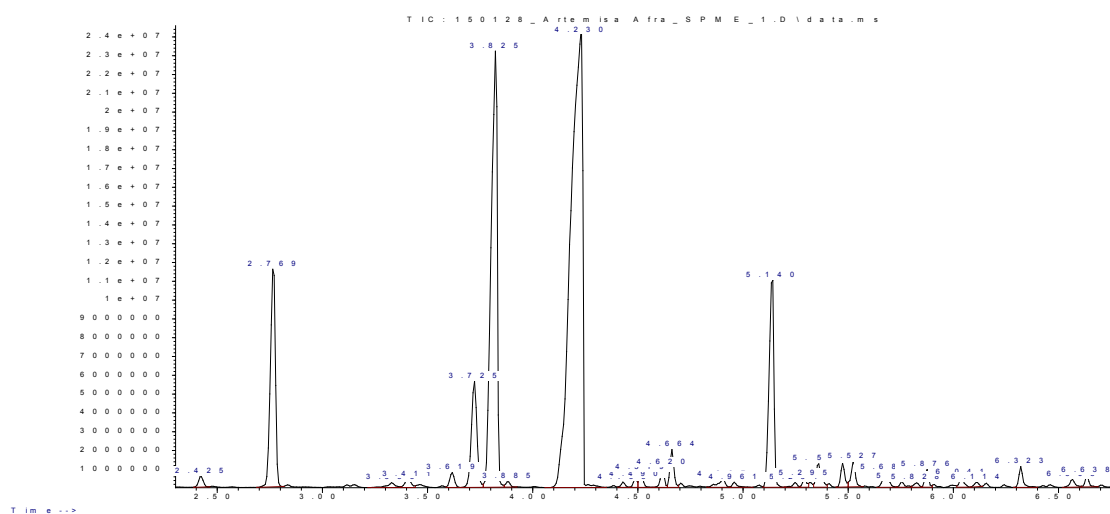
ArtemisaAfra Extract			
RT	Library/ID	Qual	Area Pct
2.4254	tricyclene (Tricyclo[2.2.1.0.(2,6)]heptane, 1,7,7-trimethyl)	96	0.35
2.7712	Camphene	97	7.53
3.3356	1,3,5-Trimethylbenzene (Mesitylene)	97	0.25
3.4132	2,5,5-trimethyl-3,6-heptadien-2-ol (yomogi alcohol)	87	0.36
3.6178	alpha-Terpinene	98	0.47
3.7236	Benzene, 1-methyl-2-(1-methylethyl)- (p-Cymene)	97	3.97
3.8224	1,8-CINEOLE	99	18.15
3.8859	CIS-OCIMENE	97	0.17
4.2316	ARTEMISIA KETONE	87	36.05
4.4362	ARTEMISIA ALCOHOL	72	0.15
4.4926	alpha-terpinolene	98	0.20
4.6196	ISOAMYL-2-METHYL BUTYRATE	86	0.53
4.662	2-Methylbutyl 2-methylbutyrate	72	1.01
4.9089	Unkown	47	0.42
4.9583	CIS-SABINENEHYDRATE	35	0.17
5.1417	CAMPHOR	98	6.78
5.2476	Verbenyl ethyl ether	55	0.15
5.3605	endo-Borneol	94	0.83
5.4733	4-Terpineol	96	0.64
5.5298	Verbenyl ethyl ether	50	0.76
5.685	Unkown	94	0.62
5.7555	2-(1-Methylpropyl)-5-methylcyclohexanone	43	0.18
5.8261	6-methylene-6,7-dihydro-4H-thiazolo[2,3-c][1,2,4]triazin-4-one	52	0.15
5.8755	1,5-dimethyl-2-pyridithione	22	0.45
6.0448	Unkown	50	0.31
6.1154	Cuminic aldehyde	91	0.13
6.32	CHRYSANTHENYL ACETATE	64	0.58
6.5669	iso-bornyl acetate	99	0.28
6.6375	Nonanoic acid, ethyl ester	91	0.30
7.4065	.alpha.-Copaene	99	0.23
7.4276	Nonanal diethyl acetal	56	0.35
7.4912	BENZYL ISOVALERATE	83	0.24
7.5405	ETHYL CAPRATE	93	0.16
7.5617	N-TETRADECANE	93	0.19
7.8016	beta.-Caryophyllene	99	0.43
7.9568	beta-Phenylethyl butyrate	78	0.12

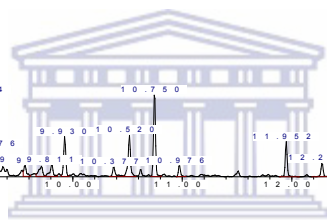
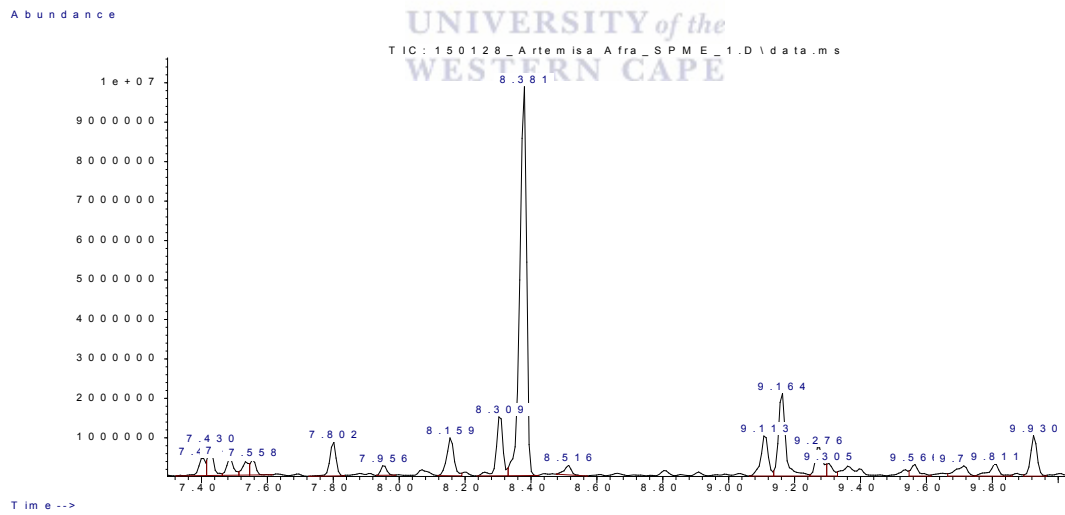
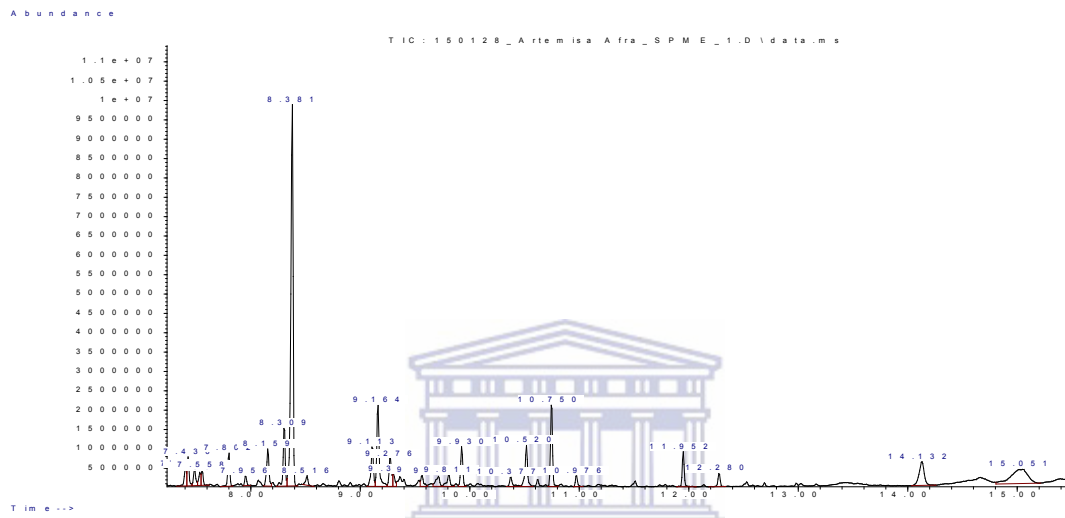
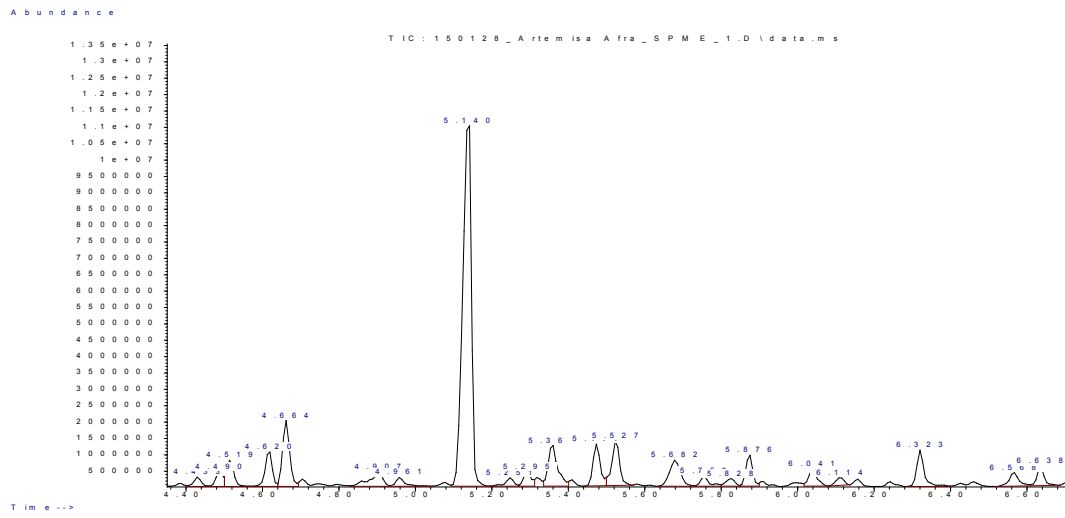
8.1614	ALLOAROMADENDRENE	99	0.56
8.3096	AR-CURCUMENE	98	0.82
8.3801	beta-Selinene	99	5.46
8.5142	beta-Bisabolene	97	0.14
9.1139	(+) spathulenol	93	0.61
9.1632	CARYOPHYLLENE OXIDE	95	1.24
9.2761	n-Butyl-.beta.-phenylpropionate	49	0.44
9.5654	Tetracyclo[6.3.2.0(2,5).0(1,8)]tridecan-9-ol, 4,4-dimethyl-	64	0.20
9.8123	alpha-Cedrene oxide	38	0.25
9.9323	NOOTKATONE	96	0.54
10.3768	Unkown	50	0.18
10.5179	Unkown	90	0.65
10.7507	Unkown	90	1.08
10.9765	6,10,14-trimethyl-2-Pentadecanone	97	0.15
11.9501	Ethyl palmitate	99	0.46
15.0544	beta-Amyrin (beta.-Amyrenol)	90	1.54

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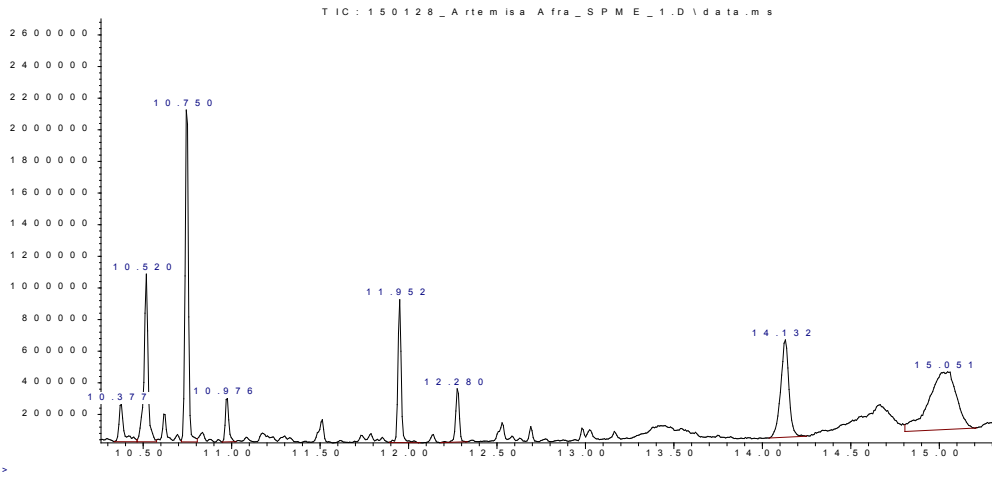
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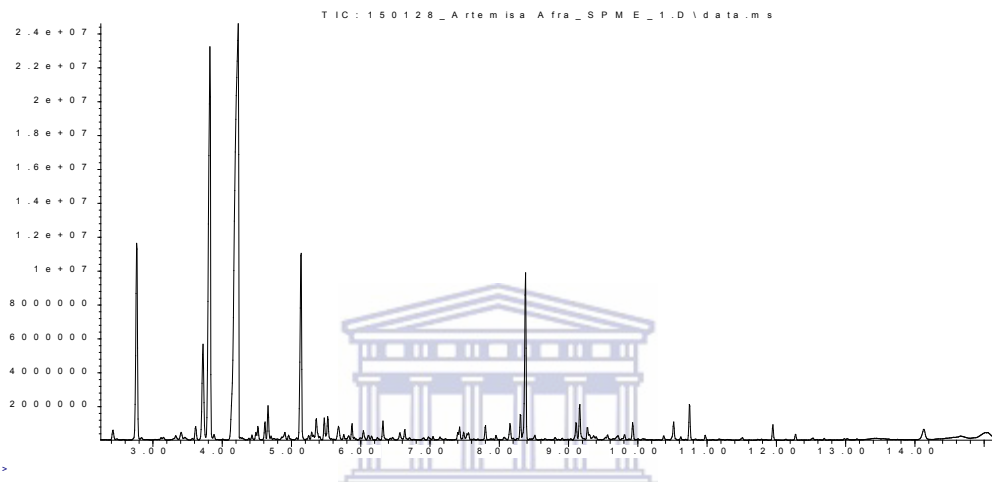


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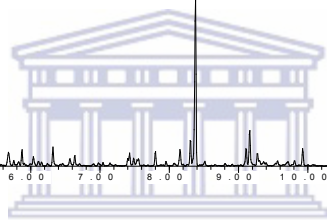
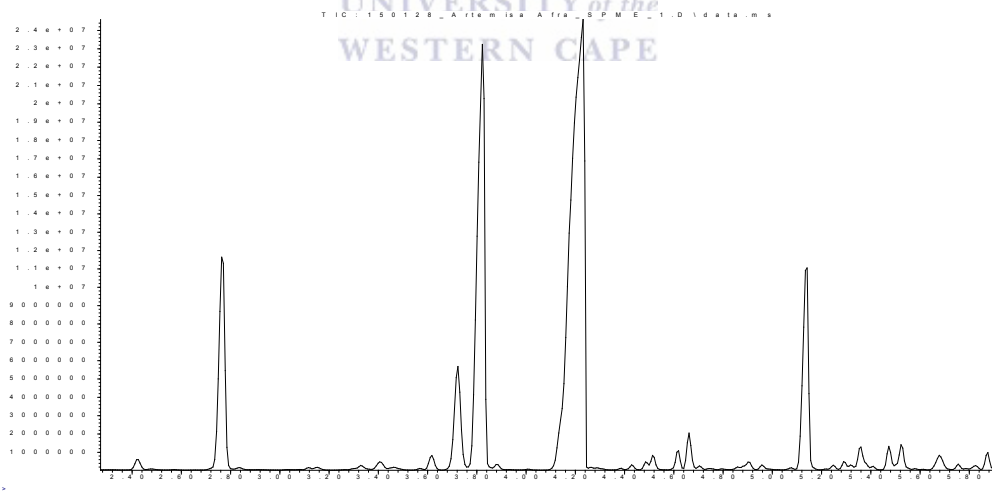
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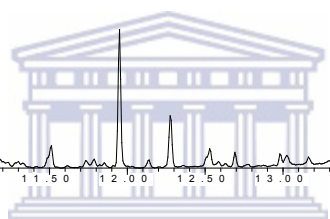
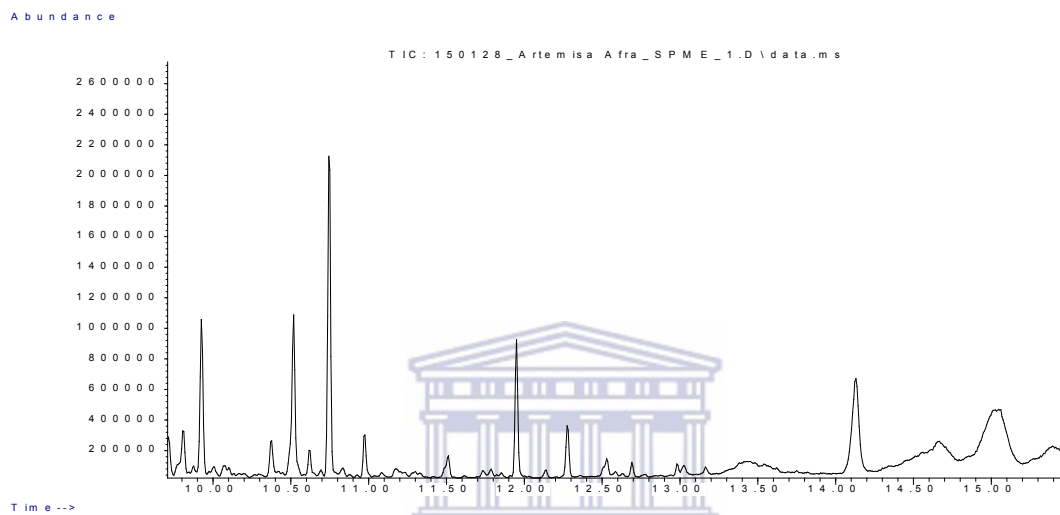
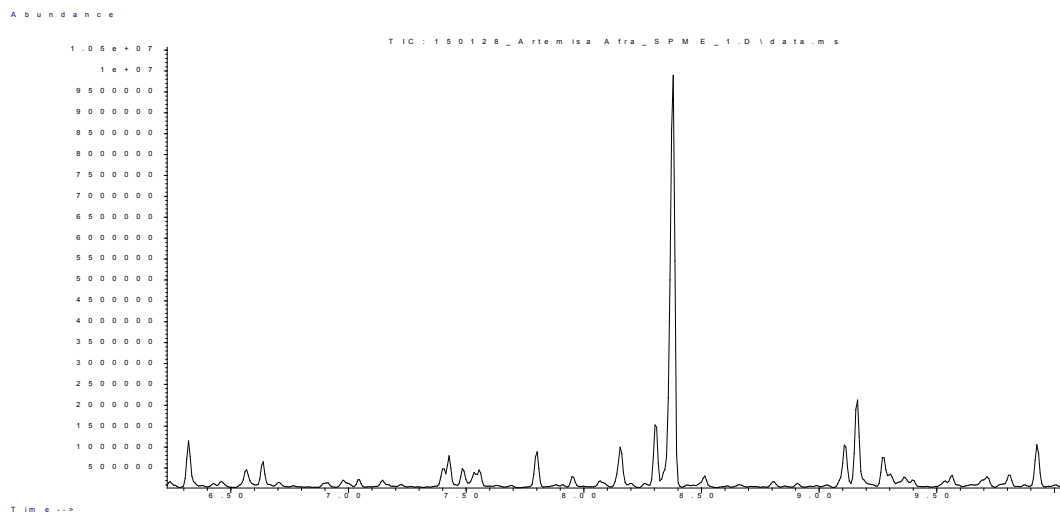
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