THE EFFECT OF *PHELA*, A TRADITIONAL MEDICINE ON THE IMMUNE SYSTEM OF A RAT MODEL

By

'MAKHOTSO ROSE LEKHOOA

[B.Sc, B.med.Sc. (Hons) Pharmacology, B.med.Sc. (Hons) Physiology]

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Supervisor: Prof. A. Walubo

Co-Supervisors: Dr. JB Du Plessis

Dr. MG Matsabisa

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ABSTRACT

The Human immunodeficiency virus (HIV) has grown to pandemic proportions, resulting in an estimated 25 million deaths globally, whereas in South Africa almost 1000 deaths occur daily due to Acquired immunodeficiency syndrome (AIDS). HIV infects the immune system, making the host unable to control the virus and more susceptible to infections. Treatment of HIV with Antiretroviral (ARV) drugs only prevents viral replication but does not stimulate the failing immune system. Thus, there is a need to incorporate an immune booster together with the HIV treatment, in order to enhance the immune system.

Phela is a herbal traditional medicine prepared using well defined parts of four African medicinal plants. It is currently under development for use as an immune booster in immune compromised individuals. The aim of the study was to determine the mechanism of action by which phela boosted the immune system of a rat model.

Unfortunately, subsequent literature research revealed that very little was known about Phela. As such there was a need to develop a method by which to identify Phela. Method development included Thin Layer Chromatography (TLC) for rapid screening. Phela was extracted by the salting-out method and eluted with ethyl acetate: ammonia: methanol (17:1:2; v/v/v) as mobile phase. Phela had a brown color during extraction and three bands with a retention factor (RF) of 0.23, 0.57 and 0.92 respectively. During Preparative Column Chromatography (CC) extraction of phela, fractions were collected every minute from time of elution. Together TLC and CC were used as preparative steps for HPLC and GC_MSD analysis.

Thereafter, a High Performance Liquid Chromatography (HPLC) method using a UV (HPLC_UV) and fluorescence detector (HPLC_FL) for identification of phela were developed. For HPLC_UV, crude phela was acidified with Hydrochloric acid (HCl) and extracted by hexane. Mobile phase was 70 % acetonitrile in water eluting at 0.5ml/min over a C_{18} (250 mm x 4.6 mm x 5µm) column coupled to a guard column and UV set at 245 nm wavelength. Two marker peaks were selected and standardized by retention

CHAPTER 1

GENERAL INTRODUCTION

The human immunodeficiency virus (HIV) is a retrovirus that leads to acquired immunodeficiency syndrome (AIDS**).** HIV infects the immune system, making the host unable to control the virus and at the same time more susceptible to infections by other pathogens. It targets the major immune cells, CD_4 T cells, macrophages and microglial cells. Symptomatic HIV infection is characterized by the emergence of opportunistic infections and cancers in different body systems that the immune system would normally prevent.

Since the first cases of acquired immunodeficiency syndrome (AIDS) were reported in 1981, the infection has grown to pandemic proportions, resulting in an estimated 65 million infections and 25 million deaths globally. In 2008, about 33.4 million people were living with HIV, of whom 2.7 million more people were newly infected with HIV and 2.0 million died of AIDS (UNAIDS, 2009). Although HIV and AIDS are found in all parts of the world, some areas are more afflicted than others. The worst affected region is sub-Saharan Africa where, in some countries, more than one in five adults is infected with HIV. South Africa has experienced one of the most severe AIDS epidemics in history in that by the end of 2008, there were 5.7 million people living with HIV in South Africa (UNAIDS, 2008).

On the other hand, the use of anti-retroviral (ARV) drugs has led to decreased HIV related morbility and mortality (WHO et al., 2009). Unfortunately, the ARV drugs do not cure the disease and are not accessible to all patients that need them**.** In addition, anti-retrovirals (ARV) drugs can cause adverse effects that include metabolic disorders and these worsen with chronic therapy. This implies that ARV drugs are not the sole solution to eradication of the virus. However, it is well established that improvement during ARV drug therapy is paralleled by improved immune response, which highlights the need for boosting the immune system in controlling the progression of HIV disease (Vicenzi and Biswas, 1997).

There is a need for immune boosters that can enhance and stimulate the immune system and, if possible, can be used in combination with ARV drugs.

Phela is a traditional medicine prepared from four African medicinal plants. An observation clinical study using Phela as an immune booster was conducted by the MRC-IKS unit in 500 HIV positive and AIDS patients **(**Matsabisa M et al., 2006)**.** The results showed an increase in the patients' appetite, 23% weight gain, 80% decrease in viral load and 200% increase in CD_4 cell counts. The overall quality of life of patients increased, some from as low as 30% to 100%. These results were indicative of the immune boosting properties of Phela. As such, the MRC is developing Phela for use as an immune booster in HIV patients.

Unfortunately, despite the worldwide use of traditional herbal medicines, quality evaluation of herbal medicines remains a challenge as there is still no reliable and acceptable strategy for their quality evaluation. Direct quantification of as many as possible components is impossible owing to the fact that most reference components are not commercially available, while the procedures for complete separation, identification and determination of their components are almost unattainable. Therefore, chromatographic fingerprinting was recommended by the WHO and other agencies as the most preferable technique to use in the quality control of herbal medicines [WHO et al., 2001]. Specifically, it is used for, among other things, authentication of raw materials and final products, testing for stability, consistency in manufacturing and fighting against counterfeits and adulteration. It involves systemic characterization of some of the components of herbal medicines under standardized chromatographic and extraction conditions.

Fingerprinting methods should incorporate methods that are affordable to most laboratories, particularly laboratories in poor resourced countries. Therefore, it was envisaged that evaluating various chromatographic techniques would lead to a chromatographic fingerprint for Phela identification.

Unfortunately, the mechanism of action of Phela on the immune system is not known. However, it is well known that the immune cells are regulated by cytokines and that the cytokine profile determines the type of immune response to any antigen (Romagnani, 1991; Muller, 2003). Activation of type 1 helper T cells (TH_1) leads to formation and activation of cytotoxic T-cells thereby conferring cell mediated immunity (CMI), while activation of type 2 helper T cells (TH_2) leads to formation and activation of B-cells for antibody response, which confers humoral immunity. The communication between the TH cells and the effector cytotoxic T cells or B cells is through cytokines. The TH_1 cells produce interferon-γ (IFN-γ) and interleukin-2 (IL-2), while TH_2 cells produce interleukin-4 (IL-4) and interleukin-10 (IL-10**).** Therefore, it was envisaged that by observing changes in cytokine profiles, important information on the status of the immune system could be revealed and used to determine the mechanism of action of Phela. Specifically, the aim of this study was to determine the effect of Phela on the immune system by observing for subclinical changes in TH_1 cytokines (IL-2, IFN-γ, TNF-α) that would enhance cell mediated immunity and TH_2 cytokines (IL-4 and IL-10) that would enhance the humoral response, with the hope that understanding the mechanism of action would help in designing immune booster therapy.

The scope of the thesis:

Chapter 1 is the general introduction, while the literature review is presented in four chapters: chapter 2 is an overview of the immune system, chapter 3 deals with HIV pathogenesis, chapter 4 concerns the use of immune boosters in HIV, chapter 5 is an overview of the methods used for fingerprinting of traditional medicines and chapter 6 provides a summary of observations from the review and study aims and objectives.

The experimental chapters comprise the development of methods for fingerprinting Phela by Thin Layer Chromatography (TLC; chapter 7), High Performance Liquid Chromatography with UV detector (HPLC_UV; chapter 8) and a High Performance Liquid Chromatography with a fluorescence detector

(HPLC_FL; chapter 9). The before-mentioned methods were then compiled into a single comprehensive approach for authentication of phela (chapter 10).

Chapter 11 is devoted to the development of an HPLC method for analysis of phela in spiked plasma, while chapter 12 describes the use of the developed method to detect and study pharmacokinetics of Phela in rats after oral administration.

Lastly, chapter 13 is an investigation of the mechanism by which Phela boosts the immune system via cytokines and chapter 14, draws the conclusion and suggests themes for future studies.

CHAPTER 2

OVERVIEW OF THE IMMUNE SYSTEM

2.1 INTRODUCTION

The immune system is a collection of mechanisms within an organism that protect it against infection, by identifying and killing pathogens and tumour cells (Ganong, 1985). It detects a wide variety of pathogens, such as bacteria, viruses, parasitic worms and foreign particles known as antigens because they can elicit an immune response (Sherwood, 2001). The immune system protects organisms from infection with layered defences of increasing specificity. The first line of defence is physical barriers that prevent pathogens from entering the body (e.g. skin) (Montaga, 1976). If a pathogen breaches these barriers, the innate immune system provides an immediate, but non-specific response. If pathogens successfully evade the innate response, a third layer of protection, the adaptive immune system, is activated. This improved response is then retained after the pathogen has been eliminated, in the form of an immunological memory, and allows the adaptive immune system to mount faster and stronger attacks each time this pathogen is encountered (Ganong, 1985; Sherwood, 2001; Lappin et al., 2000).

2.1.1 Innate Immune system

The Innate immune system defences are non-specific, they recognize and respond to pathogens in a generic way and do not confer long-lasting immunity against a pathogen. The responses include inflammation, the complement system, different leukocytes and cytokines (Ganong, 1985; Lappin et al., 2000).

2.1.1.1 Inflammation

Inflammation is the first response of the immune system to infection. The goal of inflammation is to bring phagocytes and plasma proteins to the injured area, to isolate, destroy or inactivate the invaders, to remove debris and prepare for

subsequent healing and repair (Sherwood, 2001). Inflammation can be either acute or chronic.

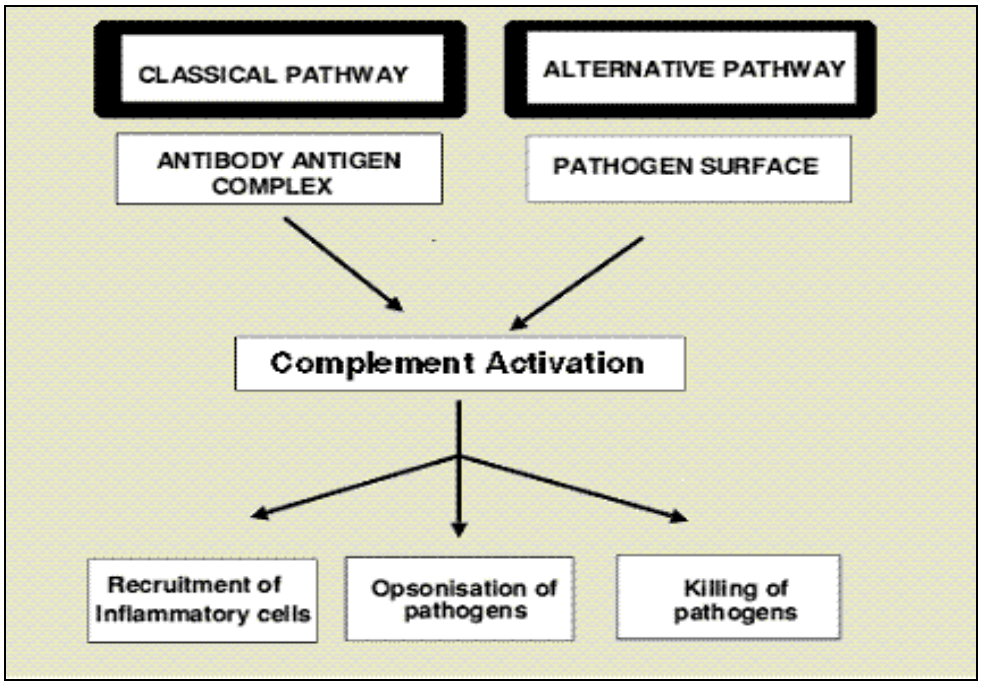
Acute inflammation is the initial short term response of the body to harmful stimuli which is characterized by the following symptoms: redness, swelling, pain, heat and loss of function. The symptoms are caused by an increased blood flow into a tissue, brought about by histamine release by mast cells and basophils. Inflammation is mediated by eicosanoids (prostaglandins and leukotrienes) and cytokines, released by either injured or infected cells. The cytokines and other chemicals recruit immune cells to the site of infection and promote healing of damaged tissue following the removal of pathogens (Lapping et al., 2000).

Chronic inflammation is a pathological condition characterized by concurrent acute inflammation, tissue destruction, and attempts at repair. Chronically inflamed tissue is characterized by the infiltration of mononuclear immune cells (monocytes, macrophages, lymphocytes, and plasma cells), tissue destruction, and attempts at healing, angiogenesis and fibrosis. Endogenous causes include persistent acute inflammation, whereas exogenous causes vary from bacterial infection, especially by mycobacterium tuberculosis, prolonged exposure to chemical agents or autoimmune reactions (Ganong, 1985; Sherwood, 2001; Lappin et al., 2000; Pinto, 2000).

2.1.1.2 Complement system

The complement system is a biochemical cascade that attacks the surfaces of foreign cells. Complement is the major humoral component of the innate immune response. Figure 2.1 illustrates how the response can be activated either through the alternate or classical pathway.

Figure 2.1: The classical and alternative pathways of the complement system [Sheerwood, 2001]



2.1.2 Leukocytes

Leukocytes are white blood cells forming the second arm of the innate immune system. They comprise phagocytes (macrophages, neutrophils and dentric cells), mast cells, eosinophils, basophils, and natural killer cells. The above named cells identify and eliminate pathogens, by attacking larger pathogens through contact or by engulfing and killing micro-organisms. Innate cells are also important mediators in the activation of the adaptive immune system (Ganong, 1985; Sherwood, 2001; Lappin et al., 2000). Leukocytes are classified into granulocytes and agranulocytes.

Granulocytes are made up of neutrophils, basophils and eosinophils. Neutrophils facilitate chemo taxis during inflammation. Basophils and eosinophils secrete chemical mediators that are involved in defending against parasites and play a role in allergic reactions, such as asthma. Mast cells reside in connective tissues

and mucous membranes, and regulate the inflammatory response. They secrete histamine and are associated with allergy and anaphylaxis. Natural killer (NK) cells are leukocytes that attack and destroy tumour cells, or cells that have been infected by viruses.

Agranulocytes comprise lymphocytes, monocytes and macrophages (Picker, 2006). Macrophages are versatile cells residing within tissues and producing enzymes, complement proteins, and regulatory factors. Macrophages also act as scavengers, ridding the body of worn-out cells and other debris, and as antigen-presenting cells that activate the adaptive immune system. Dendritic cells (DC) are phagocytes in tissues that are in contact with the external environment, therefore, they are located mainly in the skin, nose, lungs, stomach, and intestines. Dendritic cells serve as a link between the innate and adaptive immune systems, as they present antigen to T cells, one of the key cell types of the adaptive immune system.

Phagocytes circulate the body searching for pathogens, and are attracted to specific locations by cytokines. Phagocytosis is an important feature of cellular innate immunity.

2.1.3 Adaptive Immune system

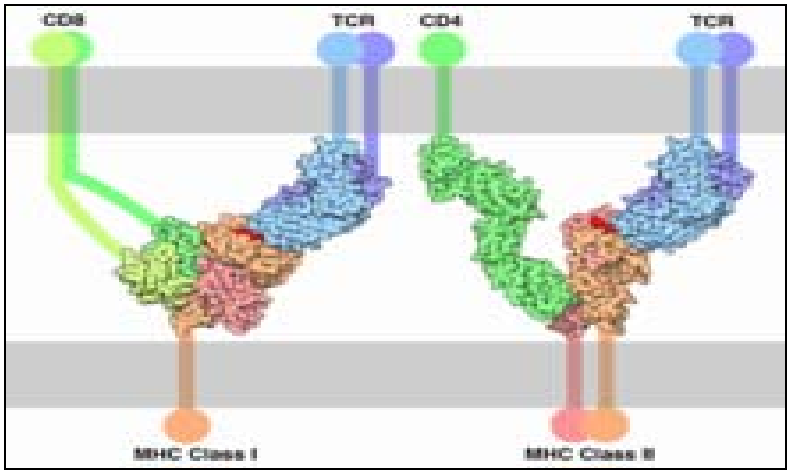
The adaptive immune response is antigen-specific and requires the recognition of specific “non-self” antigens during antigen presentation mediated by lymphocytes. B cells and T cells are the major types of lymphocytes and are derived from haematopoietic stem cells in the bone marrow. B cells mature in the bone marrow whereas T cells mature in the thymus (Sherwood, 2001). B cells are involved in the humoral immune response, whereas T cells are involved in cell-mediated immune response. Both B cells and T cells carry receptor molecules that recognize specific targets. The ability to mount these tailored responses is maintained in the body by memory cells, thus ensuring a quicker

response to eliminate the pathogen with a recurrent infection (Ganong, 1985; Sherwood, 2001; Lappin et al., 2000).

2.1.3.1 T-Lymphocytes

The different types of T cells include cytotoxic killer cells (CD_8), helper T cells (CD_4), and suppressor and γδ cells (Lappin et al., 2000). Cytotoxic T cells only recognize antigens coupled to Class I MHC molecules, while helper T cells only recognize antigens coupled to Class II MHC molecules (figure 2.2). T cells recognize a "non-self" target, such as a pathogen, only after antigens have been processed and presented in combination with a major histo-compatibility complex (MHC) molecule.

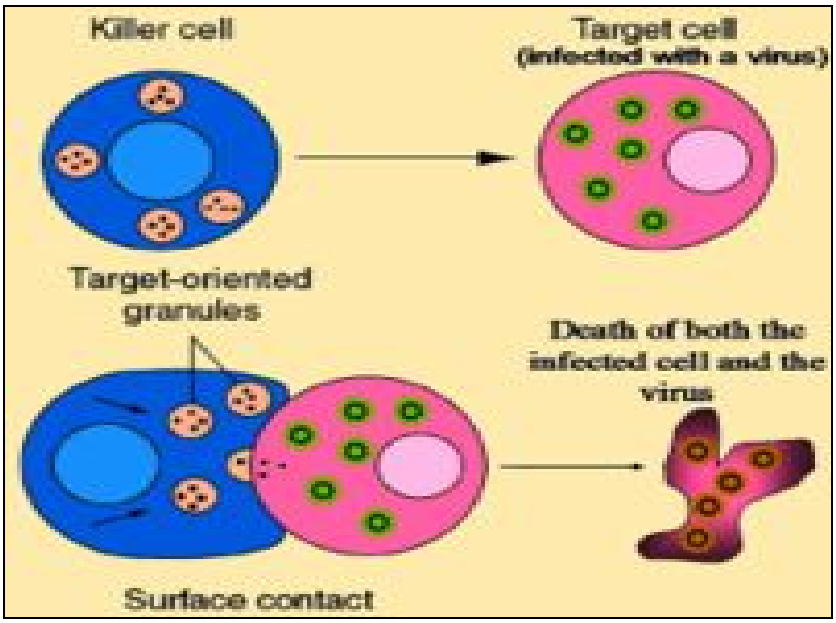
Figure 2.2: Association of a T cell with MHC class I or class II and antigen. [http://en.wikipedia.org/wiki/immune_system/image:TCR-MHC bindings (Accessed 20th March 2008)]



Cytotoxic Killer T cells (CD8) are a sub-group of T cells that kill cells that are damaged, mutated or infected with viruses and other pathogens. Each type of T cell recognizes a different antigen. Killer T cells are activated when their T cell

receptor (TCR) binds to this specific antigen in a complex formed with the MHC Class I receptor of another cell. Recognition of this MHC-antigen complex is aided by a co-receptor on the T cell, called CD_8. T cell responds by releasing cytotoxins that form pores to enhance apoptosis. T cell killing of host cells is particularly important in preventing the replication of viruses (Figure 2.3).

Figure 2.3: Killer T cells directly attacking other cells carrying foreign or abnormal antigens on their surfaces.
[http://en.wikipedia.org/wiki/immune_system/image:Cytotoxic T cell (Accessed 20th March 2008)]



Helper T cells (CD_4) regulate both the innate and adaptive immune responses and have no cytotoxic properties. They regulate the immune response by secreting cytokines. The MHC-antigen complex is also recognized by the helper cell's CD_4 co-receptor, which recruits molecules inside the T cell that are responsible for the T cell's activation. Helper T cell activation also requires longer duration of engagement with an antigen-presenting cell. The activation of a resting helper T cell causes it to release cytokines that influence the activity of many cell types. Cytokine signals produced by helper T cells enhance the microbicidal function of macrophages and the activity of killer T cells. Furthermore, helper T cell activation causes an up-regulation of molecules expressed on the T cell's surface, which provides extra stimulatory signals required to activate antibody-producing B cells.

γδ T cells possess an alternative T cell receptor (TCR) and share the characteristics of helper T cells, cytotoxic T cells and natural killer cells on the one hand. γδ T cells are a component of adaptive immunity as they re-arrange TCR genes to produce receptor diversity and can also develop a memory phenotype. On the other hand, the various subsets are also part of the innate immune system, as restricted TCR or NK receptors may be used as pattern recognition receptors (Lappin et al., 2000).

Suppressor T cells do not need to be presented with an antigen to become active. In a negative feedback mechanism, helper T cells activate suppressor T cells into action, and they in turn inhibit the helper T cells and other cells. This inhibition prevents excessive immune reaction that might be detrimental to the body. (Sherwood, 2001).

2.1.3.2 B-Lymphocytes and antibodies

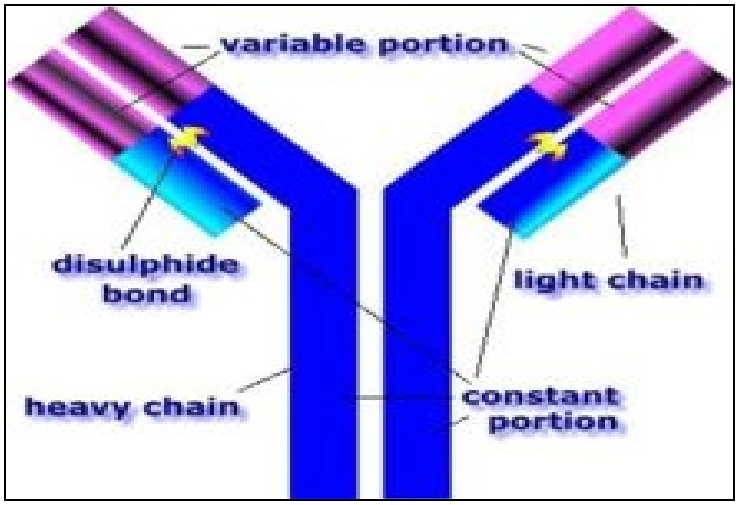
When the B cell binds to an antigen it differentiates into a plasma cell which produces antibodies. Antibodies are secreted into the blood and are classified into different groups: Ig M, Ig G, Ig E, Ig A and Ig D according to their biological

activity. B cell identifies pathogens when antibodies on its surface bind to a specific foreign antigen.

The B cell then displays antigenic peptides on its surface MHC class II molecules. This combination of MHC and antigen attracts a matching helper T cell, which releases lymphokines and activates the B cell. As an activated B cell, it begins to divide into plasma cells, and secrete millions of copies of the antibody that recognizes this antigen. These antibodies circulate in blood plasma and lymph, bind to pathogens expressing the antigen and mark them for destruction by complement activation or for uptake and destruction by phagocytes. Moreover, antibodies can physically hinder antigens from exerting their effects through neutralization, agglutination, precipitation or by interfering with the receptors that viruses and bacteria use to infect cells.

Figure 2.4: The Y structure of an antibody.

[http://en.wikipedia.org/wiki/immune_system/image:Antibody (Accessed 20th March 2008)]



Ig M serves as the B cell surface receptor for antigen attachment and is secreted in the early phase of plasma cell response. Ig G is the most potent immuno-globin in the blood; It is produced copiously when the body is subsequently exposed to the same antigen. Furthermore, Ig M and Ig G antibodies are both responsible for the most specific response against bacterial invaders and a few types of viruses. Ig E helps protect against parasitic worms and is a mediator for common allergic responses such as hay fever. Ig A is found in secretions of digestive, respiratory and genoto-urinary systems. Ig D is present on the surface of B cells, but its function is uncertain.

2.1.4 Cell-Mediated immunity

Cell-mediated immunity is directed primarily at microbes that survive in phagocytes and microbes that infect non-phagocytic cells. It is also referred to as TH_1 response. It is most effective in removing virus-infected cells, furthermore participating in defending against fungi, protozoans, cancers and intracellular bacteria. Cellular immunity protects the body by activating antigen-specific cytotoxic T-lymphocytes, that are able to induce apoptosis in body cells displaying epitopes of foreign antigen on their surface, such as virus-infected cells, cells with intracellular bacteria, and cancer cells displaying tumour antigens. They also activate macrophages and natural killer cells, enabling them to destroy intracellular pathogens and stimulating cells to secrete a variety of cytokines that influence the function of other cells involved in adaptive immune responses and innate immune responses (Lappin et al., 2000).

2.1.5 Humoral Immunity

Humoral immunity involves substances found in the humors, or body fluids and It is also known as TH_2 response. Humoral Immune Response (HIR) is the aspect of immunity that is mediated by secreted antibodies, which are produced by the B cells that have differentiated into plasma cells. Secreted antibodies bind to antigens on the surfaces of invading microbes which flag them for destruction. Humoral immunity refers to antibody production, and the accessory processes that accompany it, including: TH_2 activation and cytokine production, germinal center formation and isotope switching, affinity maturation and memory cell generation. It includes the effector functions of antibodies, which include pathogen and toxin neutralization, classical complement activation, and opsonin promotion of phagocytosis and pathogen elimination (Lappin et al., 2000)

2.1.6 Cytokines

Cytokines are a group of proteins and peptides that are used in organisms as signalling compounds. They are particularly important in immunological, inflammatory and infectious diseases; however, not all their functions are limited to the immune system, as they are also involved in several developmental processes during embryogenesis. Cytokines are produced by a wide variety of cell types, both haemopoietic and non-haemopoietic, and can have effects on nearby cells or throughout the organism. These effects are strongly dependent on the presence of other chemicals and other cytokines. Each cytokine binds to a specific cell-surface receptor. Subsequent cascades of intracellular signalling then alter cell functions. This may include the up-regulation and/or downregulation of several genes and their transcription factors, which result in either the production of other cytokines, an increase in the number of surface receptors for other molecules, or the suppression of their own effect by feedback inhibition (Dinarello, 2000).

The effects of a particular cytokine on a given cell depend on the cytokine, its extracellular abundance, the abundance of the complementary receptor on the cell surface, and downstream signals activated by receptor binding. Cytokines are characterized by considerable redundancy, in that many cytokines appear to share similar functions. Clinically and experimentally, cytokines are classified according to their immunological response, as pro-inflammatory or anti-inflammatory.

Cytokines that regulate innate immunity are produced primarily by mononuclear phagocytes such as macrophages and dendritic cells, although they can also be produced by T lymphocytes, natural killer cells and other cells. They are produced in response to pathogen-associated molecular patterns. They also act on leukocytes and the endothelial cells that form blood vessels in order to promote and control early inflammatory responses. This includes TNF-α and IL-1 to mediate acute inflammation.

Chemokines are a family of small cytokines that have the ability to induce chemotaxis in nearby responsive cells. Chemokines enable the migration of leukocytes from the blood to the tissues at the site of inflammation. IL-12 induces cell mediated immunity. Type 1 interferons induce uninfected cells to produce enzymes capable of degrading mRNA. Interleukin 6 (IL-6) stimulates the liver to produce acute phase proteins. It also stimulates B cell proliferation and increases neutrophil production. IL-10 inhibits activated macrophages and dendritic cells, thus regulating innate and cell mediated immunity. IL-15 stimulates natural killer cell proliferation and T cells. IL-18 stimulates production of IFN-γ (Kenneth et al., 2005). Formation of granulation, to localize the infection, is also mediated by cytokines.

2.1.6.1 Pro-inflammatory and TH_1 cytokines

Pro-inflammatory cytokines are cytokines that are secreted by immune cells to mediate inflammatory response during infection. These include the following cytokines: IL-1, IL-3, TNF- (α,β) and IL-18 . The TH_1 cytokines are cytokines that are secreted by T cell subtypes and facilitate the cell mediated immune response. They include IL-2, IL-12, IL-15, and IFN-γ. These cytokines activate macrophages and promote cell-mediated immune responses against invasive intracellular pathogens, enhancing fever and tissue destruction (Dinarello, 2000).

Tumour necrosis factor-alpha (TNF-α) is the principal cytokine that mediates acute inflammation, but in excessive amounts it causes systemic complications like shock cascade (Bahbouhi, 2004). TNF-α is produced by monocytes, macrophages, dendritic cells, TH_1 cells and other cells. It acts on endothelial cells to stimulate inflammation, coagulation pathway, selectins production and leukocyte ligands from diapedeis. Acute phase proteins by the liver and catabolism for energy conversion are also activated. TNF-α is cytotoxic for tumour cells and enhance fever. Macrophages are activated to produce chemokines and IL-1.

IL-1 and TNF- α synergism triggers inflammation and fever through activation of various genes (Dinarello, 2000). They both recruit macrophages, monocytes, poly-morphonuclear leukocytes to the site of infection, and enhance the expression of adhesion molecules for the cells to bind. They are both produced by activated macrophages. TGF-β inhibits proliferation of T and B cells, inhibiting their functionality. It is produced by macrophages, T cells and other cells.

IL-2 is a growth factor for antigen stimulated T and B cells. It increases the killing ability of natural killer cells and synthesis of other cytokines. It is a protein produced by activated T cells that regulate immune responses against infection and release of interferon gamma (http://en.wikipedia.org/wiki/interleukin; Accessed 24 August 2007). It controls the induction of both TH_1 and TH_2

responses by promoting T cell division and antibody synthesis by B cells (Keeneth, 2005).

IL-12 is produced by different antigen presenting cells (APC) and It is a powerful inducer of IFN-γ production by T cells. It suppresses IL-4, thus promoting TH_1 responses by down-regulating TH_2 cytokines. It also stimulates natural killer cells (NK).

Interferon-gamma (IFN-γ) is produced by NK and T cells which are induced by IL-12, IL-15 and IL-18. It possesses antiviral activities. It modulates the immune response by activating the pathway that leads to induction of cytotoxic T cells and augments TNF activity. It induces nitric oxide (NO) by NO synthase (Dinarello, 2000). Nitric oxide acts as a messenger and it induces apoptosis to tumour cells and the killing of bacteria by macrophages when bound to the reactive oxygen species (www.unaids.org/en/HIV_data/2006GlobalReport; Accessed 26 July 2007).

2.1.6.2 Anti-inflammatory and TH_2 cytokines

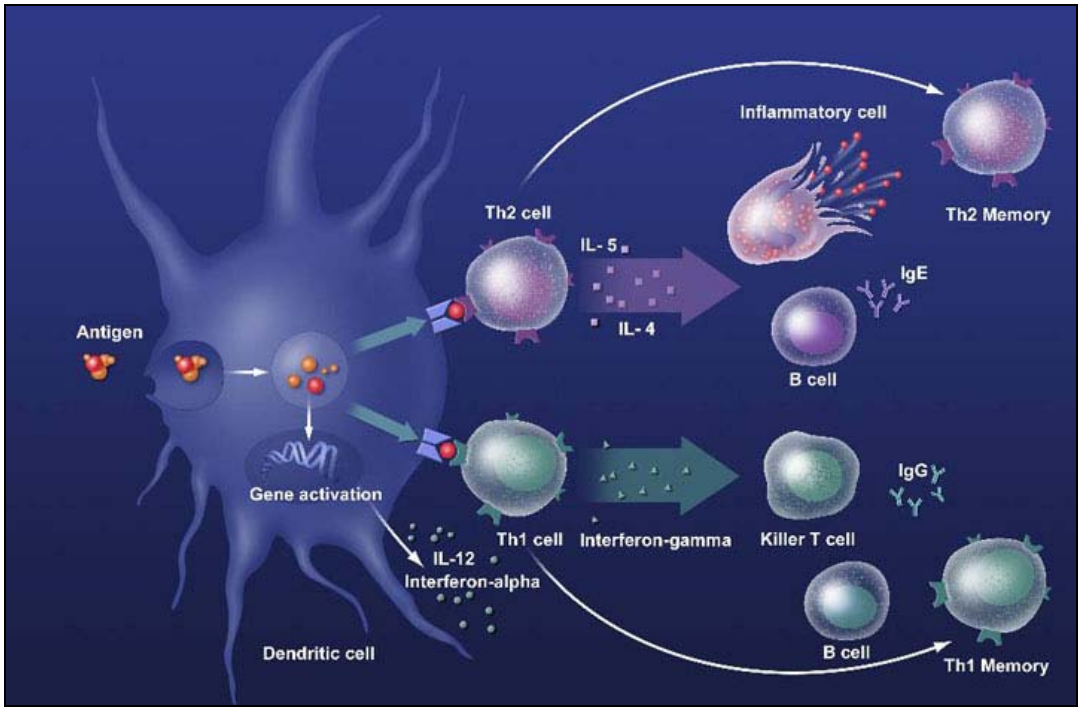
The anti-inflammatory cytokines (figure 2.5) are a series of immuno-regulatory molecules that control the pro-inflammatory response and are associated with B-cell activity, humoral immune response and Ig class switching to Ig E (Opal, 2000). These cytokines include IL-1 receptor antagonist (IL-1ra), IL-4, IL-5, IL-6, IL-10, IL-11, IL-13, TNF-α, soluble cytokine receptors and pro-inflammatory cytokine inhibitors. These cytokines are responsible for the TH_2 response.

IL-1ra is produced by monocytes and macrophages and is released into the systemic circulation in excess. IL-1ra inhibits the binding of IL-1 by competitively binding to IL-1 receptors, preventing its effects.

IL-10 is produced by T cells and monocytes, and is a very potent anti-inflammatory. IL-10 synergism with IL-4 suppresses IL-12 activity, thus inhibiting

IFN-γ production, allowing more IL-4 to be produced favouring TH_2 cytokines that promote humoral response (Opal, 2000). *IL-11* has similar properties to IL-10.

Figure 2.5: The TH_1 and TH_2 immune response diagram [Pujol, 2004]



IL-4 is secreted by TH_2 cells, mast cells and basophils. It drives the TH_2 response by activating mast cells and stimulating the production of Ig E antibodies. It has inhibitory effects on the expression and release of IL-1 and TNF-α through suppression of monocytes (Pujol, 2004). It suppresses the macrophage's cytotoxic activity, parasite killing and nitric oxide production. It stimulates synthesis of IL-1ra.

IL-13 also has similar properties to that of IL-4 of regulating IgE production. It inhibits TH_1 response and macrophage activation (http://en.wikipedia.org/wiki/interkeukin; Accessed 30 August 2007). *IL-6* is produced by T cells, antigen presenting cells, endothelial cells and fibroblasts.

IL-6 stimulates the production of acute phase proteins, proliferation of B cells and increased neutrophil production.

Soluble cytokine receptors act as anti-inflammatory agents and are found in the extracellular fluid. They compete with membrane bound receptors for binding, thus inhibiting activity on target tissue. The identified soluble receptors include receptors for the following cytokines: TNF- α, IL-1 and IL-18.

2.1.6.3 Cytokines with dual effects

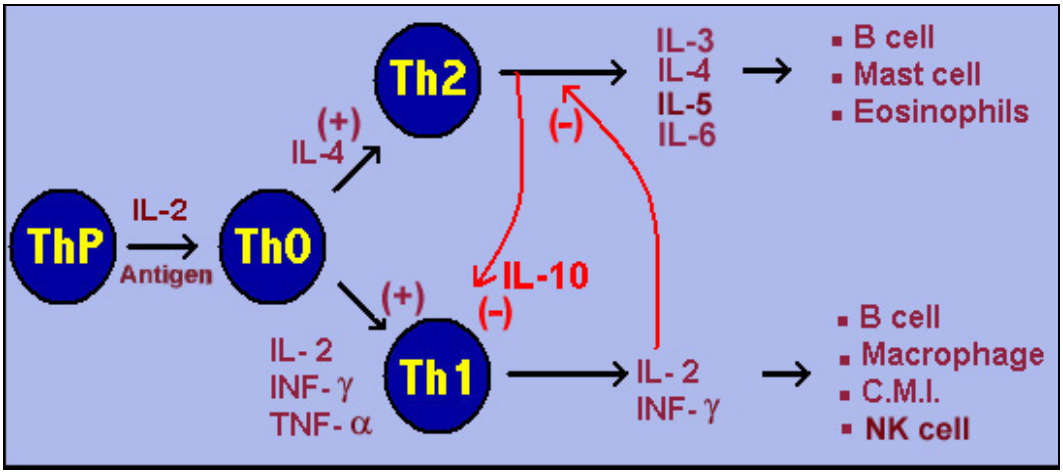
Some cytokines have both pro- and anti-inflammatory effects. *IL-2* is essential for growth for all T cells; the concentration can lead to either TH_1 or TH_2 response. IL-3 and *TNF-α* can be produced in both responses. *IL-10* is essential for TH_2 response but can synergize with TH_1 cytokines. *IL-18* is a strong inducer of IFN-γ, a TH_1 response but can synergize with IL-13 (Opal, 2000). IL-6 is a potent inducer of acute phase proteins and also has anti-inflammatory properties. It down-regulates the synthesis of IL-1 and TNF and promotes synthesis of IL-1ra and soluble TNF receptors and inhibits the production of IFN- γ (Dinarello, 2000).

2.1.6.4 TH_1 versus TH_2 response

The T helper cells can differentiate into functional subsets of TH cells depending on the micro-environment of the cell. The cytokines are classified into TH_1 and TH_2 type cells on the basis of the cytokines produced (Figure 2.6). TH_1 cells produce IL-2, IFN-γ and TNF-β, which activate T cells and macrophages to stimulate cellular immunity and inflammation. TH_2 cells also secrete IL-3 and GM-CSF to stimulate the bone marrow to produce more leukocytes. TH_2 cells secrete IL-4, IL-5, IL-6, and IL-10, which stimulate antibody production by B cells.

Figure 2.6: The T cell differentiation into TH_1 and TH_2 cells.

[http://sprojects.mmi.mcgill.ca/immunology/Th1&Th2.htm(Accessed 6 November 2007]



T cells are initially activated as Th_0 cells (Figure 2.6), which produce IL-2, IL-4 and IFN-γ. The nearby cytokine environment then influences differentiation into either TH_1 or TH_2 cells. IL-4 stimulates TH_2 activity and suppresses TH_1 activity, while IL-12 promotes TH_1 activities. TH_1 and TH_2 cytokines are antagonistic in activity. TH_1 cytokine IFN-γ inhibits proliferation of TH_2 cells, while IFN-γ and IL-2 stimulate B cells to secrete Ig G_2 and inhibit secretion of Ig G_1 and Ig E. TH_2 cytokine IL-10 inhibits TH_1 secretion of IFN-γ and IL-2, and suppresses Class II MHC expression and production of bacterial killing molecules and inflammatory cytokines by macrophages. IL-4 stimulates B cells to secrete Ig E and Ig G_1. The balance between TH_1 and TH_2 activity may steer the immune response in the direction of either cell-mediated immunity or humoral immune response.

CHAPTER 3

HUMAN IMMUNODEFICIENCY VIRUS AND THE IMMUNE SYSTEM

3.1 HUMAN IMMUNODEFICIENCY VIRUS

HIV infects the immune system, making the host unable to control the virus and at the same time more susceptible to infections by other pathogens (Jimere, 2005). During primary HIV infection, viral replication results in a depletion of CD_4 effector memory cells and persistent hyper activation state of memory T cells with a shortened lifespan (Gokhale, 2007). Immune abnormalities that occur in HIV infection include altered cytokine expression, decreased cytotoxic and natural cell killing, decreased humoral and proliferative response to antigens, decreased MHC-II expression, decreased monocyte chemotaxis and depletion of CD_4 cells. The result is underactive T cells, while B lymphocytes are hyperactive in persons with AIDS (Vicenzi, 1997).

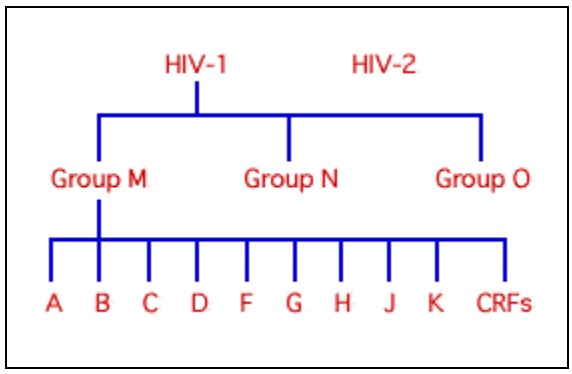
3.1.1 Structure of HIV

HIV is a retrovirus that can lead to AIDS. HIV is composed of two copies of positive single-stranded ribonucleic acid (RNA) that codes for the virus's nine genes enclosed by a conical capsid with 2000 copies of the viral protein p_{24}. Inside the core are three enzymes required for HIV replication, and they are reverse transcriptase, intergrase and protease. A matrix composed of the viral protein p_{17} surrounds the capsid ensuring the integrity of the virion particle. It is surrounded by the viral envelope, consisting of two layers of phospholipids taken from the membrane of a human cell when a newly formed virus particle buds from the cell. The glycol-protein (gp) complex consists of a cap made up of three molecules of gp_{120} and a stem made up of three molecules of gp_{41}, anchoring the structure into the viral envelope. The glyco-protein complex enables the virus to attach and fuse with target cells to initiate the infectious cycle (http://wikipedia.org/wiki/AIDS; Accessed 24 September 2007).

3.1.2 Classification and transmission of HIV

There are two types of HIV, namely HIV_1 and HIV_2. HIV_1 is more virulent. It is easily transmitted and is the cause of the majority of HIV infections globally while HIV_2 is less transmittable and is largely confined to West Africa (www.mayoclinic.com/health/hiv-aids; Accessed 24 August 2007). The strains of HIV_1 can be classified into three groups: the major group M, the outlier group O and the new group N. Within group M there are at least nine genetically distinct subtypes or clades of HIV_1 (Figure 3.1)

Figure 3.1: HIV classification: Each group is divided into groups, and each group is divided into subtypes and CRFs.
[www.mayoclinic.com/health/hiv-aids; Accessed 24 August 2007)].



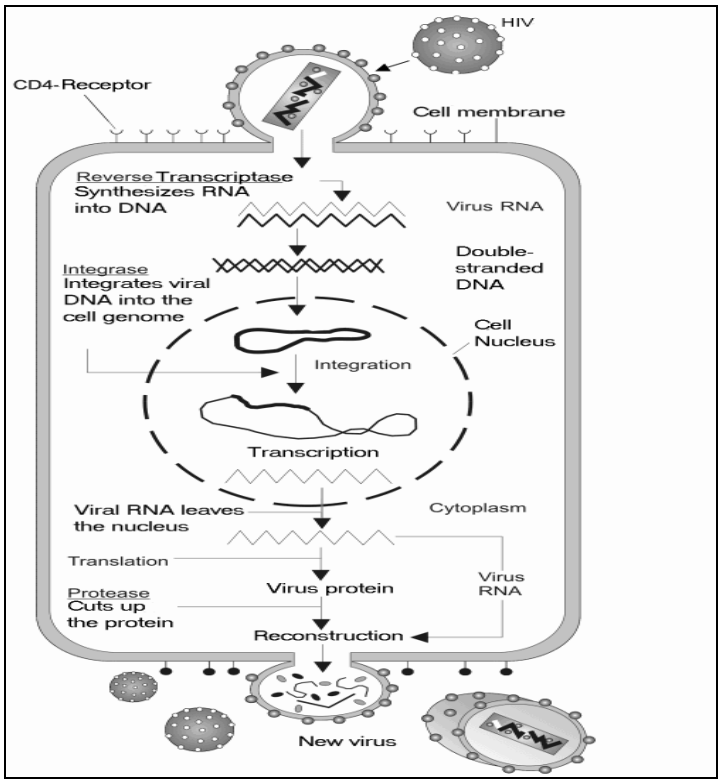
HIV infection occurs through the transfer of blood, semen or vaginal fluids from an infected person. Another method of infection is from mother to child, during pregnancy, during labour or through breast milk. HIV is present as both a free virus and within infected cells.

3.1.3 HIV Replication cycle

HIV enters macrophages and CD_4^+ T cells by the adsorption of glyco-proteins on its surface to receptors on the target cell, followed by fusion of the viral envelope with the cell membrane and the release of the HIV capsid into the cell. In Figure 3.2 (p 24) the first step in fusion involves the high-affinity attachment of the CD_4 binding domains of gp_{120} to CD_4. The virus and cell membranes close together, allowing fusion of the membranes and entry of the viral capsid. Once HIV has bound to the target cell, the HIV RNA and reverse transcriptase, integrase, ribonuclease and protease, are injected into the cell.

Thereafter, the viral capsid enters the cell, reverse transcriptase liberates the single-stranded (+) RNA from the attached viral proteins and copies it into a complementary DNA.This process of reverse transcription is extremely error-prone and it is during this step that mutations may occur. Such mutations may cause drug resistance. The reverse transcriptase then makes a complementary DNA strand to form a double-stranded viral DNA intermediate (vDNA). vDNA is transported into the cell nucleus. The integration of the viral DNA into the host cell's genome is carried out by another viral enzyme called integrase. This integrated viral DNA may then lie dormant, in the latent stage of HIV infection. To actively produce the virus, certain cellular transcription factors need to be present, the most important of which is NF-κB (NF kappa B), which is unregulated when T cells become activated. This means that those cells most likely to be killed by HIV are in fact those currently fighting infection (http://wikipedia.org/wiki/HIV; Accessed 24 September 2007).

Figure 3.2: HIV replication cycle.
[http://en.wikipedia.org/wiki/HIV; Accessed 24 September 2007]



The integrated provirus is copied to mRNA, and then spliced into smaller pieces. These small pieces produce the regulatory proteins which encourage new virus production. HIV_1 and HIV_2 appear to package their RNA differently. HIV_1 infection progresses to AIDS faster than HIV_2. During maturation, HIV proteases cleave the polyproteins into individual functional HIV proteins and enzymes. The various structural components then assemble to produce a mature HIV virion. This cleavage step can be inhibited by protease inhibitors (http://wikipedia.org.wiki/HIV; Accessed 10 January 2008).

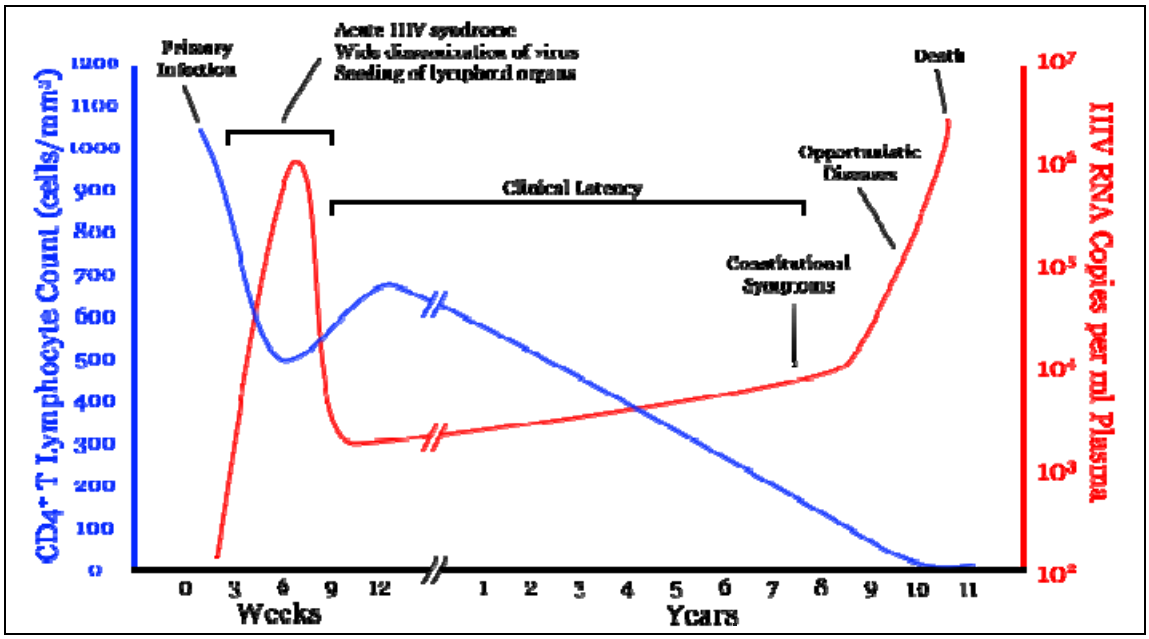
3.1.4 Clinical stages in HIV and AIDS

Infection with HIV_1 is associated with a progressive decrease of the CD_4 cell count and an increase in viral load. The stages of infection can be determined by measuring the patients' CD_4 T cell count and the level of HIV in the blood (http//:en.wikipedia.org/wiki/aids; Accessed 1 August 2007). In 1990, the World Health Organization (WHO) grouped the infections and conditions together by introducing a staging for patients infected with HIV_1 based on clinical symptoms, which may be used to guide medical decision making (Table 3.1; p 28) (Kedzierska, 2001).

The initial infection with HIV is known as primary HIV infection and the majority of patients remain asymptomatic, whereas a small part of the infected patients may develop a rash, fever, lymphadenopathy and flu-like symptoms within 2 to 3 weeks of infection. Opportunistic infections are not seen at this stage. During this subclinical stage, there is a large amount of HIV in the peripheral blood and the immune system begins to respond to the virus by producing HIV antibodies and cytotoxic lymphocytes. This process is known as sero-conversion (www.manbir-online.com/std/hiv.1.htm; 10 August 2007). The WHO staging system is described as follow:

Stage 1 is the asymptomatic phase, (Figure 3.3; p 26) in which most of the patients maintain normal health and are unaware of the disease. This phase has median duration of 10 years. The peripheral blood CD_4 T cell is above 500 cells/mm^3 (Levy, 2003). The level of HIV in the peripheral blood drops to very low levels but people remain infectious and HIV antibodies are detectable in the blood. Research has shown that HIV is not dormant during this stage, but is very active in the lymph nodes. A test is available to measure the small amount of HIV that escapes the lymph nodes. This test, which measures HIV RNA, is referred to as the viral load test, and it has an important role in the treatment of HIV infection (Levy, 2003; Fauci, 2007).

Figure 3.3: A generalized graph of the relationship between HIV copies (viral load) and CD_4 counts over the time course of HIV
[http://en.wikipedia.org/wiki/HIV; Accessed 24 September 2007]



Stage 2 is characterized by the early symptomatic phase and the continually decreasing CD_4 count (Primagi, 2005; Levy, 2003). The symptoms include fever, unexplained weight loss, recurrent diarrhoea, fatigue and headache. Cutaneous manifestations, recurrent herpes simplex infections and oral hairy leukoplakia may occur. Anti-retroviral therapy is started at this stage

Stage 3 is characterized by the late symptomatic phase and is marked by a CD_4 count lower than 200cell/mm^3 and the risk of developing AIDS related opportunistic infections is very high. Pneumocystis Carinii Pneumonia (PCP), toxoplasma encephalitis, esophageal candidiasis, lymphoma and kaposi sarcoma develops.

Stage 4, is the advanced HIV phase and is a state in which the CD_4 cell count is less than 50 cells/mm^3. The patients have multiple opportunistic infections and malignancies (Table 3.1).

AIDS stands for Acquired Immune Deficiency Syndrome. It is a collection of a symptoms and infections resulting from the specific damage to the immune system caused by the human immunodeficiency virus (HIV) in human (Kedzierska, 2001).

Over time, the immune system becomes severely damaged by HIV. This is thought to happen for three main reasons: the lymph nodes and tissues become damaged and burnt out because of the years of activity; HIV mutates and becomes more pathogenic, leading to more T helper cell destruction, and the body fails to keep up with replacing the T helper cells that are lost. As the immune system fails, symptoms develop. Initially, many of the symptoms are mild, but as the immune system deteriorates the symptoms worsen. Symptomatic HIV infection is mainly caused by the emergence of opportunistic infections and cancers that the immune system would normally prevent. These can occur in almost all the body systems [http://en.wikipedia.org/wikiAIDS; Accessed 24 September 2007]

Table 3.1: Different opportunistic infections and cancers that occur due to HIV infections

Body System	Opportunistic infections and cancers
Respiratory	Pneumocystis Carinii Pneumonia (PCP)
	Tuberculosis (TB)
	Kaposis's Sarcoma (KS)
Gastro-intestinal (GIT)	Cryptosporidiosis
	Candida
	Cytomegolavirus (CMV)
	Isosporiasis
	Kaposi's Sarcoma
Central and Peripheral nervous	Cytomegolavirus
	Toxoplasmosis
	Cryptococcosis
	Non Hodgkin's lymphoma
	Varicella Zoster
	Herpes Simplex
Skin	Herpes simplex
	Kaposi's Sarcoma
	Varicella Zoster

3.1.5 Treatment of HIV

The goal of managing HIV patients is to prevent the replication process, to lower the risk of clinical progression of the disease to AIDS and to prevent occurrence of opportunistic infections ([http://en.wikipedia.org/wiki/aids; Accessed 24 September 2007]. Anti-retrovirals (ARVs) are drugs that inhibit the multiplication of the virus using different mechanisms of action. The enzymes protease and reverse transcriptase are targets for some classes of anti-retrovirals. Reverse transcriptase is

inhibited by either nucleotide reverse transcriptase inhibitors (NtRTI's), nucleoside analogue reverse transcriptase inhibitors (NRTIs) or non-nucleoside reverse transcriptase inhibitors (NNRTI's), whereas protease is inhibited by protease inhibitors (PIs) and the last class of drugs are fusion inhibitors. Because HIV has the ability to develop resistance against one drug, a combination therapy consisting of three or more drugs is used. [Levy, 2003; http://en.wikipedia.org/wiki/HIV and www.manbir-online.com/std/hiv.1.htm]

One anti-retroviral drug from each group makes up a combination therapy and is known as HAART (highly active anti-retroviral therapy; Table 3.2; p 29). It has been shown to inhibit viral replication to levels below the limit of detection. The antiretroviral therapy is started during the early symptomatic phase. The effectiveness of the ARV therapy is reflected in a decline of the HIV related mortality and mobility, increase in CD_4 count and decrease in viremia.

Table 3.2: Combination of ARVs that make up highly active anti-retroviral therapy.

NRTIs	NNRTIs	PIs
Zidovudine	Nevirapine	Indinavir
Stavudine	Efavirenz	Nelfinavir
Lamivudine	Delavirdine	Ritonavir

3.2 CYTOKINE PROFILES IN HIV

The immune system is homeostatically regulated by a complex of immuno-regulatory cytokines which are pleiotropic and redundant and operate in an autocrine and paracrine manner. Cytokines are divided into pro-inflammatory and anti-inflammatory cytokines and are functionally called the $TH_1$and TH_2 subtypes, respectively. The reciprocal regulation in the cytokines subtypes bring about a desired immune response. Immune abnormalities that occur in HIV infection include altered cytokine

expression, decreased cytotoxic and natural cell killing, and decreased humoral and proliferative response to antigens, decreased MHC-II expression, decreased monocyte chemotaxis and depletion of CD_4 cells.

3.2.1 Immune response in HIV infection

It was shown that CD_4 T cells decline in number and function and lose the ability to serve as helper cells to the rest of the immune system (Levy, 2003). It was also observed that T cells were underactive, whereas B lymphocytes were hyperactive in persons with AIDS, as indicated by elevated levels of immuno-globin and increased numbers of activated B cells in circulation (http//:en.wikipedia.org/wiki/HIV). The observation of T cell deficiency and B cell hyperactivity in AIDS could be found in HIV infected patients long before the clinical development of AIDS. This was shown through the dysregulated activity and production of different cytokines (Agarwal, 2002; Pinto, 2000).

T cell-mediated immunity plays a major role in host defence against viral infections and it is an important component of the host immune response to HIV. It is mediated by helper CD_4 T cells and cytotoxic CD_8 T cells. During high viral loads, CD_4 cells respond to HIV antigens by shifting from proliferation to IFN-γ production, which activates cytotoxic T cells and TNF-α activity. The CD_8 cells, through their HIV specific antigen receptors, bind to and cause lytic destruction of target cells bearing MHC class I molecules associated with HIV antigens. CD_8 can express cytokines such as IFN-γ. CD_8 cell-mediated suppression of HIV is the ability of the CD_8 to inhibit HIV replication in a noncytolytic manner. Natural killer cells also kill HIV infected cells. Another way of killing is associated with antibody dependent cell-mediated cytoxicity, whereby the antibody binds to the target antigen, the natural killer binds via F_c receptor and the target cell is killed. Antibodies to HIV appear within six weeks of the primary infection. These antibodies appear before the appearance of neutralizing antibodies, which follow initial decrease in plasma viremia (Levy, 2003).

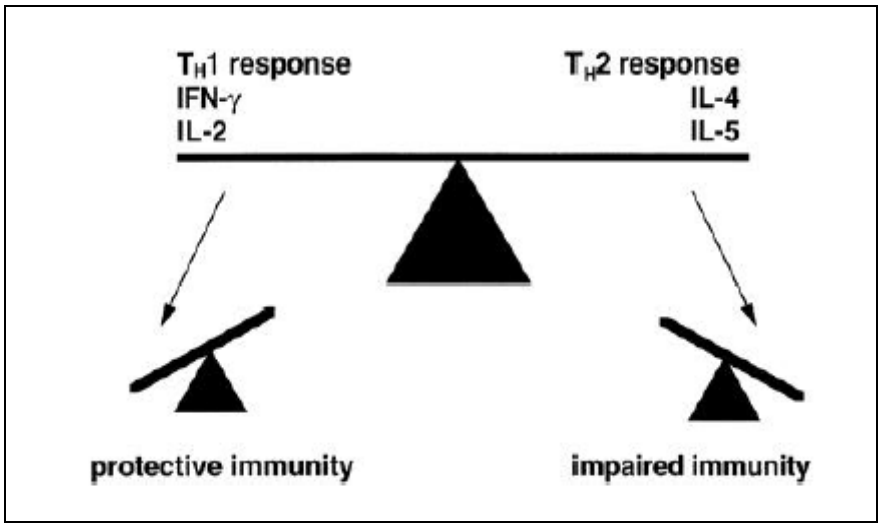
Increased secretion of TNF-α, IL-1 and IL-6 by macrophages and monocytes correlated with viral load and polymorphism in chemokine receptors and gene expression is suggested to be associated with disease susceptibility and progression. IL-10 over-expression contributes to B cell hyperactivity and the risk of AIDS lymphoma (Bahbouhi, 2004).

3.2.2 Cytokines responses to HIV

There is growing evidence that HIV infection disrupts the production of some of the pro-inflammatory (IL-2, IL-12, IFN-γ) and anti-inflammatory cytokines (IL-4, IL-5, IL-6, IL-10, TNF -α) and this disruption plays a role in the progression of HIV to AIDS (2006 UNAIDS report; http://en.wikipedia.org/wiki/HIV; Breen, 2002). TH_1 cytokines induce cell mediated immune response and Ig G antibodies, whereas TH_2 cytokines induce humoral immunity and antibodies Ig A, Ig E. (2006 AIDS epidemic report).

During early HIV infection and the asymptomatic phase, TH_1 responsive T cells predominate and are effective in controlling but not eliminating the virus (http:en.wikipedia.org/wiki/HIV). This is achieved by the secretion of TH_1 cytokines (IL-2, IL-12 and IFN-γ) which activate the cell mediated immune response and inhibit the humoral immune response. It involves direct killing by natural killer cells and macrophages, and activation of cytotoxic T lymphocytes that induce apoptosis of the cells with foreign antigens.

Figure 3.4: The reciprocal regulation of the TH_1 and TH_2 cytokines in maintaining a desired immune response [Breen, 2002].



This means that the TH_1 cytokines are higher, whereas the TH_2 cytokines remain lower. During the asymptomatic period of the HIV infection, the TH_1 cytokines remain high and the TH_2 cytokines remain low. During the clinically latent phase, as the disease progresses, a shift occurs resulting in lower TH_1 cytokines and increased TH_2 cytokines (Breen, 2002). Low TH_1 levels are associated with HIV disease progression, loss of cell-mediated immunity, and high TH_2 levels are associated with either high antibody production or cellular self-destruction, also leading to HIV progression through modulation of antigen induced cell death occurring in TH_1 cells. This change is more pronounced in patients with opportunistic infections than those without (http://en.wikipedia/wiki/AIDS).

HIV progression to AIDS in patients is linked to the TH_1/TH_2 shift that begins before the symptomatic phase. Patients that have HIV related lymphoma often have high levels of IL-6, a TH_2 cytokine. A study suggested that lowering IL-6 with antibodies seemed to reverse AIDS- related wasting, fevers and night sweats (Kedzieerska, 2001).

IL-2 is one of the most studied cytokines. According to other researchers and studies that have been done, IL-2 is often used as an immune booster in conjunction

with HAART therapy, which results in an increase in the CD_4 count, decrease in the viral load and a decreased occurrence of opportunistic infections, leading to a slow progression towards AIDS in patients infected with HIV (Rosenberg, 1991).

3.2.3 Cytokine impact on HIV

Analysis of the impact of cytokines on HIV_1 replication in vitro have generally demonstrated that a number of TH_1 cytokines up-regulate HIV replication while TH_2 cytokines down-regulate HIV. This relationship is not simplistic, given that a combination of TH_1 and TH_2 cytokines may lead to a synergistic or antagonistic effect on HIV replication. These results depend on the combination of cytokines and on the cell type examined, whether lymphotic or monocytic (Clerici, 1999).

Some of these cytokines have been demonstrated to play a major role in the regulation of HIV expression in vitro (Levy, 2004). Cytokines that are potent and consistent inducers of HIV expression are the pro-inflammatory cytokines TNF-α, IL-1 and IL-6. IFN (α and β) suppress HIV replication whereas IL-4, IL-10 and IFN-γ can either induce or suppress HIV expression, depending on the system involved. TNF α activates proteins that function as transcriptional activators of HIV expression and IL-1 activates at viral transcription level (Levy, 2004).

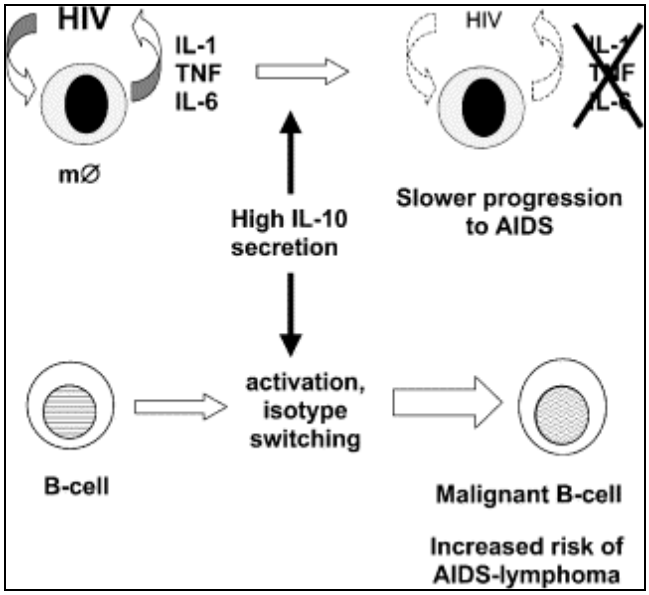
3.2.4 Interleukin 10 (IL-10) as a bi-functional mediator of HIV disease progression

Under normal conditions, one type of cross-regulation that can be clearly demonstrated is the ability of IL-10 to suppress the production of the pro-inflammatory cytokines such as IL-1, IL- 6 and TNF- α. According to Breen (2002), elevated levels of IL-1, IL-6 and TNF-α in serum and culture of cells from HIV positive patients, provided evidence that these cytokines are being over-produced in association with HIV infection. Furthermore, observation indicated that cytokines can synergize with one another and up-regulate HIV replication and production. The molecular mechanism of HIV expression is best characterized for TNF α which activates a transcriptional factor (NF κB). TNF-α and IL-1 activate NF κB, which

translocates to the nucleus, binds near the transcription start site of HIV and enhances HIV expression and viral production. IL-6 up regulates HIV replication by transcriptional and post transcriptional mechanisms (Breen, 2002).

It has been demonstrated that when IL-10 was used *in vitro* at low concentrations, it did not affect the production of inflammatory cytokines, but at high concentrations, it clearly blocked the HIV induced IL-1, IL-6 and TNF-α. HIV replication within primary cells of monocytes lineage was inhibited. The ability of IL-10 to inhibit or reduce HIV replication in monocytes, but not in T cells under similar conditions, was also observed. It was observed that under different experimental conditions *in vitro*, IL-10 served to synergize with IL-1, IL-6 and TNF-α to enhance HIV replication. Contradictory observations led to the bi-functionality of IL-10, that is , it's capability to enhance and suppress HIV replication. IL-10 can also act as B cell hyperactivity seen in association with HIV infection (Figure 13), increasing the risk of AIDS lymphoma (Breen, 2002; Pinto, 2000 and Picker, 2006).

Figure 3.5: The balance of IL-10 as an anti inflammatory and B cell stimulatory cytokine in AIDS (Breen, 2002).



This emphasizes as with IL 10 and other cytokines that are dysregulated in HIV infection and AIDS, that it is important to remember that it is the balance between synergistic and antagonistic actions that may determine the ultimate effect of cytokine changes in an HIV positive patient. This might be indicative that treatment with cytokines could reverse the TH_1/TH_2 shift, which has the potential of either reversing some of the AIDS related diseases or decreasing the occurrence of opportunistic infections. It can be achieved by inducing the production of TH_1 cytokines thus inhibiting TH_2 cytokines, or by inhibiting the production of TH_2 cytokines to increase TH_1 cytokines. The expected outcome would be restoration of cell-mediated immune response, thus, a need to use an immune booster that will induce the immune system through the production of TH_1 cytokines (IL-2, IL-12 and IFN- γ) and/or inhibition of TH_2 cytokines (Breen, 2002).

CHAPTER 4

IMMUNE BOOSTERS FOR HUMAN IMMUNODEFICIENCY VIRUS

4.1 INTRODUCTION

An immune booster is an additional immunizing agent given to increase and sustain the immune response of the body. Immune boosting works by introducing an agent into the body that boosts specific areas of the immune system, most notably the T Lymphocytes cells. In HIV studies, there is a need to look at immune boosters that have either antiviral properties, or that can enhance and stimulate the immune system capacity, or both. Immune boosters can target either the body's natural defensive response or the virus particles, but they are rarely capable of both properties in HIV patients (Babakhnian, 2000).

Anti-retrovirals (ARVs) have the potential to increase life expectancy of immune compromised patients, however they do not offer a cure nor stimulate the failing immune system (www.mcb.uct.ac.za/cann/355/AIDSI.html). Furthermore, ARVs have adverse effects which are associated with disease progression, metabolic disorders and adverse reactions with chronic and acute therapy. To date, no agent has been recommended to boost the immune system in HIV patients. Thus, there is a need for other treatments that can complement the highly active anti-retroviral therapy (HAART) in treating HIV patients to improve their quality of life. Hence, the immune booster must have the potential of boosting the suppressed immune system and reverse/decrease susceptibility to opportunistic infections.

4.2 OTHER IMMUNE BASED THERAPIES

Immune-based therapies comprise various therapies such as vaccines, cell transfer and immune modulators, and immune boosters. Immune boosters have the ability to protect cells from HIV infection and enhance immune cell

functionality. Furthermore, they are capable of reversing the progressive immune deficiency, and restoring the lost immune responses in chronic infections (www.thebody.com/content/art14513.html; Accessed 30 October 2007).

Results from different studies with different immune boosters indicated an improvement in the HIV status of different patients. Furthermore, adding an immune booster to standard HIV therapy seems to help handle the AIDS virus better than drugs alone. Moreover, in a study that involved 164 patients, an increase in new CD_4 cells and fewer episodes of AIDS related illnesses were observed (Bahbouhi, 2004; Yarnell, 2001).

Another immune booster studied is "*Impi". Impi,* consists of different blends of African herbs, and it has been used to assist the immune system in patients with viral infections and ailments. The results from an HIV hospice of patients taking *Impi*, indicated an increase in the CD_4 count, and a simultaneous drop in the viral load. According to the researchers, it has been proven that *Impi* has immune boosting and anti-viral properties, through *in vivo* results and plant *in-vitro* data (www.herbalafrica.co.za/products/impi.htm; Accessed 20 November 2007). The mechanism of action for this immune booster is unknown.

Furthermore, research has shown that some cytokines also have immune boosting properties **(**Barouch, 2004). IL-2 is one of the most studied immune booster cytokines. According to other researchers and studies that have been done, IL-2 is often used as an immune booster in conjunction with HAART therapy, which results in an increase in the CD_4 T cell count, a decrease in the viral load and a decreased occurrence of opportunistic infections, leading to a slow progression towards AIDS in patients infected with HIV. In high levels, IL-2 induces CD_4 T cells to become active, whereas at low lower doses it causes cytotoxic cells to reproduce. This would lead to fewer occurrences of opportunistic infections and less progression of HIV into AIDS resulting in a better quality of life for HIV patients.

4.3 "*PHELA*," A TRADITIONAL HERBAL MEDICINE PRODUCT

Phela is the code name for a herbal mixture of four African traditional medicinal plants [*Clerodendrum glabrum, Polianthes tuberosa, Rotheca myricoides and Senna occidentails*]**,** that has been used for decades in wasting conditions and for increasing energy in patients. Recently, Phela was reported to benefit immune compromised individuals due to its immune stimulant effects. Evidence of its efficacy was obtained from anecdotal reports by patients and traditional healers, and these were supported by the subsequent findings in observational studies involving medical doctors in the Western Cape and Gauteng provinces.

The controlled observation clinical studies using *Phela* as an immune booster were conducted on 500 HIV positive and AIDS patients. The results showed an increase in the patients' appetites, 23% increase in weight gains, 80% decrease in viral loads and 200% increase in CD_4 cell counts. The overall quality of life of the patients increased, some from as low as 30% to 100%. These results are indicative of the immune boosting properties of Phela **(**Matsabisa et al., 2006).

Therefore, the Indigenous Knowledge Systems (IKS) Lead Programme of the Medical Research Council (MRC) and the Department of Health of South Africa, embarked on investigating these claims in scientifically controlled pre clinical and controlled clinical studies. At the MRC laboratories, Phela is prepared in exactly the same way as is made traditionally but in accordance with strict Good Manufacturing Practices (GMP). The plants are dried and milled into a homogeneous powder of uniform particle size, and sterilized by gamma irradiation after being filled into standardized 250 mg unit dose capsules.

Results from the *in vitro* studies of phela extract indicated no effect on the activity and no significant interaction with the different iso-forms of CYP 450 enzymes. A sub-chronic toxicology study of Phela in vervet monkeys over 3 months, using 10 times the traditionally recommended adult dose, showed that it did not have toxic effects on the test animals. In a phase I clinical trial in healthy human participants, no toxicity was exhibited [*Medical Research Council, Indigenous Knowledge Systems Lead Programme report, 2009*]. The results of the different studies have been presented at numerous national[1-2] and international[3] conferences. Further research on Phela is necessary to investigate the effect it has on the immune system and its possible mechanism of action thereof.

4.4 AN IDEAL IMMUNE BOOSTER FOR HIV MANAGEMENT

Several studies carried out in patients with opportunistic infections have demonstrated that, the immune system response can be enhanced by regulation of cytokines (Hess et al., 1989) stimulating the TH_1 cytokines and/or inhibiting TH_2 cytokines. TH_1 response restores cell mediated immunity, and is effective in fighting viral infections.

Thus, an ideal immune booster should regulate the TH_1/TH_2 balance to enhance cell mediated immunity and to complement HAART without adverse drug reactions.

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[1] M.G. Matsabisa. *IKS Exhibition and workshop, Department of Science and Technology*, Republic of South Africa, August 2008.

M.G. Matsabisa. *INNO4DEV workshop of Chemical, Mining, Financial and other Industries that work with Grassroots Communities*, Pretoria, January 2010

[3] M.G. Matsabisa. *The Association of University Technology Managers 2010 Conference: Top 4 Bio-plan competition on Traditional Medicinal Product Development for HIV, AIDS and malaria*, New Orleans USA, March 2010.

- M.G. Matsabisa. *International Conference on Herbal Medicine Evaluation of Quality, Efficacy and Safety*, Bangalore, India, February 2009.
- M.G. Matsabisa. *FAPRONATURA 2009 Second International Symposium on Pharmacology of Natural Products,* Matanzas, Cuba June 2009.
- M.G. Matsabisa. *Conference on Research in Molecular Medicine based on Natural Science and Traditional Knowledge*, Pune India, November 2009.
- M.G. Matsabisa. *FIP-WSMI symposium on Traditional Medicines and Complementary Medicines*, Beijing, November 2008.
- M.G. Matsabisa. *WHO Congress on Traditional Medicines, Beijing*, November 2008.

CHAPTER 5

AN OVERVIEW OF METHODS FOR FINGERPRINTING TRADITIONAL MEDICINES

5.1. INTRODUCTION

Traditional herbal medicines (THM) and their preparations have been widely used for years in the entire African continent and many oriental countries. The World Health Organization (WHO), in pursuance of its goal of providing affordable, accessible and culturally acceptable health care to the global population, has encouraged the rational use of traditional, plant-based medicines by member states of the world health assembly (WHO, 1998), and to this purpose, developed technical guidelines for assessment for herbal medicines (WHO, 2000). Thus, it is necessary to develop a type of quality assessment system that adequately meets the complex-multiple characteristics of traditional herbal medicines (Xie et al., 2006) by developing a fingerprint. This includes methods for evaluation (identification and authentication) of the product for quality assurance purposes.

5.2. QUALITY CONTROL AND FINGERPRINTING

Quality control of raw materials and finished products from medicinal plants is a crucial part of the production of traditional herbal medicines (Khan et al., 2006). Although there has been an increase of interest in science-based research into THM, much of the research to date has been plagued by studies conducted using unauthenticated, uncharacterized products (Smillie, 2010). A study cannot be considered scientifically valid if the material tested was not authenticated and characterized so that the material can be reproduced, and these results cannot be extrapolated to other products on the market or compared with other studies, due to inconsistencies in the identity of the THM. Furthermore, failure to fingerprint products has resulted in a considerable amount of published work that is inconsistent, contradictory and irreproducible due to either misidentification of the collected plant, adulteration with other species and/or

contamination with extraneous ingredients. In traditional medicine the issue, of plant substitutes further poses a challenge to quality notification of traditional herbal medicines.

Authentication tools range widely, depending on the plant and processes involved, from straightforward THMs, morphological identification of a plant, to very elaborate genetic or chemical approaches. Each identification method uses different techniques and requires different levels of prior information, infrastructure and skill sets to achieve proper authentication of traditional medicines products. For the most part, the THMs have utilized the application of chemical “fingerprinting” techniques for identity purposes (Smillie, 2010; Khan et al., 2006). Two major approaches applied for quality control are analytical fingerprinting and marker compounds.

5.2.1. Analytical/chromatographic fingerprinting

Analytical fingerprinting is a method to measure and represent the entire composition of a herbal product, with some common chemical components of pharmacologically active and/or chemical characteristics (Liang et al., 2004). It is the most reliable and applicable authentication and identification method based on chemical and chromatographic techniques (Smillie, 2010). The chromatographic profile should be featured by the fundamental attributes’ of “integrity” and “fuzziness” or “sameness” and “differences” so as to chemically represent the THM investigated (Liang et al, 2004).

An analytical fingerprint is considered valid when the researcher uses statistically significant representatives of reference sample from multiple geographical locations to establish a “fingerprint” profile. The identified and selected “marker” compounds that make up an analytical fingerprint should be unique to selected species and preferably represent the health-relevant principles, although this is not always possible.

A fingerprinting pattern is mainly based on qualitative analysis demonstrating the general characteristics of herbal materials and herbal preparations with regard to quality

consistency and stability (Xie et al., 2007). A fingerprinting multi-pattern is used for complex preparations derived from a combination of many plants. Under these circumstances, a novel fingerprint is based on various chromatographic approaches or different detections providing comprehensive information for quality control purposes (Li, 2008). Furthermore, fingerprinting provides improved correlation of bioactivity, phyto-chemical properties and sometimes quantitative analysis. Disadvantages of fingerprinting are that it is time consuming, its data evaluation is complex and the process is not an easy or trivial job.

5.2.2. Marker compounds

Marker compounds are one or more constituents that occur naturally in the traditional herbal medicines and are selected by the researcher for identification and/or quality control purposes in fingerprints (i.e HPLC chromatograms), especially when the active constituents are not known or identified (Eisner, 2001; Ong, 2004) . The amount and identity of marker compounds are often chosen arbitrarily. This approach is also termed a multi-component approach and the analysis is based solely on one or two compounds (Li, 2008). Marker compound analysis can either be qualitative or quantitative. The advantages of marker compound analysis are that data evaluation is simple and it is less time consuming, whereas the disadvantage is that there may be no correlation exists with bioactivity.

5.2.3. Other fingerprinting techniques

The manufacturers conduct the initial step in fingerprinting of traditional herbal medicines entailing classical botanical methodologies for collection and documentation of the plant at its source. Vital information, such as the proper Latin binominal nomenclature of the plant, person collecting the plant, date of collection, location of collection, collection number, list of the plant part (s) collected and habitat information is obtained. In addition, organoleptic characteristics (e.g. smell, taste, and colour, angle of

response, moisture content, particle size extractability etc.), processing steps and visual representative image, are documented (Smillie, 2010).

Macroscopic and microscopic fingerprinting is applied on plant material that has been dried and ground. Macroscopic characteristics that may be examined are the morphological traits of all parts of the plant. Macroscopic techniques discriminate between desired plant species, plant parts and morphologically similar, yet distinguishable, species that occur as potential adulterants. Moreover, a microscopic approach distinguishes variability in characteristics for identification utilizing techniques such as scanning electron microscopy.

Genetic and antimicrobial fingerprinting use molecular biology techniques to identify species or characterize isolates within a species.

5.2.4. Evaluation of fingerprints

5.2.4.1. Marker peaks

Evaluation of chromatographic fingerprints is achieved by selecting a few marker peaks for identification amongst all peaks observed. The marker peaks are selected based on a peak with a high response (e.g. high level of peak area), consistency and stability. However, there is no set method for marker peak selection.

5.2.4.2. Two-dimensional data for fingerprints

Hyphenated procedures use an on-line spectrum result with a two-dimensional data. The two-way data matrix contains both information from spectra and chromatograms, making on-line structure of some compounds of interest possible. On-line spectrum also enhances separation ability by the additional spectral information, and a useful selectivity component.

The spectral correlative chromatogram (SCC) states that the same chemical component should be of the same spectrum no matter how it is eluted through diverse

chromatographic columns. The procedure evaluates peak purity of a compound of interest, its on-line spectrum, and assesses the similarity and/or differences of the chromatographic fingerprints under investigation.

5.2.4.3. Retention time and Retention time shifts

Retention time application for evaluation of similarity or differences in a chromatographic fingerprint is done by setting a retention time window in both sample and test chromatograms, and then a range of retention times for the peak of interest (Liang et al., 2004). Peak retention time limits were statistically calculated. Similarity and a low standard deviation was observed when comparing the retention time data of the selected peaks in different chromatograms to indicate co-identity (Springfield, 2005). Though the evaluation method is simple, retention time shifts are a common occurrence.

Retention time shifts are caused by various complex factors. The first one might be due to the deterioration of the stationary phase, especially the low stability silica and silica-based supports at high pH values and the collapse of the C_{18} bonded phase because of a high polar mobile phase. In addition, the minor changes in mobile phase composition caused by temperature and pressure fluctuations, variations in flow rate and gradient dispersion also have an effect. Detector based changes such as wavelength shift, intensity variation and the misalignment of monochromator are also common. Column overloading due to a great injected amount or some components with high concentrations, or possible interactions amongst the analytes and other unknown shifts in the instrument (Liang et al., 2004; Gong, 2004), can contribute to retention time shifts.

The correction of retention time shift of the THM should be taken into consideration as some types of variation sources are inevitably encountered due to the factors mentioned earlier. Extensive literature study revealed that various warping methods (Eilers, 2004; Gong, 2003; Kassida, 1998; Walczak, 2005) based on complex mathematical equations are applied to correct the retention time shift, but unfortunately

the statistically based programs used are not readily available in developing laboratories and require prior skills/understanding of the programs.

5.2.5. Approach for chromatographic fingerprinting of traditional herbal medicine products

Chromatographic fingerprinting methods for quality control of traditional herbal medicines involve analytical inspection using instrumental techniques such as Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), Gas Chromatography Mass Spectrometry (GC-MS), Liquid Chromatography – Mass Spectrometry (LC-MS), Near Infrared (NIR), Capillary Electrophoresis (CE) and Spectrophotometer (Liang, 2004)

5.2.5.1. Sample preparation

Sample preparations often include various extraction methods such as sonication, liquid-liquid extraction (LLE), solid phase extraction (SPE) and preparative liquid chromatography and TLC fractionation (Ong, 2004). Pre –column derivatization methods are recommended for pre-treatment to enhance sensitivity for chromatographic analysis. In order to achieve a reproducible derivatization, the selection of an appropriate derivatizing reagent and optimizing the reaction conditions is critical.

5.2.5.2. Thin Layer Chromatography (TLC)

TLC is used to construct the fingerprint of herbal medicines because of its simplicity, versatility, high velocity, specific sensitivity and simple sample preparation. TLC is still the first method used to provide first characteristics during fingerprinting. Furthermore, it is semi-quantitative and capable of analysing multiple samples simultaneously on lone plate, saving time and resources. It is a convenient method for determining the quality

and possible adulteration of herbal products. However, TLC has known disadvantages such as low sensitivity and poor reproducibility (Lin, 2001).

5.2.5.3. Gas chromatography (GC)

GC effectively detects compounds in all the volatile chemical compounds. Furthermore, the high selectivity of capillary columns enables separation within a comparatively short time. However, the most serious disadvantage of GC is that it is not convenient for the analysis of the samples of polar and non-volatile compounds. For this, it requires the use of tedious sample preparation which may include derivatization. Therefore, liquid chromatography becomes another necessary tool for comprehensive analysis of the herbal medicines (Xie, 2006).

5.2.5.4. High Performance Liquid Chromatography (HPLC)

HPLC is a popular method for the analysis of THM because it is easy to use and it is not limited by the volatility or stability of the sample compound. Time after time HPLC continues to receive the most extensive application in the analysis of THM. Reversed phase columns are the most popular columns used in the analytical separation. Optimal separation conditions involve many factors, such as different composition of the mobile phase, their pH adjustment and flow rates, etc. Thus, a good experimental design for the optimal separation is necessary. To date, new techniques used include micellar electro- kinetic capillary chromatography (MECC), high speed counter-current chromatography (HSCCC), high performance thin layer chromatography (HPTLC) and hyphenated techniques: ultra-violet (UV), photo-diode array (PDA), diode-array detector (DAD), capillary electrophoresis (CE), fluorescence (FL) and mass-spectrometry (MS) detection (Fan et al., 2006; Hoai et al., 2009; Ji, 2005).

5.2.5.5. Hyphenated procedures

Hyphenation procedures are evaluated for either identification or confirmation of the identity of target and unknown compounds. For most trace-level analysis, the

combination of column liquid chromatography or capillary gas chromatography (HPLC-DAD, CE-DAD, GC-MS and LC-MS respectively) is the preferred approach for the analysis of herbal medicines. Complementary information can be provided by atomic emission, Fourier-transform infrared (FTIR), fluorescence emission (FE) or nuclear magnetic resonance (NMR) spectrometry. The data obtained from such hyphenated instruments are so-called two-data, with one way for chromatography and the other way spectrum which could provide much more information than the classic one-way for chromatography (Khan et al., 2006).

In developing countries seeking to promote the rational use of herbal medicines, correct species identification is of paramount importance for quality assurance as very few of these herbs are cultivated and almost all raw materials are obtained from natural stands of vegetation. HPLC_DAD is a sensitive, rapid and economical technique that can be used for establishing chromatographic fingerprint (Springfield, 2005). Moreover, combined HPLC_DAD_MS techniques take advantage of chromatography as a separation method and of both DAD and MS as identification methods. With the help of this hyphenation, in most cases, one could identify the chromatographic peaks directly on-line by comparison with the literature data or standard compounds, this hasmade the LC-MS-DAD become a powerful approach for the rapid identification of phyto-chemical constituents in botanical extracts, and it can be used to avoid time-consuming isolation of all compounds to be identified (Malmquist, 1994).

5.2.5.6. Challenges for chromatographic fingerprinting of traditional herbal medicine products

In most cases, traditional herbal medicine products especially African traditional medicines, under development have no published analytical/fingerprinting methods. An extensive literature study revealed that traditional medicine fingerprint development involves evaluation of a combination of chromatographic techniques for optimal separation of compounds (Xiao-Hui, 2005).

Even though a fingerprinting method may exist for the resourced plants, this does not guarantee that the method will successfully identify the product. This is mainly due to a combination of complex plant material in THM products and additional components added during manufacturing process. Hence, the approach for evaluation of the entire product should begin with the simplest sample extraction methods (Liquid-Liquid Extraction using organic/aqueous solvents) and techniques such as TLC and column chromatography. Thereafter, advanced techniques such as HPLC or GC, depending on the need, may be used. The choice of the technique is primarily dependent on the availability of the equipment and cost-benefit rationale.

Presence of multiple compounds in the THM products poses a challenge of failure to detect all compounds when using a single detector. Thereafter, usage of multiple detectors such as photo-diode-array, mass spectrometry and fluorescence will improve sensitivity and increase the possibility of detecting most compounds present in THMs products.

5.3. METHOD DEVELOPMENT OF THM PRODUCTS IN BIOLOGICAL MATRIX

5.3.1. Introduction

Availability of a method for analysis of THM products in plasma for monitoring their use, and during poisoning, is of utmost importance. However, the fingerprinting methods are not always applicable to plasma monitoring because the peaks observed in spiked plasma may not be present in test plasma from *in vivo* samples. Therefore, when dealing with THM products plasma analysis, a different approach is required such as frequent analysis of the test plasma from *in vivo* application samples, throughout the development process. This approach is relevant for phela monitoring in rat plasma.

5.3.2. Method development

A bio-analytical method is a set of all of the procedures involved in the collection, processing, storing and analysis of a biological matrix for an analyte (Shah et al., 1992). Method development involves evaluation and optimization of the various stages of

sample preparation, chromatographic separation, detection and quantification. Pre-development entails consideration of analytical variables. Firstly, it is the choice of biological matrix and in this project plasma was chosen. The choice of instrument is based on availability and suitability for the compound under investigation and the choice of either an internal/external standard is also relevant.

The assumption for use of an internal standard is for it to serve as qualitative marker, to monitor detector stability, and to correct errors in dilution and pipetting (Karnes et al., 1991). Quantification by external standard is the most straightforward approach since the peak response of the standard is compared to the response of the sample. The standard solution concentration should be close to that expected in the sample solution (Anonymous, 2001). The choice of the detector depends on the chemical structure of the sample, the requirements of the method and availability of the instruments. An extraction procedure needs to be timely and economical with the highest possible recovery without interference.

5.4. GENERAL METHOD VALIDATION (STANDARDISATION)

5.4.1. Introduction

According to Food and Drug Administration (FDA) 2001 guidelines, method validation is a process for establishing that the performance of characteristics of the analytical method is suitable for the intended use. Moreover, validation is also proof of the repeatability, specificity and suitability of the method.

5.4.2. Calibration line

A calibration line is a curve showing the relation between the concentration of the analyte in the sample and the detected response. It is necessary to use a sufficient number of standards to define adequately the relationship between response and concentration. In many cases five to eight concentrations (excluding blank values) may define the standard curve. It is important to use a standard curve that will cover the entire range of the concentration of the unknown samples. Estimation of the unknown

by extrapolation of standard curve below the lower standard and above the higher standard is not recommended. Acceptability of the linearity data is often judged by examining the correlation coefficient and y-intercept of the linear regression line for the response versus concentration plot. A correlation coefficient of > 0.999 is generally considered as evidence of an acceptable fit of the data to the regression line (Green, 1996).

5.4.3. Selectivity/Specificity

The terms selectivity and specificity are used interchangeably. A method is said to be specific if it produces a response for only a single analyte. Selective refers to a method which produces a response of chemical entities which may not be distinguished by other methods. Specificity can be established by comparing the chromatographic retention time of the analyte in extracted matrix samples, with retention time of at least one reference solution. Processing blank samples from different sources will help to demonstrate lack of interference from a substance native to the biological sample but not from the analyte metabolite (Dadgar et al., 1995). If analyte concentration is sufficiently high, and the chromophores differ sufficiently, the use of photodiode array (PDA) or scanning UV detection under the regular condition can give evidence of peak purity.

5.4.4. Accuracy

The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentration is recommended. The mean value should be within 15 % of the actual value except at lower limit of quantification (LLOQ), where it should deviate by more than 20 %. The deviation of the mean from the true value serves as the measure of accuracy (Anonymous, 2001).

5.4.5. Coefficient of variance (Precision)

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogenous volume of biological matrix. Precision should be measured using a minimum of five determinations per concentration and three minimum concentrations in the range of expected concentrations. The precision determined at each concentration level should not exceed 15 % of the coefficient variation percentage (CV %) except for the LLOQ, where it should not exceed 20 % of the CV % (Anonymous, 2001).

$$[CV\%] = \left[\frac{S\,\text{tan}dard - deviation}{Mean}\right] x100 \qquad \text{E.q 5.1}$$

5.4.6. Stability

Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept from 4 to 24 hours (based on the expected duration of that samples will be maintained at room temperature in the intended study) and analysed. Storage time in a long-term stability evaluation should exceed the time between the date of the first sample collection and the date of the last sample analysis. The concentrations of all stability samples should be compared to the mean of back-calculated values for the standards at the appropriate concentrations from the day of long-term stability testing.

CHAPTER 6

OBSERVATIONS FROM THE REVIEW, AIMS AND OBJECTIVES

6.1 OBSERVATIONS FROM THE REVIEW.

6.1.1 General Observation

- HIV/AIDS is a pandemic with an estimated 25 million deaths globally in 2006 and almost 1000 AIDS related deaths occur every day in South Africa.
- Anti-retroviral drugs do not cure HIV/AIDS and have adverse effects.
- HIV suppresses the immune system, making the host more susceptible to opportunistic infections.
- Stimulating the immune system is associated with improved quality of life in HIV/AIDS patients.
- There is no immune booster for HIV treatment that has been scientifically proven or approved by the Medicines Control Council.
- There is a need for an immune booster that can be used together with anti-retroviral drugs or other therapy.
- A traditional herbal medicine product, Phela**,** has exhibited immune boosting features in an observational clinical trial study but its mechanism of action is unknown.
- Furthermore, understanding of the mechanism of action of a THM product (i.e. Phela) will enable optimal use of the product.

6.1.2 Regarding fingerprinting of traditional herbal medicine (THM) products

- A study cannot be considered scientifically valid if the traditional herbal medicine product is not authenticated and characterized.
- Quality control of raw materials and finished products from medicinal plants is done by chromatographic fingerprinting for identification and authentication purposes.

- Unfortunately, little information is available on THM products' (i.e. Phela) fingerprinting.
- Various chromatographic fingerprinting techniques need to be evaluated to characterize and authenticate THM products.

6.1.3 Regarding plasma analysis/monitoring of traditional herbal medicine products

- A method for analysis/monitoring of THMs in plasma must be available prior to undertaking *in vivo* studies.
- Frequent analysis of plasma from *in vivo* samples is necessary throughout the development process.
- Knowledge of a THM product plasma profile gives proof of absorption of any component of the product.

6.1.4 Regarding the mechanism of action of traditional herbal medicine products

- Although immune stimulation could be monitored by changes in the blood and lymphatic cell, these cellular changes are late and can be signalled by many stimuli.
- Monitoring of early signals is the most revealing because they also occur at sub-clinical stage.
- Cytokines are the earliest signals of the immune system, which also define the type of immune system, cell mediated immunity (TH_1 cytokines) or humoral immune response (TH_2 cytokines).
- An ideal immune booster should stimulate TH_1 cytokines (IL-2, IFN-γ, TNF-α) and/or suppress TH_2 cytokines (IL-4, IL-10).
- Knowledge of the change in the levels of TH_1 and TH_2 cytokines during treatment with Phela may offer a better understanding of its mechanism of action on the immune system.

6.1.5 Summary

To summarise observations from the review:

- Traditional herbal medicine products need a method for fingerprinting to ensure authentication, consistency in quality of manufactured/supplied product, and to help in accounting for variation in our research observations with subsequent batches or suppliers.
- There is also a need for a method to analyse/monitor traditional herbal medicine (THM) products in plasma for proof of absorption of any component of THM products and to define the conditions/parameters (plasma THM product levels) under which particular observations and results would be obtained.
- Cytokines are the earliest signals of the immune system, which also define the type of immune system.
- Evaluation of changes in TH_1 and TH_2 cytokines will define the mechanism by which the THM product (i.e. Phela) boosts the immune system

6.2 AIM

To study the effect of a traditional medicine product, Phela*,* on some TH_1 cytokines and TH_2 cytokines in a rat model.

6.3 ORIGINAL OBJECTIVES

- To develop a High Performance Liquid Chromatography method to fingerprint Phela in rat plasma*.*
- To study the effect of Phela on the immune system by analyzing some TH_1 (IL-2, IFN-γ, TNF-α) and TH_2 (IL-4, IL-10) cytokines to understand its mechanism of action.

6.4 ORIGINAL EXPECTED OUTCOME

- To have a chromatographic fingerprint for Phela identification and its monitoring in plasma.
- To understand the effect of Phela on the immune system and therefore its mechanism of action. The results will be useful in designing *Phela* as an immune boosting therapy for HIV treatment.

Decision: Since Phela is a medicinal preparation product, its characteristics could not be presumed to be similar to the parent plants owing to changes during its preparation/manufacturing. As such a complete evaluation of its properties (identification and analysis defines its characteristics) was necessary. Therefore, the original objectives and expected outcomes were modified accordingly and ultimate objectives and ultimate expected outcome established. Thus, the ultimate objectives of the study are presented as follows**:**

6.5 ULTIMATE OBJECTIVES

i. To develop chromatographic method(s) [fingerprint] for identifying and authenticating phela.

- **a)** A characterized product ensures reproducibility and consistency in results;
- **b)** Fingerprinting acts as a reference for subsequent products;
- **c)** It may be helpful in explaining variation in results amongst researchers;
- **d)** In the current study, it will ensure that claims regarding the mechanism of action of Phela are made on a characterized product, and
- **e)** Lastly, when the product is released the fingerprint can be used for quality control purposes.

ii. To develop a method for analysis of phela in plasma by High Performance Liquid Chromatography.

- **a)** This would help to determine proof of absorption of phela, and
- **b)** Help to define the conditions/parameters under which particular observations and results would be obtained. For instance, by relating concentration of marker compounds (levels) and/or size of mark peaks to the dose administered.

iii. To investigate pharmacokinetics of Phela after oral administration in a rat.

- **a)** This will enable understanding of:
  - The extent of phela's exposure to the body;
  - The extent of drug accumulation;
  - Elimination characteristics of phela.

iv. To study the mechanism of action of Phela on the immune system by observing the changes in some TH_1 cytokines (IL-2, IFN-γ, TNF-α) and TH_2 cytokines (IL-4, IL-10).

- **a)** This will help in achieving the ultimate aim of the study, and
- **b)** Enable optimal use of phela as an immune booster.

6.6 ULTIMATE EXPECTED OUTCOME

i. A method for fingerprinting of phela as well as identification and quality control purposes.
ii. An HPLC method for detecting and monitoring of phela in plasma.
iii. Understanding Phela pharmacokinetics after oral administration in a rat.
iv. Knowledge of the effect of Phela on the immune system and therefore its mechanism of action.
v. The results will be useful in designing Phela as an immune boosting therapy for HIV treatment.

CHAPTER 7

DEVELOPMENT OF A METHOD FOR IDENTIFICATION OF PHELA BY THIN LAYER CHROMATOGRAPHY (TLC)

7.1 SUMMARY

The development of a TLC method for identifying phela is described. It involved testing different solvents for extraction of phela and furthermore trying them as TLC mobile phases to separate bands. The salting-out extraction method and ethyl acetate: ammonia: methanol (17:1:2; v/v/v) were selected for use. The method is as follows: Phela is treated with ammonia sulphate and extracted with iso-propanol. The extract is horizontally shaken for 10 minutes and centrifuged at 2500 rpm (1 251 g) at 4°C for 10 minutes. The organic layer is evaporated under nitrogen at 47°C. The residue is reconstituted with methanol, run with ethyl acetate: ammonia: methanol (17:1:2; v/v/v) as mobile phase and visualized under UV light. The final fingerprint of phela consisted of three distinct bands with retention factors of 0.24, 0.31 and 0.92 for band 1 to 3, respectively. Band 1 and 2 were brownish while band 3 was orange in colour. In conclusion, a TLC method for identifying phela was developed.

7.2 INTRODUCTION

A thin layer chromatography was the first chromatographic method evaluated for initial screening of phela. Method development involved evaluating various solvents for extraction and as mobile phases to compile a TLC profile with which to identify phela with.

7.3 MATERIALS AND METHODS

7.3.1 Apparatuses

A vortex mixer (Scientific Industries Inc, U.S.A) was used for mixing. A 5810 R centrifuge and mini spin (Eppendorf, Germany) were used for separating samples. Precision balances SBC 31 and SPB 52 (Scaltec Instruments, Germany) were used for weighing milligram and gram quantities respectively. A nitrogen evaporator with a heating block was used for evaporating samples. TLC aluminium oxide glass plates 60 F_{254} (type E) pre-coated (Merck, Germany) were used. The TLC system consisted of a TLC tank (5L capacity) with a glass lid, glass cutter and laboratory spray gun (Shandon Scientific Co, England). Micro capillary pipettes (Einmal, Germany) were used to load the samples. A ruler in centimetres, pencil, hair dryer, timer, calculator and a UV light were all used during TLC analysis.

7.3.2 Chemicals and reagents

Phela was manufactured and supplied by the Indigenous Knowledge System Lead Programme (IKS) of the South African Medical Research Council. Extraction solvents were: dichloromethane, hexane, methanol, acetonitrile, diethyl ether, toluene (Burdick and Jackson, U.S.A). Furthermore acetic acid, iso-propanol (2-propanol), ethyl acetate, n-butanol and ammonia (Merck, Germany) were evaluated as mobile phases. Sodium hydroxide and ammonia sulphate were purchased from Sigma –Aldrich (Germany). Visualizing agents (iodine, iodoplatinate, dragendorff and copper chloride) were a donation from the toxicology laboratory of the University of the Free State. Deionised and distilled water was prepared by Millipore water system (Milli –Q^{Tm}).

7.3.3 Optimization of sample extraction conditions

7.3.3.1 Liquid-liquid extraction method

Phela (10 mg/ml) was prepared by extracting 50 mg of phela with 5 ml of either dichloromethane (extract AA), hexane (extract FF), water (extract GG) , varying pH, either acidic (extract DD) or basic (extract EE) and varying concentrations of methanol [60 % (extract BB), 100 % (extract CC), respectively. The extracts were left overnight at 4°C. The following day centrifugation was done for 10 minutes at 2500 rpm (1 251 g) at 4°C. The organic layer was used for TLC analysis.

7.3.3.2 Salting-out extraction method

Phela (200 mg) in 3 ml of iso-propanol was treated with 1 g of ammonia sulphate. The extract was horizontally shaken for 10 minutes and centrifuged at 2500 rpm (1251 g) at 4°C for 5 minutes. The supernatant was evaporated under nitrogen at 47 °C and the residue was reconstituted with 200 µl methanol. The resultant extract was used for TLC analysis.

7.3.4 Selection of mobile phases

The above-mentioned extracts were analyzed by TLC using different mobile phases. The included 8 mobile phases were: methanol:ethyl acetate (5:95; v/v), hexane, acetonitrile: water (70:30; v/v), methanol: ethyl acetate (50:50; v/v), hexane: ethyl acetate (95:5; v/v), methanol: water: acetic acid (70:25:5; v/v/v), ethyl acetate: ammonia: methanol (17:1:2; v/v/v), and n-Butanol : acetic acid: water (14: 2: 1; v/v/v).

7.3.5 Thin Layer Chromatography (TLC) analysis

For the *liquid-liquid extractions* a bigger TLC (20 cm x 20 cm) TLC plate was cut into 4 equal pieces (10 cm x 10 cm). A baseline was drawn 1 cm from the base of each plate and pencil point marks for loading spots were made on the line 1 cm apart, and labelled according to the extract names. Each extract (20 µl) was spotted on the point that corresponded with the extract's code name. The samples were dried with

a hairdryer. The sample-loaded TLC plates were each placed in a humid TLC tanks with a different mobile phase. All the samples were run for 30 minutes. A pencil line was drown at the solvent front and plates were left to dry before visualization under UV light. Visualized bands were marked by pencil under UV light.

The salted-out extracts were loaded similarly, however they were run on a big plate (20 cm x 20 cm) with ethyl acetate: ammonia: methanol (17:1:2; v/v/v) as mobile phase for 1 hour. These samples were stained with Iodine, Iodoplatinate, dragendorff and copper chloride to further develop the bands. They were also visualized under UV light and bands marked with a pencil.

7.3.6 Retention factor calculations

The distance travelled by the solvent front and by each individual band was measured in centimetres (cm) and the retention factor (RF) was calculated with the following equation:

$$[RF] = \left[\frac{Band - distance(cm)}{Solventfront - distance(cm)} \right] \qquad \text{Eq. 7.1}$$

7.4 RESULTS

Preliminary results showed that the tested sprays (Iodine, Iodoplatinate, dragendorff and copper chloride) did not enhance the bands. Therefore, all the chromatograms reported in this chapter were not treated with any spraying reagent.

7.4.1 Ethyl acetate: ammonia : methanol as mobile phase

Figure 7.1 (p 64) is a chromatogram with ethyl acetate: ammonia: methanol (17:1:2; v/v/v) as mobile phase, in which all extracts exhibited several bands. There were eight bands in total with retention factors of 0.12 for band 1, 0.23 for band 2, 0.31 for band 3, 0.41 for band 4, 0.57 band 5, 0.87 for band 6, 0.93 for band 7 and 0.96 for band 8 (Table 7.1; p 65). Band (compound) 2 and 7 luminated with an orange, while other bands had a brown–yellowish colour that varied in intensity. Each band had its own characteristics, some more frequent than the others. Methanolic extract (extract CC) had all bands present. The best separation was achieved with this mobile phase.

7.4.2 N-Butanol: ammonia: water as mobile phase

Figure 7.2 (p 66) shows the 6 separated bands with n-Butanol: acetic acid: water (14: 2: 1; v/v/v). Their retention factors were 0.15 for band 1, 0.23 for band 2, 0.28 for band 3, 0.50 for band 4, 0.75 for band 5 and 0.90 for band 6 (Table 7.2; p 67). Although the mobile phase was run for 30 minutes, the solvent only travelled half way through the TLC plate. The observation was bands that overlapped each other and further analysis was not possible.

7.4.3 Other mobile phases

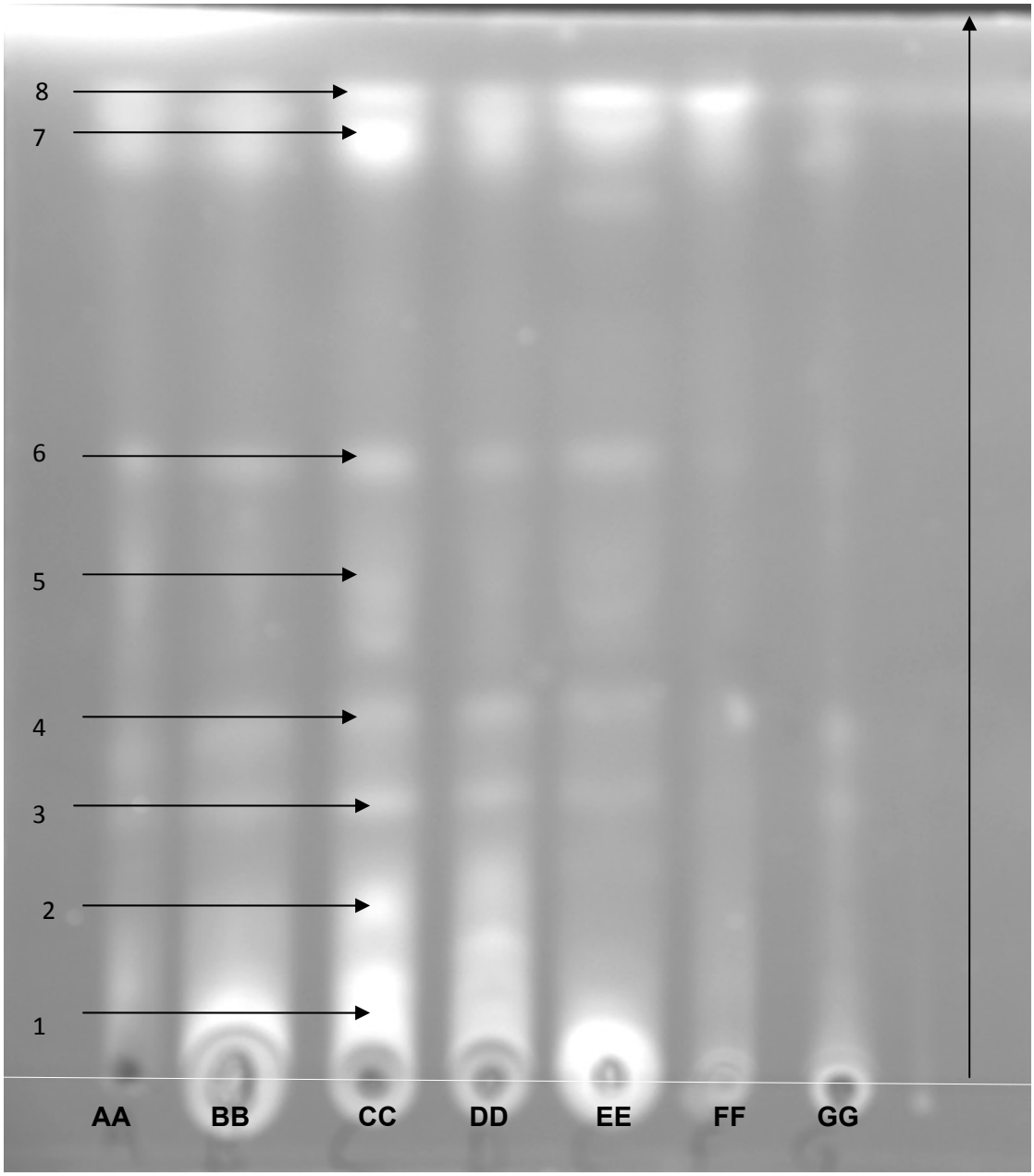
Figure 7.3 (p 68) is A TLC chromatogram showing separation with the 70 % acetonitrile in water mobile phase. Separation was poor, in that very faint bands were visible in the methanol extract (extract CC) and other extracts had no compounds separated (Table 7.3; p 69). All the remaining 5 mobile phases failed to separate compounds when all the extracts were run. Visualization under UV light showed no bands, even after visualizing reagents were sprayed on the TLC plate.

Decision: Based on the results from all observations, ethyl acetate: ammonia: methanol (17: 1: 2; v/v/v) was preferred as mobile phase.

7.4.4 Phela extraction by salting-out method

Figure 7.4 (p 70) shows the TLC chromatogram of the sample extracted using the salting-out procedure was run with ethyl acetate: ammonia: methanol (17: 1: 2; v/v/v) mobile phase. There are three distinct bands with retention factors of 0.24 for band 1, 0.31 for band 2 and 0.92 for band 3 (Table 7.4; p 70). Bands 1 and 2 had a brownish colour and the furthest band, band 3 was orange in colour.

Figure 7.1: A picture of a TLC plate with various phela extracts run with ethyl acetate: ammonia: methanol (17: 1: 2; v/v/v) as mobile phase. [By author]



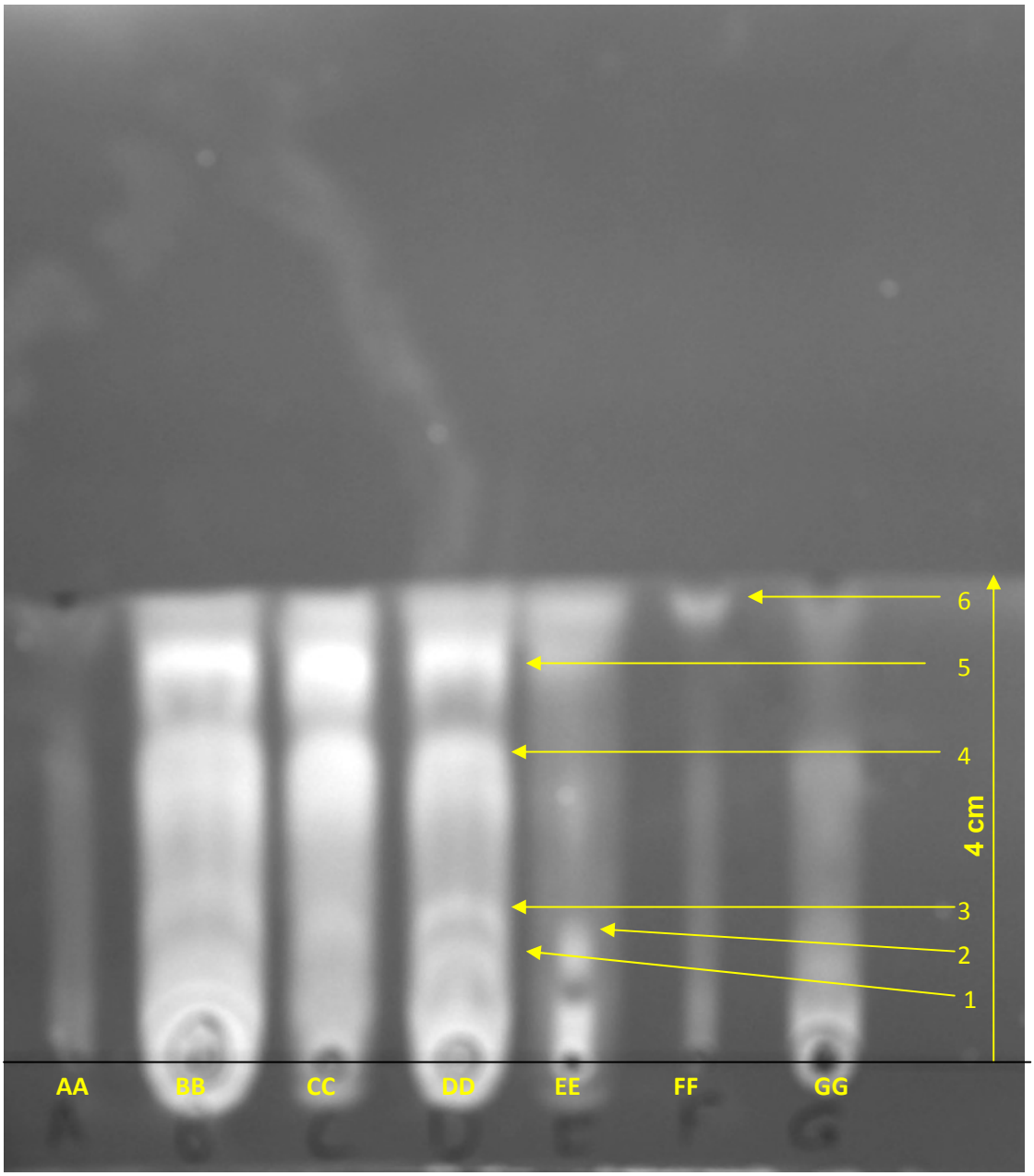
Key: Dichloromethane (extract AA), hexane (extract FF), water (extract GG) and varying methanolic acidic (extract DD) or basic (extract EE) mixtures and varying concentrations of methanol, i.e., 60 % (extract BB) and 100 % (extract CC).

Table 7.1: A summary of separated compounds and their retention factors from phela extracts run with ethyl acetate: ammonia: methanol (17: 1: 2; v/v/v) as mobile phase.

Extracts	**Compounds**							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
AA				x	x	x	x	x
BB		x	x		x	x	x	x
CC	x	x	x	x	x	x	x	x
DD		x	x		x	x	x	
EE		x	x		x	x	x	x
FF		x	x				x	x
GG			x				x	x
Distance travelled by each compound (cm)	1	2	2.7	3.5	4.9	7.5	8	8.3
Total distance at the solvent front (cm)	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6
RF	**0.12**	**0.23**	**0.31**	**0.41**	**0.57**	**0.87**	**0.93**	**0.96**

Key: Dichloromethane (extract AA), hexane (extract FF), water (water GG) and varying methanolic acidic (extract DD) or basic (extract EE) mixtures and varying concentrations of methanol, i.e., 60 % (extract BB) and 100 % (extract CC).

Figure 7.2: A picture of a TLC plate with various phela extracts run with N-Butanol: acetic acid: water (14: 2: 1; v/v/v) as mobile phase. [By author]



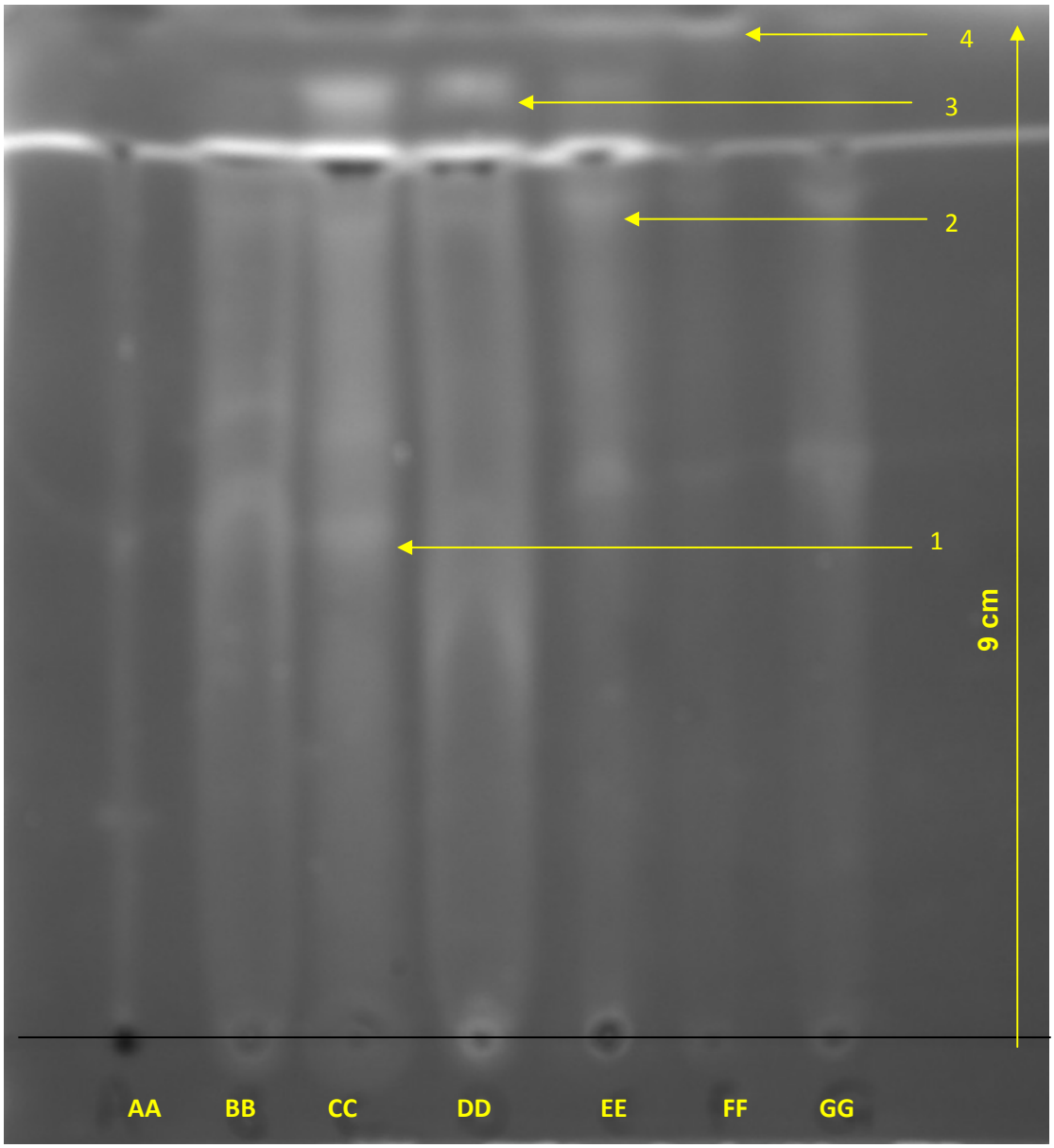
Key: Dichloromethane (extract AA), hexane (extract FF), water (extract GG) and varying methanolic acidic (extract DD) or basic (extract EE) mixtures and varying concentrations of methanol, i.e., 60 % (extract BB) and 100 % (extract CC).

Table 7.2: A summary of separated compounds and their retention factors from phela extracts run with N-Butanol: acetic acid: water (14: 2: 1; v/v/v) as mobile phase.

Extracts	**Compounds**					
	1	**2**	**3**	**4**	**5**	**6**
AA						
BB				x	x	
CC				x	x	
DD	x		x	x	x	x
EE		x				x
FF						
GG						
Distance travelled by each compound (cm)	0.6	0.9	1.1	2	3	3.6
Total distance At the solvent front (cm)	4	4	4	4	4	4
RF	**0.15**	**0.23**	**0.28**	**0.50**	**0.75**	**0.90**

Key: Dichloromethane (extract AA), hexane (extract FF), water (extract GG) and varying methanolic acidic (extract DD) or basic (extract EE) mixtures and varying concentrations of methanol, i.e., 60 % (extract BB) and 100 % (extract CC).

Figure 7.3: A picture of a TLC plate with various phela extracts run with 70 % acetonitrile in water as mobile phase. [By author]



Key: Dichloromethane (extract AA), hexane (extract FF), water (extract GG) and varying methanolic acidic (extract DD) or basic (extract EE) mixtures and varying concentrations of methanol, i.e., 60 % (extract BB) and 100 % (extract CC).

Table 7.3: A summary of separated compounds and their retention factors from phela extracts run with 70 % acetonitrile in water as mobile phase**.**

Extracts	**Compounds**			
	1	**2**	**3**	**4**
AA				x
BB	x			x
CC	x	x	x	x
DD		x	x	x
EE				x
FF				x
GG	x	x		x
Distance travelled by each compound (cm)	4.0	6.9	8	8.6
Total distance at the solvent front (cm)	9	9	9	9
RF	**0.44**	**0.77**	**0.89**	**0.96**

Key: Dichloromethane (extract AA), hexane (extract FF), water (extract GG) and varying methanolic acidic (extract DD) or basic (extract EE) mixtures and varying concentrations of methanol, i.e., 60 % (extract BB) and 100 % (extract CC).

Figure 7.4: A picture of a TLC plate of a salted-out phela extraction run with ethyl acetate: ammonia: methanol (17: 1: 2; v/v/v) as mobile phase. [By author].

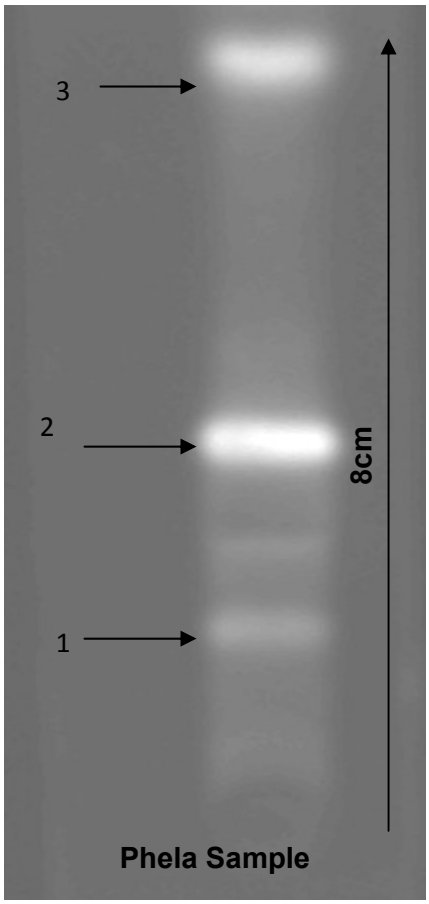


Table 7.4: A summary of 3 bands present in Phela salted-out extract and their retention factors run with ethyl acetate: ammonia: methanol (17:1:2; v/v/v) as mobile phase

Band Name	Distance travelled		Retention factor
	Band (cm)	solvent front (cm)	
1	1.9	8	**0.24**
2	2.5	8	**0.31**
3	7.3	8	**0.92**

7.5 DISCUSSION

The results indicate that a TLC method for screening and identification of phela has been developed. The visualized bands (n = 3), along with their retention factors and their colour have established the first characteristic fingerprint/TLC profile for phela identification. This method is simple, quick and inexpensive. According to Liang et al. (2004), TLC is still the common method of choice for herbal analysis before instrumental chromatography (GC and HPLC) methods are applied.

During the preliminary experiments, poor separation (e.g. diffuse and overlapping bands) with most evaluated mobile phase was observed, with the exception of ethyl acetate: ammonia: methanol (17:1:2; v/v/v) mobile phase, that is commonly used during TLC analysis of medicinal herbs (Lin, 2001).

The TLC method has successfully separated various compounds found in phela extract. Furthermore, TLC can be used as a preparative step for analysis by other chromatographic methods (e.g. high performance liquid chromatography and gas chromatography analysis) described in the chapters that follow.

7.6 CONCLUSION

A TLC screening method for phela identification and its TLC profile has been successfully developed and can be used as a preparative step for other chromatographic methods.

CHAPTER 8

DEVELOPMENT OF A METHOD FOR IDENTIFICATION OF PHELA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH A UV DETECTOR (HPLC_UV)

8.1 SUMMARY

The development of a high performance liquid chromatography with a UV detector method to fingerprint phela entailed evaluating various sample extractions; pH based extractions methods; different solvents as extraction solvents and optimizing HPLC parameters. The final conditions for sample extraction were as follows: Phela was acidified with 0.5 M HCl (100 µl), shaken horizontally for 15 minutes, extracted with hexane (5 ml) and left to shake for further 10 minutes. The organic layer was evaporated under nitrogen at 47 °C. Thereafter, the residue was reconstituted with mobile phase and 50µl was injected on the HPLC. The optimized HPLC conditions were mobile phase of 70 % acetonitrile eluting at a flow rate of 0.5 ml/min for thirty minutes. Separation was done on a C_{18} (250 mm x 4.6 mm x 5 µm) phenomenex column coupled to a C_{18} guard column and detected at 245 nm wavelength. The retention time for marker peak (1) was at (9.35 ± 0.71) minutes and marker peak (2) (24.5 ± 0.71) minutes with a time difference of (15.2 ± 0.54) minutes. Ultimately, an HPLC_UV method for identification of phela was developed.

8.2 INTRODUCTION

In this chapter, an HPLC_UV method for fingerprinting phela was developed by evaluating various sample extraction methods. The HPLC conditions evaluated comprised different concentrations of acetonitrile in water as mobile phases, different flow rates for better peak elution and determining the most selective wavelength to work with.

8.3 MATERIALS AND METHODS

8.3.1 Apparatuses

A vortex mixer (Scientific Industries Inc, U.S.A) was used for mixing. A 5810 R centrifuge and mini spin (Eppendorf, Germany) were used for centrifuging and quick spinning of samples. Precision balances SBC 31 and SPB 52 (Scaltec Instruments, Germany) were used for weighing milligram and gram quantities respectively. A nitrogen evaporator with a heating block was used for evaporating samples. Finnpipettes from Thermo Labsystems (Canada) were used for spiking volumes. Glass pipettes were used for adding extraction solvents. Sterile Millipore HA 0.45 µm filter unit (Millex®) was used for filtration.

8.3.2 Chemicals and reagents

Phela was manufactured and supplied by the Indigenous Knowledge Systems Lead Programme (IKS) of the South African Medical Research Council. HPLC graded: dichloromethane, hexane, methanol, acetonitrile, diethyl ether, toluene (Burdick and Jackson, U.S.A) and hydrochloric acid, iso-propanol (Merck, Germany) were evaluated as extraction solvents. Sodium hydroxide and ammonia sulphate were purchased from Sigma –Aldrich (Germany). Deionised and distilled water was prepared by Millipore water system (Milli –Q^{Tm}).

8.3.3 Chromatographic system and conditions

Chromatographic system: The HPLC system consisted of a Hewlett-Packard 1100 series system comprised of an isocratic pump, an auto-sampler and a UV detector. Data was collected with LC Chem station program.

Chromatographic conditions: Chromatographic separation was achieved with a reverse phase C_{18} (250 mm x 4.6 mm x 5 microns) coupled to a C_{18} guard column (Phenomenex, U.S.A). Different mobile phases, flow rates and wavelengths were evaluated to establish the final HPLC conditions. Preliminary conditions were achieved with 20 % acetonitrile in water eluting at a flow rate of 1 ml/min with UV detection set at 210 nm and 40 minutes run time.

8.3.4 Optimization of sample extraction conditions

8.3.4.1 Organic solvents extraction method

Phela (100 mg) was extracted with 10 ml of 3 different organic solvents [Ethyl Ether, Chloroform and Hexane] in order to determine the solvent with best extraction properties. The samples were horizontally shaken and left over night in the refrigerator at 4°C. The samples were centrifuged at 4 °C and 2 500 rpm (1 251g) for 5 minutes. The organic layers were evaporated under nitrogen at 47°C, and residues reconstituted with 5 ml of mobile phase (20 % acetonitrile in water). The samples were filtered with a sterile millipore HA 0.45 µm filter unit to get rid of impurities and 50 µl of each sample was injected on the HPLC.

8.3.4.2 Aqueous and methanolic extraction method

Varying percentages of methanol [20 %, 60 % and 90 %] and water were also evaluated. The extracts were horizontally shaken for 1 hour and left over-night at 4°C. The supernatant of each extract was filtered through a sterile millipore HA 0.45 µm filter unit and 50 µl of the filtrate was injected on the HPLC. All the pure solvents used were run as blank on the HPLC under the same conditions.

8.3.4.3 pH based extractions

8.3.4.3.1 Acidic liquid-liquid extraction

Phela (100 mg) was extracted with 5 ml of either ether or hexane and shaken horizontally for 15 minutes at room temperature. The extract was acidified with 500 µl of 0.1 M HCl (Table 8.1; p 76; sample C and D) and allowed 10 minutes of shaking. The samples were centrifuged at 4 °C and 2 500 rpm (1 251g) for 10 minutes. The organic layer was evaporated under nitrogen at 47°C, and residues reconstituted with mobile phase.

8.3.4.3.2 Basic liquid-liquid extraction

Phela was extracted with 5 ml of either ether or hexane and shaken horizontally for 15 minutes at room temperature. The samples were basified by addition of 500 µl 20 % NaOH (Table 8.1; p 76; Sample B and E) and shaken for 10 minutes. The samples were centrifuged at 4 °C and 2 500 rpm (1 251g) for 10 minutes. The organic layer was evaporated under nitrogen at 47°C, and residues reconstituted with mobile phase.

8.3.5 Mobile phase Optimization

The following mobile phases were prepared with acetonitrile in de-ionised water and degassed with helium gas.

1. 20 % acetonitrile [Acetonitrile : water (20:80; v/v)]
2. 50 % acetonitrile [Acetonitrile : water (50:50; v/v)]
3. 70 % acetonitrile [Acetonitrile : water (70:30; v/v)]

Phela was extracted as described in section 8.3.4.1 and 8.3.4.2. The first evaluated mobile phase was 20 % acetonitrile and all the HPLC parameters were kept constant as described in section 8.3.3. Thereafter, the extracts of hexane, chloroform, ether and 90 % methanol were run on 50 % acetonitrile as mobile. The last mobile phase tested was 70 % of acetonitrile in water mobile phase.

Table 8.1: A table showing how the pH based extractions with hexane and ether were carried out.

				Extraction Solvent		
Sample name	**Conc. (mg/ml)**	**20 % NaOH (µl)**	**0.1 M HCl (µl)**	**Name**	**volume (ml)**	**MP (µl)**
A (blank)		**500**		**Ether**	**5**	**100**
A 1 + A 2	**100**	**500**		**Ether**	**5**	**100**
B (blank)			**500**	**Ether**	**5**	**100**
B 1 + B 2	**100**		**500**	**Ether**	**5**	**100**
C (blank)				**Ether**	**5**	**100**
C 1 + C 2	**100**			**Ether**	**5**	**100**
D (blank)		500		Hexane	5	100
D 1 + D 2	100	500		Hexane	5	100
E (blank)			500	Hexane	5	100
E 1 + E 2	100		500	Hexane	5	100
F (blank)				Hexane	5	100
F 1 + F 2	100			Hexane	5	100

Key: **Conc. =** concentration, **NaOH =** Sodium Hydroxide, **HCl =** Hydrochloric acid**, MP =** mobile phase

8.3.6 Selection of UV wavelength

UV spectrum of 6 mg/ml of phela in water was measured using a Biochrom Libra S12 spectrophotometer over a wavelength range of 200 – 900 nm in order to determine the wavelength at which the HPLC detector system must operate.

8.3.6.1 Optimization of wavelength sensitivity

An HP HPLC 1090 series with a Diode-array-detector was used for this part of the study because the detector can read simultaneously at multiple wavelengths. The detector was set at wavelengths of 210 nm and 245 nm to evaluate wavelength sensitivity. The samples (ether and hexane extracts) were prepared with the methods described in section 8.3.4.3.1 (for acidification) and 8.3.4.3.2 (for basification) and analysed on the HPLC with 70 % of acetonitrile as mobile phase.

8.3.7 Optimization of the flow rate

The different flow rates tested were 1ml/min, 0.8 ml/min and 0.5 ml/min.

8.3.8 UV Spectra analysis of marker peaks with HPLC with photo-diode-array detector (HPLC_PDA)

The UV spectra of the selected marker peaks were determined using a photo-diode-array detector

8.3.9 Marker peaks analysis by Gas Chromatography – Mass spectrometry (GC_MSD)

Marker peaks one and two were collected as fractions during HPLC_UV analysis were further analysed by GC_MSD.

8.4 RESULTS

8.4.1 Mobile phase and extraction solvent selection

8.4.1.1 *Mobile phase of 20 % acetonitrile*

Figure 8.1 (p 79) to 8.5 (p 80) are chromatograms of extracts run on HPLC_UV with 20 % acetonitrile mobile phase. Figure 8.1, 8.2 and 8.3 (p 79) shows chromatograms of organic solvent extracts (ether, chloroform and hexane).

Figure 8.1 A (p 79) is a chromatogram of blank ether, in which there were too many peaks. Figure 8.1 B (p 79) is a chromatogram of ether extract, in which peaks overlapped, making it difficult to choose marker peaks.

Figure 8.2 A (p 79) is a chromatogram of blank chloroform, in which peak separation was good. Figure 8.2 B (p 79) is a chromatogram of a chloroform extract, in which separation was poor as peaks overlapped each other and they were not symmetrical.

Figure 8.3 A (p 79) is a chromatogram of blank hexane, with all peaks eluting early. Figure 8.3 B (p 79) is a chromatogram of hexane extract; there were fewer peaks in comparison with the extracts discussed above: two peaks with a retention time of 7.9 minutes and 18.3 minutes were symmetrical and well spaced, however, solvent drifting was observed.

Figure 8.4 (p 80) is chromatogram of a 90 % methanolic extract; peaks overlapped with each other making further analysis difficult. All the various methanolic concentration (20 % and 60 %; Appendix A, figure A-2, and A-3; p 236 - 237) extracts had a similar chromatographic separation to Figure 8.4 (Blank chromatogram of the methanol are in the appendix A, Figure A-1; p 236).

Figure 8.5 (p 80) is chromatogram of an aqueous extract; here peaks were not symmetrical, making further analysis difficult.

Decision: Hexane extract had a better peak separation than other extracts. Separation with the 20 % acetonitrile mobile phase showed too many peaks that were overlapping, making if difficult to establish marker peaks. Furthermore, in some extracts analysis the baseline drifted. Hence higher concentrations of acetonitrile in mobile phase were further evaluated.

Mobile phase of 20 % acetonitrile

Figure 8.1: Chromatogram of blank ether (A) and phela ether extract (B) eluted with 20 % acetonitrile as mobile phase.

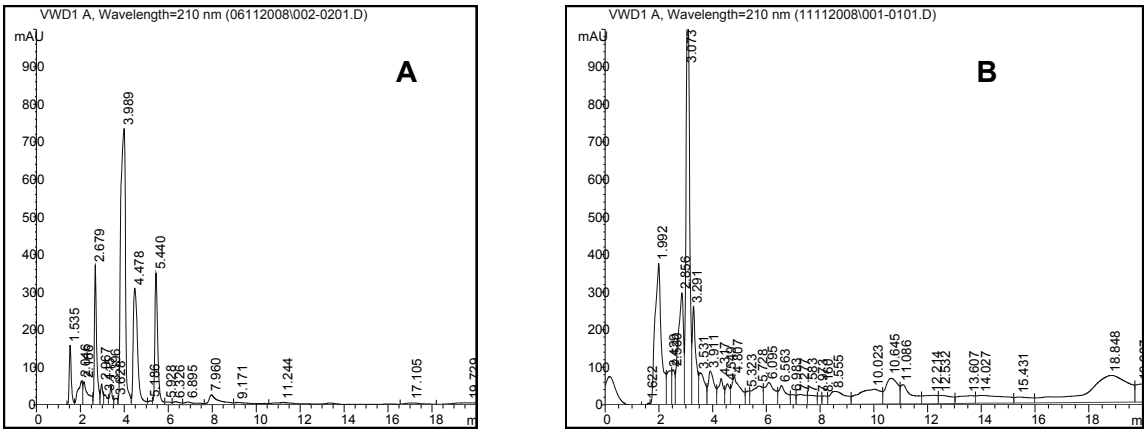


Figure 8.2: Chromatogram of blank chloroform (A) and phela chloroform extract (B) eluted with 20 % acetonitrile as mobile phase.

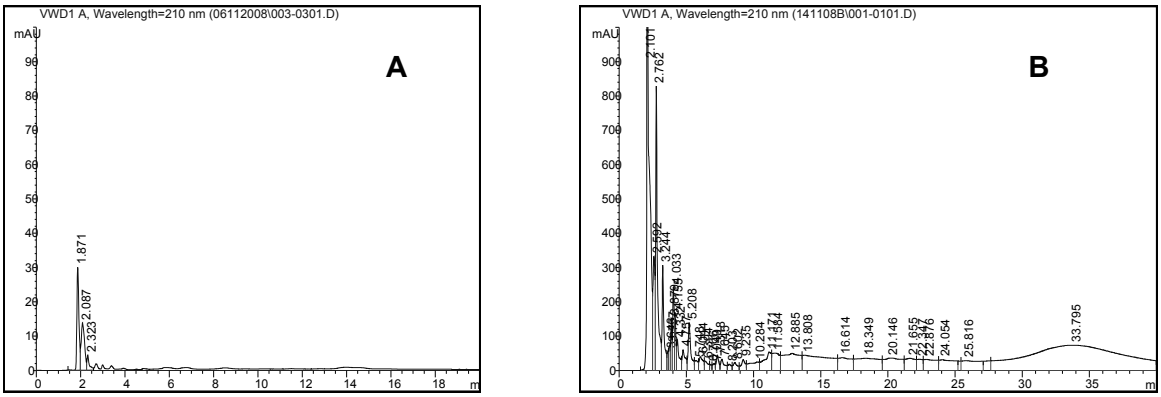


Figure 8.3: Chromatogram of blank hexane (A) and phela hexane extract (B) eluted with 20 % acetonitrile as mobile phase.

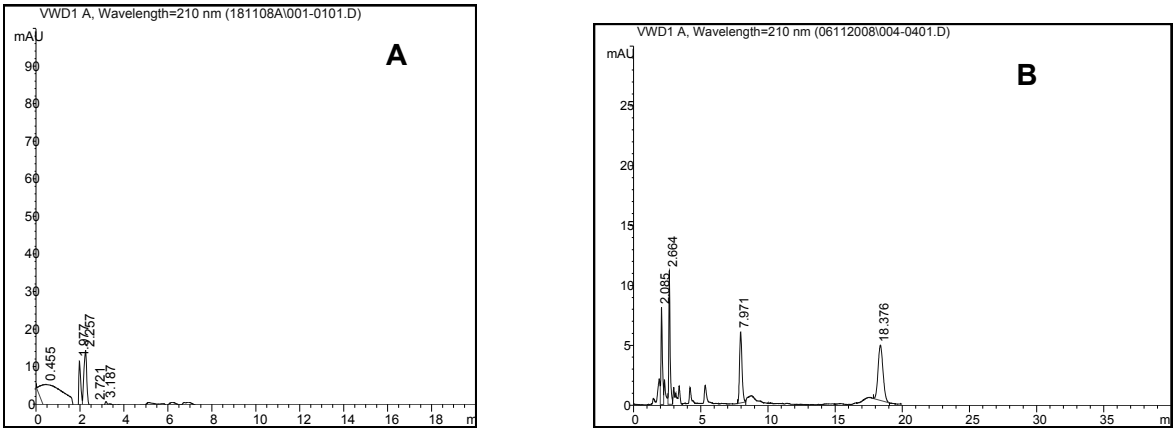


Figure 8.4: Chromatogram of phela 90 % methanol extract eluted with 20 % acetonitrile as mobile phase.

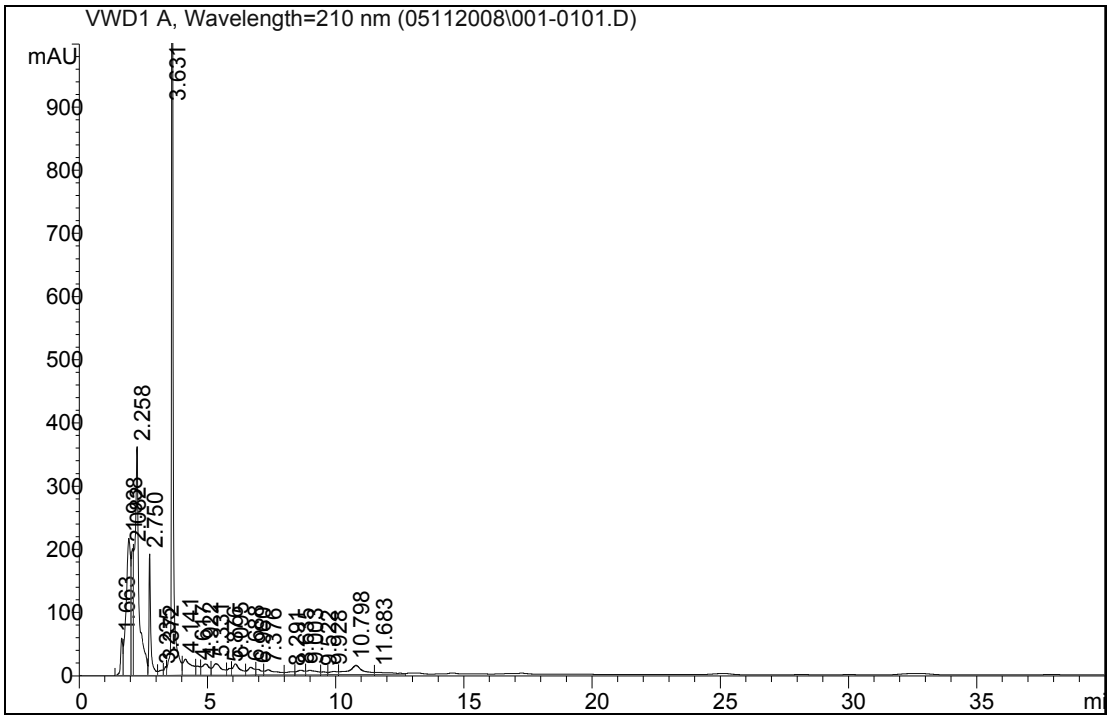
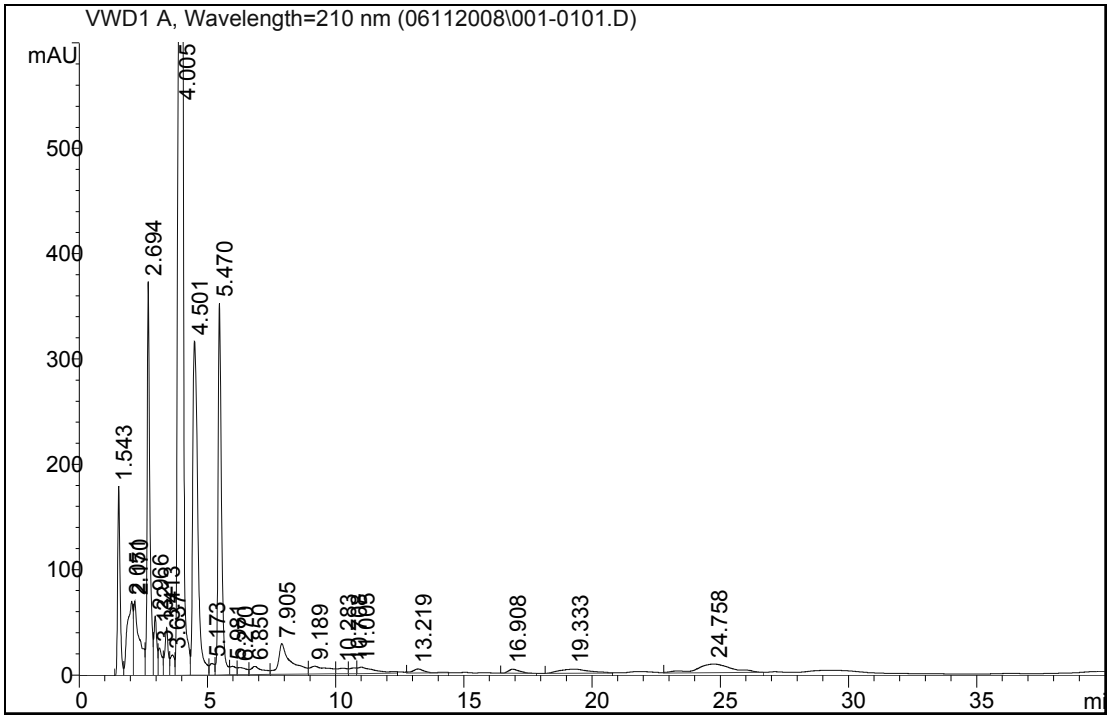


Figure 8.5: Chromatogram of phela water extract eluted with 20 % acetonitrile as mobile phase.



8.4.1.2 *Mobile phase of 50 % acetonitrile*

Decision: Evaluation of 50 % of acetonitrile resulted in poor chromatographic separation (Appendix A; methanol: fig A-4, p 237; ether: fig A-5 – A-6, p 238; hexane: fig A-7 – A-8, p 239). Furthermore, the baseline of some extracts started drifting, and a decision was taken to increase the concentration of acetonitrile in the mobile phase.

8.4.1.3 *Mobile phase of 70 % acetonitrile*

Figure 8.6, 8.7 8.8 (p 82) illustrates chromatographic peak elution for all 3 organic solvents (ether, chloroform and hexane) with 70 % acetonitrile as mobile phase.

Figure 8.6 A (p 82) is a chromatogram of blank hexane. Figure 8.6 B (p 82) is a chromatogram of hexane extract, with distinct high peaks at 5.2, 18.6 and 24.5 minutes. The presence of at least two symmetrical peaks made hexane a preferred extraction solvent.

Figure 8.7 A (p 82) is a chromatogram of blank chloroform. Figure 8.7 B (p 82) is a chromatogram of chloroform extract, with distinct peaks at 22.9 and 26.0 minutes. Chloroform had a good peak elution; however chloroform is dense and slow to evaporate, making it difficult to work with and it was not further analysed.

Figure 8.8 A (p 82) is a chromatogram of blank ether. Figure 8.8 B (p 82) is a chromatogram of ether extract, with distinct peaks at 5.8, 18.9 and 29.9 minutes. There were too many peaks that overlapped one another. However, because ether evaporates quickly and it is easier to work with, it was evaluated further.

Decision: Hexane and ethyl ether extracts were further investigated to establish the extraction solvent. Methanol (appendix A; A-4; p 237) and chloroform extracts failed to separate and were not investigated further. The extraction volume of the solvents was reduced to 5 ml; the chromatographic results remained the same. The elution with 70 % acetonitrile in water mobile phase had the best, sharpest and symmetrical peaks, thus this mobile phase was the preferred one. In conclusion, the extraction volume was 5 ml and the mobile phase 70 % acetonitrile in water.

Mobile phase of 70 % acetonitrile

Figure 8.6: Chromatogram of blank hexane (A) and phela hexane extract (B) eluted with 70 % acetonitrile as mobile phase.

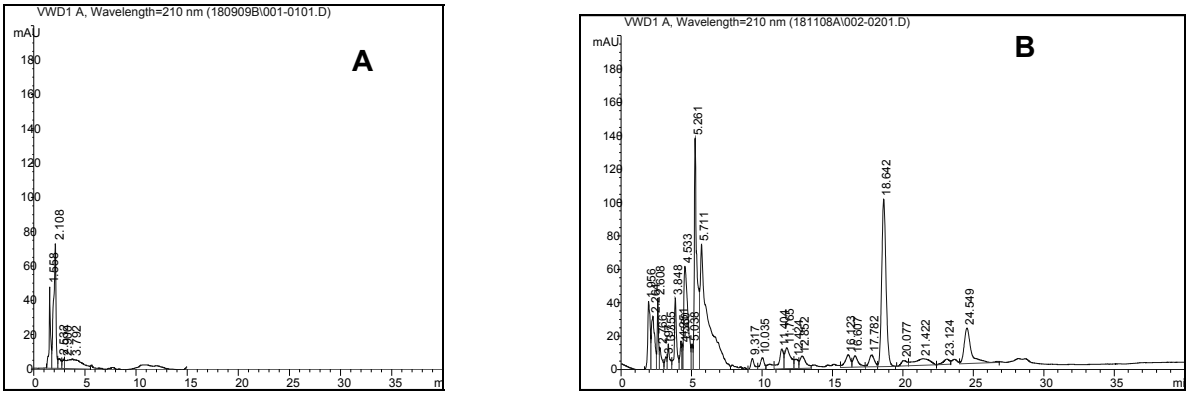


Figure 8.7: Chromatogram of blank chloroform (A) and phela chloroform extract (B) eluted with 70 % acetonitrile as mobile phase.

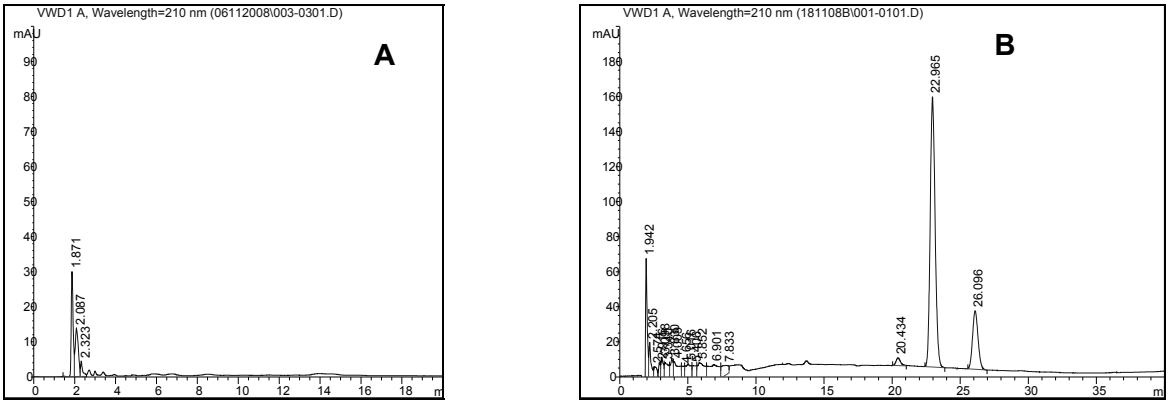
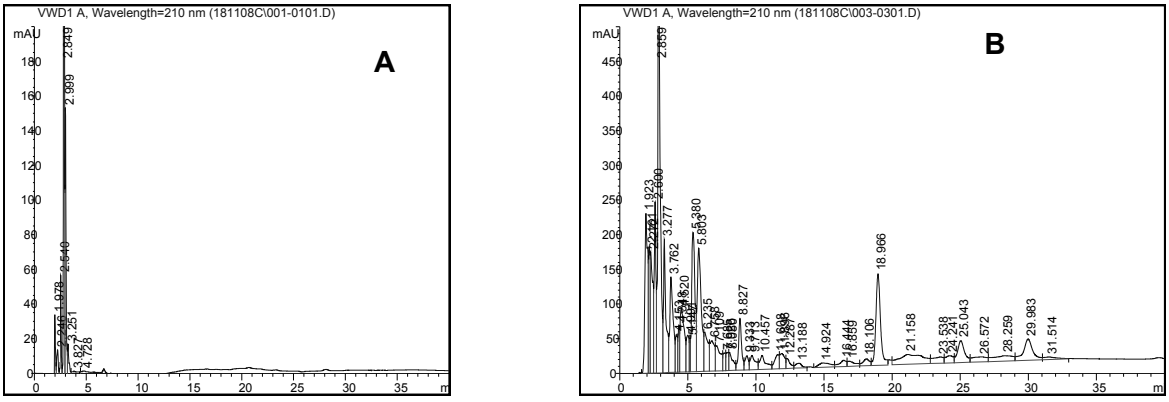


Figure 8.8 Chromatogram of blank ethyl ether (A) and phela ethyl ether extract (B) eluted with 70 % acetonitrile as mobile phase.



8.4.2 UV wavelength

Figure 8.9 (p 85) illustrates the UV spectra of phela with absorbance high between 240 – 300 nm. The absorbance peak was highest around 240 and 260 nm. Hence 245 nm wavelength was evaluated on the HPLC.

8.4.3 Selection of extraction solvent

Figures 8.10 (p 86) and 8.11 (p 87) are the chromatograms of the blank and acidic extracts detected at 210 nm and 245nm for hexane and ether respectively. Each extract was characterized by the eluted peaks and two peaks per extract were chosen as marker peaks.

Figure 8.10 A (p 86) reflects the chromatograms of the acidic blank hexane detected simultaneously at 210 and 245 nm wavelengths.. The chromatogram detected at 210 had too many peaks that could interfere with the extract peaks. The chromatogram detected at 245 nm had fewer peaks.

Figure 8.10 B (p 86) reflects the chromatograms of the acidic hexane extracts detected at a wavelength of 210 and 245 nm. The chromatogram detected at 210 had symmetrical peaks 4.8 and 11.6 minutes and many peaks that were overlapping with one another. The chromatogram detected at 245 nm had fewer peaks; two symmetrical peaks were selected as marker peaks, with the retention time of 4.8 minutes and 11.6 minutes (6.8 minutes time difference between the marker peaks).

Figure 8.11 A (p 87) reflects the chromatograms of the blank ether detected at a wavelength of 210 and 245 nm. Both chromatograms had peaks eluting early.

Figure 8.11 B (p 87) reflects the chromatograms of the acidic ether extracts detected at a wavelength of 210 and 245 nm. The chromatogram detected at 210 had peaks at 3.8, 9.5 and 12.0 minutes, and many overlapping peaks were observed. The chromatogram detected at 245 nm had fewer peaks; two symmetrical peaks were selected as marker peaks; at the retention time of 4.9 minutes and 12.0 minutes (7.1 time distance between the marker peaks).

The marker peaks in both acidic ether (Fig 8.10 B; p 86) and acidic hexane (Fig 8.11 B; p 87) extracts had similar marker peaks. Figures 8.12 and 8.13 show the UV spectra analysis of the marker peaks (from figure 8.10 B and 8.11 B). The spectra confirmed the similarity of the marker peaks of the ether and hexane extracts. Furthermore, the hexane extract peaks had a higher absorbance reading (in maU) than ether extract for both peaks.

The chromatograms of all basic extractions (ether and hexane) detected at both the wavelengths (210 nm and 245nm) had no peaks. (Appendix A; ether: fig A-9, p 240; hexane: fig A-10, p 241).

Decision: The 245 nm was the most sensitive wavelength to work with. Acidic extractions with hexane had a higher concentration of marker peaks when compared with acidic ether extracts. Hence the extraction method of choice is the acidification by HCl with hexane as extraction solvent.

8.4.4 UV spectra of marker peaks

Figures 8.12 A and B (p 88) show the UV spectra of marker peak 1 after hexane (A) and ether (B) extraction. The spectrum is the same with a higher absorbance (maU) in the hexane extract. Figures 8.13 A and B (p 89) show the UV spectra of marker peak 2 after hexane (A) and ethyl ether (B) extraction. The spectrum is the same with a higher concentration in the hexane extract. This confirms that the marker peaks from the two extracts were the same.

UV spectra

Figure 8.9: A graph showing phela UV spectrum

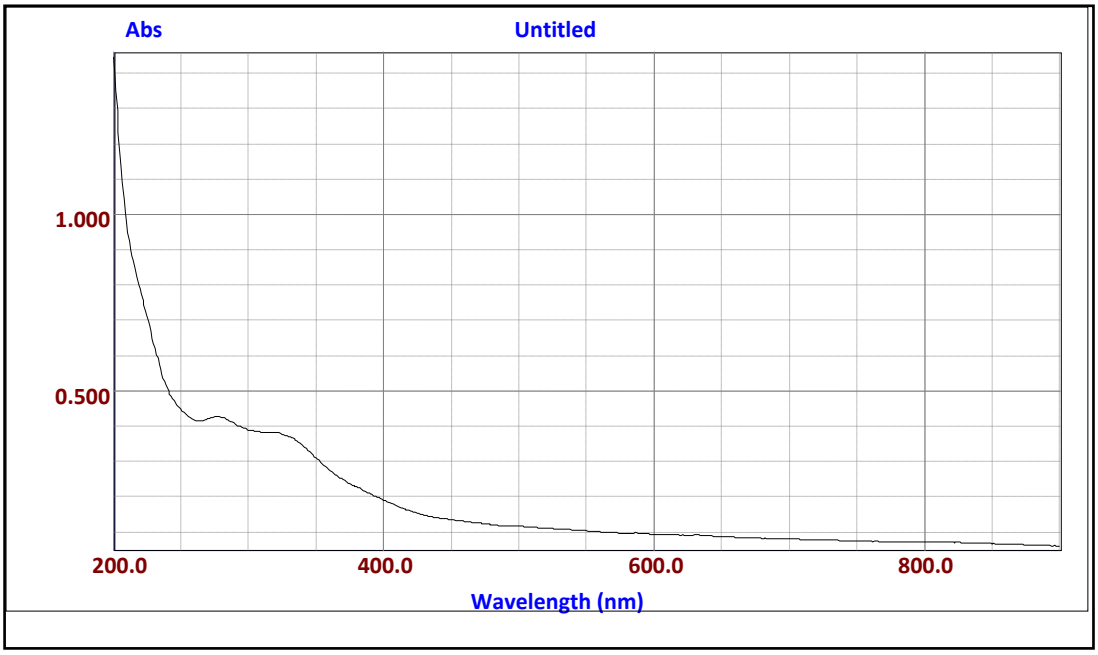
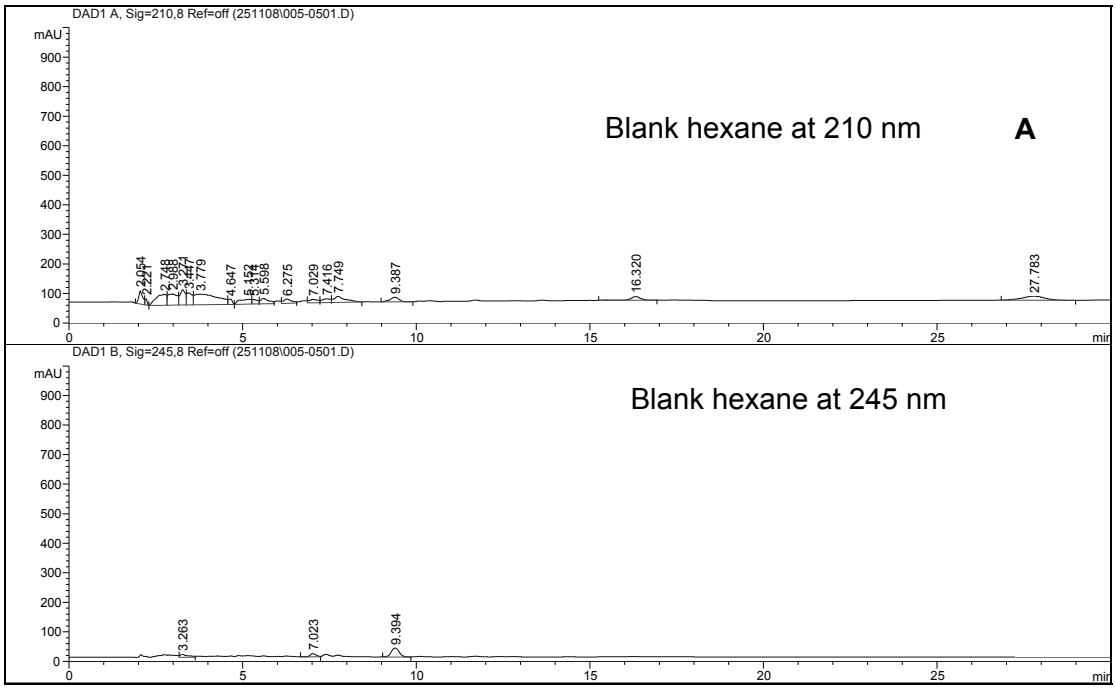


Figure 8.10: Chromatograms of blank hexane (A) acidic hexane extraction measured at 210 nm and 245 nm wavelength (B)



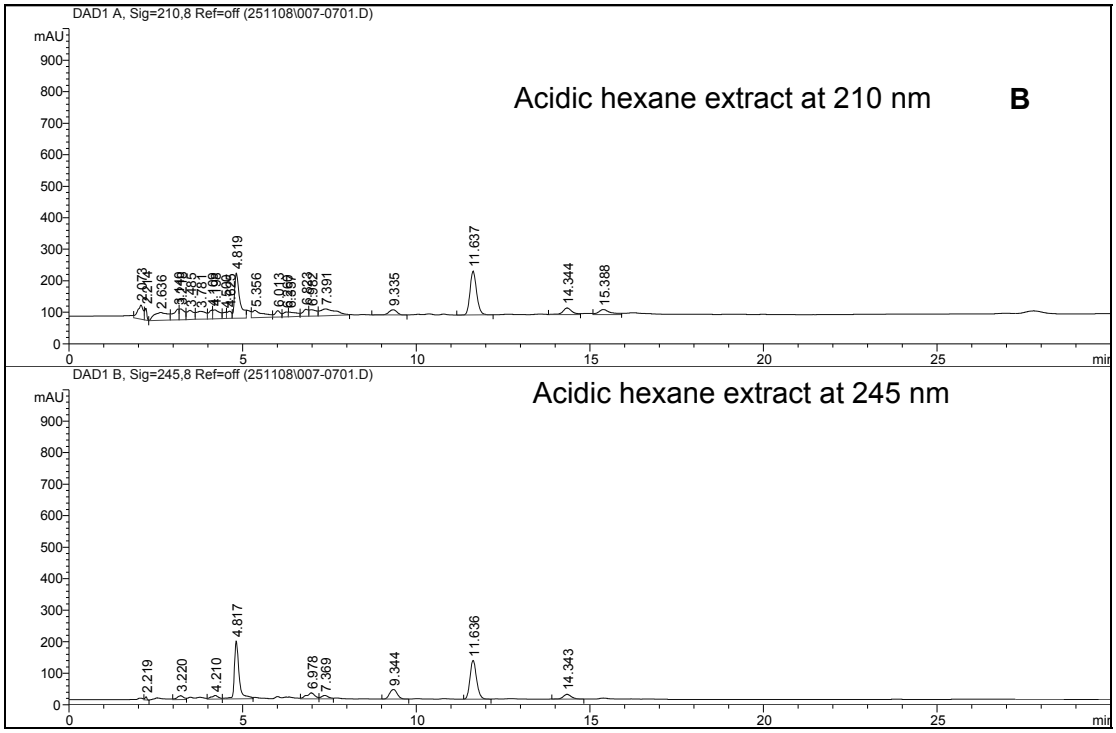


Figure 8.11: Chromatograms of blank ether (A) acidic ether extraction measured at 210 nm and 245 nm wavelength (B)

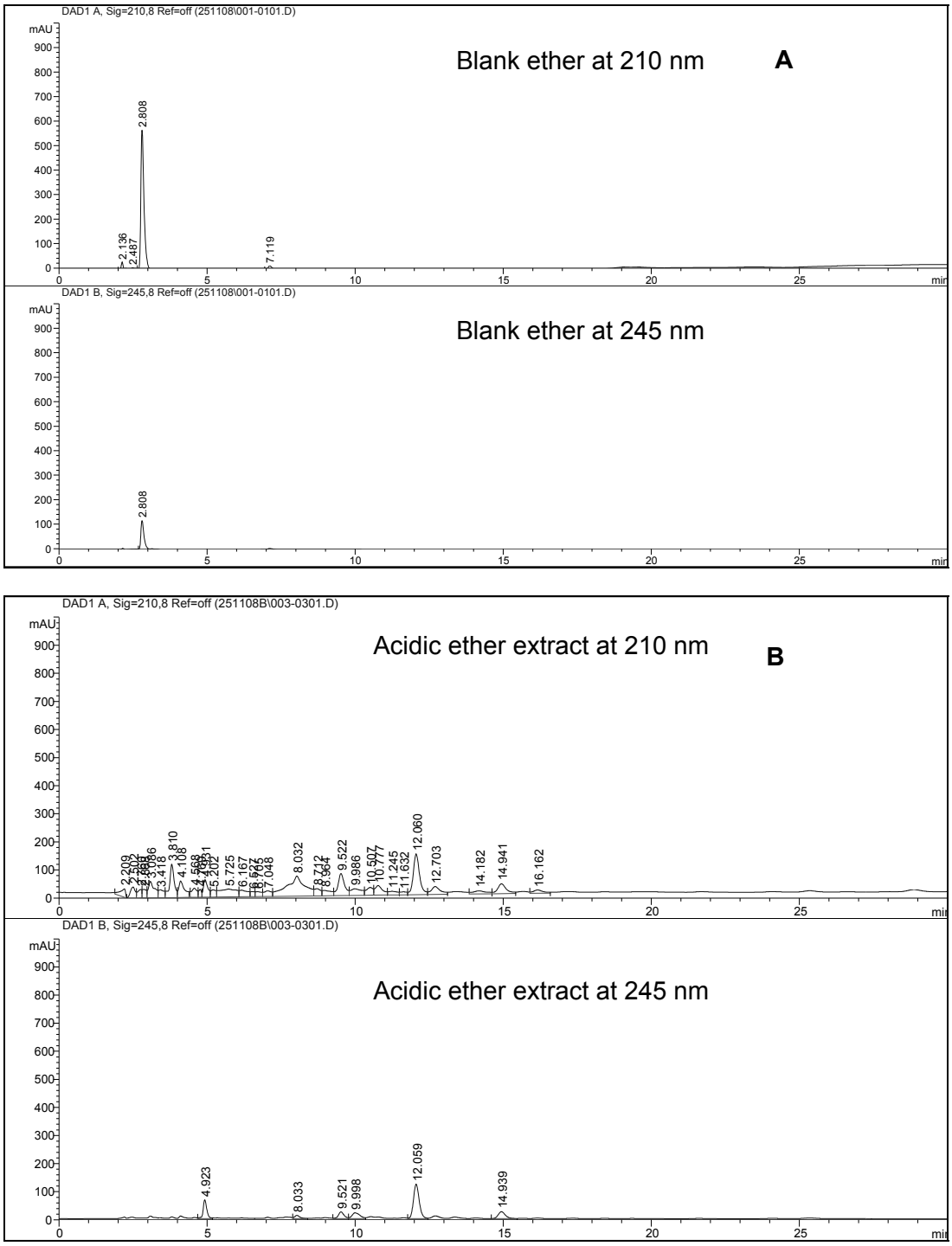
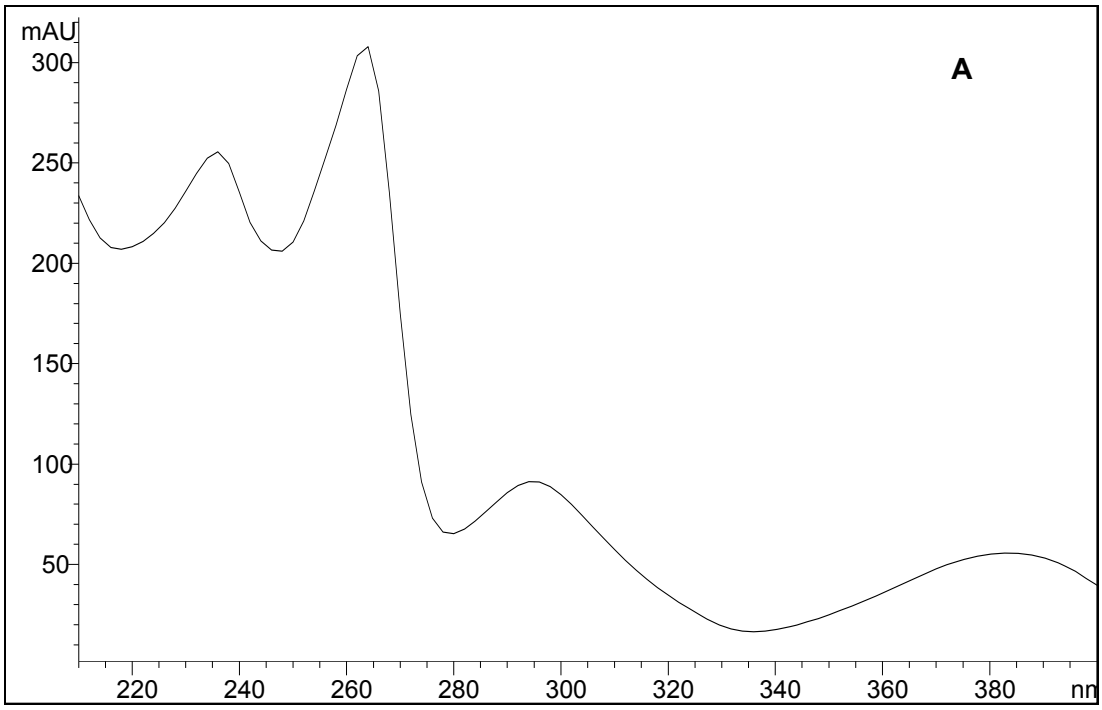


Figure 8.12: UV spectra of marker peak 1 at 4.8 minutes for hexane (A) and 4.9 minutes for ether extract (B)



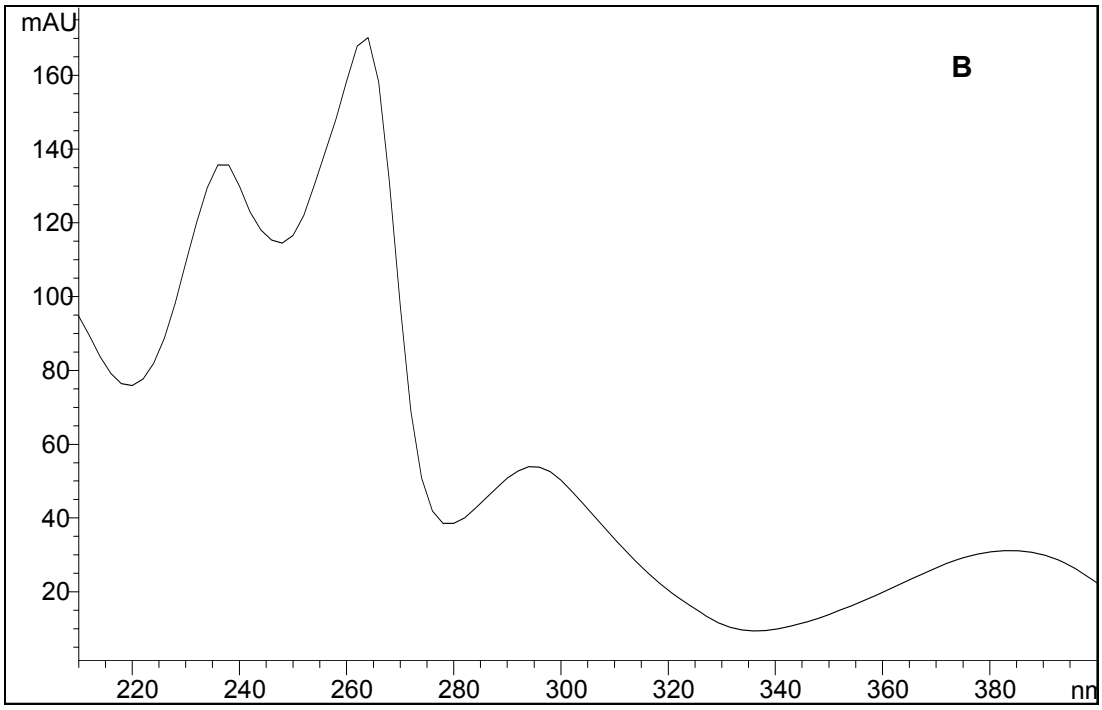
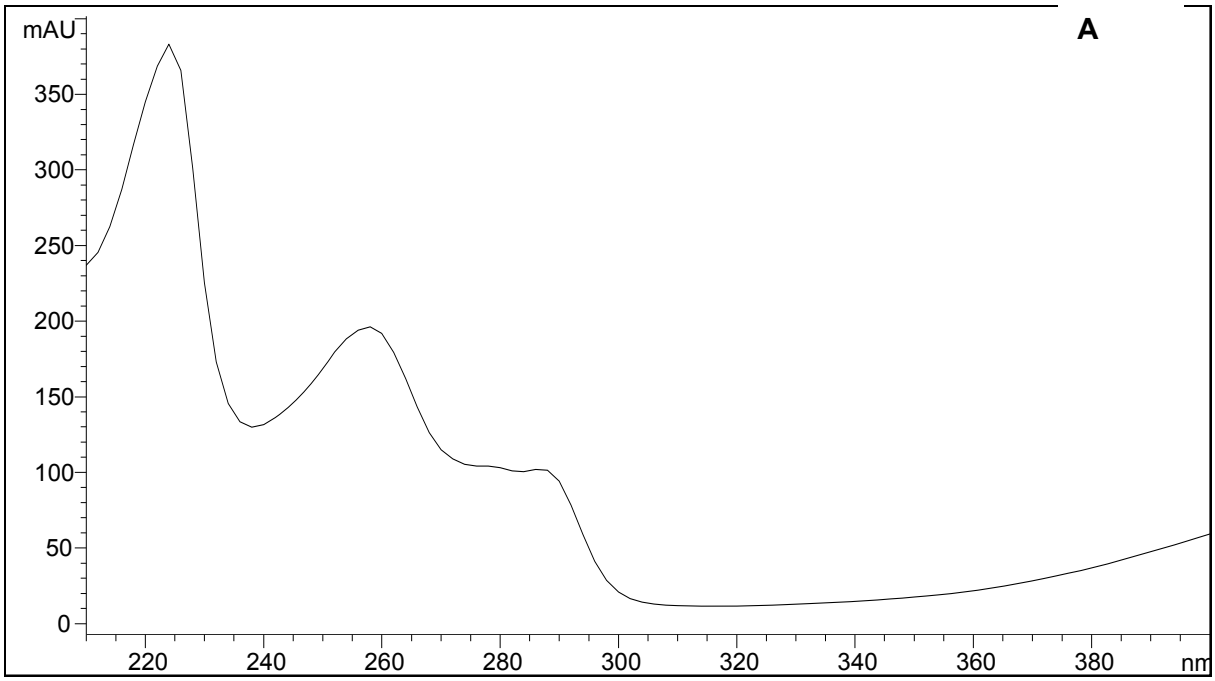
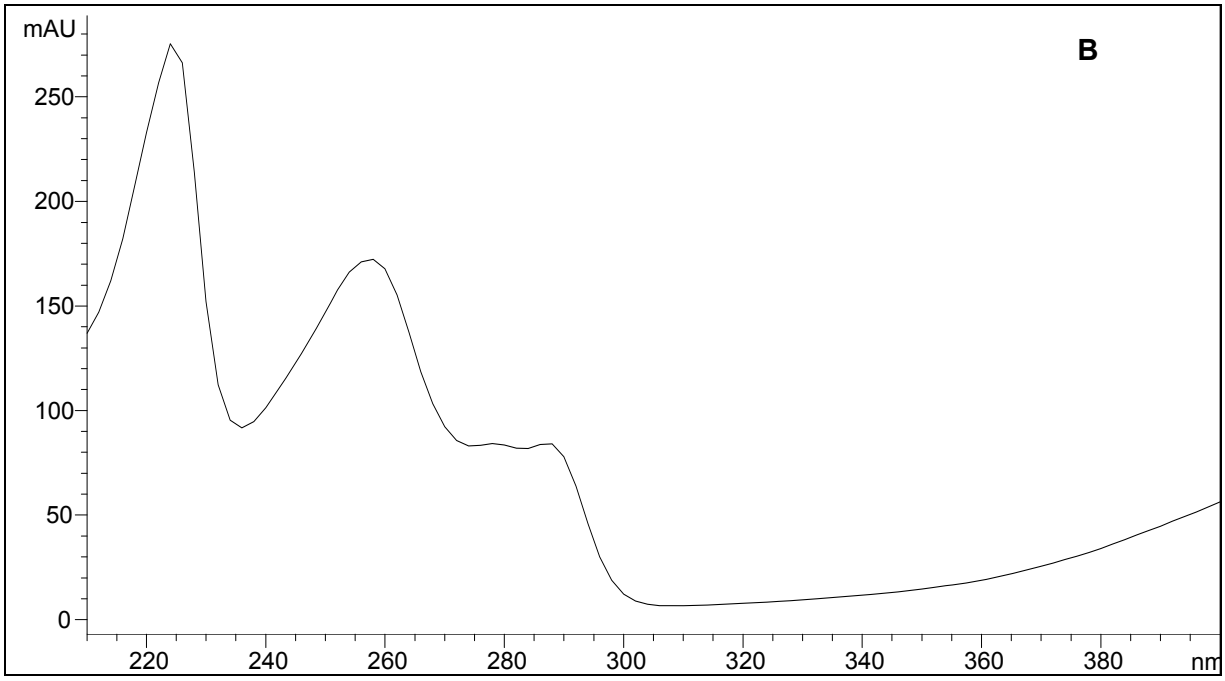


Figure 8.13: UV spectra of marker peak 2 at 11.6 minutes for hexane (A) and 12. 0 minutes for ethyl ether extract (B).





8.4.5 Flow rate optimization and retention time shifts

The flow rate was set at 0.5 ml/min. Figures 8.14 A and B (p 91) are chromatograms of a blank and hexane extract with the retention time for marker peak (1) at 9.8 minutes and marker peak (2) at 24.9 minutes. The time difference is 15.1 minutes.

The chromatograms for 1 ml/min and 0.8 ml/min are in appendix A, Fig A-11; p 242 and Fig A-12; p 242 respectively. The peaks with the highest absorbance are evenly spaced out.

Table 8.2 (p 92) is a summary of HPLC_UV retention time shifts of marker peaks at the final flow rate of 0.5 ml/min. The marker peak means are 9.35 ± 0.71 minutes (marker peak 1), 24.5 ± 0.71 minutes (marker peak 2) and 15.2 ± 0.54 minutes (the time difference in peaks).

8.4.6 The recommended method conditions

Sample preparation

Phela (200 mg) is extracted with 5 ml of hexane, acidified with 0.1M HCl (100 µl) and horizontally shaken for 15 minutes. The samples are centrifuged at 4 °C and 2 500 rpm (1 251g) for 10 minutes. The organic layer is evaporated under nitrogen at 47°C, and residues reconstituted with 100 µl of mobile phase and 50 µl is injected onto HPLC for analysis

HPLC conditions

The sample is eluted with 70 % of acetonitrile in water and separated on a reverse phase a C_{18} (250 mm x 4.6 mm x 5 µm) column coupled to a C_{18} guard column at a flow rate of 0.5 ml/min. The wavelength is set at 245 nm and sample run for 30 minutes.

HPLC_UV Chromatogram Profile

The retention time for marker peak (1) is at (9.35 ± 0.71) minutes and marker peak (2) (24.5 ± 0.71) minutes with a peak time difference of 15.15 ± 0.54.

Figure 8.14: A chromatogram of blank (A) and hexane extract (B) prepared with the final method conditions marker peak 1 at 9.8 minutes and marker peak 2 at 24 minutes

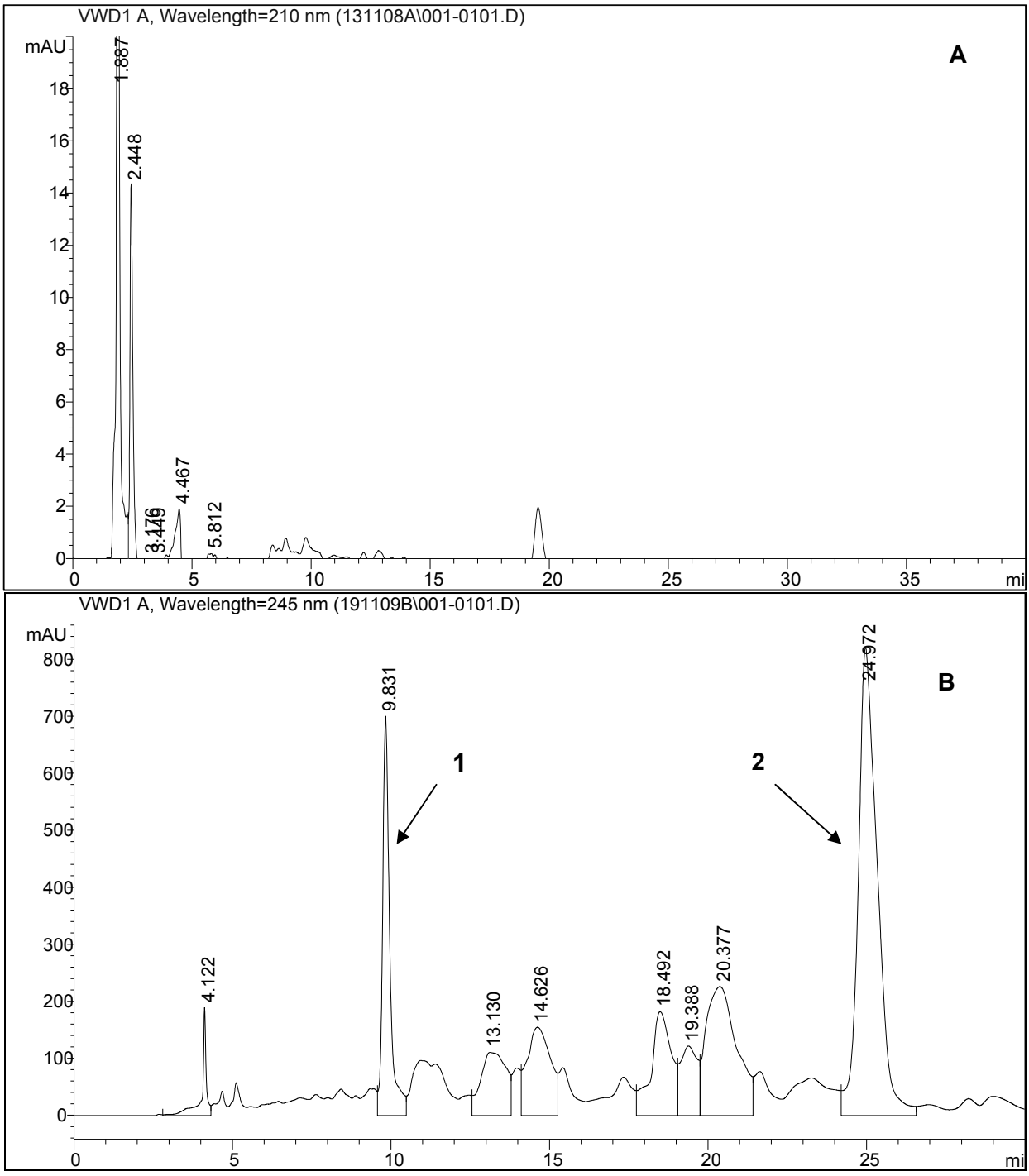


Table 8.2: A summary of statistical analysis of marker peak retention time shifts

Runs	Peak 1	Peak 2	Peak RT difference
1	9.8	24.9	15.1
2	9.5	23.9	14.4
3	9.8	25.3	15.5
4	8.3	23.9	15.6
Mean	**9.35**	**24.5**	**15.15**
SD	**0.71**	**0.71**	**0.54**

Key: **Peak RT (Retention time) difference** = Marker peak 2 retention time (-) marker peak 1 retention time

8.5 DISCUSSION

The findings suggest that High performance liquid chromatography (HPLC) using a UV detector can be used to identify and quantify crude phela. The retention time was within the set limits; marker peak (1) was 9.35 ± 0.71 minutes and marker peak (2) 24.5 ± 0.71 minutes with a distance of 15.15 ± 0.51 between the two peaks. This method is versatile as it can identify phela at lower concentration and quantify it. According to Springfield (2005) and co-workers, when reporting results of pharmacological research on herbal medicines often based on whole plant or partially fractionated extraction, HPLC_UV profile of active extracts is useful, where identity of individual compound is not known or finally clarified. Furthermore in the review by Liang et al (2004), "HPLC and reverse-phase columns are the most popular in the analytical separation of herbal medicines". He further states that retention time shifts are common during herbal medicine fingerprinting, and the source is very complex, varying from analytical processing to the instrumentation, etc.

Nevertheless, a retention time shift of marker peaks was observed throughout the study. Due to a lack of the software often used for retention time shift correction it was opted to standardize the marker peaks by preparing the extracts in replicate. Thereafter, calculation was done of the mean and standard deviation of each marker peak and the time difference of the two marker peaks. UV spectra analysis was evaluated to confirm any similarities or differences in the peaks' spectra. Despite a good separation achieved with this method, single wavelength detectors are sometimes unable to detect all compounds due to non-chromophoric compounds found in herbal medicines. Hence a need to further develop an HPLC method with other available detectors, using a fluorescence detector. This method is described in the following chapter. Moreover, the described method will be adopted when developing an HPLC_UV method for monitoring of phela in plasma in the chapters that follow.

8.6 CONCLUSION

An HPLC_UV chromatographic profile by which to identify phela has been successfully developed.

CHAPTER 9

DEVELOPMENT OF A METHOD FOR IDENTIFICATION OF PHELA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH A FLUORESCENCE DETECTOR (HPLC_FL)

9.1 SUMMARY

High performance liquid chromatography with a fluorescence detector (HPLC_FL) method for identifying phela is presented. Various pre-column derivatization methods were evaluated, also the different wavelengths were tested for emission and excitation during the development process. The recommended method is described. Phela is treated with 1 g Ammonia sulphate {$(NH_4)_2SO_4$} “salting-out.”, Iso–propanol (3 ml) was added and left to shake for 10 minutes, then centrifuged at 4 °C and 2 500 rpm (1 251g) for 10 minutes. Then it was evaporated under nitrogen at 47 °C and the residue reconstituted with mobile phase, and 50µl was injected onto the HPLC. The HPLC conditions were as follows: The eluting mobile phase was 70 % acetonitrile at a flow rate of 0.5 ml/min. Separation was done on a reverse phase C_{18} (250 mm x 4.6 mm x 5µm) column coupled to a C_{18} guard column and detected by a fluorescence detector set at 210 nm and 290 nm for emission and excitation and 40 minutes run time. The HPLC retention time for marker peak (a) is at (12.34 ± 0.36) minutes, for marker peak (b) at (22.3 ± 1.18) minutes, for marker peak (c) at (32.96 ± 0.88) and for marker peak (d) (38.32 ± 0.88) with a time difference of (10.12 ± 1.13) minutes between peaks a and b, (20.68 ± 1.22) minutes between peaks a and c and (25.98 ± 1.42) minutes between peaks a and d . In conclusion a HPLC_FL method for phela identification has been developed.

9.2 INTRODUCTION

In this chapter, a hyphenated procedure (HPLC_FL) is applied to further develop a method to fingerprint phela. Various pre-column derivatization methods were evaluated during development, to increase selectivity/sensitivity and to identify other compounds not visible under UV detection.

9.3 MATERIALS AND METHODS

9.3.1 Apparatuses

A vortex mixer (Scientific Industries Inc, U.S.A) was used for mixing. A 5810 R centrifuge and mini spin (Eppendorf, Germany) were used for centrifuging and quick spinning of samples. Precision balances SBC 31 and SPB 52 (Scaltec Instruments, Germany) were used for weighing milligram and gram quantities, respectively. A nitrogen evaporator with a heating block was used for evaporating samples. Finnpipettes from Thermo Labsystems (Canada) were used for spiking volumes. An attached glass pipette attached was used for adding extraction solvents.

9.3.2 Chemicals and reagents

HPLC graded solvents: hexane, methanol, acetonitrile, diethyl ether, and toluene (Burdick and Jackson, U.S.A) iso-propanol, acetic acid and hydrochloric acid (Merck, Germany) were used during extraction. Ammonia sulphate was purchased from Sigma–Aldrich (Germany). Deionised water was prepared by Millipore water system (Milli –Q^{Tm}).

9.3.3 Chromatographic system and conditions

Chromatographic system: The HPLC system consisted of a Hewlett-Packard 1100 series system that comprised of an isocratic pump, an auto-sampler and an hp 1046 fluorescence detector. Data were collected with LC Chem stations software.

Chromatographic conditions: Chromatographic separation was achieved with a reverse phase C_{18} (250 mm x 4.6 mm x 5 microns) coupled to a C_{18} guard column

(Phenomenex, U.S.A). Acetonitrile: water (70:30; v/v) was mobile phase, the flow rate was 0.5 ml/min and the run time was 40 minutes. Different fluorescence wavelengths were evaluated during development.

9.3.4 Optimization of sample extraction

9.3.4.1 Acidic extraction method

Phela (50 mg) was extracted with 5 ml of hexane, acidified with 0.1M HCl (100 µl) and horizontally shaken for 15 minutes. The samples were centrifuged at 4 °C and 2 500 rpm (1 251g) for 10 minutes. The organic layer was evaporated under nitrogen at 47°C, and residues reconstituted with 100 µl of mobile phase and 50 µl was injected onto HPLC for analysis.

9.3.4.2 Derivatization method (1)

Sample a: 50 mg of phela was sonicated with 5 ml of extraction solvent [methanol: water acetic acid, (70:25:5; v/ v/ v)] for 30 minutes. It was centrifuged for 10 minutes at 4000 rpm (3 202 g) at 4°C. The supernatant was analysed by HPLC_FL.

9.3.4.3 Derivatization method (2): Addition of Zinc powder

The first part was prepared similarly to samples a, above: To 1 ml of sample a supernatant was derivatized by adding 10 mg of zinc powder at room temperature for 15 min, whilst vortexing periodically. The sample was centrifuged for 10 minutes at 4000 rpm (3 202 g) at 4°C and analysed with HPLC_FL.

9.3.4.4 Derivatization method (3): Salting out procedure

Phela (100 mg) was treated with 1 g Ammonia sulphate {$(NH_4)_2SO_4$} “salting-out.” Iso–propanol (3 ml) was added and left to shake for 10 minutes. Centrifugation of the sample for 10 minutes at 2500 rpm (1 251 g) at 4°C followed. The supernatant was evaporated under nitrogen at 47 °C and the residue reconstituted with 200 µl of

mobile phase. It was run on HPLC. Various concentrations in the range of 30 – 100 mg/ml of crude product were extracted with the salting-out method and analysed.

9.3.5 Optimization of wavelength

The 1046 Fluorescence detector was set at 393nm for excitation and 455 nm for emission wavelength (Chan et al, 2007). The other wavelengths tested were 210 nm for excitation and 290 nm for emission.

9.4 RESULTS

9.4.1 Optimization of extraction method and wavelength

Extraction with the acidic method did not produce any peaks. This led to evaluation of derivatization methods that could enhance fluorescencing molecules in extract mixtures. The fluorescence detector settings at 393nm for excitation and 455 nm for emission wavelength gave no peaks. The settings at 210 nm for excitation and 290 nm for emission were evaluated further.

9.4.2 Chromatographic performance

Figure 9.1 (p 100) is a blank chromatogram. Figure 9.2 (p 100) is a chromatogram of extracts prepared by the derivatization methods 1 and 2. Peaks were visible with retention times of 12.1, 23.7 and 32.8 minutes and had a low absorbance (%*F). The chromatogram peak profile was similar in both derivatization methods 1 and 2.

Figure 9.3 (p 101) is a blank chromatogram. Figure 9.4 (p 101) is chromatogram of an extract prepared by the salting-out extraction method. Four peaks were selected and set as marker peaks. Four marker peaks were identified as peak a, b, c and d at a retention time of 12.7, 22.8, 32.3 and 38.0 minutes, respectively. The higher concentration (30 – 100 mg/ml) had a similar peak profile with increasing peak heights (%*F). Figure 9.5 is a chromatogram of higher concentration (80 mg/ml) of phela extract.

Table 9.1 (p 102) is a statistical summary of the retention time shifts of marker peaks a, b, c and d observed throughout the study and the time difference of peaks.

The standardized retention time in minutes for peak a to d are as follows (12.34 ± 0.36) for a, (22.3 ± 1.18) for b, (32.96 ± 0.88) for c, and (38.22 ± 0.98) for d. The time differences for a & b are 10.12 ± 1.13, a & c are 20.68 ± 1.22 and a & d are 25.98 ± 1.42. Table 9.1 (P 102) is a summary of marker peak retention time shifts and the marker peaks time differences.

9.4.3 The recommended method conditions

Sample preparation

Phela (100 mg) is treated with 1 g Ammonia sulphate {$(NH_4)_2SO_4$} “salting out.” Iso–propanol (3 ml) is added and left to shake for 10 minutes, then centrifuged at 4 °C and 2 500 rpm (1 251g) for 10 minutes. The organic layer is evaporated under nitrogen at 47°C, and residues reconstituted with 100 µl of mobile phase and 50 µl is injected on to HPLC for analysis.

HPLC conditions

The sample is eluted with 70 % of acetonitrile in water and separated on a reverse phase a C_{18} (250 mm x 4.6 mm x 5 µm) column coupled to a C_{18} guard column at a flow rate of 0.5 ml/min. The fluorescence wavelength is set at 210 nm for emission and 290 nm for excitation for 40 minutes.

HPLC_FL Chromatogram Profile

The retention time for marker peak (a) is at (12.34 ± 0.36) minutes, marker peak (b) (22.3 ± 1.18) minutes, marker peak (c) (32.96 ± 0.88) and marker peak (d) (38.32 ± 0.88) with a time difference of (10.12 ± 1.13) minutes between peak a and b, (20.68 ± 1.22) minutes between peak a and c and (25.98 ± 1.42) minutes between peak a and d.

Figure 9.1 A blank chromatogram after HPLC_FL analysis

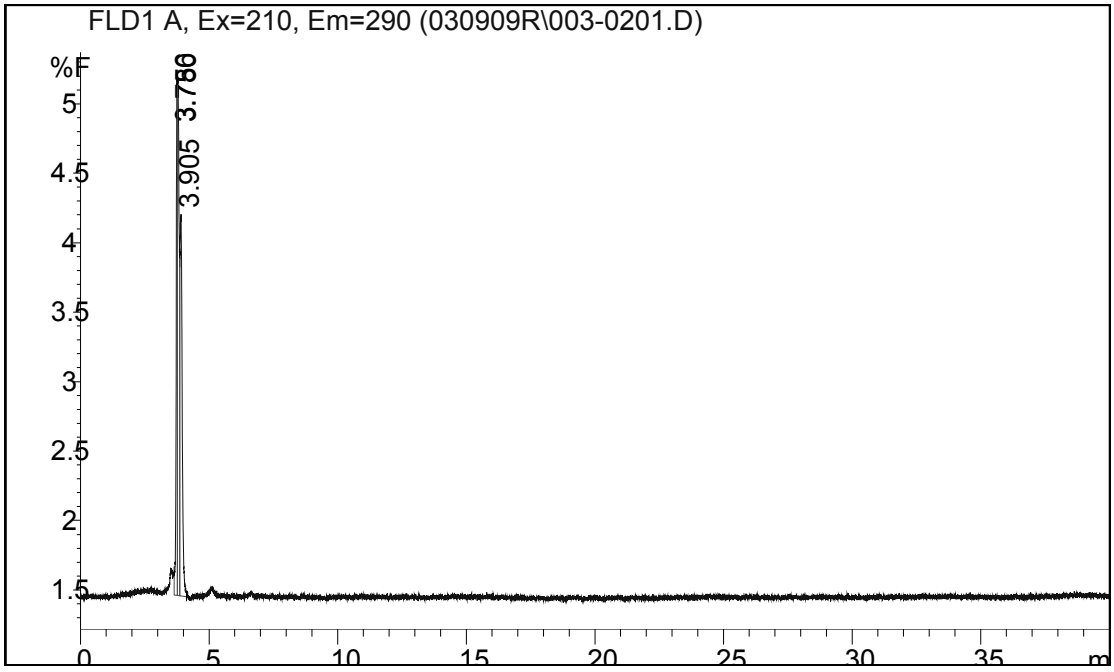


Figure 9.2 A chromatogram of derivatization methods 1 and 2 extracts after HPLC_FL analysis with peak A, B and C at 12.1, 20.6 and 32.8 minutes

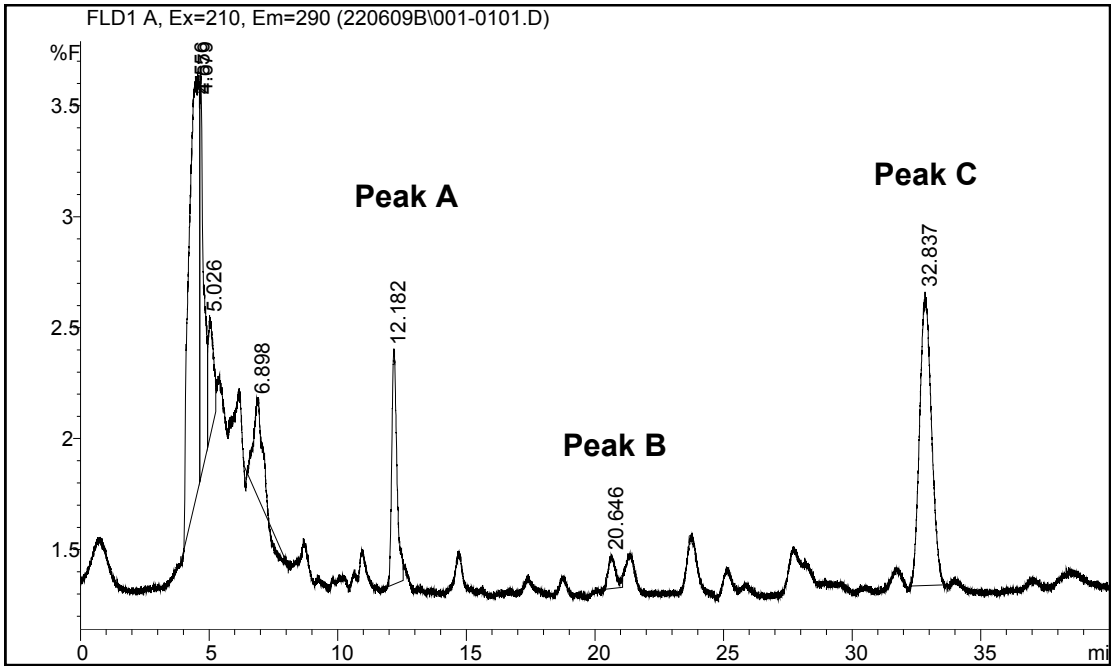


Figure 9.3: A blank chromatogram after HPLC_FL analysis

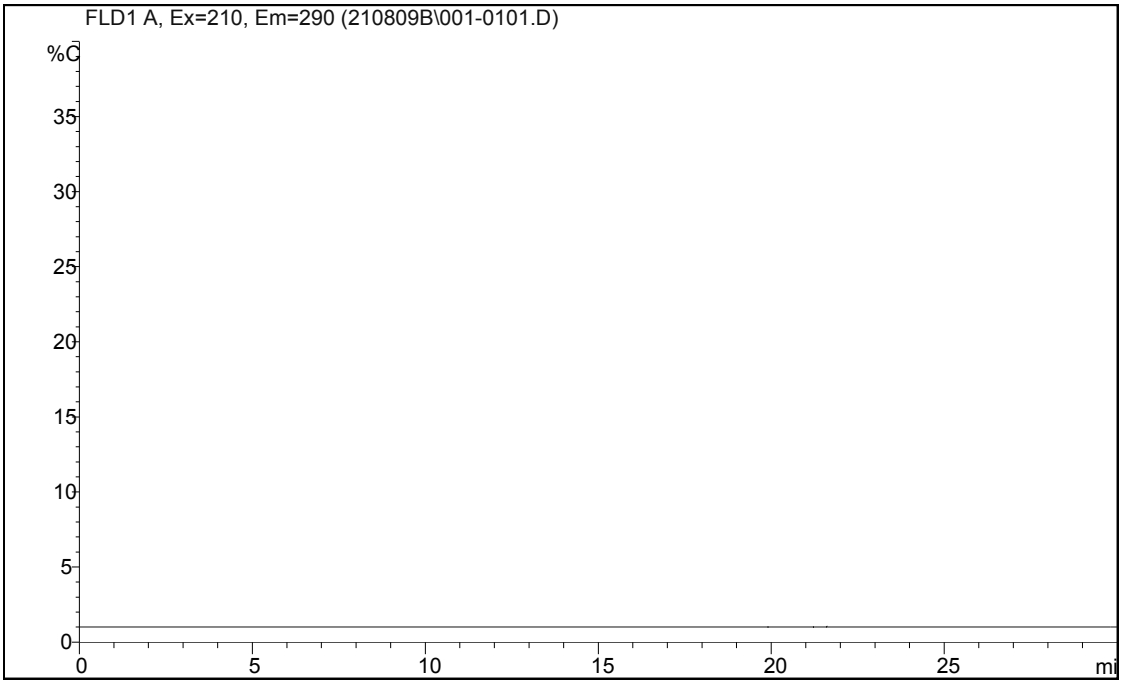


Figure 9.4: A chromatogram a salting-out method extract (20 mg/ml), with marker peaks a to d at a retention time of 12.7, 22.8, 32.3 and 38.0 minutes

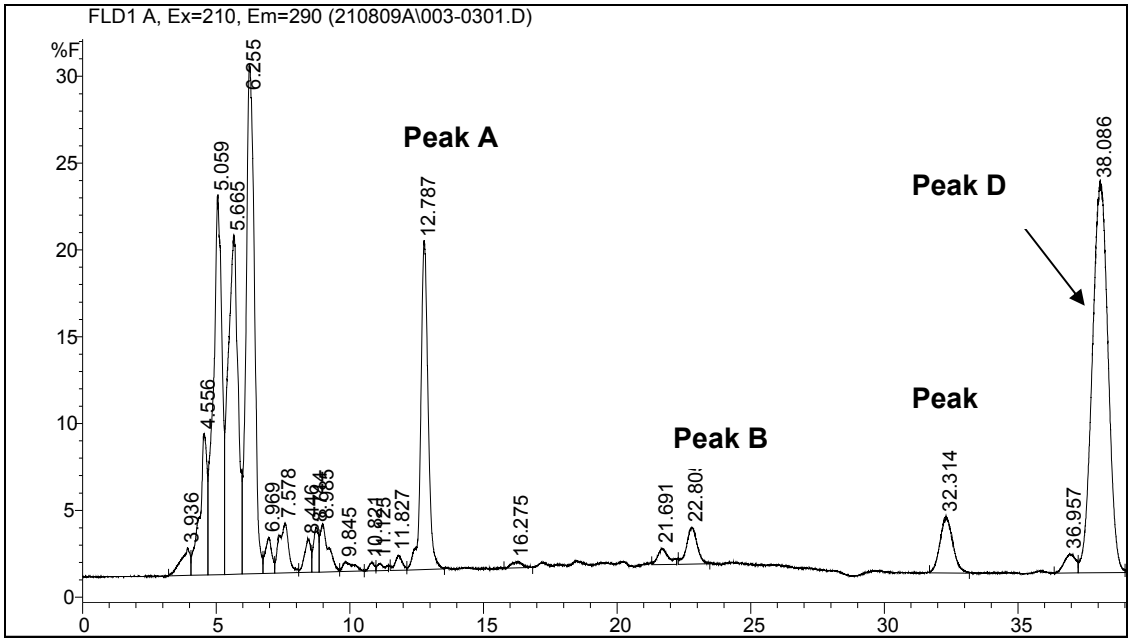


Figure 9.5 Chromatogram of an 80 mg/ml sample prepared by salti- out method; with marker peaks a to d at a retention time of 12.1, 22.8, 32.3 and 38.0 minutes

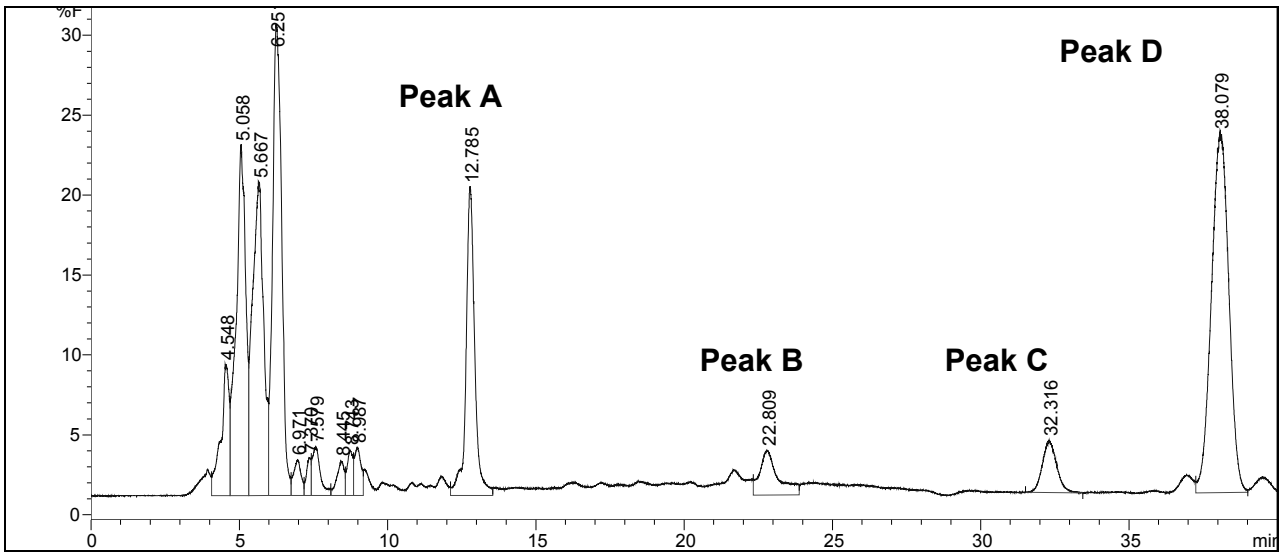


Table 9.1: A summary of statistical analysis of marker peak retention time shifts after HPLC_FL analysis.

Runs	**Marker peaks retention time (min)**				**Peaks RT difference (min)**		
	a	**b**	**c**	**d**	**a & b**	**a & c**	**a & d**
1	12.1	23.7	32.8	38.3	11.6	20.7	26.2
2	12.7	22.8	32.3	38.0	10.7	19.6	25.3
3	12.0	20.6	33.3	38.0	8.6	21.6	25.6
4	12.7	22.8	32.3	37.7	10.1	19.6	25.4
5	12.2	21.8	34.1	39.6	9.6	21.9	27.4
Mean	**12.34**	**22.3**	**32.96**	**38.32**	**10.12**	**20.74**	**25.98**
SD	**0.36**	**1.18**	**0.88**	**0.88**	**1.13**	**1.01**	**1.42**

Key: **Peak RT (Retention time) difference** = Marker peak b retention time - marker peak a retention time. **Min** = Minutes, **SD** = Standard deviation.

9.5 DISCUSSION

The results suggest that High performance liquid chromatography with a fluorescence detector (HPLC_FL) was used to develop a fingerprinting method for identifying phela. The salting out pre-column derivatization method increased sensitivity and selectivity for fluorescence detection, whereby more peaks were observed with the fingerprint. Several reviews (Liang et al, 2004 and Lin et al, 2001) have been published for the analysis of the bioactive chemical compounds in plants and herbal medicines, in which HPLC is the most used technique, especially hyphenated techniques, in our context, fluorescence (HPLC_FL) for fingerprinting. HPLC_FL successfully developed a time profile for phela identification and the method conditions will be used to develop an HPLC_FL for phela monitoring in plasma.

9.6 CONCLUSION

An HPLC_FL chromatographic profile by which to identify phela has been successfully developed.

CHAPTER 10

A COMPREHENSIVE APPROACH FOR AUTHENTICATION OF PHELA

10.1 SUMMARY

Here, a comprehensive approach for authentication of Phela is described. The approach comprises the developed fingerprinting methods for Phela presented in previous chapters 7 – 9. It involved extraction of phela by either acidic extraction or a simple 'salting-out' method, followed by Thin Layer Chromatography (TLC) and/or preparative Column Chromatography (CC) that was supported by High Performance Liquid Chromatography with UV-detector (HPLC-UV), HPLC with fluorescence-detector (HPLC-FL), HPLC with Photo Diode Array detector (HPLC-PDA) and Gas-Chromatography- Mass Selective Detector (GC-MSD) spectrometry. The fingerprints were successfully used to differentiate Phela from another herbal product made from *Hypericum perforatum* (St. John's Wort), thereby illustrating its potential for use in fingerprinting other herbal medicines in development. By validating the different chromatographic techniques on a standardized extraction process, this approach will enable wide application in quality control and the use of internationally acceptable procedures, thereby promoting effective monitoring of the finished product in all countries that will use it.

10.2 INTRODUCTION

In this chapter a comprehensive chromatographic fingerprint method for phela identification and used for quality control purposes is explained. Two extraction methods were used followed by thin layer chromatography (TLC), and/or preparative column chromatography (CC), high performance liquid chromatography with UV (HPLC_UV), HPLC with fluorescence detectors (HPLC_FL), HPLC with photo diode array (HPLC_PDA) and Gas -chromatography mass selective detector spectrometry (GC_MSD). This approach was used to differentiate between phela and a herbal product of *hypericum perforatum* (St john's wort).

10.3 MATERIALS AND METHODS

10.3.1 Apparatuses

A vortex (Scientific Industries Inc; U.S.A) was used for mixing. A 5810 R centrifuge and mini spin (Eppendorf; Germany) were used for separating samples. Precision balance (SBC 31 and SPB 52) (Scaltec Instruments; Germany) were used for weighing miligrams and grams respectively. A nitrogen evaporator with a heater block was used for evaporation.

TLC: TLC plates pre-coated with silica gel 60 F 254, a TLC tank (5L) with a glass lid, glass cutter and a laboratory spray gun (all Shandon Scientific Co; England). Micro capillary pipettes (Einmal; Germany) were used to load the sample. A ruler in centimeters, pencil, hair dryer, timer, calculator. UV light were all used during TLC analysis.

CC*:* A 25 ml glass pipette (Silberbrand; Germany), test tubes for collection and a stop watch for keeping time.

10.3.2 Chemicals and reagents

Phela (Medical research council Indigenous Knowledge System Lead Programme; S.A). *Hypericum perforatum* (St. John's wort) from Zauberkrant, harvested in December 1999. Extraction solvents: dichloromethane, hexane, methanol,

acetonitrile, diethyl ether, toulene (Burdick and Jackson; U.S.A). Acetic acid, iso-propanol (2-propanol), ethyl acetate, n-butanol and ammonia (Merck; Germany). Sodium hydroxide, ammonia sulphate were purchased from Sigma –Aldrich (Germany). Visualizing agents (Iodine, Iodoplatinate, dragendorff and copper chloride) were a donation from the toxicology laboratory. Deionised and distilled water was prepared by Millipore water system (Milli –Q^{Tm};).

10.3.3 Sample Extraction

10.3.3.1. Acidic extraction method

Phela (200 mg) was extracted with 5 ml of hexane by shaking horizontally for 15 minutes at room temperature and acidified with 100 ul of 0.1M of HCl. The sample was then centrifuged at 2 500 rpm (1 251 g) at 4°C for 10 minutes. The organic layer was evaporated under nitrogen at 47°C and the residue was reconstituted with 100ul of mobile phase, specific for each chromatographic process.

10.3.3.2. Salting-out method

Phela (200 mg) in 3 ml of iso-propanol was treated with 1 g of ammonia sulphate, centrifuged at 2500 rpm (1251 g) at 4°C for 5 minutes. The supernatant was evaporated under nitrogen at 47 °C and the residue was reconstituted with 200 µl methanol. This method was used for samples analysed by TLC, HPLC-FL and GC/MSD.

10.3.4. Chromatographic method

All chromatographic methods were developed and validated in our laboratory.

10.3.4.1. TLC analysis

The TLC consisted of a glass TLC plate coated with silica gel (stationary phase), a 5 L capacity TLC tank with a lid and ethyl acetate: ammonia: methanol (17: 1: 2; v/v/v) as mobile phase. A capillary pipette was used to spot (load) samples on the TLC plate 1 cm apart and 1 cm from the bottom of the plate. Each sample was loaded at

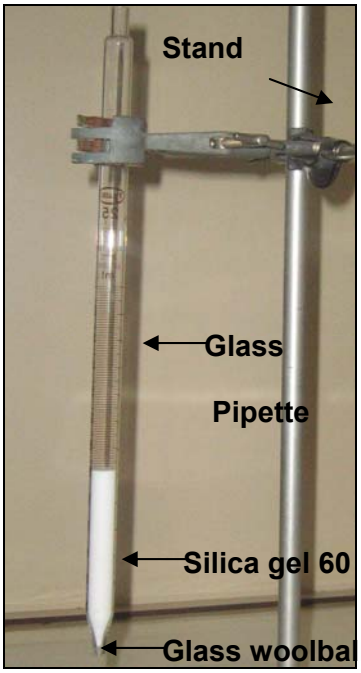
a specified point marked with the respective sample's code-name. The samples were dried with a hair dryer. The sample-loaded TLC plate was then placed in the humid tank with 20 ml of the mobile phase and left to run for one hour. Thereafter, the bands were visualized under UV light during which time they were marked with a pencil. Therefore, a pencil-line was drawn marking the solvent front, and the retention factors for each band (compound) were calculated as distance (cm) travelled by each band divided by distance (cm) travelled by the solvent front. Thereafter, the marked bands were scraped off, reconstituted in 1 ml of 70% acetonitrile and kept for analysis by HPLC- UV detector (HPLC_UV) or HPLC-fluorescence detector (HPLC_FL) and Gas-chromatography – Mass spectrometry (GC_MSD).

10.3.4.2. Column chromatography

Column chromatography consisted of a 25 ml glass pipette that was blocked with a small glass woolball at the tip to prevent spillage and then filled with 13 ml of silica gel 60. The filled glass pipette, now the column, was held upright with a clamp illustrated in figure 10.1; p 108. A methanolic solution of phela (200 ul) was loaded on the silica gel and eluted under gravity with 20 ml of ethyl acetate: ammonia: methanol (17:1:2; v/v/v) mobile phase and fractions of the eluent were collected every minute (elution started after 9 minutes of adding the mobile phase) in different test tubes.

Thereafter, 46 fractions were collected. The fractions were labelled according to number of minutes elapsed after mobile phase addition. They were evaporated under nitrogen at 47 °C and the residue was reconstituted with 70 % acetonitrile. Preliminary experiments indicated that only the first 12 fractions (collected between 9 – 20 minutes) contained major peaks, and as such only these 12 were taken for further analysis by HPLC_UV, HPLC_FL and GC_MSD.

Figure 10.1: A picture illustrating a column chromatography apparatus set up [by author]



10.3.4.3. HPLC analysis

10.3.4.3.1. HPLC_UV

For HPLC_UV analysis, the HPLC system was a HP (Hewelt-Parkard) 1100 series consisting of an isocratic pump, a UV detector set at 245 nm and computer (Chem-data station) software. The mobile phase consisted of acetonitrile: deionised water (70:30 v/v) and was run at a flow rate of 0.5 ml/min for 40 min (run time) on a reverse phase C_{18} column (250 mm x 4.6 mm x 5 µm) coupled to a C_{18} guard column and run for 30 minutes. Samples for HLPC_UV detection were prepared by the 'acidic extraction method' as described earlier (section 10.3.3.1) and the HPLC Injection volume was 50µl.

10.3.4.3.2. HPLC_PDA

The HPLC_PDA was used to obtain the UV-absorption spectra of Phela's marker peaks, and the same conditions as under HPLC_UV were used. This was because this system (HPLC_PDA) was not fully available for this project. Furthermore, the HPLC_UV could not generate UV spectra.

10.3.4.3.3. HPLC_FL

For HPLC_FL analysis, the HPLC-system and chromatographic conditions were the same as for HPLC_UV except for the programmable fluorescent detector (HP-1046A; made in Germany) with Emission and Excitation wavelengths set at 210 nm and 290 nm, respectively. Samples for HLPC_FL detection were prepared by the salting-out method described earlier and the HPLC injection volume was 50µl.

10.3.4.4. GC_MSD analysis

10.3.4.4.1. GC_MSD system and conditions

The GC_MSD model came from Agilent Technologies (made in China) equipped with gas chromatography, a mass selective detector (MSD) and auto-injector of 6890, 5973 and 7883 Network series, respectively. The MSD was equipped with a commercial library of compounds by which the analytes were screened. The carrier gas was helium and the column was an HPS MS cross linked 5 % Phenyl methanol silicone (15 m x 0.75mm) and ID 0.75 µm film thickness, with the oven temperature set at 300 °C.

10.3.4.4.2. Sample preparation

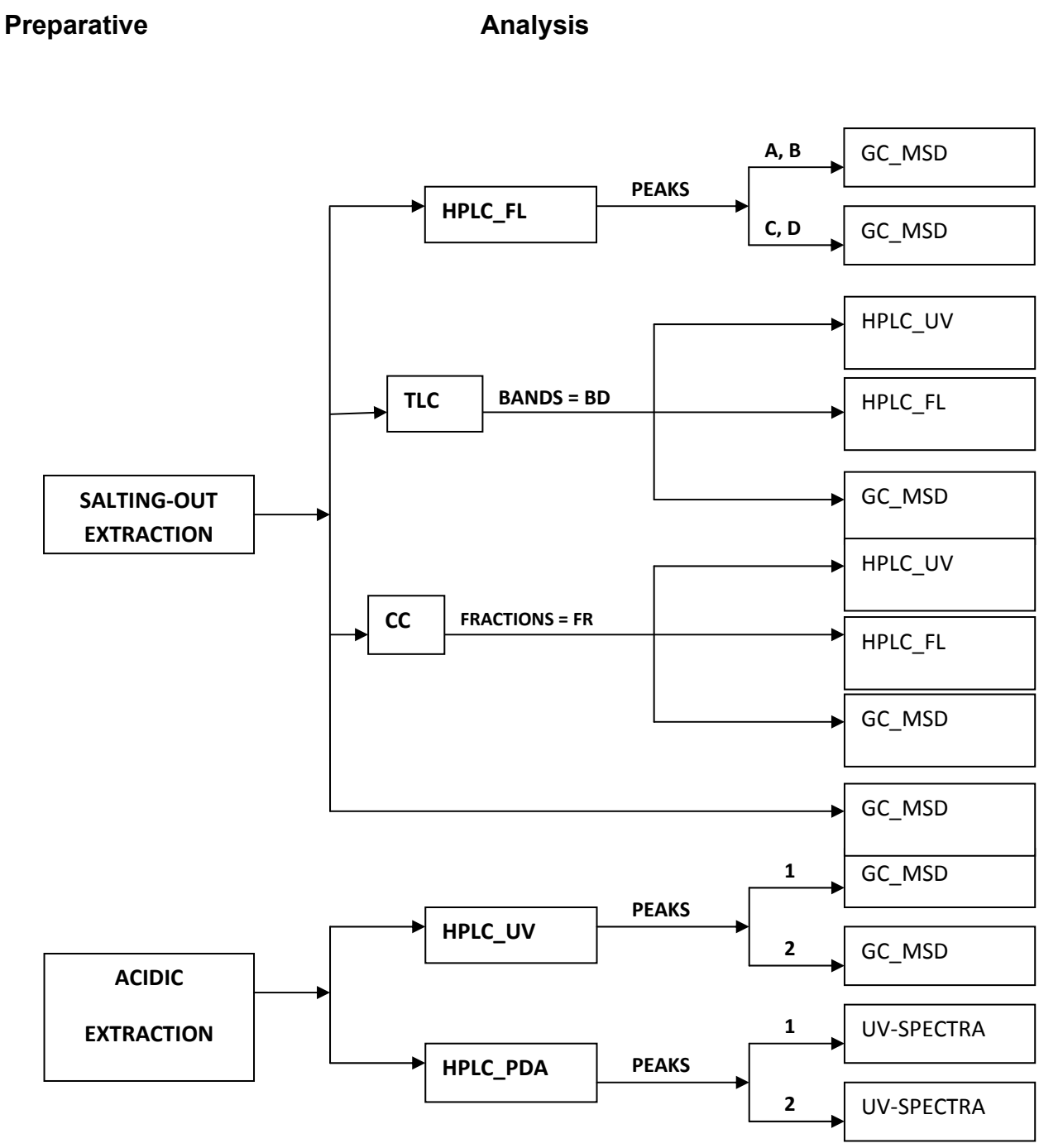
All the samples of the TLC collected bands, as well as fractions from CC and HPLC, were processed further before GC_MSD analysis. The samples were evaporated under nitrogen at 47 °C and the residue reconstituted into 5 ml of water, followed by 0.5 ml of 20 % NaOH. The mixture was extracted with 5 ml diethyl ether by shaking gently for 5 minutes on a horizontal shaker. The supernatant was transferred into 5 ml ampoules and evaporated under nitrogen without heating. The residue was reconstituted into 100 µl toluene followed by vigorous vortexing, after which it was transferred into a GC –Vial and loaded on the GC_MSD for analysis. Only 2 µl was injected.

10.3.5. Study design

Figure 10.2 (p 111) illustrates the study design, i.e how the different chromatographic techniques were integrated. The TLC, HPLC_UV, HPLC_FL and GC_MSD fingerprints of the crude phela were obtained by extracting phela powder either by the 'acidic extraction' method (n = 5) for HPLC_UV detection, or by the 'salting out' method for HPLC_FL and GC_MSD methods as well as for TLC and CC processes (n = 5). Thereafter each of the TLC bands were analysed further by HPLC_UV, HPLC_FL and GC_MSD, while the marker peaks of crude phela were collected and analyzed further. The marker peaks in the HPLC_UV chromatograms were analysed for UV-absorption spectra and GC_MSD, while those of HPLC_FL were analysed by GC_MSD only.

For CC, the different collected fractions were analysed by HPLC_UV, HPLC_FL and GC_MSD. The chromatograms of TLC, HPLC_UV, HPLC_FL and GC_MSD analysis were evaluated and, in each case, the most appropriate chromatogram for the respective TLC-band, CC-fraction (s), marker peaks and or crude extract was selected for making a fingerprint. Thereafter, the retention time for the fingerprint chromatograms were standardized by determining the average retention times of marker peaks in the respective chromatograms of samples prepared and run on 5 different days. For the GC_MSD, abundance as indicated by the peak-height was also used to differentiate extracts.

Figure 10.2: Study design of chromatographic methods used for phela fingerprinting



10.3.6. Comparison of Phela and Hypericum Perforatum

The afore-mentioned fingerprints were used to differentiate phela from a preparation of *Hypericum perforatum* products (St John's wort; SJW), a traditional medicine commonly used for depression and many other ailments. Phela (200 mg) and *hypericum perforatum* (200 mg) were extracted and processed as already explained. Figure 9.3 shows the chromatographic techniques that were selected for comparison. The retention times of major peaks in SJW chromatograms were compared with those of peaks in phela fingerprint chromatograms.

10.3.7. Data analysis

For qualitative analyses of chromatographic fingerprints, marker peaks (peaks that showed relatively high content) were selected and used due to a lack of known standards that could have been used as markers and calculation of time differences amongst the peaks. Secondly, the low standard deviation and mean value were indicative of co-identification amongst the peaks. Quantitave analysis was based on abundance, peak area or %*F (units on the HPLC_FL chromatograms) where applicable.

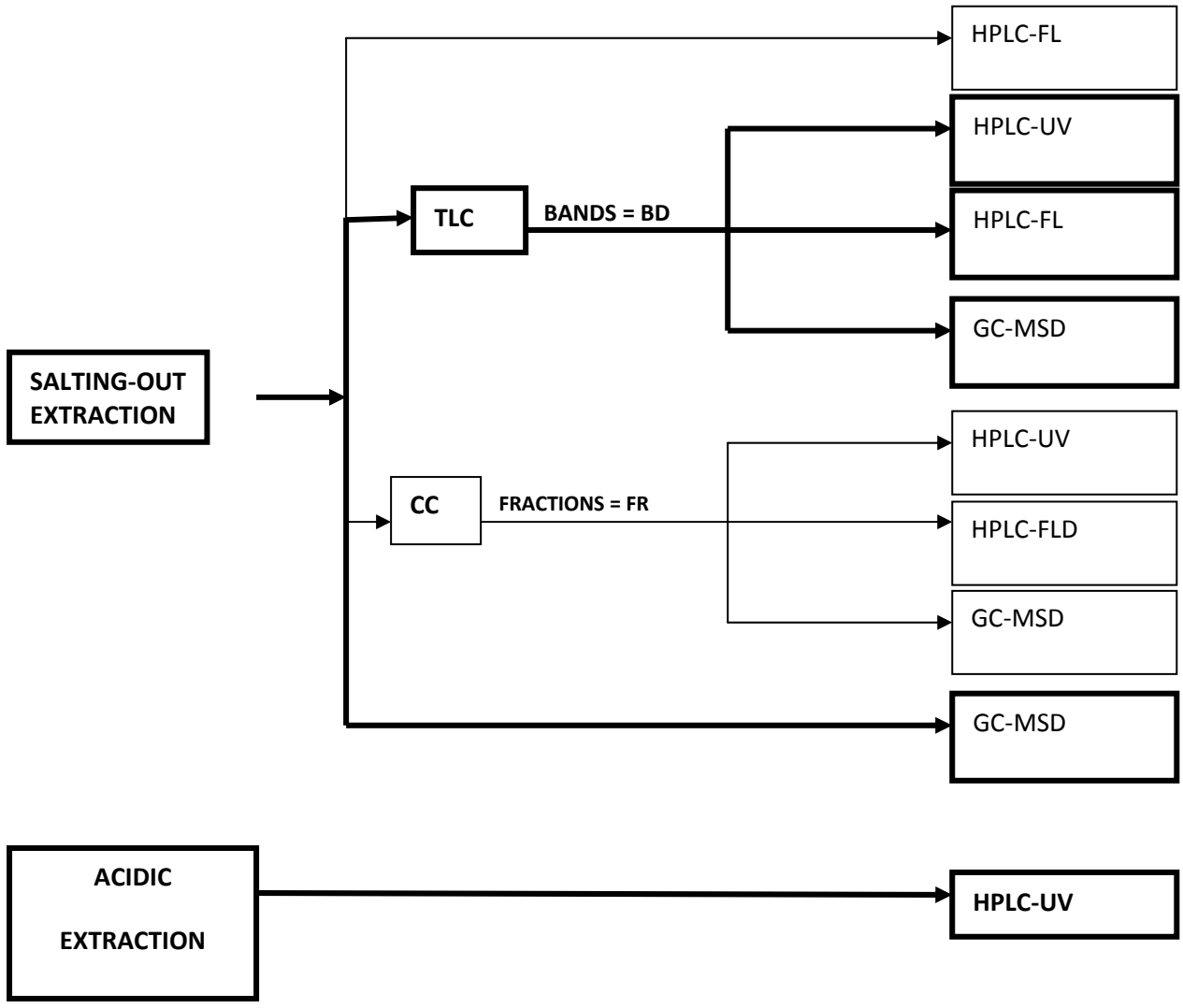
TLC analysis was done on the bands observed and their derived retention factors. Only two bands were further analysed by other methods.

CC analysis was made based on the observation of separated bands during the experiment, and only the 12 fractions (collected between 9 - 20 minutes) collected from the start of elution were further supported by other methods.

During ***HPLC*** analysis, marker peaks were selected based on their stability and higher peak area and/abundance. Primarily marker peaks (2 for HPLC_UV/HPLC_PDA analysis; 4 for HPLC_FL analysis) were established during the crude extract analysis from amongst many other peaks. For both bands and fractions HPLC analysis, all the observed peaks were used as the chromatogram had fewer peaks present.

GC_MSD analysis was based on all peaks observed and their abundance.

Figure 10.3: Study design of selected chromatographic methods to compare phela and St John's wort



10.4 RESULTS

10.4.1. Thin Layer Chromatography (TLC) plus HPLC_UV HPLC_FL & GC_MSD

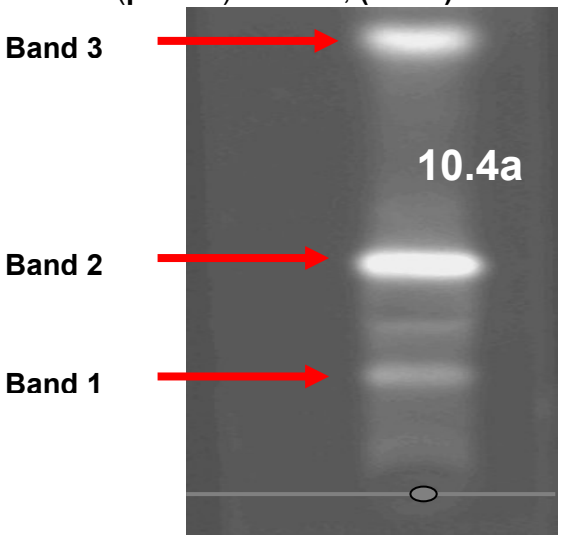
10.4.1.1. Analysis of crude phela

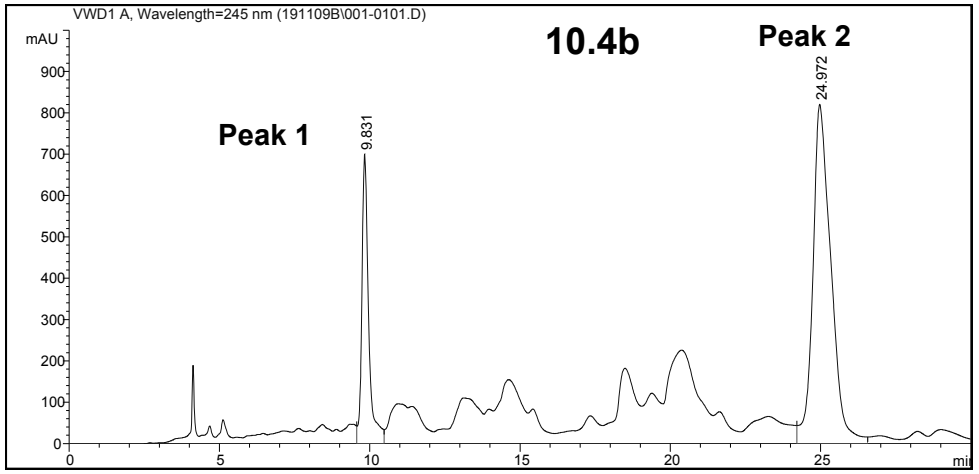
Figure 10.4 (p 115) shows the TLC, HPLC_UV, HPLC_FL and GC_MSD fingerprints of crude Phela. The representative TLC chromatogram (figure 10.4 a; p 115) exhibited three distinct bands with retention factors of 0.24 for band 1, 0.31 for band 2, and 0.92 for band 3, which are within the standardized range of 0.23±0.01, 0.31±0.02, 0.91±0.02, respectively (Table 10.1; p 116). Of note, bands 1 and 2 were brownish while band 3 was orange in color.

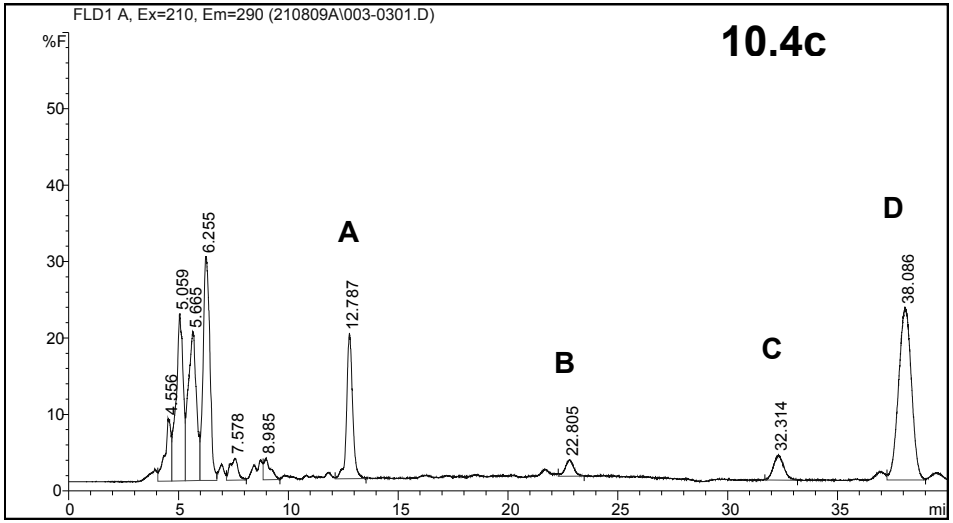
The HPLC_UV chromatogram had two marker peaks at 9.8 (peak 1) and 24 (peak 2) (Fig. 10.4b; p 115), and the established range for these peaks was 9.35±0.71 minutes for peak 1, and 24.5±0.71 peak 2 (Table 10.2; p 116). In figure 10.4c (p 115), the HPLC_FL chromatogram had four marker peaks at 12 (peak A), 22.81(peak B), 32.31(peak C) and 38.0 (peak D) minutes, and the standardized range for this fingerprint was 12.34 ± 0.36, 22.3±1.18, 32.96±0.88, 38.32±0.98 minutes, respectively (Table 10.3; p 117).

The GC_MSD chromatogram of the salted-out extract of crude Phela had marker peaks at 2.26, 6.3, 6.74, 7.25, 7.78 and 7.93 minutes (Fig. 10.4d; p 115), and the standard retention times were set at 2.23±0.04, 6.25±0.05, 6.74±0.07, 7.24±0.02, 7.77±0.05 and 8.01±0.09 minutes (Table 10.4; p 117).

Figure 10.4: **(10.4a) -** A TLC chromatogram of *Phela* extract run with ethyl acetate: ammonia: methanol (17: 1: 2) mobile phase. Retention factors (RF) were: band 1 = 0.24, band 2 = 0.31 and band 3 = 0.92; **(10.4b) -** An HPLC-UV chromatogram of hexane extract of crude *Phela* with marker peaks at 9.8 (**Peak 1**) and 24 (**peak 2**); **(10.4c) -** An HPLC-FL chromatogram of salted extract of crude *Phela* with marker peaks 12 (**peak A**), 22.81(**peak B**), 32.31(**peak C**) and 38.0 (**peak D**) minutes; **(10.4d)** - GC-MSD chromatogram of salted extract of crude *Phela* with marker peaks at 6.3, 6.74, 7.25, 7.78 and 7.93 minutes.







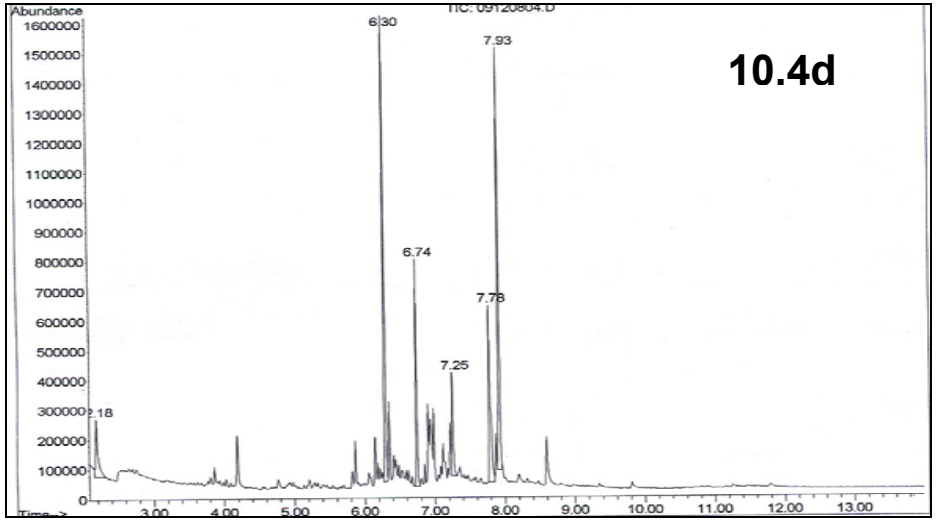


Table 10.1: A summary of retention factors (**RF)** of marker Bands on the TLC chromatogram of the crude extract of Phela in Fig 10.4a.

Run (n)	Band 1	Band 2	Band 3
1	0.24	0.31	0.92
2	0.23	0.33	0.90
3	0.21	0.28	0.88
4	0.22	0.30	0.92
Mean	**0.23**	**0.31**	**0.91**
SD	**0.01**	**0.02**	**0.02**

Table 10.2: A summary of retention times (**RT)** of marker peaks (n = 4) on the HPLC-UV chromatogram of the crude extract of Phela in (Fig 10.4b)

Run (n)	Peak 1	Peak 2
1	9.8	24.9
2	9.5	23.9
3	9.8	25.3
4	8.3	23.9
Mean	**9.35**	**24.5**
SD	**0.71**	**0.71**

Table 10.3: A summary of retention times (**RT)** of marker peaks in the HPLC-FL chromatogram of the crude extract of Phela in (Fig 10.4c)

Run (n)	Peak A	Peak B	Peak C	Peak D
1	12.1	23.7	32.8	38.3
2	12.7	22.8	32.3	38.0
3	12.0	20.6	33.3	37.6
4	12.7	22.8	32.3	38.1
5	12.2	21.8	34.1	39.6
Mean	**12.2**	**22.3**	**32.96**	**38.32**
SD	**0.28**	**1.18**	**0.88**	**0.98**

Table 10.4: A summary of retention times (**RT)** of marker peaks in the GC_MSD chromatogram (n = 3) of the crude extract of Phela in (Fig 10.4d)

Run	Peak a	Peak b	Peak c	Peak d	Peak e	Peak f
1	2.18	6.3	6.74	7.25	7.78	7.93
2	2.26	6.1	6.68	7.12	7.72	8.01
3	2.24	5.93	6.81	7.26	7.81	8.10
Mean	**2.23**	**6.25**	**6.74**	**7.24**	**7.77**	**8.01**
SD	0.04	**0.05**	**0.07**	**0.02**	**0.05**	**0.09**

10.4.1.2. Further Analysis of TLC-Bands

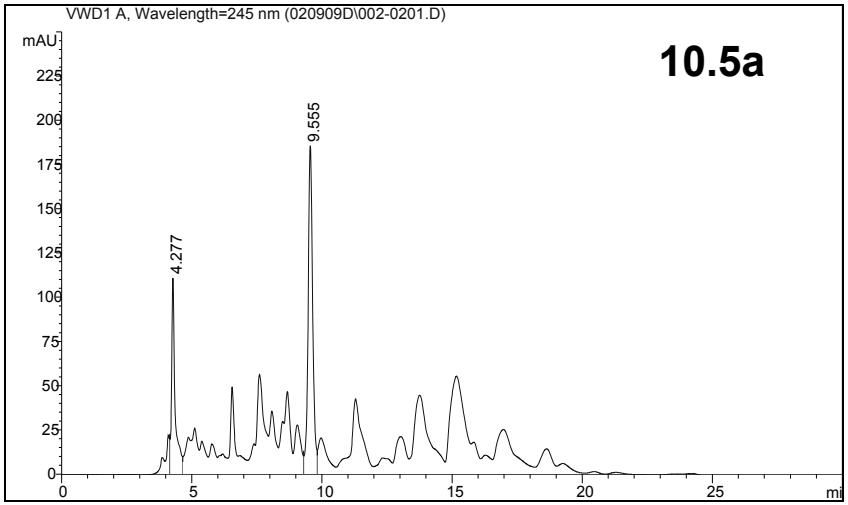
Figure 10.5 (p 119) shows the HPLC_UV, HPLC_FL and GC_MSD fingerprints of the TLC-band 2. The HPLC_UV chromatogram had two marker peaks at 4.27 and 9 minutes (Fig. 10.5a; p 119) versus the set limits of 4.28±0.02 and 9.55±0.03 minutes 9 (Table 10.5; p 120), while the HPLC_FL chromatogram had one marker peak at 6.02 minutes (Fig. 10.5b; p 119), which was within the limit of 6.60±0.01 (Tables 10.6; p 120).

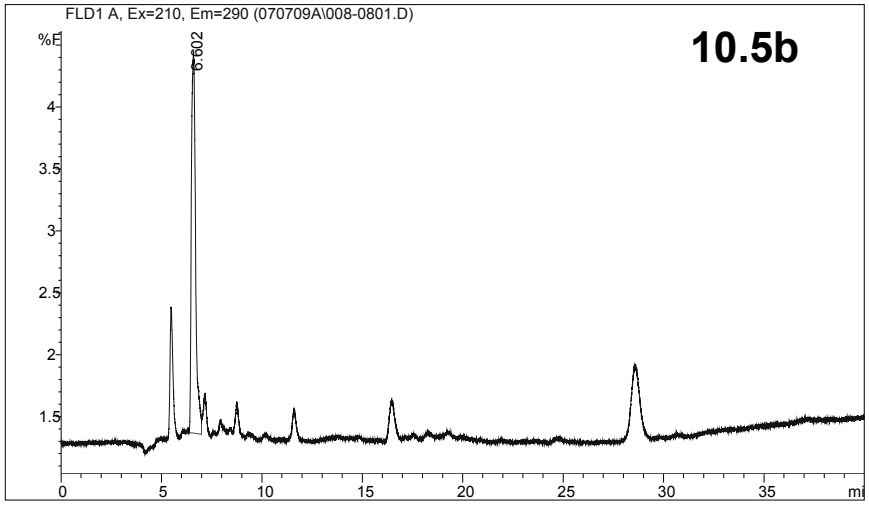
In figure 10.5c (p 119), the GC_MSD had six marker peaks at 2.26, 5.13, 5.89 7.98, 8.03 and 8.20 minutes. The respective range was set at 2.25±0.02, 5.13±0.02, 5.87±0.05, 7.90±0.09, 8.01±0.05 and 8.21±0.04 minutes (Table 10.7; p 121).

In figure 10.6 (p 122), the HPLC_UV, HPLC_FLD and GC_MSD fingerprints for TLC-Band 3 are showed. The HPLC_UV chromatogram had four marker peaks at 8.46, 14.43, 17.89 and 23.13 minutes (Fig. 10.6a; p 120), which corresponds to the set range of 8.45±0.04, 14.42±0.02, 17.87±0.08, 21.13±0.32 minutes (Table 10.8; p 123). The HPLC_FL chromatogram had three marker peaks at 8, 20, and 33 minutes (Fig. 10.6b; p 122), and the set range was 8.58±0.07, 20.69±0.04, 33.69±0.04 minutes (Table 10.9; p 123).

The GC-MSD marker peaks were at 2.26, 5.88, 6.35, 7.43, 8.01 and 9.9 minutes (Fig 10.6c; p 122), and set limits were at 2.25±0.02, 5.88±0.03, 6.35±0.11, 7.45±0.13, 8.01±0.08 and 9.86±0.05 minutes (Table 10.10; p 124). Of note, two of the peaks of band 2 were identified as diisopropylnaphthalene (DIPN) at 5.13 minutes and ferrigunol at 8.01 minutes, while for band 3, only one peak at 8.01 minutes was identified as ferrigunol by use of the MSD commercial library. Moreover, band 2 had a much higher signal at 5.88 than band 3 (abundance was 1,400, 000 vs. 400,000), which in turn had very high signal at 8.01(abundance was 2,800, 000) vs. none for band 2 (Table 10.11; p 124).

Figure 10.5: (10.5a) - HPLC-UV chromatograms of Phela TLC-Band 2 with marker peaks at 4.27 and 9 minutes; **(10.5b)** - HPLC-FL chromatograms of Phela TLC-band 2 with a marker peak at 6.02 minutes**; (10.5c)** - a GC_MSD chromatogram of Phela TLC-band 2 with marker peaks at 2.26, 5.13, 5.89 and 8.01 minutes.





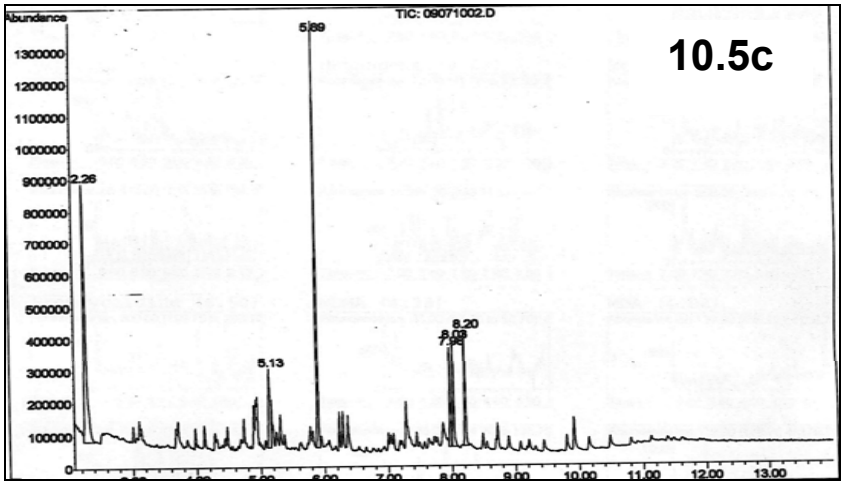


Table 10.5: A summary of retention times (**RT)** of marker peaks in the HPLC_UV chromatogram of Phela TLC-band 2 in (Fig 10.5a)

Run (n)	Peak 1	Peak 2
1	4.28	9.56
2	4.25	9.54
3	4.30	9.58
4	4.28	9.51
Mean	**4.28**	**9.55**
SD	**0.02**	**0.03**

Table 10.6: A summary of retention times (**RT)** of marker peaks in the HPLC_FL chromatogram of Phela TLC-band 2 in (Fig 10.5b)

Run (n)	Peak A
1	6.6
2	6.59
3	6.61
4	6.6
Mean	**6.60**
SD	**0.01**

Table 10.7: A summary of retention times (**RT)** of marker peaks in the GC_MSD chromatogram (n = 3) of Phela TLC-band 2 in (Fig 10.5c)

Run	Peak a	Peak b	Peak c	Peak d	Peak e	Peak f
1	2.26	5.13	5.89	7.98	8.03	8.20
2	2.23	5.14	5.91	7.91	8.01	8.25
3	2.27	5.11	5.82	7.80	7.98	8.17
Mean	**2.25**	**5.13**	**5.87**	**7.90**	**8.01**	**8.21**
SD	**0.02**	**0.02**	**0.05**	**0.09**	**0.05**	**0.04**

Figure 10.6: (10.6a) - HPLC-UV chromatograms of Phela TLC-Band 3 with marker peaks at 8.462, 17.894 and 23.131 minutes **(10.6b)** - HPLC_FL chromatograms of Phela TLC-band 3 with marker peaks at 8, 20, and 33 minutes. **(10.6c)** - a GC_MSD chromatogram of Phela TLC-band 3 with the marker peaks at 2.26, 5.88 and 8.01 minutes

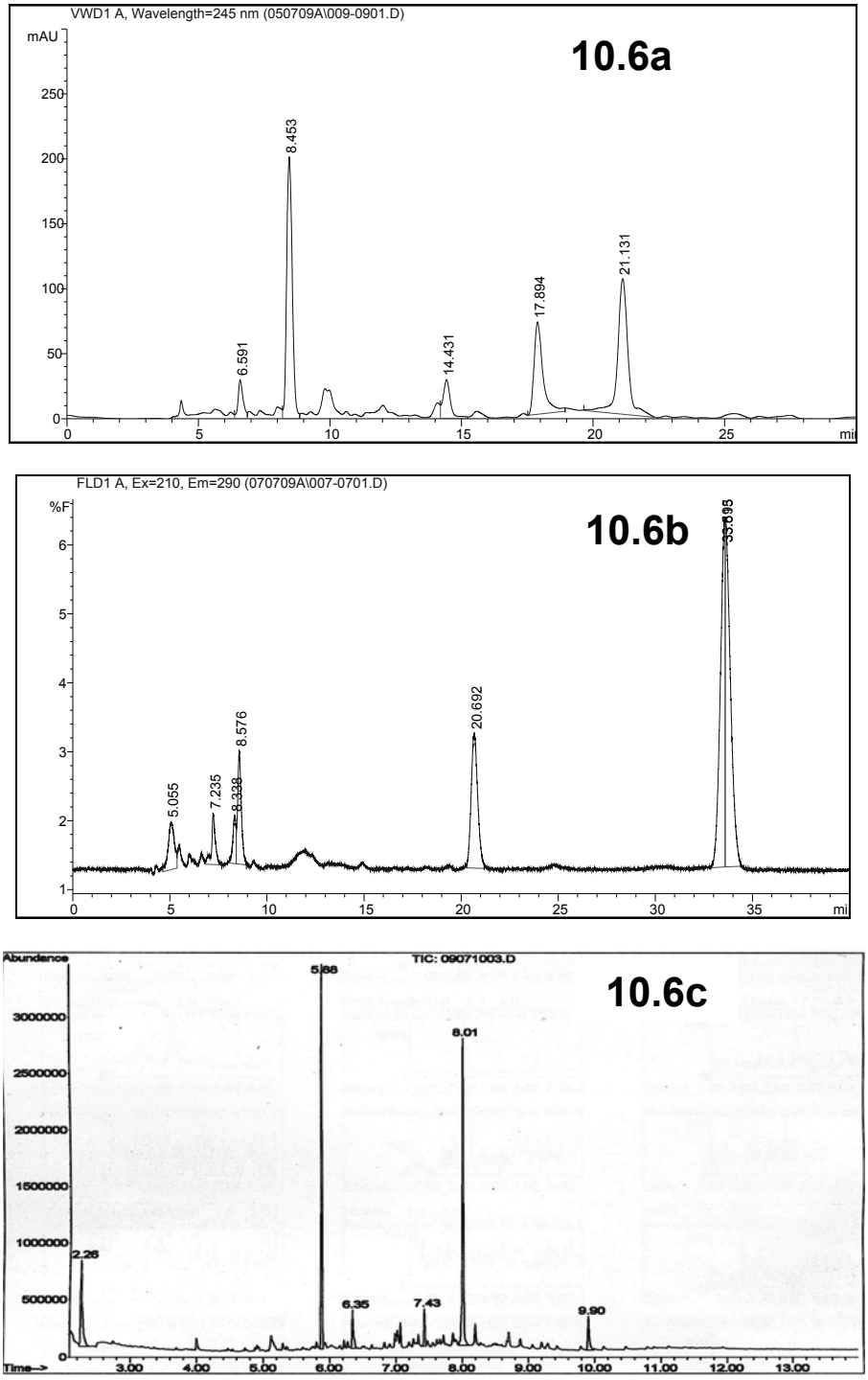


Table 10.8: A summary of retention times (**RT)** of marker peaks in the HPLC_UV chromatogram of Phela TLC-band 3 in (Fig 10.6a)

Run (n)	Peak 1	Peak 1	Peak 3	Peak 4
1	8.45	14.43	17.89	21.13
2	8.42	14.45	17.83	21.65
3	8.43	14.39	17.84	21.13
4	8.51	14.40	17.79	22.90
5	8.43	14.43	18.01	20.83
Mean	**8.45**	**14.42**	**17.87**	**21.13**
SD	**0.04**	**0.02**	**0.08**	**0.32**

Table 10.9: A summary of retention times (**RT)** of marker peaks in the HPLC_FL chromatogram of Phela TLC-band 3 in (Fig 10.6b)

Run (n)	Peak A	Peak B	Peak C
1	8.58	20.69	33.69
2	8.53	20.60	33.63
3	8.50	20.72	33.73
4	8.68	20.63	33.53
Mean	**8.58**	**20.69**	**33.69**
SD	**0.07**	**0.04**	**0.04**

Table 10.10: A summary of retention times (**RT)** of marker peaks in the GC_MSD chromatogram (n = 3) of Phela TLC-band 3 in (Fig 10.6c)

Run	Peak a	Peak b	Peak c	Peak d	Peak e	Peak f
1	2.26	5.88	6.35	7.43	8.01	9.90
2	2.23	5.91	6.46	7.58	7.93	9.80
3	2.27	5.85	6.25	7.33	8.09	9.87
Mean	**2.25**	**5.88**	**6.35**	**7.45**	**8.01**	**9.86**
SD	**0.02**	**0.03**	**0.11**	**0.13**	**0.08**	**0.05**

Table 10.11: Comparison of composition of TLC-bands by the abundance of compounds as indicated on the peaks of GC_MSD chromatogram in Fig. 10.6c and 10.6d.

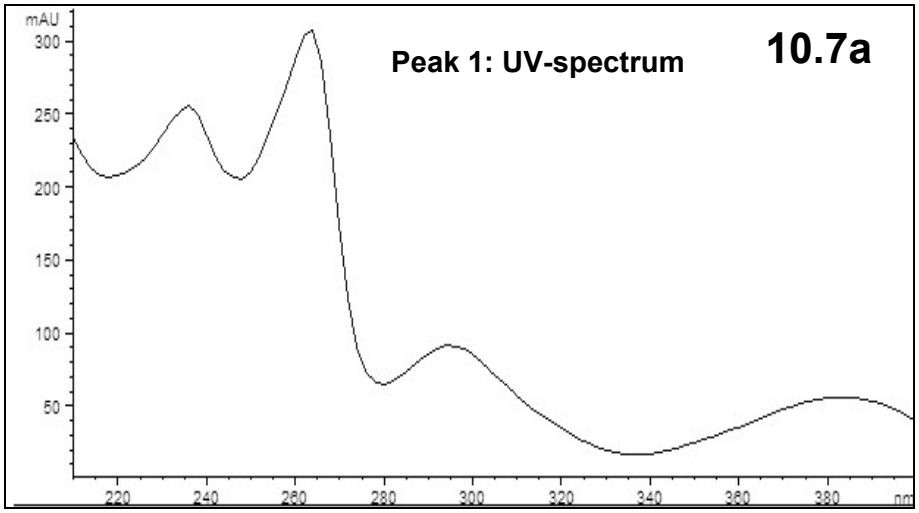
Peak RT (min)	TLC-Band 2	TLC-Band 3
2.26	900,000	800.000
5.13	300,000	------
5.88	**1,400,000**	400,000
6.35	------	500,000
7.43	------	500,000
7.98	400,000	------
8.01	------	**2,800,000**
8.03	400,000	------
8.20	400,000	------
9.90	------	400,000

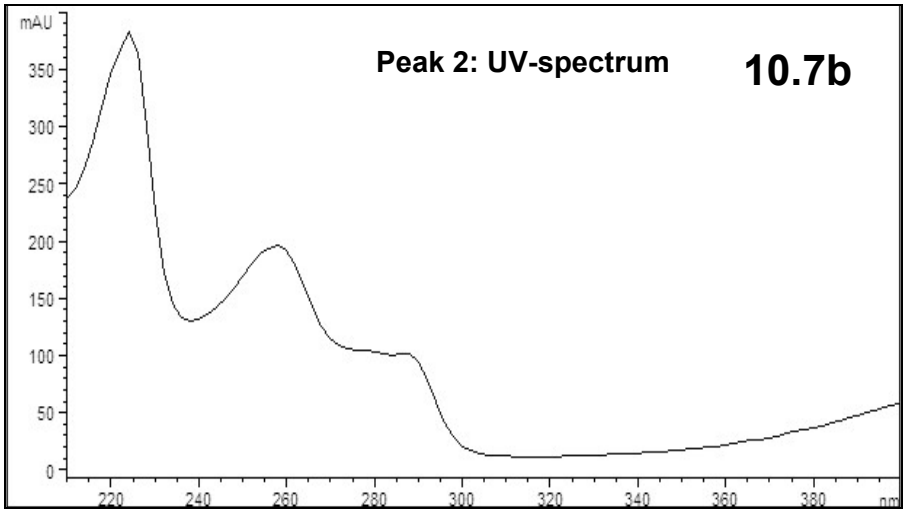
10.4.1.3. Analysis of fractions of the HPLC-peaks

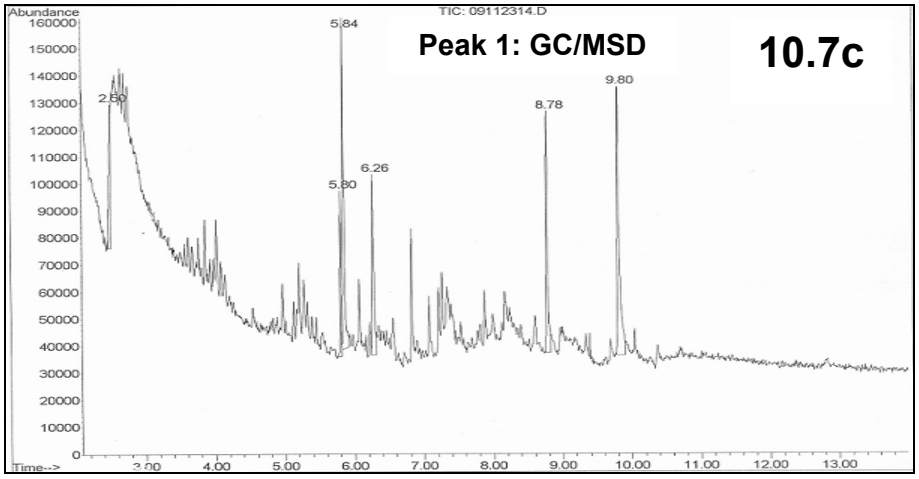
Figure 10.7 (p 126) shows the UV-absorption spectra of the two fractions of crude Phela (peak 1 and peak 2) in HPLC_UV chromatogram (Fig. 10.7b; p 126), and their respective GC_MSD chromatograms. The two fractions are definitely different in the two aspects. Regarding the GC_MSD, the two have peaks at 2.50, 5.80, 5.84, 6.26, 8.78 and 9.80 (Fig 10.7c; p 126); while fraction 2 has peaks at 2.50, 5.79, 5.84, 6.26, 7.92 and 9.80 minutes (Fig 10.7d; p 126). This implies that the two fractions differ at the 5^{th} GC_MSD peak where it is at 8.78 for fraction1, and at 7.92 for fraction two. The set limits are shown in Table 9.12 and 9.13 (p 127). Furthermore, fraction 1 had a much higher signal at 9.80 than fraction 2 (Table 10.14; p 128)

Figure 10.8 (p 129) shows the GC_MSD chromatograms of fractions of the four marker peaks of crude Phela (A, B, C and D) collected from the HPLC_FL chromatogram (Fig. 10.4c; p 115). There were six marker peaks, whereby, except for fraction C, all the marker peaks were detected in all fractions, i.e., fraction A, B and D (Tables 10.15; p 130, 10.16; p 130, 10.17; p 131, 10.18; p 131). Therefore, a further analysis required inclusion of minor peaks and this led to recognition of differences between the fractions. Fraction C had peaks at 7.34 and 8.60 minutes, which were not in the other three fractions. Most notable was that each fraction exhibited lack of at least three peaks that were detected in the other fractions (Table 10.19; p 132). In addition, fraction C had the highest abundance at 9.80 minutes, while fraction B was highest at 2.20 minutes.

Figure 10.7: **(10.7a and 10.7b)** - the UV-absorption spectra of the two marker peaks (peak 1 and peak 2) in HPLC-UV chromatogram of crude *Phela* **fig. 10.4b**; and **(10.7c and 10.7d)** - GC-MSD chromatograms of the two marker peaks (peak 1 and peak 2) in **fig. 10.4b**.







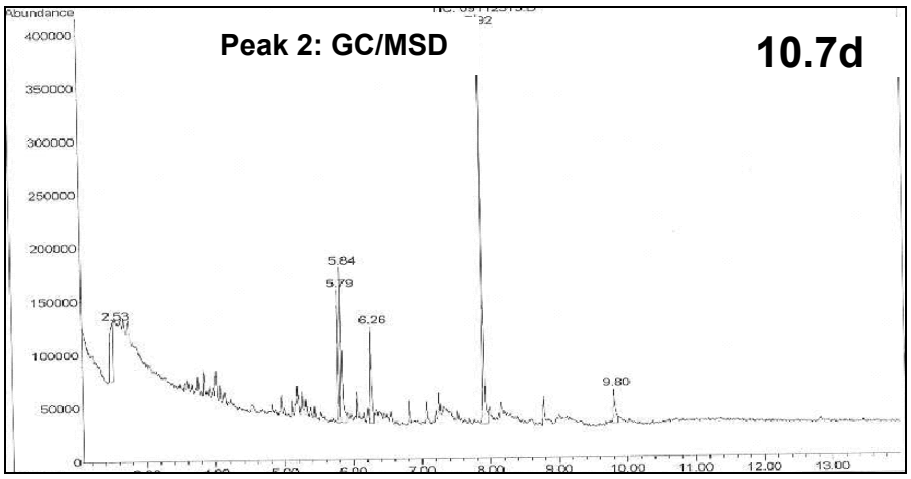


Table 10.12: A summary of retention times (**RT)** of marker peaks in the GC_MSD chromatogram (n = 3) of Peak 1 (Fig. 10.7c) collected from the HPLC_UV chromatogram of crude Phela extract (Fig 10.4b)

Run	Peak a	Peak b	Peak c	Peak d	Peak e	Peak f
1	2.50	5.80	5.84	6.26	8.78	9.80
2	2.49	5.78	5.79	6.12	8.65	9.60
3	2.55	5.85	5.9	6.40	8.89	9.87
Mean	**2.51**	**5.81**	**5.84**	**6.26**	**8.77**	**9.76**
SD	**0.03**	**0.04**	**0.06**	**0.14**	**0.12**	**0.14**

Table 10.13: A summary of retention times (**RT)** of marker peaks in the GC_MSD chromatogram (n = 3) of Peak 2 (Fig. 10.7d) collected from the HPLC-UV chromatogram of crude Phela extract (Fig 10.4b)

Run	Peak a	Peak b	Peak c	Peak d	Peak e	Peak f
1	2.50	5.79	5.84	6.26	7.92	9.80
2	2.49	5.72	5.79	6.18	7.66	9.65
3	2.55	5.80	5.88	6.35	8.02	9.88
Mean	**2.51**	**5.77**	**5.84**	**6.26**	**7.87**	**9.78**
SD	**0.03**	**0.04**	**0.05**	**0.09**	**0.19**	**0.12**

Table 10.14: Comparison of composition of HPLC-UV peaks by the abundance of compounds as indicated on the peaks of GC_MSD chromatogram in Fig. 10.7c and 10.7d.

Peak RT (min)	Peak 1	Peak 2
2.50	140,000	140.000
5.80	100,000	120,000
5.84	160,000	160,000
6.26	110,000	120,000
8.78	**130,000**	-----
7.92	------	**400,000**
9.80	**140,000**	**60,000**

Figure 10.8: GC-MSD chromatograms of the four marker peaks in the HPLC_FL chromatogram of **fig. 10.4c**; 10.8a - peak A; 10.8b - peak B, 10.8c - peak C and 10.8d - peak D.

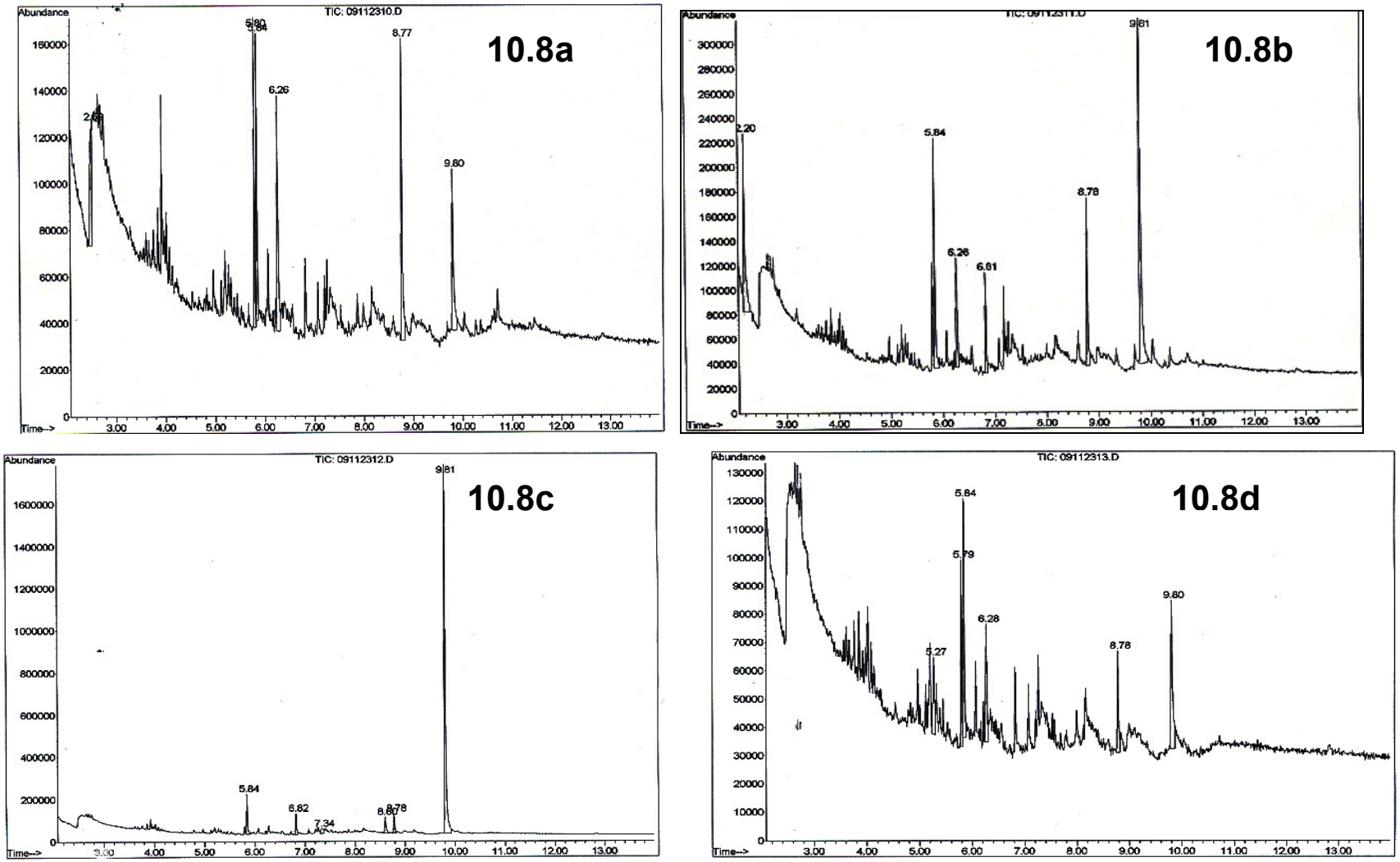


Table 10.15: A summary of retention times (**RT)** of marker peaks in the GC_MSD chromatogram (n = 3) of Peak A (Fig. 10.8a) collected from the HPLC_FL chromatogram of crude Phela extract (Fig 10.4c)

Run	Peak a	Peak b	Peak c	Peak d	Peak e	Peak f
1	2.50	5.80	5.84	6.26	8.78	9.80
2	2.47	5.84	5.80	6.32	8.69	9.70
3	2.56	5.80	5.88	6.20	8.8	9.80
Mean	**2.51**	**5.81**	**5.84**	**6.26**	**8.76**	**9.77**
SD	**0.05**	**0.02**	**0.04**	**0.06**	**0.06**	**0.06**

Table 10.16: A summary of retention times (**RT)** of marker peaks in the GC/MSD chromatogram (n = 3) of Peak B (Fig. 10.8b) collected from the HPLC_FL chromatogram of crude Phela extract (Fig 10.4c)

Run	Peak a	Peak b	Peak c	Peak d	Peak e	Peak f
1	2.50	5.80	5.84	6.26	8.78	9.80
2	2.51	5.79	5.81	6.22	8.75	9.81
3	2.53	5.82	5.9	6.30	8.78	9.87
Mean	**2.51**	**5.80**	**5.85**	**6.26**	**8.78**	**9.83**
SD	**0.02**	**0.02**	**0.05**	**0.04**	**0.02**	**0.04**

Table 10.17: A summary of retention times (**RT)** of marker peaks in the GC_MSD chromatogram (n = 3) of Peak C (Fig. 10.8c) collected from the HPLC_FL chromatogram of crude Phela extract (Fig 10.4c)

Run	Peak a	Peak b	Peak c	Peak d	Peak e	Peak f
1	2.50	5.84	6.82	8.60	8.78	9.80
2	2.50	5.84	8.80	8.62	8.78	9.80
3	2.53	5.82	6.79	8.63	8.77	9.81
Mean	**2.51**	**5.81**	**6.80**	**8.62**	**8.78**	**9.80**
SD	**0.02**	**0.01**	**0.02**	**0.02**	**0.02**	**0.01**

Table 10.18: A summary of retention times (**RT)** of marker peaks in the GC_MSD chromatogram (n = 3) of Peak D (Fig. 10.8d) collected from the HPLC_FL chromatogram of crude Phela extract (Fig 10.4c)

Run	Peak a	Peak b	Peak c	Peak d	Peak e	Peak f
1	2.50	5.79	5.84	6.28	8.78	9.80
2	2.53	5.78	5.83	6.26	8.78	9.81
3	2.48	5.79	5.84	6.26	8.78	9.80
Mean	**2.50**	**5.79**	**5.84**	**6.27**	**8.78**	**9.80**
SD	**0.03**	**0.01**	**0.01**	**0.01**	**0.00**	**0.01**

Table 10.19: Comparison of composition of HPLC_FL peaks by the abundance of compounds as indicated on the peaks of GC_MSD chromatogram in Fig. 10.8a – 10.8d.

Peak RT (min)	Peak A	Peak B	Peak C	Peak D
2.20	-----	**210,000**	-----	-----
2.50	130,000	-----	150,000	120,000
5.27	70,000	-----	-----	60,000
5.80	140,000	-----	-----	100,000
5.84	150,000	210,000	210,000	120,000
6.26	140,000	130,000	-----	80,000
6.81	-----	120,000	150,000	-----
7.34	-----	-----	60,000	-----
8.60	-----	-----	**160,000**	-----
8.78	150,000	180,000	160,000	70,000
9.80	110,000	**350,000**	**1,800,000**	90,000

10.4.2. Column-Chromatography plus HPLC_UV, HPLC_FL & GC_MSD

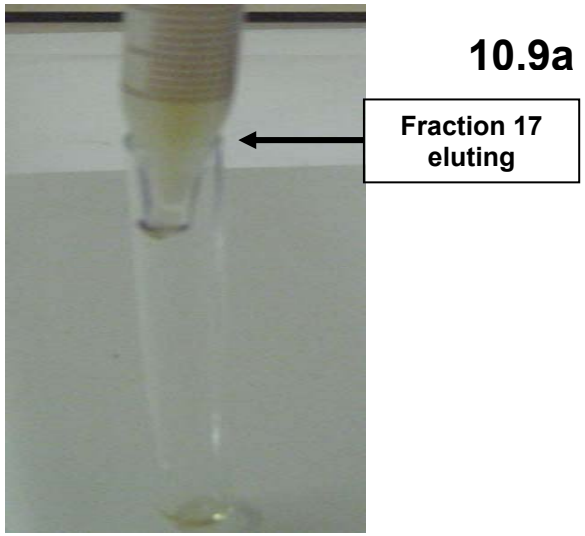
The fractions started eluting from the column after 9 minutes of adding mobile phase. Fractions are labelled according to the time elapsed, after mobile phase addition, i.e , the first fraction is CC-FR-9 and so forth). Figure 10.9 (p 134) shows *Phela's* colored fraction 17 (CC-FR 17) eluting from the column (Fig. 10.9a; p 134) and the respective HPLC_UV, HPLC_FL & GC_MSD fingerprints (Fig. 10.9b-d; p 134). Only CC-FR-17 was selected because the coloured band made its collection very easy, and anybody can be taught to collect the band.

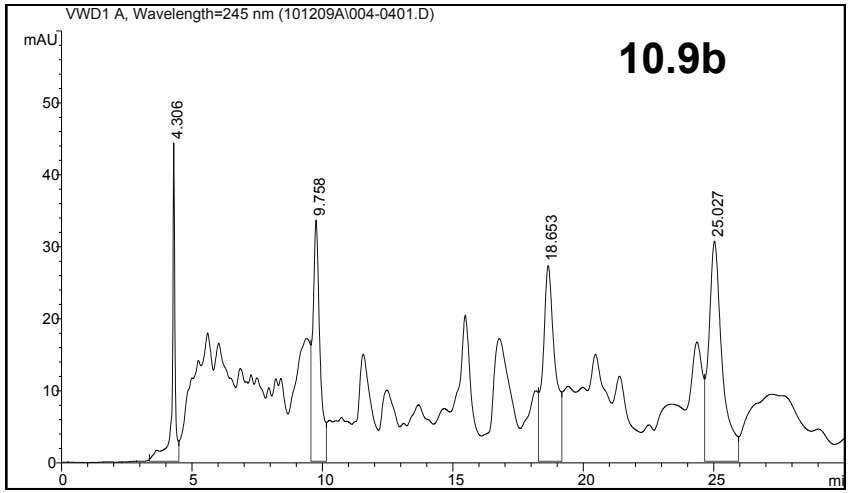
On the HPLC_UV chromatogram, the marker peaks were at 4.3, 9.8, 18.7 and 25 minutes (Fig. 10.9b; p 134), and the standardized limits were 4.25±0.09, 9.78±0.02, 18.65±0.02 and 25.10±0.68 minutes (Table 10.20). On the HPLC_FL chromatogram, the marker peaks were at 4.60, 10.3 and 19.56 minutes and they are within the limits of 4.36±0.16, 9.73±0.38 and 19.34±0.25 minutes (Table 10.21; p 136). The GC_MSD chromatogram of CC-FR had marker peaks at 2.14, 5.92, 6.63, 6.90, 7.95 and 8.86 minutes, and they are within the standardized range of 2.15±0.03, 5.88±0.02, 6.56±0.09, 6.96±0.12, 8.01±0.04 and 9.81±0.02 minutes (Table 10.22; p 136).

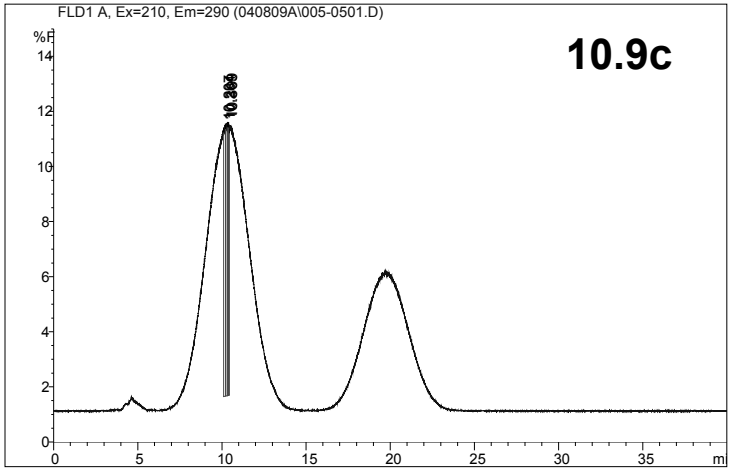
10.4.3. Summary of phela fingerprinting results

Figure 10.10 (p 137) is an overview of phela fingerprinting results with various chromatographic methods.

Figure 10.9: (10.9a) - Column-chromatography (CC) of Phela coloured fraction 17 (CC-FR 17) eluting from column. **(10.9b)** - HPLC_UV chromatograms of Phela CC-FR 17 with marker peaks at 4.3, 9.8, 18.7 and 25 minutes; **(10.9c)** - HPLC_FL chromatograms of Phela CC-FR 17 with marker peaks at 10 and 20 minutes. **(10.9d)** - a GC_MSD chromatogram of Phela CC-FR 17with the marker peaks at 2.14, 5.92, 6.8 , 7.95 and 8.86 minutes







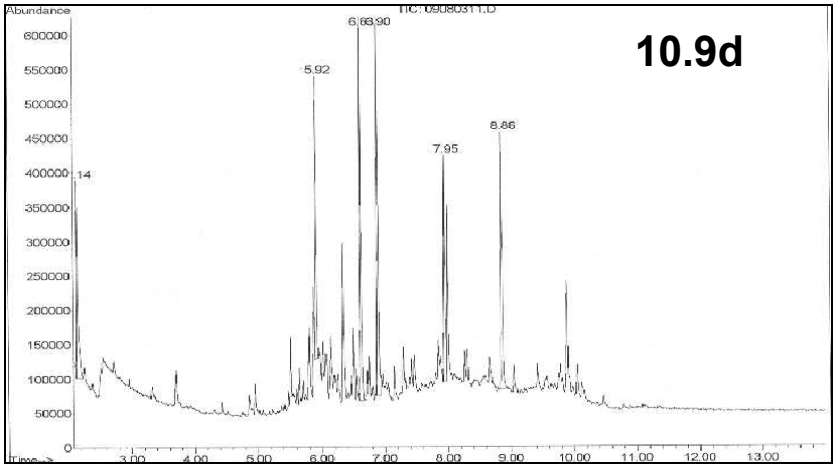


Table 10.20: A summary of retention times (**RT)** of marker peaks in the HPLC_UV chromatogram of Phela Column Chromatography fraction 17 (CC-FR-17) in (Fig 10.9b)

Run (n)	Peak 1	Peak 1	Peak 3	Peak 4
1	4.31	9.76	18.67	26.03
2	4.16	9.78	18.65	24.98
3	4.18	9.81	18.65	24.40
4	4.34	9.77	18.62	25.00
Mean	**4.25**	**9.78**	**18.65**	**25.10**
SD	**0.09**	**0.02**	**0.02**	**0.68**

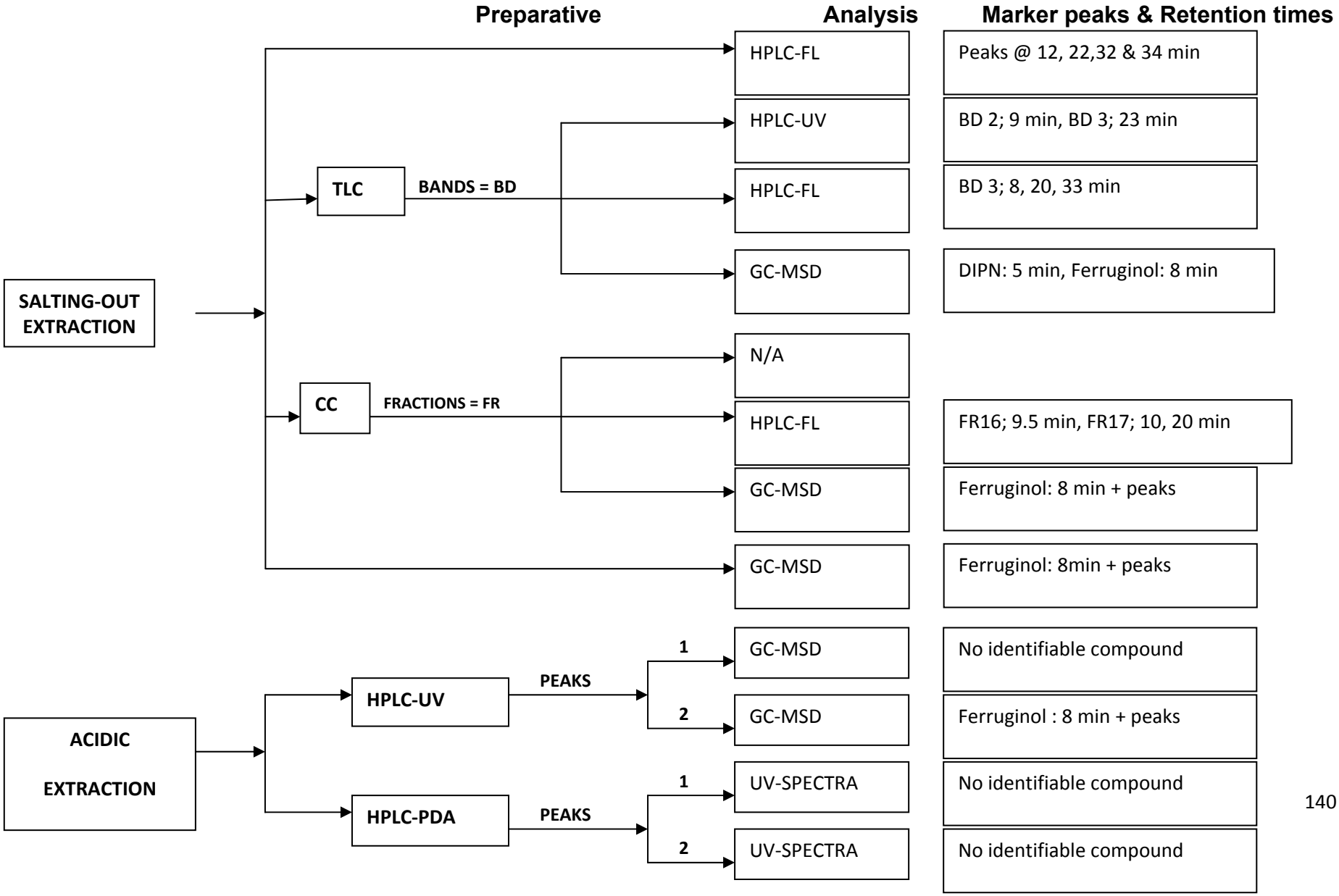
Table 10.21: A summary of retention times (**RT)** of marker peaks in the HPLC_FL chromatogram of Phela Column Chromatography fraction 17 (CC-FR-17) in (Fig 9.9c)

Run (n)	Peak A	Peak B	Peak C
1	4.60	10.3	19.56
2	4.25	9.54	19.00
3	4.30	9.58	19.48
4	4.28	9.51	19.30
Mean	**4.36**	**9.73**	**19.34**
SD	**0.16**	**0.38**	**0.25**

Table 10.22: A summary of retention times (**RT)** of marker peaks in the GC_MSD chromatogram (n = 3) of Phela Column Chromatography fraction 17 (CC-FR-17) in (Fig 10.9d)

Run	Peak a	Peak b	Peak c	Peak d	Peak e	Peak f
1	2.14	5.92	6.63	6.90	7.95	8.86
2	2.18	5.91	6.46	7.10	7.93	9.80
3	2.13	5.88	6.58	6.88	8.01	9.83
Mean	**2.15**	**5.88**	**6.56**	**6.96**	**8.01**	**9.81**
SD	**0.03**	**0.02**	**0.09**	**0.12**	**0.04**	**0.02**

Figure 10.10: Overview of the results of phela fingerprint derived from the various chromatographic methods



10.4.4. Comparison of *Phela* and *hypericum perforatum* (SJW) Extracts

10.4.4.1. Direct Inspection of the crude products

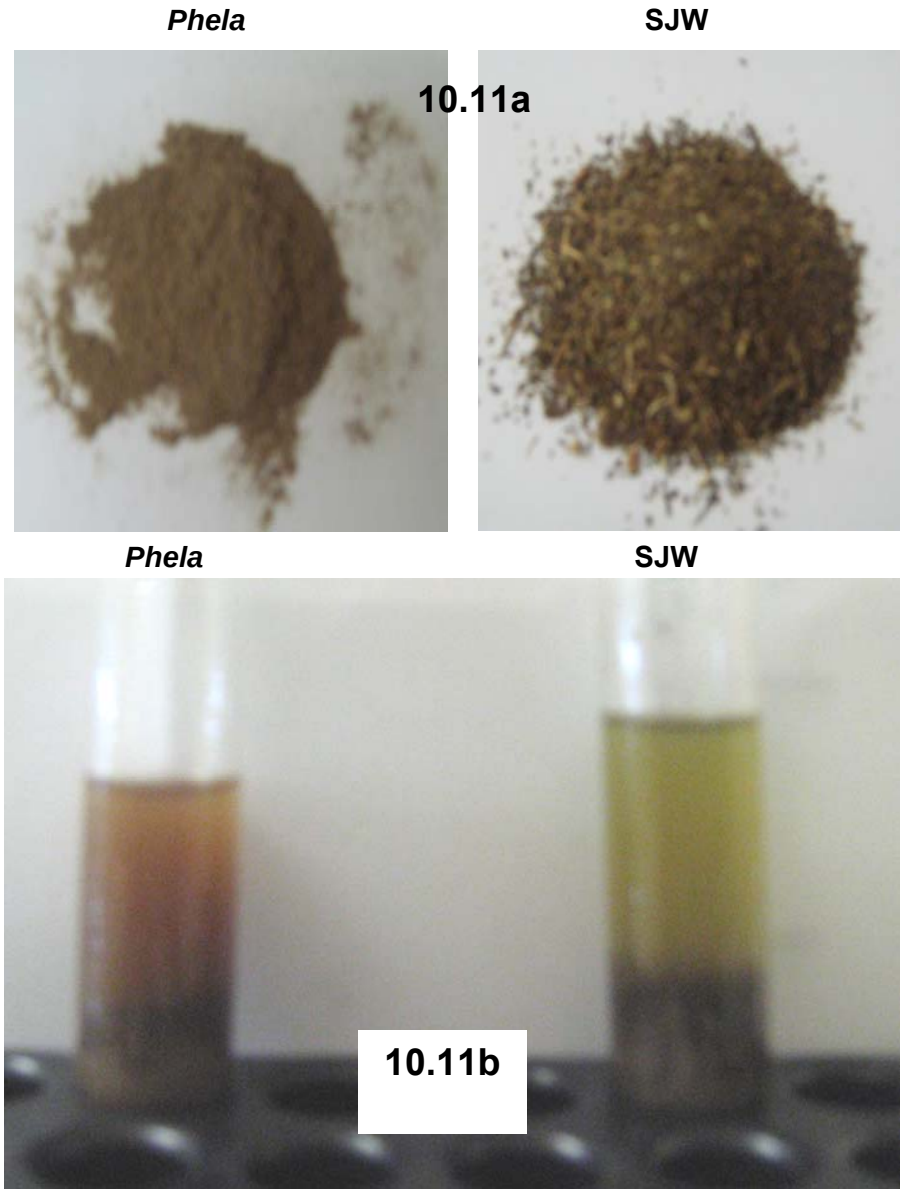
Figure 10.11a (p 139) shows the original products of Phela and SJW. It confirms that the two are different in preparation in that Phela is more finely ground than SJW. Figure 10.11b (p 139) illustrates the colour changes when the two products were treated with ammonia sulphate in iso-propanol (salting-out method). The solvent of Phela extract was dark brown, while the SJW extract was light green. Also in both extractions, there were also three layers: ammonia salt at the bottom, followed by the plant solid material in the middle and the organic solvent was at the top.

10.4.3.2. Chromatographic fingerprints of the crude products

Figure 10.11c (p 139) shows the TLC chromatogram for crude Phela and SJW. The three bands 1, 2, and 3 of Phela were clearly identified with RF of 0.23, 0.35 and 0.91, respectively. SJW has two major bands, A and B, with RF of 0.46 and 0.98. With RF of 0.46, band A is out of the range of 0.31± 0.02 for Phela's band 2, while the 0.98 for band B is also out of the range for Phela's band 3 at 0.91± 0.02 (Table 10.1; p 116).

Figure 10.12 (p 140) is a comparison of HPLC_UV and GC_MSD fingerprints of crude Phela and those of SJW. On the HPLC-UV chromatogram, SJW has five marker peaks at 4.02, 4.66, 6.96, 12.62 and 16.08 minutes (Fig 10.12a; p 140), as opposed to only two for Phela. Furthermore, none of the SJW marker peaks coincided with those of Phela at 9.35±0.71 and 24.5±0.71 (Table 10.2; p 116). On the GC_MSD chromatogram, SJW has six marker peaks at 5.67, 5.85, 6.05, 6.82, 9.33 and 10.04 minutes. Phela too has six marker peaks at 2.23±0.04, 6.25± 0.05, 6.74±0.07, 7.24±0.02, 7.77± 0.05 and 8.01±0.09 minutes (Table 10.4; p 117). Therefore, one of the SJW peaks at 6.82 minutes is likely to coincide with the Phela marker peak at 6.74±0.07 minutes. Of note, on the GC_MSD chromatogram, Phela was characterized by a major peak at 7.93 minutes, while SJW was characterized by a major peak at 10.04.

Figure 10.11: (10.11a) - a picture of Phela powder and St John's Wort (SJW) powder**; (10.11b)** - a picture illustrating the brown colour of Phela; the green color of St John's Wort when the respective powders were treated with ammonia and iso-propanol during the salting-out extraction; and **(10.11c)** - a TLC chromatogram of Phela extract and St John's wort.



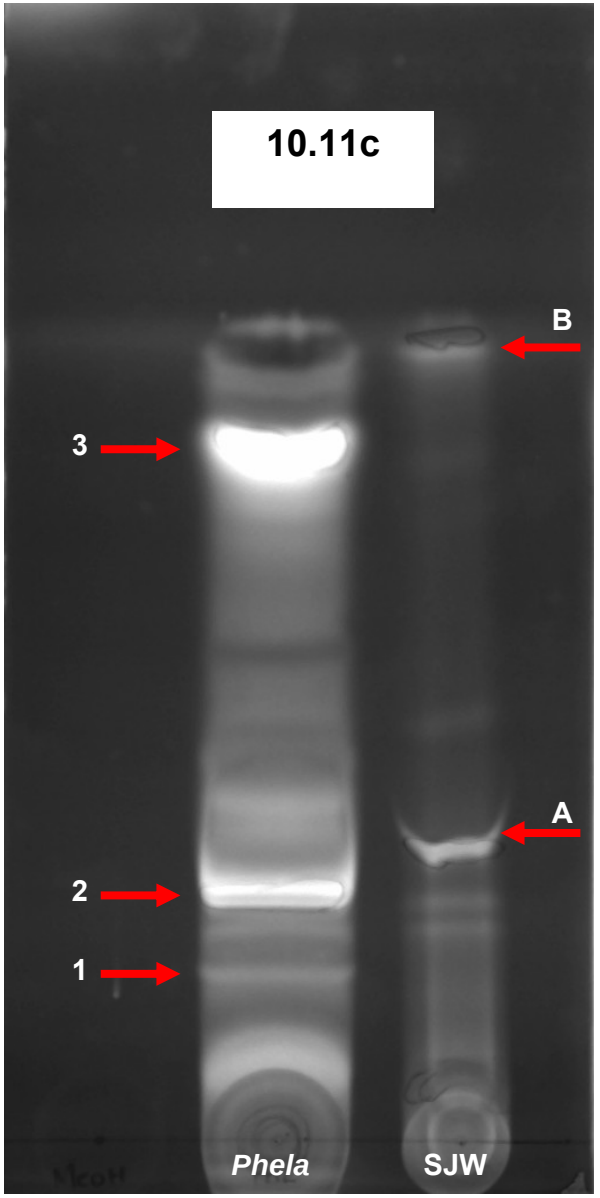
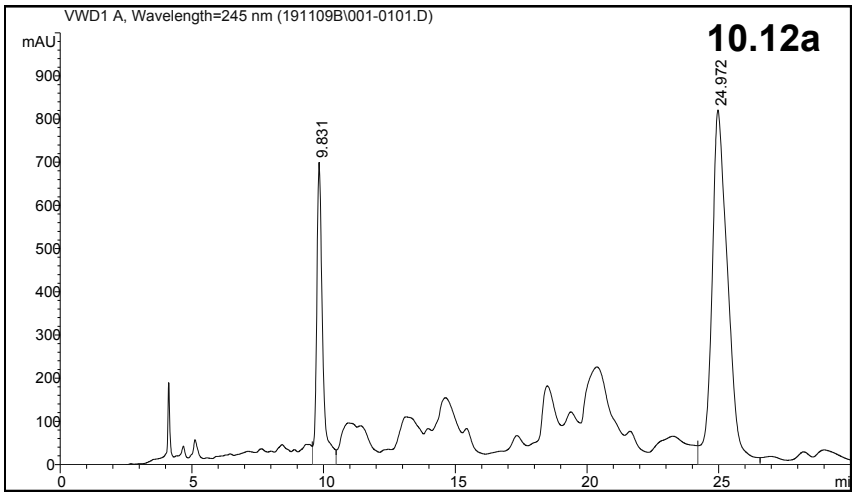
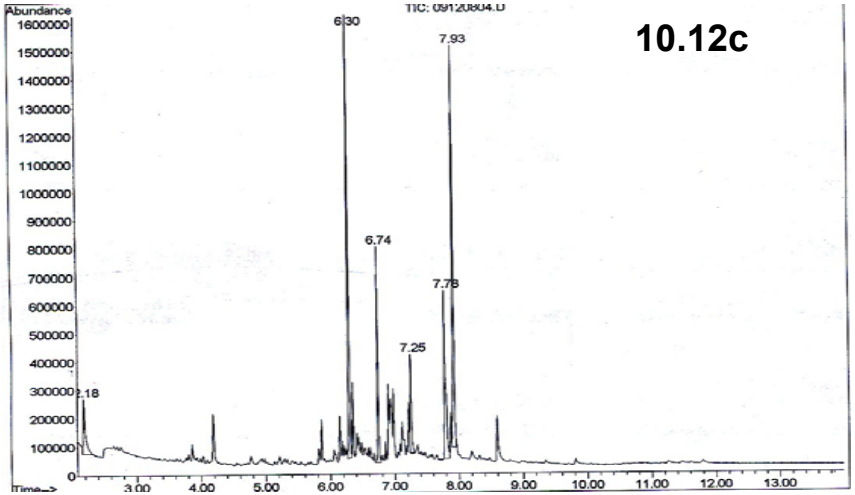
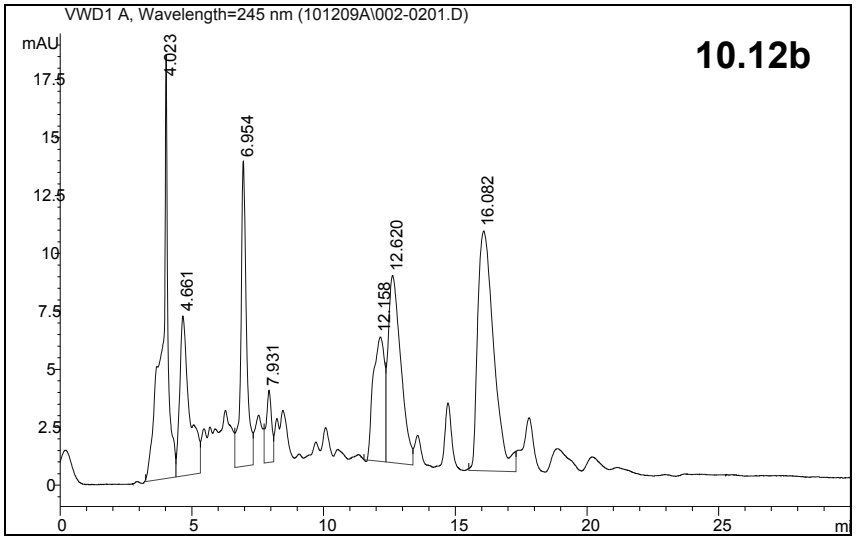


Figure 10.12: **(10.12a)** - HPLC_UV chromatogram of the crude Phela extract versus **(10.12b)** - HPLC_UV chromatogram of the crude SJW extract. **(10.12c)** - GC_MSD chromatogram of the crude Phela extract versus **(10.12d)** - GC_MSD chromatogram of the crude SJW extract.







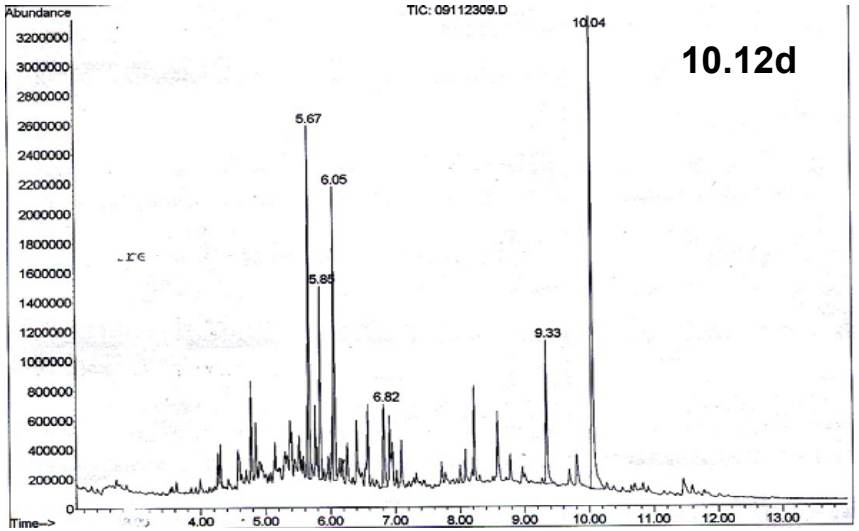
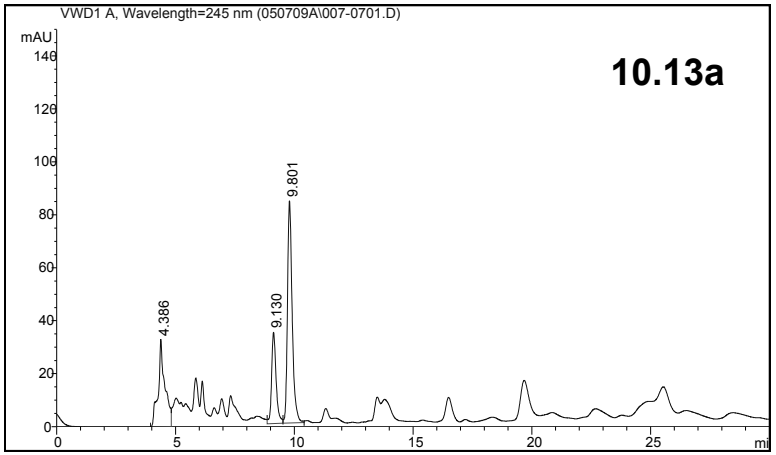
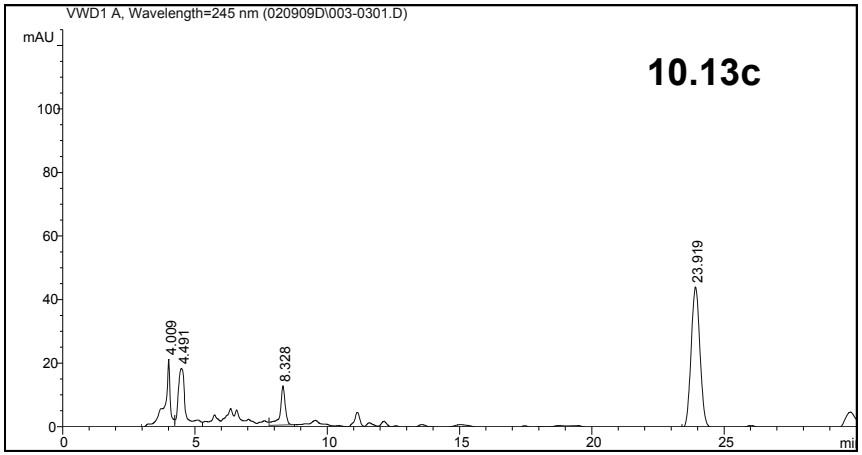
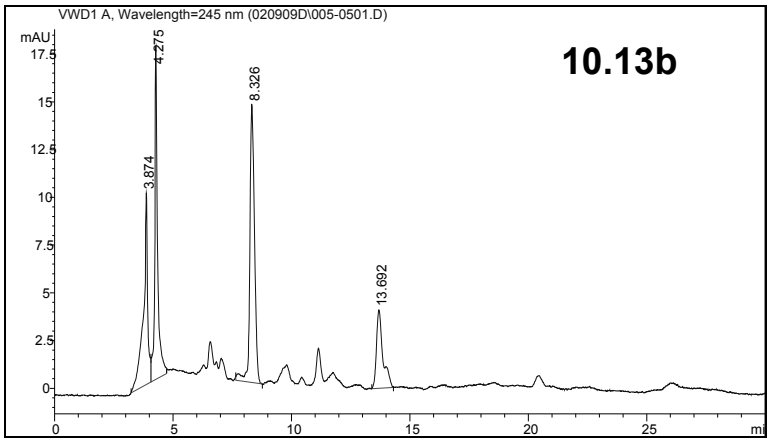
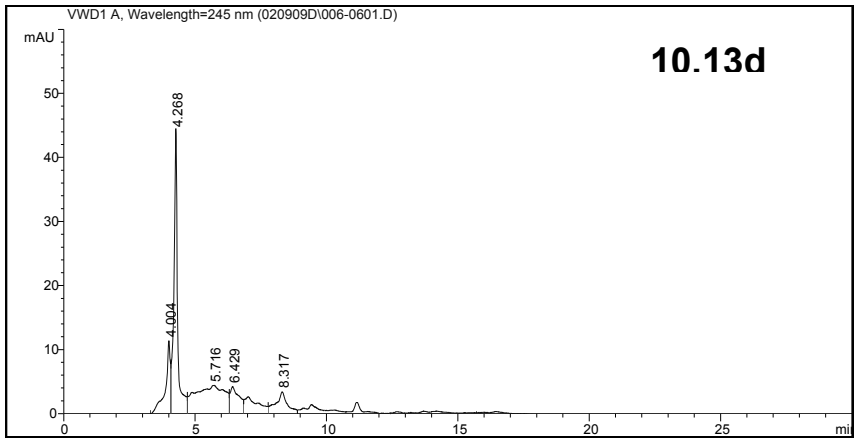


Figure 10.13: (10.13a) - HPLC-UV chromatograms of TLC-band 2 from Phela with marker peaks at 4.386, 9.13 and 9.8 minutes, versus **(10.13b)** - HPLC-UV chromatogram of St John's Wort TLC-band A with marker peaks at 3.874, 4.275, 8.326 and 13.693 minutes; **(10.13c)** - HPLC-UV chromatogram of TLC-band 3 from Phela with marker peaks at 4.009, 4.489, 8.328 and 23.919 minutes, versus **(10.13d)** - HPLC-UV chromatogram of St John's Wort TLC-band B with marker peaks at 4.268 and 8.317 minutes.









10.4.3.3. Comparison of TLC-bands

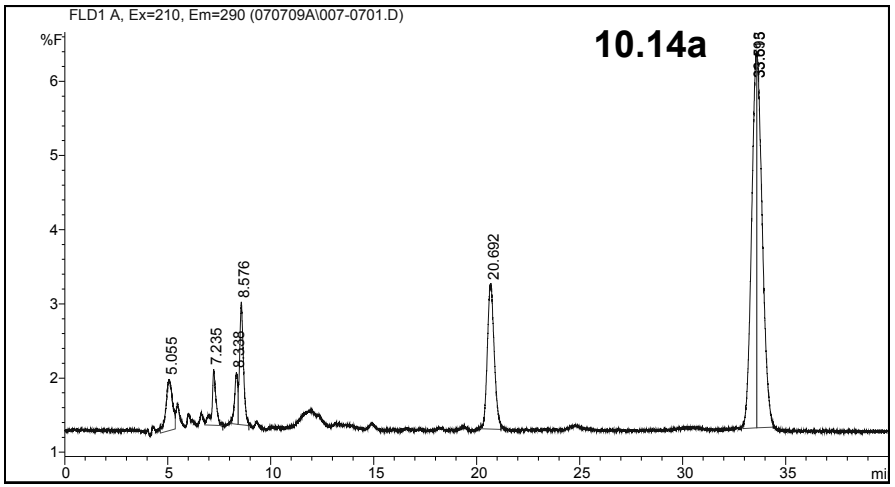
Figure 10.13 (p 141) illustrates a comparison of HPLC_UV chromatograms of TLC-band 2 from Phela with bands A from St John's Wort, as well as band 3 and band B. On the HPLC_UV chromatogram, SJW band A has four marker peaks at 3.87, 4.28, 8.33, and 13.69 minutes, while Phela band 2 has two marker peaks at 4.28±0.02 and 9.55±0.03 minutes. Therefore, one SJW peak at 4.28 minutes is likely to coincide with the marker peak for Phela band 2 at 4.28±0.02 minutes.

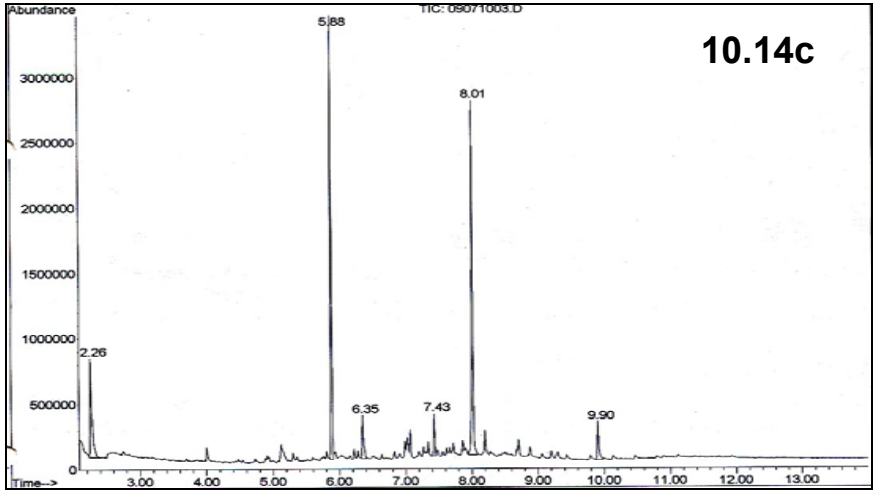
Figure 10.14 (p 143) shows a comparison of band 3 of Phela and band B of SJW by their HPLC_FL and GC_MSD chromatogram**.** On the HPLC_FL chromatogram, SJW band B has two marker peaks at 7.82 and 32 minutes, while Phela band 3 has three marker peaks at 8.58± 0.07, 20.69±0.04 and 33.69±0.04 minutes. Since the SJW peak at 32 minutes is broad, it is likely to coincide with the marker peaks for Phela band 3 at 33.69±0.04 minutes. On the GC_MSD chromatogram, SJW band B has six marker peaks at 3.62, 3.95, 4.34, 4.75, 5.80 and 6.26 minutes. Phela too has six marker peaks at 2.25±0.02, 5.88±0.03, 6.35±0.11, 7.45±0.13, 8.01±0.08 and 9.86±0.05 minutes. Therefore, only one SJW peak at 6.26 minutes is likely to coincide with one Phela marker peak at 6.35±0.11 minutes.

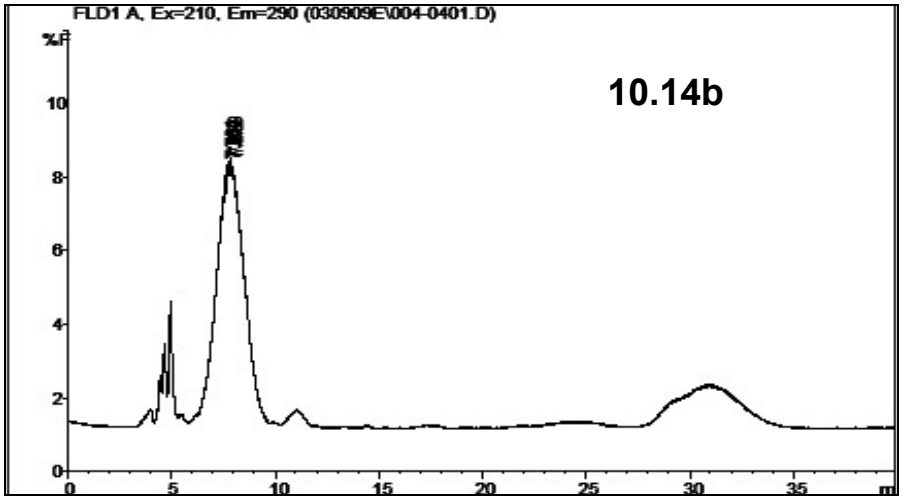
10.4.3.4. Impression

Table 10.23 (p 144) summarizes the major differences between Phela and SJW. There was no relationship at any point between the two products. The methods successfully differentiated Phela from SJW's product.

Figure 10.14: **(10.14a)** - HPLC-FL chromatogram of TLC-band 3 of Phela, versus **(10.14b)** - HPLC_FL chromatogram of St John's Wort TLC-band B; **(10.14c)** - GC_MSD chromatogram of TLC-Band 3 of Phela, versus **(10.14d)** - GC_MSD chromatogram of TLC_Band B of SJW.







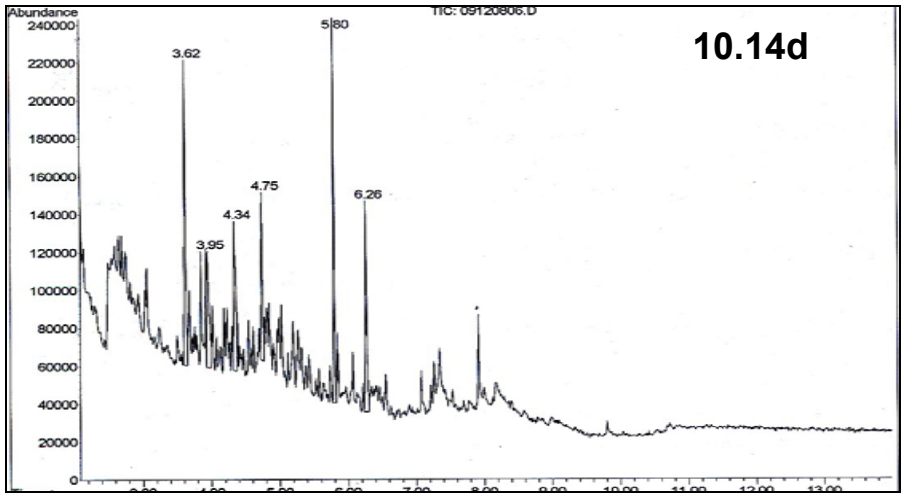


Table 10.23 A summary of major findings between Phela and SJW.

	Phela	**SJW**
1. Powder	Refined	Harsh
2. Salting-out	Brown	Green
3. TLC	3 bands	2 bands
• RF	0.23, 0.57, 0.92	0.27, 0.98
4. HPLC-UV		
• Marker peaks	Band 2: 9 min Band 3 23 min	Band A: 4 & 8 min Band B: 4 & 8 min
5. HPLC-FL		
• Marker peaks	Band 3: 8, 20, 33 min	Band A: 7 min
6. GC-MSD		
i) Crude		
• Marker peaks	2.18, 6.3 & 7.93 min	5.67, 10.04 & 11.04 min
• Identification	7.93 min = ferruginol	11min = clionasterol
ii) TLC bands		
• Bands	Band 2:	Band A:
- Marker peaks	2.26, 5.13, 5.89 & 8 min	3.93, 6.28 &11 min
• Bands	Band 3:	Band B:
- Marker peaks	2.26, 5.88 & 8 min	3.62, 5.80 & 7.9min
- Identification	8 min = ferruginol	7.9 min = ferruginol

10.5. DISCUSSION

A comprehensive method for identification of Phela has been developed. It involves inspection and extraction of the product by simple salting-out and/or acidic extraction methods, followed by TLC analysis and preparative column chromatography that were supported by HPLC and GC analysis using UV, PDA, and fluorescence and MSD detectors, respectively. In his review article Liang (2004) writes that chemical fingerprints obtained by chromatographic and electrophoretic techniques, especially hyphenated chromatographies, are strongly recommended for the purpose of quality control of herbal medicines, since they might represent appropriately the "chemical integrities" of the herbal medicines and therefore be used for authentication and identification of herbal products. The method was used successfully to differentiate Phela from another herbal product made from SJW, thereby illustrating its high potential in fingerprinting of other herbal medicines. Since the SJW product was authentic, a fingerprint for SJW product was also made in the process.

By integrating the simple and high tech-chromatographic techniques, this approach caters for the poor and the rich without loss of quality. It was also observed that most of the reports describe one chromatographic technique, thereby denying many who do not have such equipment from utilizing the technique. Fingerprinting methods should incorporate methods that are affordable to most laboratories, particularly laboratories in poor resource countries. It was envisaged that using multiple chromatographic methods for fingerprinting would offer users' options to correlate the need for further testing versus increasing cost (cost-benefit analysis). It would also lessen the need for intensive calculations that are coherent to single chromatographic techniques. In effect, expensive chromatographic fingerprinting should only be used where really necessary and after cost-benefit analysis. In fact, as it was in this study, where the differences between SJW and Phela were evident in the early tests of inspection and TLC analysis, it is likely that most products can be distinguished by these qualitative tests thereby leaving only a few that deserve further testing to proceed to the more expensive tests. This also obviates the use of complex calculations, but this feature can be incorporated by those who can afford it because there was considerable shift in the retention times of

the marker peaks. Furthermore, the validation of different chromatographic techniques on one standardized extraction process will enable effective monitoring of the finished product in all countries that will use it, promoting international harmonization of quality control methods.

During optimization, an extract with fewer and well separated major peaks, was preferred to those with many peaks. For instance, methanol and water extracts had many bands on the TLC leading to poor separation, while on the HPLC, many peaks would complicate marker-peak evaluation. Interestingly, the 'salting-out' method enhanced TLC and fluorescence detection but not UV-detection. On the other hand, Column Chromatography was characterized by a coloured band, which, fortunately, made fraction collection easy. The GC_MSD is essential for products that are closely related or have marker peaks of similar retention times. Furthermore, the presence of similar peaks in GC_MSD, that vary in abundance in both traditional medicines, may correlate with the multi- healing properties common in Traditional Herbal Medicines

A comprehensive method for fingerprinting Phela, a traditional herbal medicine, has been developed, and this approach can be used to fingerprint other traditional herbal medicine products. However, there is a need to compare phela with traditional medicines that are closely related to it.

The results of phela and SJW comparison using the above methods successfully differentiated the two compounds. Moreover, the chromatographic fingerprint for SJW has been generated, further illustrating that these methods can be fundamental in identifying other traditional medicines by generating a chromatographic fingerprint.

10.6 CONCLUSION

To conclude, a chromatographic fingerprint for authentication and quality control of phela has been developed.

CHAPTER 11

DEVELOPMENT OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR MONITORING OF PHELA, IN RAT PLASMA.

11.1 SUMMARY

The HPLC_UV and HPLC_FL identification methods in chapter 8 and 9, respectively, were adopted and further developed for phela monitoring in plasma. The HPLC_UV method comprised spiking of plasma (1 ml) with varying concentrations of phela and 50 µl of internal standard (40 µg/ml). Furthermore, the samples were acidified with 0.5 M HCl, extracted with hexane and evaporated under nitrogen at 47 °C. The samples were analysed by the set HPLC_UV parameters. The retention time for marker peak (1) was at 9.67 ± 0.16 minutes and marker peak (2) at 24.7 ± 0.59 minutes, with a time distance of 15.0 ± 0.43 minutes between the marker peaks, and IS eluting at 10.0 ± 0.09 minutes. The calibration range of 0 – 10 mg/ml was found to be linear with a linearity regression equation of y = 0.02 x – 0.59 and the correlation coefficient of r^2 = 0.9983. The accuracy percentages were 103, 87, 103 % for 2, 6, 10 mg/ml respectively. Thereafter, HPLC_FL method was developed due to pitfalls observed during HPLC_UV rat plasma analysis. Ammonia sulphate was added to 0.5 ml of plasma, extracted with iso-propanol and analysed by HPLC_FL. Three marker peaks were selected and standardized. Rat plasma analysis had a new peak at 9.1 ± 0.5 minutes. Ultimately, a HPLC_FL method for monitoring phela in plasma was developed.

11.2 INTRODUCTION

Here, the development of an HPLC method for analysis of phela in plasma and the associated pitfalls is described. The primary aim was to develop and standardize an HPLC_UV method for phela analysis and monitoring in plasma, however analysis of rat plasma using this method was unsuccessful. Thereafter a HPLC_FL method was developed and rat plasma analysed during the development process. It is hoped that more avenues for concentration monitoring of phela and other traditional herbal medicine products in general will be established.

11.3 MATERIALS AND METHODS

11.3.1 Apparatuses

A vortex mixer (Scientific Industries Inc, U.S.A) was used for mixing. A 5810 R centrifuge and mini spin (Eppendorf, Germany) were used for centrifuging and quick spinning of samples. Precision balances SBC 31 and SPB 52 (Scaltec Instruments, Germany) were used for weighing milligram and gram quantities, respectively. A nitrogen evaporator with a heating block was used for evaporating samples. Finnpipettes from Thermo Labsystems (Canada) were used for spiking volumes. Glass pipettes were used for adding extraction solvents.

11.3.2 Chemicals and reagents

Phela was manufactured and supplied by the Indigenous Knowledge system Lead Programme (IKS) of the South African Medical Research Council. HPLC graded: hexane, methanol, acetonitrile, diethyl ether, toluene (Burdick and Jackson, U.S.A) and hydrochloric acid, (Merck, Germany) were used during sample preparation. Sodium hydroxide and ammonia sulphate were purchased from Sigma –Aldrich (Germany). Deionised and distilled water was prepared by Millipore water system (Milli –Q^{Tm}).

11.3.3 Chromatographic system and conditions

Chromatographic system: The HPLC system consisted of a Hewlett-Packard 1100 series system that comprised an isocratic pump, an auto-sampler and a UV detector set at 245 nm. A computer with LC-Chemstation software was used. For HPLC_FL analysis, the HPLC-system and chromatographic conditions were the same as for HPLC_UV except for the programmable fluorescent detector (HP-1046A; made in Germany) with emission and excitation wavelengths set at 210 nm and 290 nm respectively.

Chromatographic conditions: Chromatographic separation was achieved with a reverse phase C_{18} (250 mm x 4.6 mm x 5 microns) coupled to a C_{18} guard column (Phenomenex, U.S.A). Acetonitrile: water (70:30; v/v) was mobile phase eluting the sample at the flow rate was 0.5 ml/min and the run time 30 minutes and 40 minutes for HPLC_UV and HPLC_FL respectively.

11.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH A UV DETECTOR (HPLC_UV) METHOD

11.4.1 Optimization of HPLC_UV method conditions

The identification method developed in chapter 8 was adopted and modified for phela monitoring in plasma. The HPLC system conditions remained the same. A working stock solution of 100 mg/ml of Phela was used for spiking throughout the development process.

11.4.1.1 Selection of the Internal Standard (IS)

A search for the appropriate internal standard was done by looking at acidic drugs with more than 2 rings in their chemical structure, and the following reference standards provided by the toxicology laboratory were evaluated: Chloroxazone, chloropropramide, flufenamic acid, tiaprofenic acid and flurbiprofen. Each reference standard (10 ug/ml) was prepared in mobile phase. Compounds with more than 3 ring structures, namely promefazine, digitoxin, colchizine, mesoridazine,

diphenylcarbazone, 2.5 diphenyloxazole, progesterone and 17 α methyltestoterone, were also tested.

11.4.2. Sample extraction: acidic extraction method

Plasma (1ml) was spiked with Phela (100 mg/ml), 50 µl of internal standard (40 µg/ml) acidified with 100 µl of 0.5 M HCl and allowed to shake horizontally for 10 minutes. The samples were extracted with 5 ml of hexane, and further shaken for 10 minutes. The samples were centrifuged at 4 °C and 2 500 rpm (1 251g) for 10 minutes. The organic layer was evaporated under nitrogen at 47°C, and residues reconstituted with 100 µl of mobile phase and 50 µl was injected onto HPLC for analysis.

11.4.3. HPLC_UV Method Standardization

11.4.3.1. Calibration curve

The calibration standards concentration in the range 0 – 10 mg/ml were prepared according to the described method (section 11.4.2) and repeated over five days. The ratio per calibration standard was calculated by using the area under the curve of marker peak 2 of phela over area under the curve of internal standard (IS) peak.

$$\left[Ratio\right] = \left[\frac{AUC - mar\,\mathrm{ker}\;peak2}{AUC - Internals\,\mathrm{tan}\;dard}\right] \qquad \text{Eq. 11.1}$$

A calibration curve was plotted with peak ratio on the y-axis versus concentration on the x-axis for each daily calibration curve. The mean calibration curve ratios, standard deviation (SD) and coefficient of variance (CV %) were derived and used to plot an average calibration curve. Linear regression was defined by correlation coefficient (r^2) and linear equation (y = mx + c).

11.4.3.2. Accuracy

Three calibration standard concentrations (2, 6, and 10) mg/ml were selected; low, medium and high concentrations respectively. Calibration standards were prepared as described in the above section. Each of the selected concentrations (2, 6, and 10) mg/ml was prepared five times (Table 11.1). Blank samples (n = 5) were prepared as well and used as quality control standards.

Table 11.1: A design of accuracy testing of 2, 6 and 10 mg/ml test samples and blank.

Accuracy samples			**Quality control samples**
2 mg/ml	**6mg/ml**	**10mg/ml**	**Blank**
2A	6A	10A	Blank_1
2B	6B	10B	Blank_2
2C	6C	10C	Blank_3
2D	6D	10D	Blank_4
2E	6E	10E	Blank_5

The ratios were calculated with equation 11.1. The ratio for the accuracy samples was used to drive concentrations from the calibration curve. Accuracy percentage (Acc %) was calculated with the following equation:

$$[Acc\%] = \left[\frac{Derived\_accuracy\_concentration x 100\%}{Known\_concentration} \right] \qquad \text{Eq 11.2}$$

The mean (concentration and accuracy percentage), standard deviation (SD) and CV % were also calculated for all three concentrations.

11.4.3.3. Stability

11.4.3.3.1. Short –term stability

Different temperature settings were used to evaluate stability over the short- term and long–term. Three calibration standards, namely low, medium and high concentration (2, 6, and 10 mg/ml) were selected. For short – term stability, 1 sample per concentration range was left at varying temperatures; at either room temperature (± 25 °C), in the fridge (4 °C) and in the freezer (-20 °C), and analysed after 24 and 48 hours respectively.

11.4.3.3.2. Long – term stability

For long-term stability 1 sample per concentration was left at 4 °C and at -20 °C and analysed after 1 week. Furthermore, 1 sample per concentration was left in at -20 °C and analysed after 4 months.

The ratios were calculated with equation 11.1. The derived ratios of the stability samples were used to derive concentrations from the calibration curve. The derived concentrations were compared with the spiked concentration to evaluate how stable phela is at different temperature settings over time.

11.5. TESTING OF RAT PLASMA

The study was approved by the Animals Ethics Committee of the University of the Free State. Fifteen (n = 15) male Sprague Dawley rats were used. Aqueous solution of phela was orally administered to rats at a dose of 15.4 mg/kg. Blood samples were collected from three rats (n = 3) after 1, 2, 4, 6 and 8 hours of dosing and kept on ice. The samples were centrifuged and plasma separated within 30 minutes after withdrawal, then stored at -20°C until the time of analysis.

A test plasma sample from a rat at 2 and 6 hours after oral administration of phela was extracted and analysed using the method described in section 11.4.2.

11.6. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH A FLUORESCENCE DETECTOR (HPLC_FL) METHOD

11.6.1. Sample preparation: Salting out extraction method

Plasma (0.5 ml) was spiked with varying volumes of phela stock solution (100 mg/ml) to achieve desired concentrations. The samples were treated with 100 mg ammonia sulphate {$(NH_4)_2SO_4$} “salting out.” Iso–propanol (1 ml) was added and the mixture left to shake for 10 minutes. The samples were centrifuged for 10 minutes at 2500 rpm (1 251 g) at 4°C. The supernatant was evaporated under nitrogen at 47 °C and the residue reconstituted with 200 µl of mobile phase. It was run on HPLC_ FL with the same parameters as above, with the exception of the detector that was a fluorescence set at a wavelength of 210 nm and 290 nm for emission and excitation respectively

11.6.2. Optimization of concentration range

Primarily, the concentration range of the calibration curve (0 – 10 mg/ml) used during HPLC_UV method analysis was adopted and prepared using the method explained above (section 11.6.1). Thereafter the higher concentrations were evaluated, namely 20, 30, 40, 60, 80 and 100 mg/ml.

11.6.3. Standardization and testing

Standardization was not done owing to the lessons learned in the HPLC_UV section. Instead, a test plasma sample from a rat 2 and 6 hours after oral administration of Phela was extracted and analysed via this method.

11.7. RESULTS

11.7.1. HPLC_UV

11.7.1.1. Selection of internal standard

All the standards evaluated as internal standard had a retention time of between 3 and 4 minutes, interfering with phela peaks. Only 17-α-methyltestoterone had a peak at 10 minutes and was selected as an internal standard (IS). The 40 µg/ml concentration of the IS along with the 50 µl spiking volume were preferred.

11.7.1.2. HPLC_UV Chromatographic performance

Figure 11.1 A and B (p 155) are chromatograms of blank plasma and spiked plasma extracted using the acidification method and analysed by HPLC_UV. In figure 11.1 B (p 155), the retention time for marker peaks 1 and 2 was at 9.8 and 25.4 minutes respectively and IS eluted at 10.5 minutes.

Table 11.2 (p 156) is a summary of the mean and standard deviation for the retention time shifts with marker peak 1 at 9.47 ± 0.43 minutes and marker peak 2 at 24.65 ± 0.75 minutes, and a time difference of 15.16 ± 0.44 minute and 10.42 ± 0.14 for the internal standard .

Figure 11.2 (p 157) is UV spectra of marker peaks (1 and 2) confirming similarity with those observed in crude Phela analysis (Figure 8.13 and 8.14; page 88 – 89).

Figure 11.1: HPLC-UV chromatogram of blank rat plasma (**A**) and spiked plasma (**B**) with marker peak (1) 9.8, internal standard at 10.5 and marker peak (2) at 25.4 minutes.

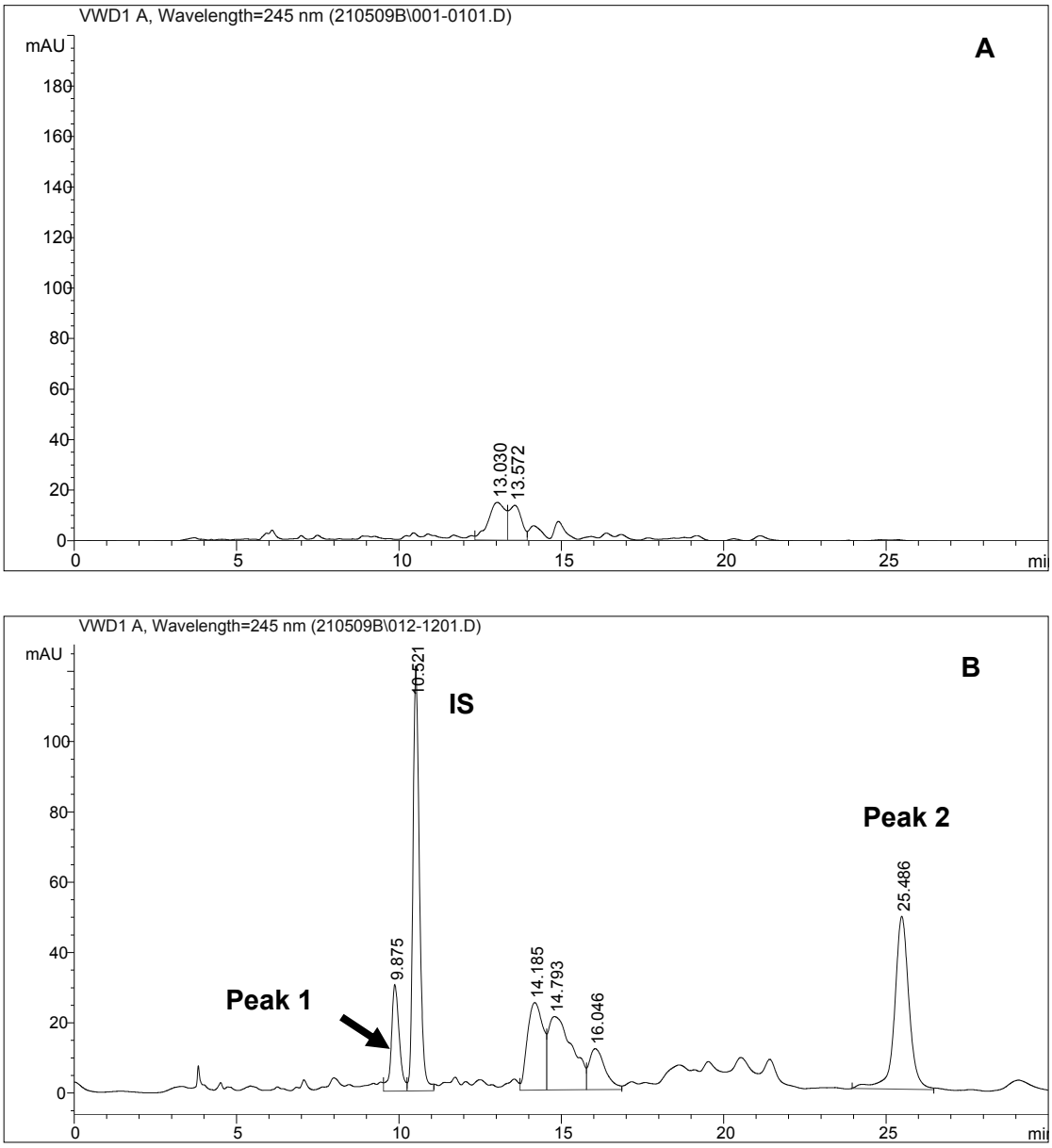
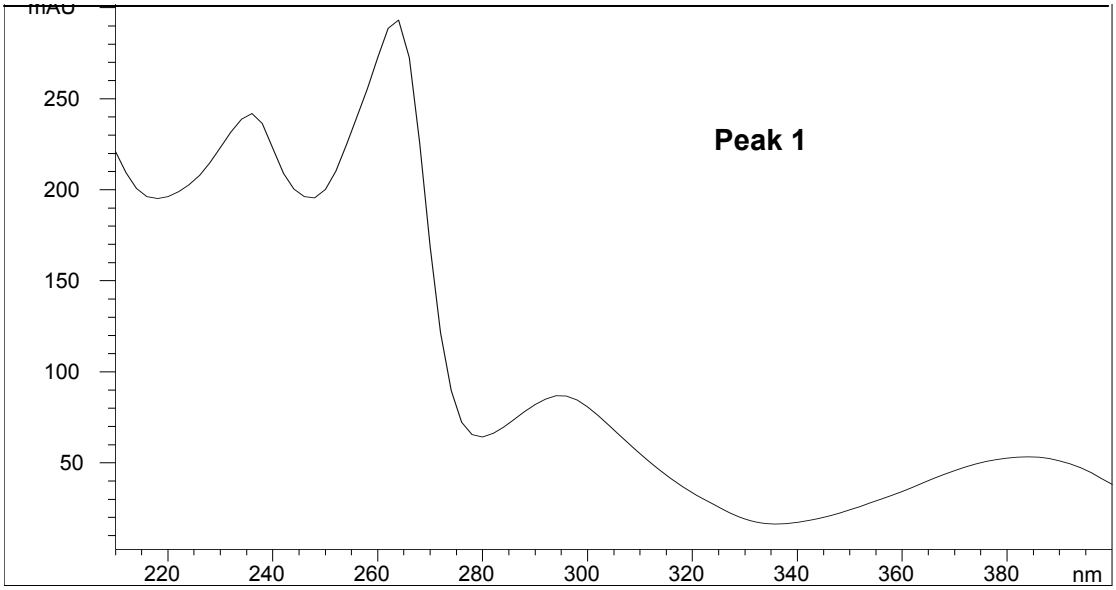


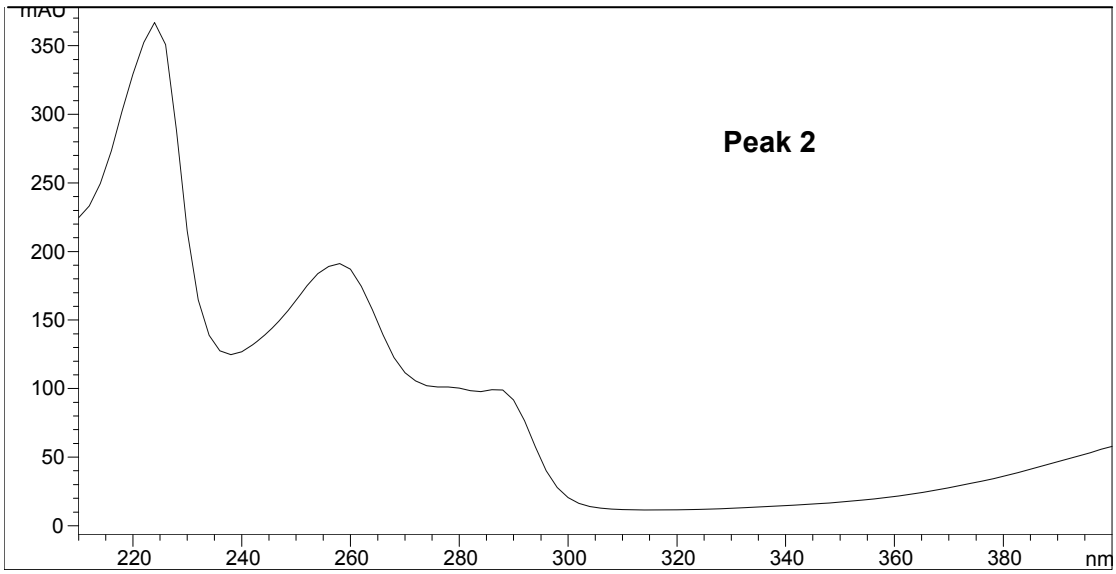
Table 11.2: A summary of statistical analysis of marker peak retention time shifts.

	Marker peaks Retention time (minutes)		**Peaks RT diff.**	**IS**
	1	**2**	**(min)**	**(min)**
	9.8	25.4	15.6	10.49
	8.3	23.9	15.6	10.40
	9.7	24.6	14.9	10.42
	9.6	23.9	14.1	10.33
	9.5	24.1	14.6	10.33
	9.9	25.4	15.5	10.56
Mean	**9.47**	**24.65**	**15.16**	**10.42**
SD	**0.43**	**0.75**	**0.44**	**0.14**

Key: **Peak RT (Retention time) diff** = Retention time difference of marker peak 2 retention time - marker peak 1 retention time. **IS** = internal standard, **Min** = Minutes

Figure 11.2: UV spectra of peak 1 (9.8 minutes) and peak 2 (25.4 minutes). [Similar to fig 8.13 and fig 8.14, UV spectra of marker peak 1 and 2 of phela crude extracts]





11.7.2. Method Standardization

11.7.2.1. Calibration curve

Table 11.3 (p 159) shows summarized data of the five days' calibration curves. The CV % was less than 20 % for concentrations below 6 mg/ml, while 8 – 10 mg/ml concentrations were less than 21 %. Figure 11.3 (p 159) shows the plot of the average 5-day calibration curve. The average calibration standards gave a linear regression of y = 0.02 x – 0.59 and the correlation coefficient of r^2 = 0.9983.

11.7.2.2. Accuracy

Table 11.3 (p 159) provides a summarized data of accuracy for all three concentrations. Mean accuracy percentages were found to be 102.87 ± 29.9 (CV % = 29 %), 86.57 ± 7.4 (CV % = 8.5%) and 102.50 ± 18.3 (CV % =17.8) % for the 2, 6 and 10 mg/ml, respectively. The CV % was high at the low concentration of 2 mg/ml.

11.7.2.3. Stability

Figure 11.4 (p 161) shows both short and long-term stability of each concentration, with (Table 11.4; sample A; p 160) showing stability for 2 mg/ml while (Table 11.4; sample B; p 160) and (Table 11.4; sample C; p 160) are stable for 6 mg/ml and 10 mg/ml.

For the 2 mg/ml concentration (Table 11.4; sample A; p 160) the sample was more stable at room temperature after 24 and 48 hours, and a similar pattern was observed after storage at -20°C after 24 hours, While the 6 and 10 mg/ml concentrations were only stable at -20°C after 24 hours. Other results were variable. Table 10.5 (p 162) is a table of stability percentages of all three concentrations. The results indicate that the samples were only stable after 24 hours storage at -20°C. Long-term stability also had variable results (Appendix C; table C-2).

Table 11.3 HPLC_UV peak ratios of phela calibration standards over 5 days, with mean, standard deviation and coefficient of variance (CV %).

	Calibration curve range (mg/ml) of phela				
Days	**2**	**4**	**6**	**8**	**10**
1	0.113	0.167	0.232	0.240	0.292
2	0.145	0.213	0.253	0.297	0.372
3	0.176	0.206	0.263	0.354	0.400
4	0.151	0.168	0.189	0.205	0.235
5	0.166	0.197	0.239	0.294	0.379
Mean	**0.150**	**0.190**	**0.235**	**0.278**	**0.336**
SD	**0.024**	**0.021**	**0.028**	**0.057**	**0.069**
CV %	**16.0**	**11.3**	**12.1**	**20.6**	**20.7**

Key: **Mean** = mean value of values **SD** = Standard deviation **CV %** = Coefficient of variance % (Precision)

Figure 11.3 An average calibration curve of phela over five days with concentration versus peak ratio and correlation line of y = 0.02x – 0.59 and r^2 = 0.9983

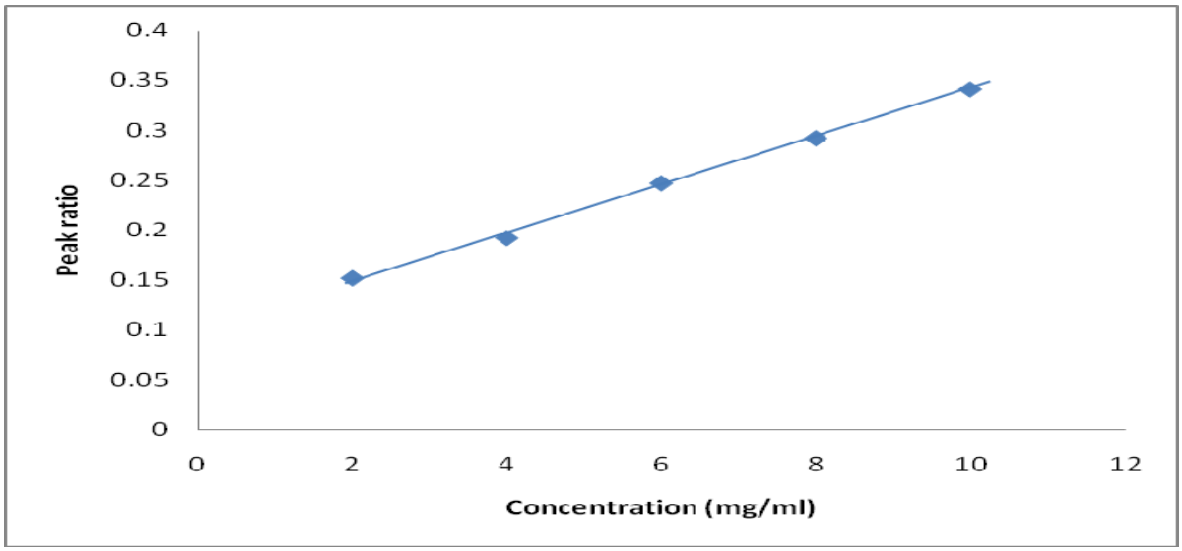
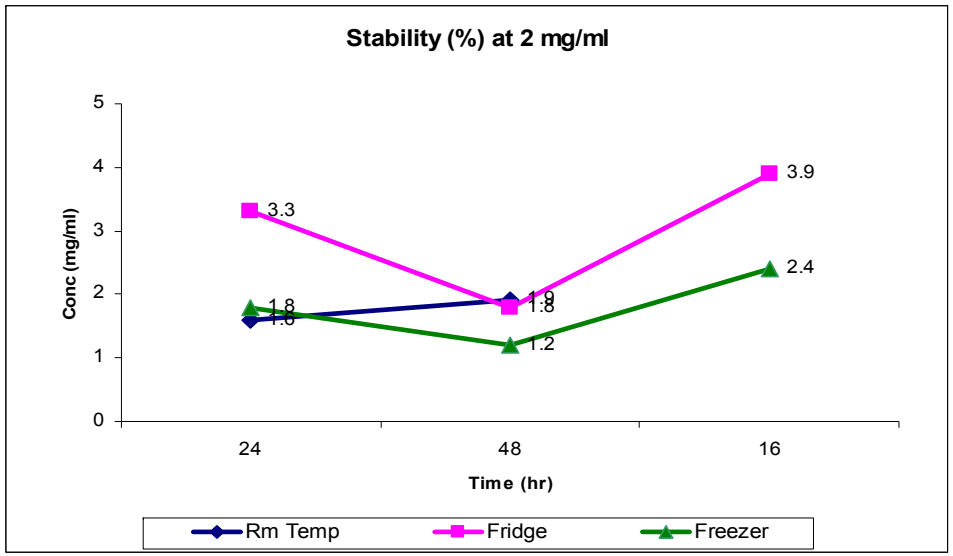


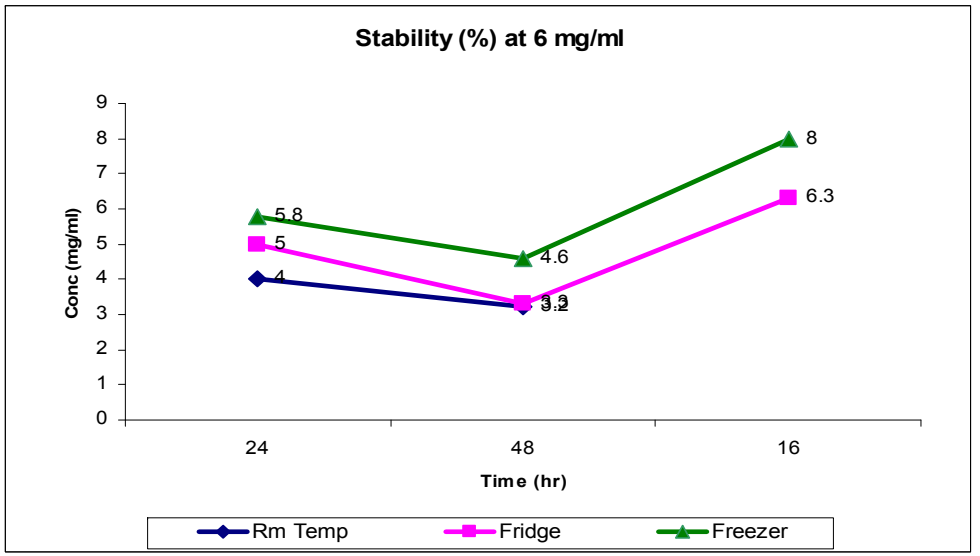
Table 11.4: Summary of accuracy testing results; Derived concentration and accuracy percentages of 2, 6 and 10 mg/ml are reported. The mean, standard deviation and coefficient of variance are also calculated for all concentrations.

	2 mg/ml		6 mg/ml		10 mg/ml	
Samples	**Derived Conc. (mg/ml)**	**Accuracy %**	**Derived Conc. (mg/ml)**	**Accuracy %**	**Derived Conc. (mg/ml)**	**Accuracy %**
A	2.400	120	5.730	95.05	12.786	127.86
B	1.500	75	4.991	83.18	10.809	108.09
C	1.704	85.2	4.716	78.6	9.900	99.00
D	2.938	146.9	5.628	93.8	7.718	77.18
E	1.745	87.25	4.933	82.22	10.036	100.36
Mean	**2.057**	**102.87**	**5.194**	**86.57**	**10.250**	**102.50**
SD	**0.597**	**29.853**	**0.443**	**7.385**	**1.825**	**18.25**
CV %	**29**	**29**	**8.5**	**8.5**	**17.81**	**17.81**

Mean = mean value of values **SD** = Standard deviation **CV %** = Coefficient of variance % (Precision) **Conc** = Concentration

Figure 11.4: Plots showing the stability of Phela 2, 6 and 10 mg/ml at room temperature (±25°C), Fridge (4°C) and Freezer (-20°C) for 24 hr, 48 hr and 1 week.





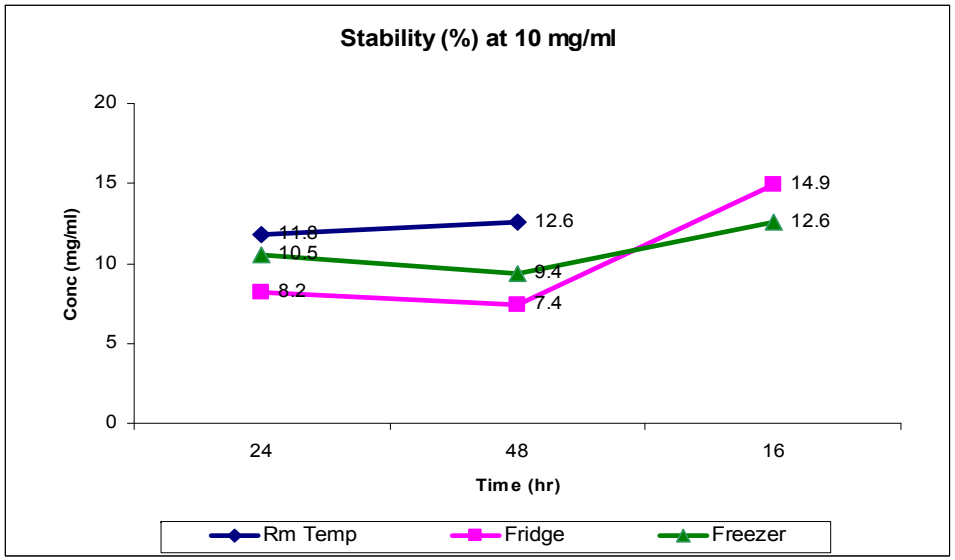


Table 11.5: Stability concentration (mg/ml) and percentage (%) of 2, 6 and 10 mg/ml after storage at room temperature (25 °C), fridge (4°C) and freezer (-20°C) for 24, 48 hours, 1 week and 4 months.

Time	**Temperature**					
	(±25 °C)		**(4 °C)**		**(-20 °C)**	
	Conc (mg/ml)	**%**	**Conc (mg/ml)**	**%**	**Conc (mg/ml)**	**%**
			2 mg/ml			
24	**1.6**	80	**3.3**	165	**1.8**	90
48	**1.9**	95	**1.8**	90	**1.2**	60
1 week	**____**	**____**	**3.9**	195	**2.4**	120
			6 mg/ml			
24	**4.0**	67	**5.0**	83	**5.8**	97
48	**3.2**	53	**3.3**	55	**4.6**	77
1 week	**____**	**_____**	**6.3**	105	**8.0**	133
			10 mg/ml			
24	**11.8**	118	**8.2**	82	**10.5**	105
48	**12.6**	126	**7.4**	74	**9.4**	94
1 week	**____**	**_____**	**14.9**	149	**12.6**	126

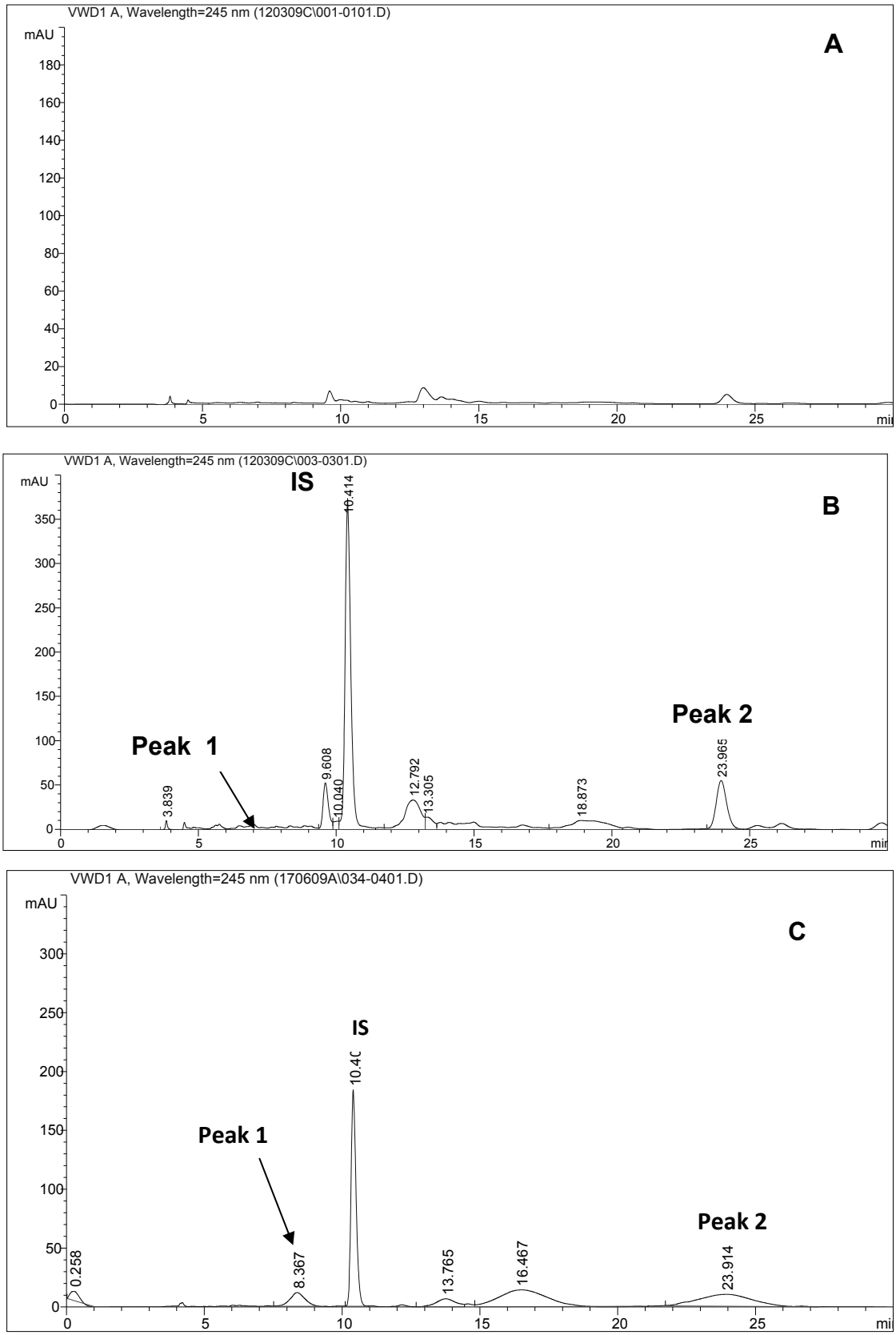
Conc = Concentration **%** = Percentage

11.7.3. Testing of rat plasma on HPLC_UV

Figure 11.5 A to C (p 164) are HPLC_UV chromatograms of blank plasma, spiked plasma and rat plasma after six hours of phela oral administration. In the blank plasma (A) there were no plasma peaks, and the marker peaks were observed in the spiked plasma at 9.6 and 23.9 minutes and internal standard eluting at 10.4 minutes. Unfortunately, the test plasma exhibited very small and poorly resolved peaks at 8.3 and 23.9 minutes. In fact, all the rat samples had similar peak patterns.

Decision: Due to unresolved phela peaks, it was impossible to derive concentrations; therefore the HPLC-UV method was considered unsuitable to measure Phela in rat plasma after oral administration. It was decided to investigate HPLC_FL method further.

Figure 11.5 HPLC_UV chromatogram of blank plasma (**A**), An HPLC_UV chromatogram of spiked plasma with marker peaks at 9.6 and 23.9 minutes and IS at 10 minutes (**B**)and rat plasma after six hours of phela administration and spiked plasma (**C**) with unresolved marker peaks at 8.3 and 23.9 minutes and a sharp symmetrical intreanal standard peak at 10 minutes.



11.7.4. HPLC_FL chromatographic performance

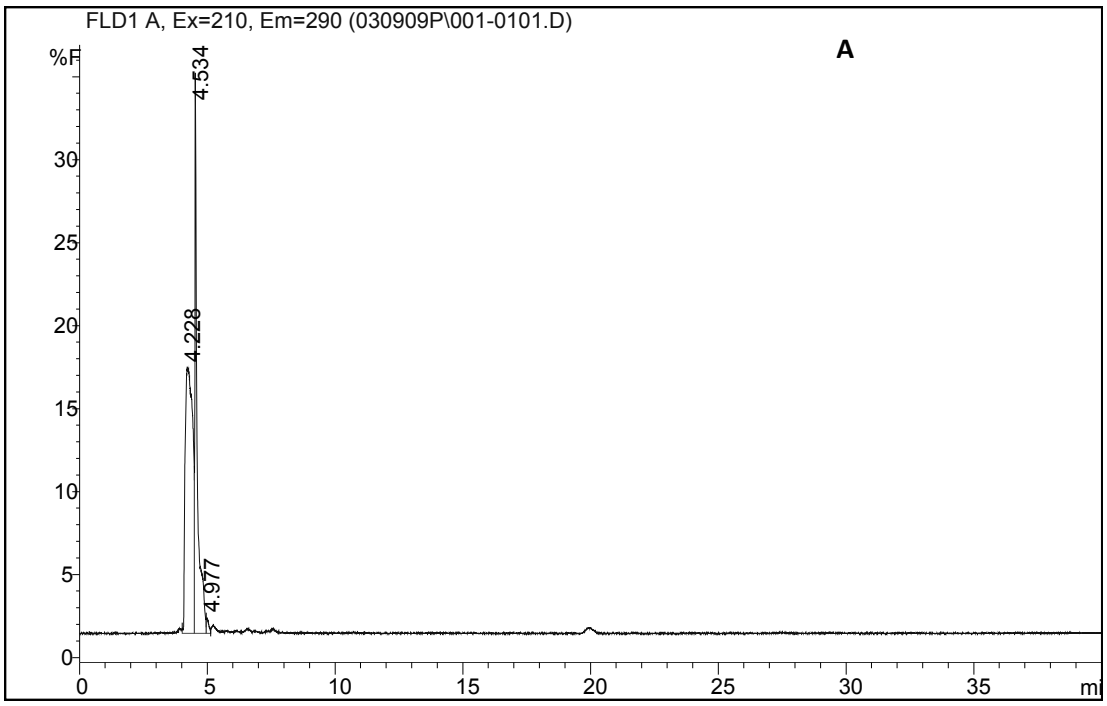
Figures 11.6 A to C (p 166 – 167) are HPLC_FL chromatograms of blank plasma, 20 mg/ml (B) and 80 mg/ml(C) spiked plasma samples. In blank plasma there was a peak at 4 minutes, while spiked plasma (fig 11.6 B and C; p 166 - 167) had peaks from 3.9 – 6.4 minutes with varying peak height (%F*S) and marker peaks, a, b, and c at 12.1, 20.0 and 33.7 minutes respectively. The retention time shift was standardized by mean and standard deviation calculation (Table11.6; p 168). The retention time for marker peak (a) is at (12.3 ± 0.31) minutes, marker peak (b) at (20.9 ± 0.50) minutes and marker peak (c) at (34.1 ± 0.54) with a time difference of at (8.5 ± 0.70) minutes between peaks a and b and (21.7 ± 0.35) minutes between peak a and c.

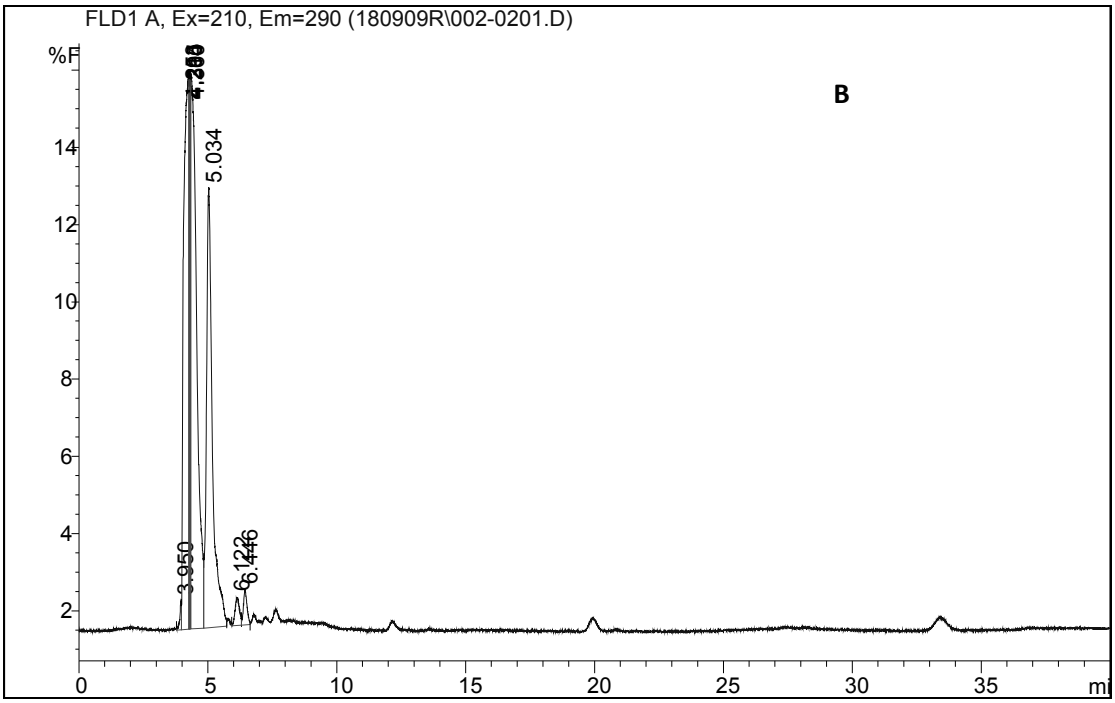
11.7.5. Testing of rat plasma on HPLC_FL

Figure 11.7 A to C (p 167) are HPLC_FL chromatograms of blank rat plasma, spiked rat plasma and rat plasma after six hours of oral administration with Phela. In the blank plasma, there were peaks at 4 minutes, and the marker peaks are shown in the spiked plasma at 12, 22 and 34 minutes. Unfortunately, the test plasma exhibited a new peak at 9.2 minutes. In fact, all the rat samples had similar peaks with varying %F*S. The retention time shift was standardized as mean and standard deviation at 9.1 ± 0.5 minutes. The peak is evaluated further in chapter 11 for pharmacokinetics.

Decision: Though the new peak was observed, it was used as a marker peak in test samples for monitoring of phela in plasma.

Figure 11.6: HPLC_FL chromatogram of blank plasma with peaks at 4.7, 4.8 and 4.9 minutes (**A**). HPLC_FL chromatogram of 20 mg/ml spiked plasma with peaks at 3.8, 5.0 and 6.1 minutes (**B**). HPLC_FL chromatogram of 80 mg/ml spiked plasma with peaks that eluted earlier, and marker peaks a, b and c at 12.1, 20.2 and 33.7 minutes respectively.




FLD1 A, Ex=210, Em=290 (180909R\002-0201.D)
%F
B
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3.950
6.122
6.446
0
5
10
15
20
25
30
35
mi

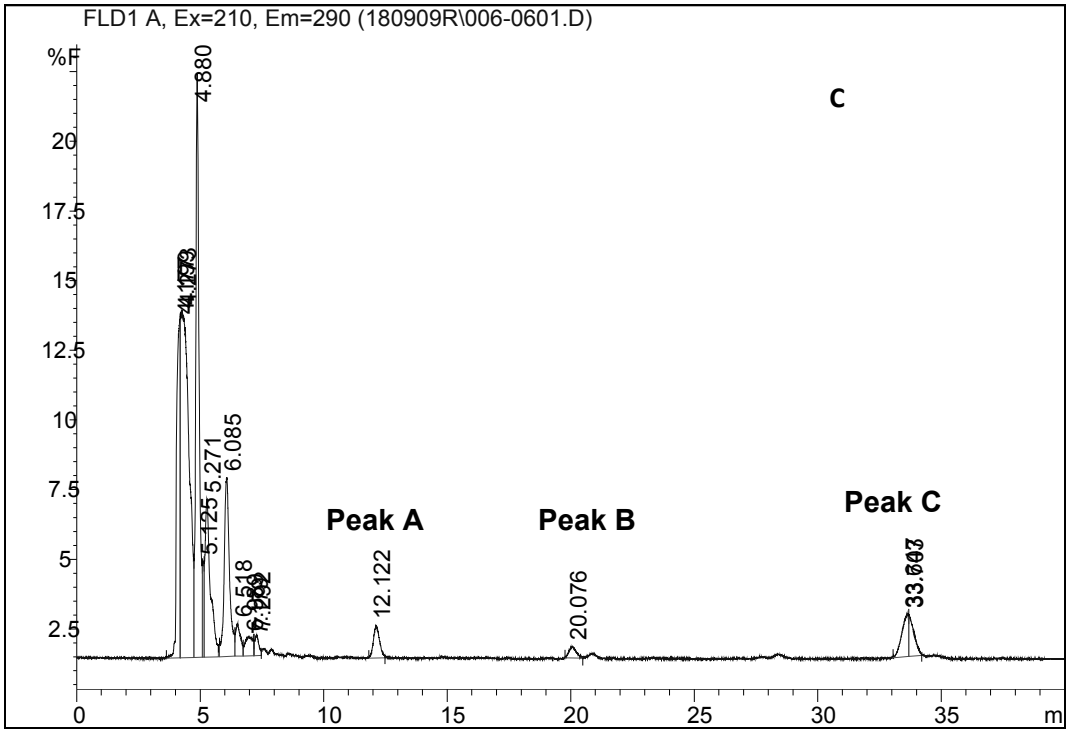
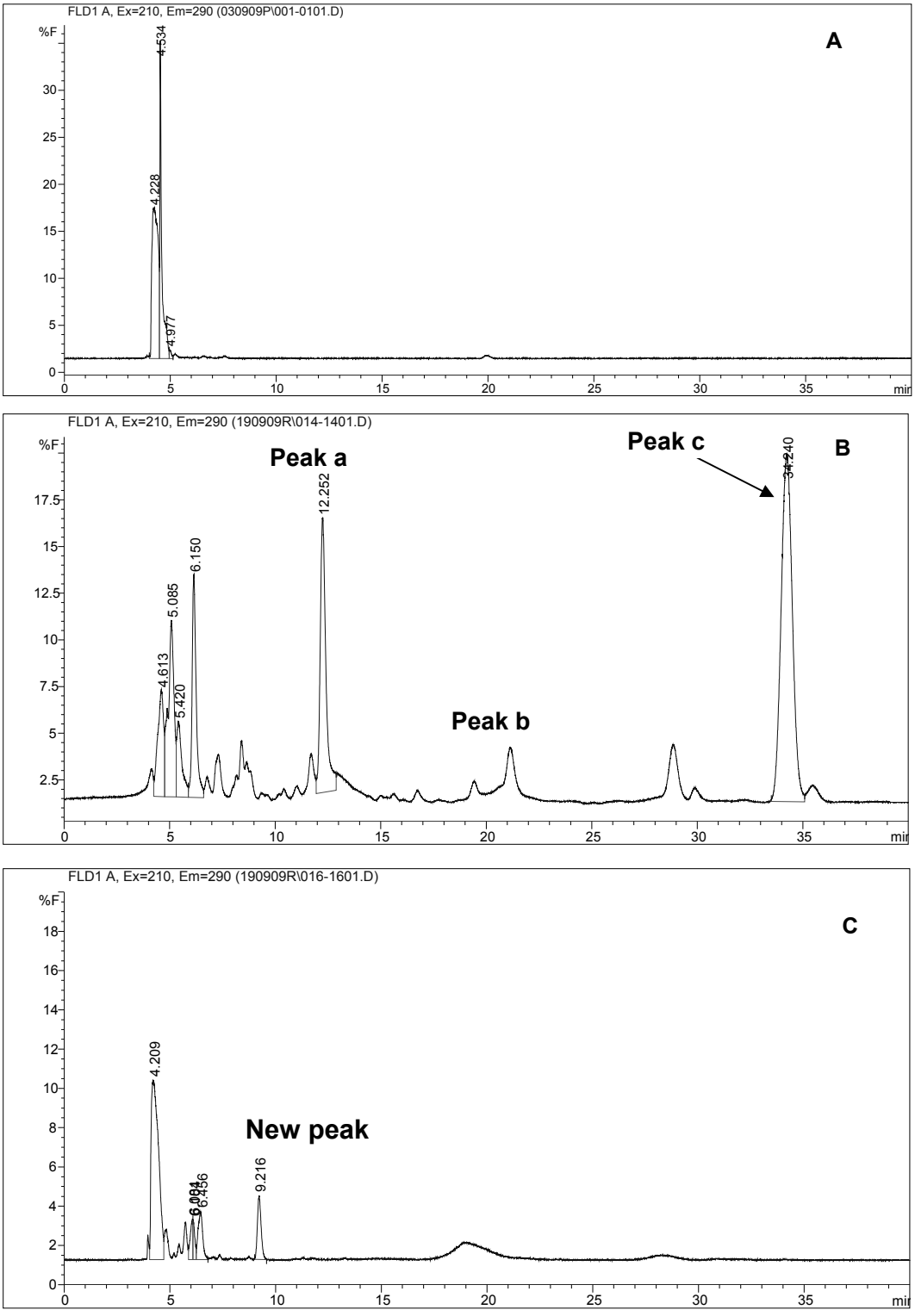

FLD1 A, Ex=210, Em=290 (180909R\006-0601.D)
%F
C
4.880
6.085
5.271
5.125
Peak A
Peak B
Peak C
12.122
20.076
0
5
10
15
20
25
30
35
m

Table 11.6: A summary of statistical analysis of marker peak retention time shifts in HPLC_FL chromatogram.

Marker peaks Retention time (minutes)			**Peaks RT diff.**	
A	**B**	**C**	**A &B (min)**	**A & C (min)**
12.3	21.1	34.1	8.8	21.8
12.8	20.9	34.4	8.1	21.6
12.3	21.1	34.0	8.8	21.7
12.2	21.4	34.2	9.2	22.0
12.1	20.0	33.7	7.9	21.6
Mean 12.3	**20.9**	**34.1**	**8.5**	**21.7**
SD 0.31	**0.50**	**0.54**	**0.70**	**0.35**

Key: **Peak RT (Retention time) diff** = Retention time difference of Marker peak 2 retention time - marker peak 1 retention time. **IS** = internal standard, **Min** = Minutes

Figure 11.7 HPLC_FL chromatogram of blank plasma with peaks at 4.7, 4.8 and 4.9 minutes (**A**). HPLC_FL chromatogram of 100 mg/ml sample with marker peaks a, b and c at 12.1, 21.4 and 34.2 minutes respectively (**B**). HPLC_FL chromatogram of rat plasma after 6 hours of phela administration with a new peak at 9.2 minutes (**C**)



11.8. DISCUSSION

A High performance liquid chromatography (HPLC_UV) with UV detector method has been optimized and standardized. The method was found to be linear and accurate. It is rapid, simple and sensitive. The linear regression is y = 0.02 x – 0.59 and the correlation coefficient r^2 = 0.9983. The CV % was less than 20 % for concentrations below 6 mg/ml and for 8 – 10 mg/ml was less than 21 %. Mean accuracy percentages were found to be within the 15 % set limit. HPLC_UV is commonly used in the analysis of traditional medicines in plasma. (Yin-Zeng, 2003; Lv, 2008).

However, the stability testing gave variable results, with the exception of the 24 hours storage at – 20 °C, that had a recovery of more than 90 % in all three tested concentrations. The samples can be left overnight at – 20 °C if immediate analysis is not possible. Retention time shifts of phela marker peaks were observed, which is common when analyzing traditional medicine by HPLC (Yi-Zeng, 2004). Due to a lack of computer based programs that calculate the retention shift error, the mean and standard deviation were used to calculate the range of shift for each marker peak.

On the other hand, the failure to analyse Phela via the HPLC_UV method was a clear indication that the orthodox approach of developing and validating a method for analysis of a pure compound before its application *in vivo* does not work with traditional medicines. As a result, some innovation was effected by testing the subsequent HPLC_FL method on samples of treated animals before validation was attempted. In fact, the testing obviated the validation process because a metabolite was found instead. It is therefore important that everybody, researchers and ethics committees, consider in good light applications for administration of traditional medicines to subjects for analytical method development.

The results suggest that high performance liquid chromatography with a fluorescence detector (HPLC_FL) method was more suitable for phela concentration monitoring in plasma. The small sample volume (0.5 ml) had optimal results. A standard extract with a concentration of 100 mg/ml was chosen as an external standard for validation and

quality control purposes. The test samples were found to have a new peak that was further analysed in chapter 12.

Concentration monitoring of traditional medicines is not common, and/or traditional practice. However, it would be useful in situations where proof of ingestion, absorption, and change in dose, indicator of potential problems, therapeutic end points, and toxicity are required.

Unfortunately, concentration monitoring of Traditional Herbal Medicines (THM) is difficult, owing not only to the complex nature of the products, but also to lack of knowledge of the active or toxic ingredients by which to monitor therapeutic response or toxicity. Furthermore, in most cases the individual compounds maybe in such low concentrations that they maybe neither detectable nor targeted by the analytical methods. On the other hand, therapeutic activity may be due to multiple compounds whereby one compound may not correlate with response.

Most important is that concentration monitoring requires a method for analysis of whatever marker is to be used. Such a method needs to be validated to ensure robustness before use. This is a challenge in THM because there is no guarantee that peaks observed in the product will be seen in plasma after administration of the phela. Neither the use of isolated intestine nor liver would replace the need for whole animal study in this respect. Furthermore, it creates a challenge in respect of the use of conventional units for concentrations measurement, given that the peaks represent unknown compounds.

Nevertheless, owing to the inaccessibility due to cost or availability of existing western medicines, the use of THM is increasingly gaining importance. Already, the WHO and most government and non-government organizations (NGOs) are championing the development of cheaper effective medicines for neglected diseases from THM. Therefore, promotion of THM calls for a paradigm shift in the manner in which we

undertake the evaluation and safety monitoring of medicines. There is a need for innovative techniques by which to ensure safety of traditional medicines.

11.7 CONCLUSION

An HPLC method for the monitoring in plasma of phela concentrations was developed and a possible new avenue for monitoring concentration of traditional medicines in plasma would be developed.

CHAPTER 12

THE ANALYSIS AND USE OF METABOLITE PEAK-KINETICS TO MONITOR PHELA IN RAT PLASMA.

12.1 SUMMARY

Fifteen (n = 15) male Sprague Dawley rats were each dosed with 15.4 mg/kg phela aqueous solution. Blood was withdrawn from rats (n = 3) after 1, 2, 4, 6 and 8 hours, respectively. Rat plasma samples were analysed by HPLC_FL and the result was a new peak at 9.1 ± 0.5 minutes, which was concluded to be phela metabolite was observed. Changes of the peak area per unit volume were used to derive the appropriate pharmacokinetics parameters, hence the term peak-kinetics. The predictive parameters show that concentration of the metabolite at steady state would be 48 peak-area/ml and would never exceed with frequent dosages, hence no accumulation of drug. In conclusion, phela concentration was monitored in plasma by HPLC_FL and pharmacokinetics parameters established.

12.2 INTRODUCTION

The South African Medical Research Council Indigenous Knowledge System Lead Programme has calculated the recommended the standard dose for phela as 15.4 mg/kg per day, given as an aqueous solution. The rats were administered with a single oral dose, hence, there is a need to monitor phela concentration by HPLC to determine whether phela is absorbed after an oral dose.

12.3 MATERIALS AND METHODS

12.3.1 Apparatuses

A 5810R centrifuge from Eppendorf (Germany) was used for centrifuging. A nitrogen evaporator with a heating block and horizontal shaker were used for shaking and evaporating respectively. A Vortex mixer (Scientific Industries Inc, U.S.A) was used for mixing of samples. Fin pipettes from Thermo Labsystems (Canada) were used for spiking volumes.

12.3.2 Chemicals and reagents

Phela was manufactured and supplied by the Indigenous Knowledge Unit (IKS) of the South African Medical Research Council. Hexane, Isopropanol and ether were purchased from Burdick and Jackson (U.S.A). Ammonia sulphate was purchased from Sigma – Aldrich (Germany). Deionised water was prepared using by the Millipore system. The oral gavage was a 16 G-3 curved with a 3.00 mm ball and used for oral dosing of phela.

12.4 ANIMAL EXPERIMENT

The study was approved by the Animals Ethics Committee of the University of the Free State. Fifteen male Sprague Dawley rats (Figure 12.1; p 175)were divided into three groups of 5 animals each. All the animals were then administered orally with an aqueous solution of Phela at a dose of 15.4 mg/kg. Thereafter, one animal from each group was sacrificed after 1, 2, 4, 6 and 8 hours of dosing. Under ether anesthesia,

blood samples were collected by cardiac puncture in a heparin tube. The samples were placed on ice, after which they were centrifuged and plasma separated within 30 minutes. Plasma was stored at -20°C until analysis.

Figure 12.1: A picture of a Sprague Dawley male rat used during the animal experiment (By author)

12.5 MONITORING OF PHELA IN PLASMA BY HPLC

12.5.1 Chromatographic system and conditions

Chromatographic system: The HPLC system consisted of a Hewlett-Packard 1100 series system that comprised an isocratic pump, and an auto-sampler, connected to a computer with LC-ChemStation software. The programmable fluorescent detector (HP-1046A; made in Germany) was set at emission and excitation wavelengths of 210 nm and 290 nm, respectively.

Chromatographic conditions: Chromatographic separation was achieved with a reverse phase C_{18} (250 mm x 4.6 mm x 5 microns) coupled to a C_{18} guard column

(Phenomenex, U.S.A). The mobile phase used for elution was 70 % acetonitrile in water at a flow rate of 0.5 ml/min and 40 minutes run time.

12.5.2 Sample preparation: Salting-out method

To 500 µl of rat plasma, 100 mg ammonia sulphate {$(NH_4)_2SO_4$} was added. The samples were extracted with 1 ml of Isopropanol and left to shake for 10 minutes at room temperature. Centrifugation was achieved at 2500 rpm (1 251 g) for 10 minutes at 4°C. The supernatant was evaporated under nitrogen at 47 °C, whilst the residue was reconstituted with 200 µl of mobile phase and 50µl was injected onto the HPLC. External standards (n = 5) of 100 mg/ml each were also analysed.

12.6 PEAK-KINETICS

For evaluation of the metabolite kinetics the concentration of the metabolite was expressed as the metabolite's peak area (PK-area) per plasma volume (ml), by dividing the peak area by sample volume (i.e., PK-area/ml). This concentration was then used to derive the necessary pharmacokinetic parameters (i.e., Peak-kinetics; the use of peaks in plasma chromatograms to determine the pharmacokinetics of a compound). In effect, the pharmacokinetic formulae remain the same except for the concentration units.

Pharmacokinetic parameters of the marker peak for each group of animals were obtained from plasma concentration (PK-area/ml) versus time plots by non-compartmental methods, whereby the area under plasma concentrations versus time (AUC) were calculated using the trapezoidal rule with extrapolation to infinity (Ct/Ke) (Gibaldi et. al., 1984). The elimination rate constant (Ke) for the decline of plasma concentrations was obtained by linear regression on log plasma concentration against time plots. Thereafter, the following equations were used to derive the relevant pharmacokinetic parameters:

Maximum concentration (C_{max}) and time to reach Cmax (T_{max}) were read off directly from the concentration time curve.

- Half-life ($T_{½\,(m)}$) = Eq.12.1
- Mean Residence Time ($MRT_{(m)}$) = Eq.12.2

Prediction of concentration of metabolite at steady state ($C_{ss(m)}$) at specific dosing interval (*τ*) and the extent of drug accumulation (Accumulation index; Acc_{index}) on multiple dosing were calculated as follows:

- Conc. of metabolite at steady state ($C_{ss(m)}$) = Eq.12.3
- Accumulation index ($Acc_{index(m)}$)= Eq. 12.4

12.6.1 Statistical data analysis:

Data was analysed by non-parametric methods using the Graph Pad™ statistical program. Accordingly, parameters were reported as median and range, and the Mann Whitney Test was used for data comparison with the level of significance set at $p < 0.05$.

12.7 RESULTS

Table 12.1 (p 179) is a summary of rat weights used throughout the study. The weights ranged from 299g to 404g. There was no significant difference in the weight of animals.

Figures 12.2 A - C (p 180) are chromatograms of blank plasma, external standard and rat plasma after HPLC_FL analysis. Figure 11.2A (p 180) is blank plasma; there was no interference with plasma. In figure 11.2B (p 180) is a chromatogram of external standard with marker peaks (a, b and c) at 12.2, 22.0 and 34.2 minutes, which is within the standardized range of 12.3 ± 0.31 minutes for peak (a), 20.9± 0.50 minutes for peak (b) and 34.10 ± 0.54 minutes for peak (c) (n =6) in table 11.6.

Figure 12.2 C (p 180) is a chromatogram of rat plasma after six hours of phela oral administration. There was a symmetrical peak at a retention time of 9.2 minutes in the rat plasma samples. However this peak does not correspond with any of the phela peaks in the external standard chromatogram (fig 12.2 B; p 180). All the analysed samples had a similar peak with varying peak heights (in %F*S units).

In Figure 12.3 (p 181) the peak area at 9.1 ± 0.5 minutes increased with time to a maximum and then fell off, typical of a time versus concentration curve: the peak areas (%F*S) were 116 at 1 hour, 156 at 2 hours, 204 at 4 hours, 156 at 6 hours and 60 at 8 hours after oral administration of Phela. Since this peak was not observed in the spiked plasma, it implied that it was a metabolite of Phela. Therefore, it was decided to use this peak as a marker for monitoring Phela in plasma.

Table 12.2 (p 182) is a summary of Phela concentrations per group after single oral administration in rats over eight hours. The mean concentration was 80 ± 16, 102 ± 34, 124 ± 31, 98 ± 44 and 45 ± 5 peak area/ml for 1, 2, 4, 6 and 8 hours respectively.

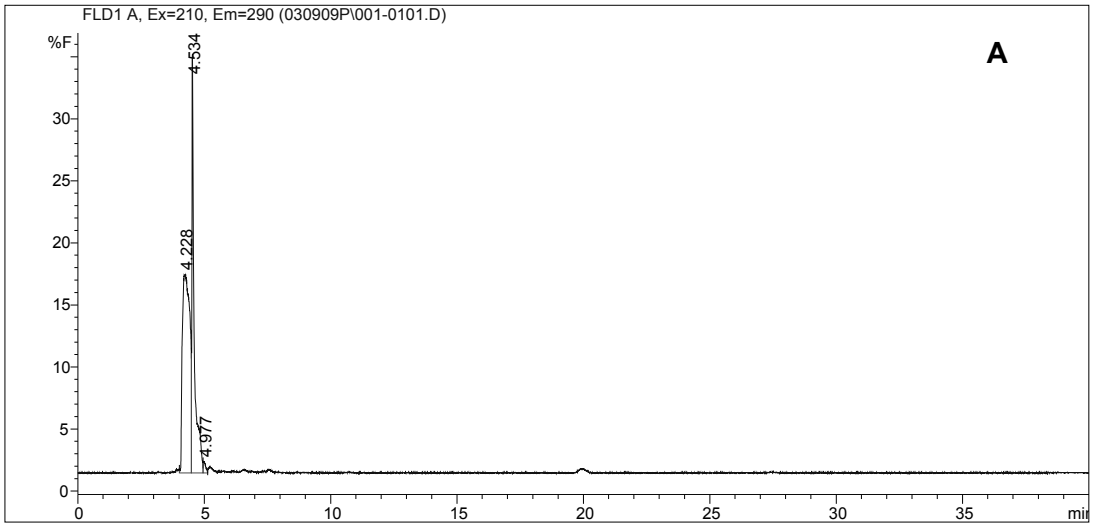
Figure 12.4 (p 182) is the concentration versus time plot for Phela's metabolite in rats after oral administration (Appendix D; fig D-1, p 253; D-2, p 254; D-3, p 255), while Table 11.3 (p 183) shows the pharmacokinetic parameters of the metabolite derived from the plasma concentration profile in the same animals. The rats' weights were within the same range and so were the doses. Except for the T_{max} of 6 hours in group 2, the rest of the kinetic parameters were similar in the three groups. Owing to lack of knowledge of the parent compound kinetic parameters, it was not possible to determine whether the metabolite's rate limiting elimination step is in the formation or elimination process.

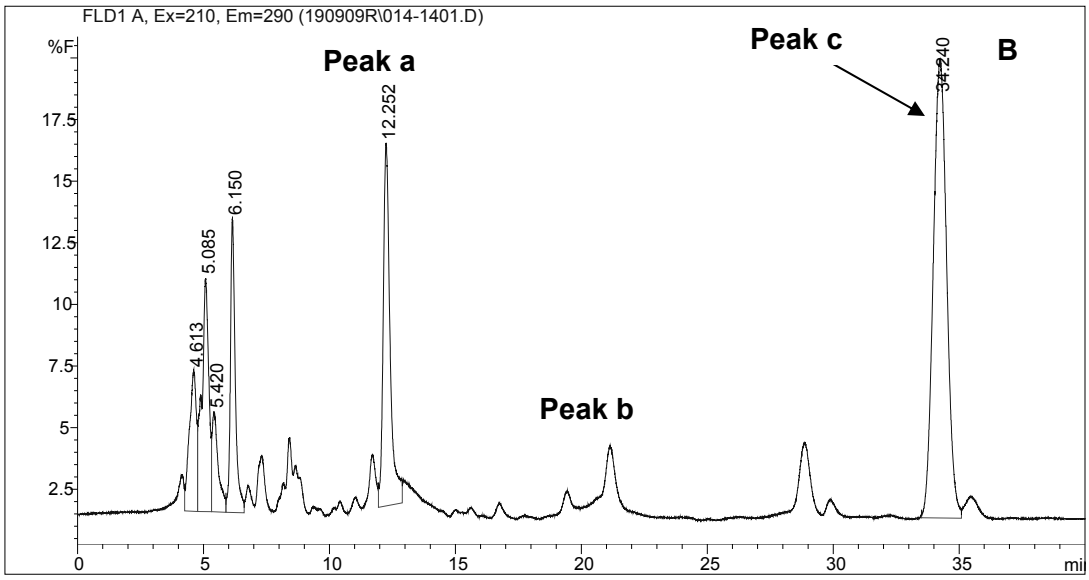
The predictive parameters show that concentration of the metabolite at steady state would be 48 PK-areas/ml, and that this concentration at steady state will be similar to that observed after single dose administration (Acc. index = 1), which implies that there will be no accumulation of the drug. Since the dose of metabolite is unknown, and urine was not collected, it was not possible to calculate clearance and volume distribution of the metabolite.

Table 12.1: Summary of rats weight (grams) per group used during the animal experiment

	Weight (grams)		
Time (hours)	**Group 1**	**Group 2**	**Group 3**
1	380	299	392
2	378	358	389
4	373	352	404
6	395	382	401
8	358	352	361
Mean	**377**	**349**	**389**
SD	**13**	**30**	**17**

Figure 12.2: HPLC_FL chromatogram of blank plasma (**A**) external standard with marker peaks at 12.2, 21.7, 34.2 minutes (**B**) and rat plasma after six hours of phela administration with a new peak at 9.2 minutes (**C**).





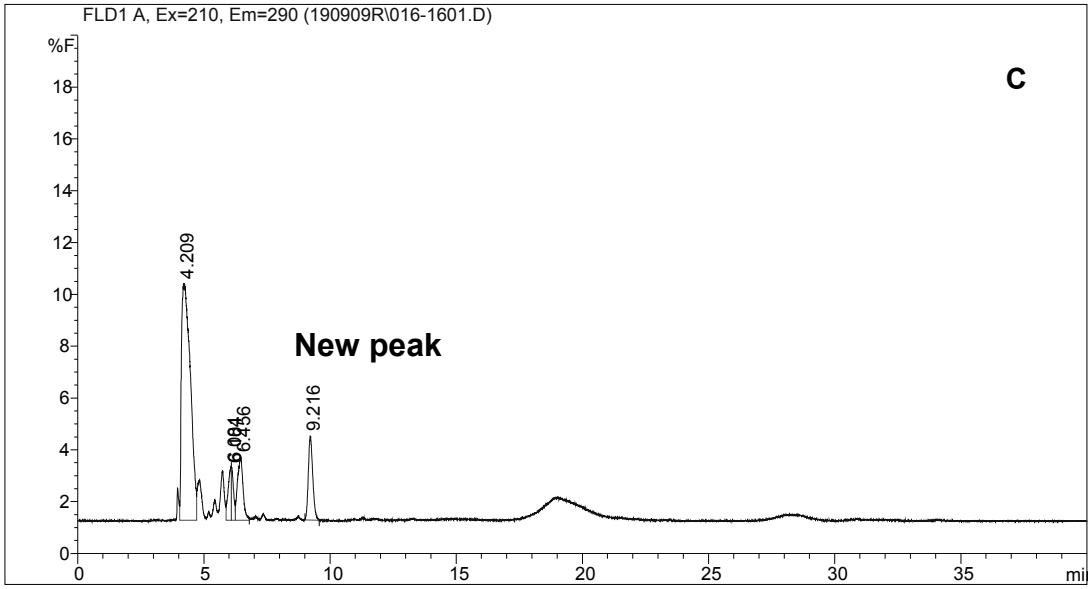
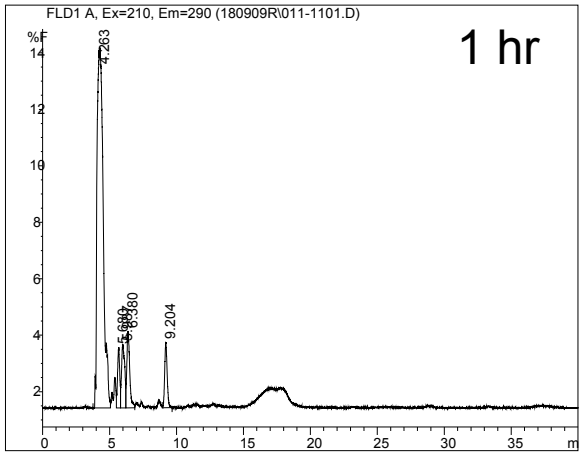
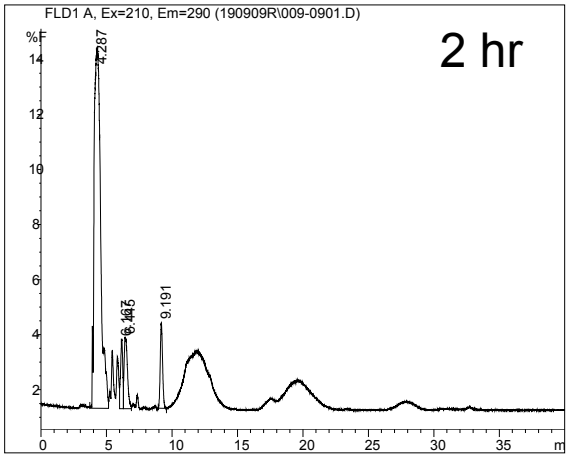
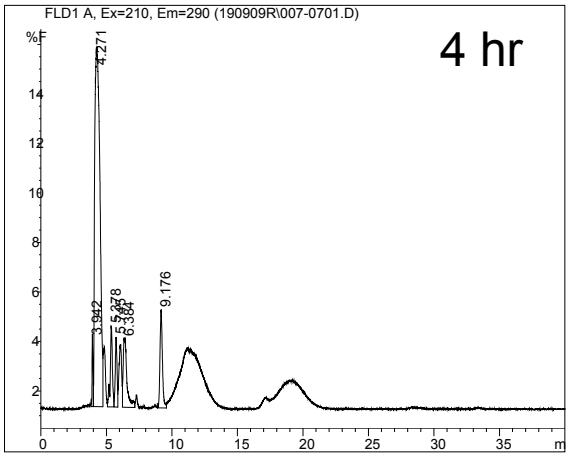
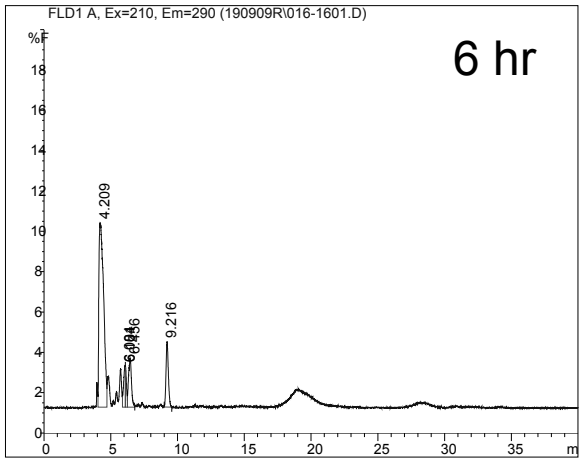


Figure 12.3: HPLC_FL chromatograms illustrating the changes in peak area of RT 9.1 -9.3 minutes, after 1 hour (58 peak area/ml), 2 hours (78 peak area/ml), 4 hours (102 peak area/ml), 6 hours (78 peak area/ml) and 8 hours (30 peak area/ml) of oral administration of Phela.









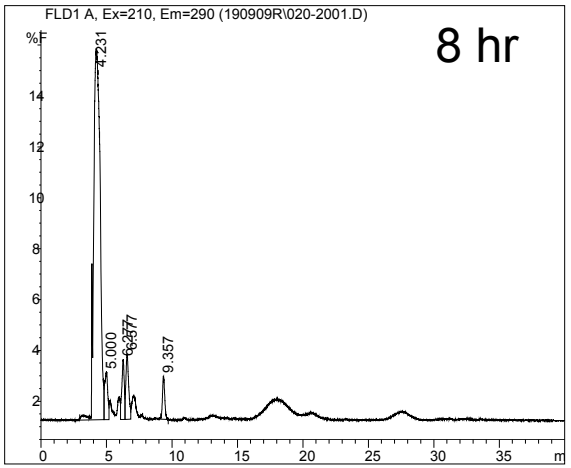


Table 12.2 Summary of phela rat plasma concentration (peak-area/L) after HPLC_FL analysis. The average plasma concentrations of Phela's metabolite in rats (n = 3) after oral administration of Phela.

	Concentration (peak-area/L)			
Time (hours)	**Group 1**	**Group 2**	**Group 3**	**Mean ± SD**
1	58	72	48	**59 ± 12**
2	100	76	50	**75 ± 25**
4	118	76	82	**92 ± 23**
6	78	104	80	**87 ± 14**
8	30	32	40	**34 ± 5**

Figure 12.4 Linear graph of rat plasma mean concentration (peak-area/L) versus time (hr) after phela single oral dose.

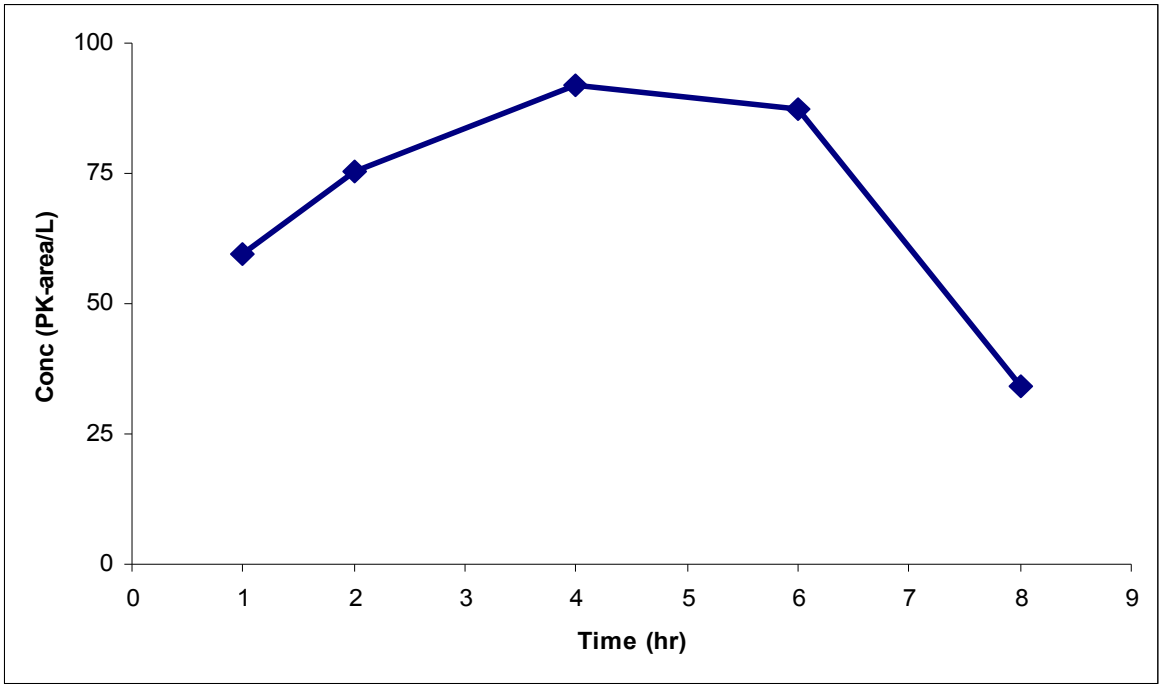


Table 12.3: Plasma pharmacokinetic parameters of Phela's metabolite in rats after oral administration of Phela.

	Group 1	**Group 2**	**Group 3**	**Mean ± S.D.**
Wt (kg)	0.377	0.349	0.389	**0.372** ± 0.02
Dose (mg)	5.80	5.37	6.00	**5.72** ± 0.32
Co$_m$ (Pk-area/L)	270.05	231.47	187.91	**229.81** ± 41.10
Ke$_m$ (/hr)	0.207	0.216	0.180	**0.201** ± 0.02
Half-life$_m$ (hr)	3.35	3.20	3.85	**3.47** ± 0.35
AUC$_m$ (PK-area.hr/L)	1304.47	1070.19	1045.90	**1140.52** ± 142.45
MRT$_m$ (hr)	4.83	4.62	5.57	**5.01** ± 0.50
F$_m$ (%)	1	1	1	1 ± 0
Tmax$_m$ (hr)	4	6	4	**4.67** ± 1.15
Cmax$_m$ (Pk-area/L)	118	104	82	**101.33** ± 18.15
Predictive parameters				
C$_{ss\ (m)}$ (Pk-area/L)	54.35	44.59	43.62	**47.52** ± 5.94
Accc$_{Index(m)}$	1.007	1.006	1.014	**1.009** ± 0.004

Key: AUC$_m$ **=** Area under the plasma concentration-time curve for the metabolite; **CLp**$_m$ = plasma clearance of the metabolite; **Cmax**$_m$ = maximum concentration; **Co**$_m$ = concentration of the metabolite after instantaneous distribution at time zero; **F** = bioavailability of the metabolite; **Ke** = elimination rate constant of the metabolite; **MRT**$_m$ = mean residence time of the metabolite; **Tmax**$_m$ = the time to reach maximum concentration of the metabolite; **Vd**$_m$ = volume of distribution of the metabolite; and **Wt** = weight.

12.8 DISCUSSION

Phela concentration was successfully monitored in rat plasma by analysis with high performance liquid chromatography with a fluorescence detector (HPLC_FL). The new peak observed was depicted as a metabolite for phela and was then utilized as the plasma marker for Phela. Since the metabolite is unknown and therefore there was no standard by which to express its plasma concentration in the conventional units, the changes in peak area per unit volume of plasma (L) were used to derive the appropriate pharmacokinetic parameters, hence the term peakokinetics. Please note, peak-kinetics is a process, while the derived parameters are true pharmacokinetic parameters of the unknown compound.

The peak area was successfully used to derive the appropriate pharmacokinetic parameters. There is no doubt that the pharmacokinetic parameters obtained will be the same even after purifying and converting to the appropriate conventional concentration units of the metabolite. This enabled the setting of experimental conditions so that, when animals are given a single dose of Phela of 15.4 mg/kg, the parameters in Table 12.3 are obtained, and that all associated therapeutic or toxic responses will require these same conditions to be reproduced. It was also established by predictive kinetics that at the indicated rate of dosing (i.e., once daily) a steady state concentration of 48 PK-area/ml would be obtained, and that there would be no accumulation of the drug. Using this information, the dosage could be varied until a desired plasma concentration at steady state is achieved. This information will be used in the subsequent experiments to investigate the mechanism of action of Phela (chapter 13; p 185).

12.9 CONCLUSION

In conclusion, the use of the peak area of phela metabolite to derive pharmacokinetics of unknown compounds as well as to monitor Phela, a traditional medicine, were demonstrated with a hope that they may be useful to those experiencing similar problems with the development of products of which little is known.

CHAPTER 13

AN INVESTIGATION ON THE EFFECT OF PHELA ON THE IMMUNE SYSTEM IN A RAT MODEL

13.1 SUMMARY

Sprague Dawley rats were used to investigate the mechanism of action of phela on the immune system. The rats were divided into six groups. The first group was not treated with anything. The four groups were treated daily with either normal-saline orally (control), cyclosporine in olive oil, subcutaneously (negative control), Phela orally (test-group 1) and phela+cyclosporine (test-group 2). The last group was inoculated once with influenza vaccine. The treatment period was 14 days and in each group rats were sacrificed after 7 and 14 days. Haematology tests, biochemistry tests, and cyclosporine level analysis were done. Serum cytokine analysis for TH_1 and TH_2 cytokine was measured by ELISA. On days 7 and 14, the concentrations of TH_1 cytokines in the Phela-only treated group were similar to those of the control. However, the TH_1 cytokines were higher in the Phela+cyclosporine-A treated group than in the cyclosporine-A group, and cyclosporine-A concentrations were similar in both groups. These results show that Phela did not stimulate TH_1 cytokines of a normal immune system but stimulated them when the immune system was suppressed by cyclosporine-A. This implies that Phela is a cyclosporine-A antagonist that may stimulate the immune system in patients with a compromised immune system. It can be concluded that Phela is an immune stimulant.

13.2 INTRODUCTION

In this chapter the aim was to determine the effect of Phela on the immune system by observing for subclinical changes in TH_1 cytokines (IL-2, IFN-γ, TNF-α) that would enhance cell mediated immunity and TH_2 cytokines (IL-4 and IL-10) that would enhance the humoral response, with the hope that understanding the mechanism of action would help in designing phela as an immune booster therapy.

13.3 MATERIALS AND APPARATUSES

13.3.1. Apparatuses

The oral gavage was a 16 G-3 curved with a 3.00 mm ball purchased from Popper and Sons inc (U.S.A), and was used for the administration of phela, and neutral saline. The 1ml and 2 ml syringes were purchased from Lasec (S.A). A 25G (5/8) needles were used to administer cyclosporine A and influenza vaccine, whereas 21G needles were used for the withdrawal of blood and were purchased from Lasec (S.A). Diethyl ether was purchased from Honeywell Burdick & Johnson (USA). Red toped tubes for serum collection were purchased from Lasec. EDTA tubes and yellow toped tubes were supplied by the PathCare laboratory at Universtas Bloemfontein. A micro plate reader with an Ascent software program was used during ELISA analysis. A tecan micro plate washer [Tecan Austria GmbH; Austria] was used for the washing of the wells.

13.3.2. Chemicals and reagents

Phela was manufactured and supplied by the Indigenous Knowledge System Lead Programme (IKS) of the South African Medical Research Council. The neutral saline (euro-med 0.9 % NaCl) [Euro–med, Philippines] was a donation from the University of the Free State toxicology laboratory. Cyclosporine A was purchased from Sigma-Aldrich (Germany). Olive oil was purchased from a local Woolworth's supermarket. The Influvac® subunit 2009 (Solvay Pharm. Pty Ltd, Johannesburg, South Africa) was purchased from a local Clicks® pharmacy. The Influvac subunit 2009 contained 30ug/ml of surface antigens of each the strains; H_1N_1 (A/Brisbane/59/2007), H_3N_2 (A/Brisbane/10/2007) and B (B/Florida/4/2006). Rat IL-2 and rat IFN-γ ELISA kits were purchased from Bender Med systems (Austria). Rat

TNF-α ELISA kit was purchased from Endogen (U.S.A). Rat IL-4 and IL-10 ELISA kits were bought from BD Biosciences (U.S.A). Cyclosporine FPIA Kit was purchased from Abbots Laboratories (U.S.A).

13.4 EXPERIMENTAL DESIGN

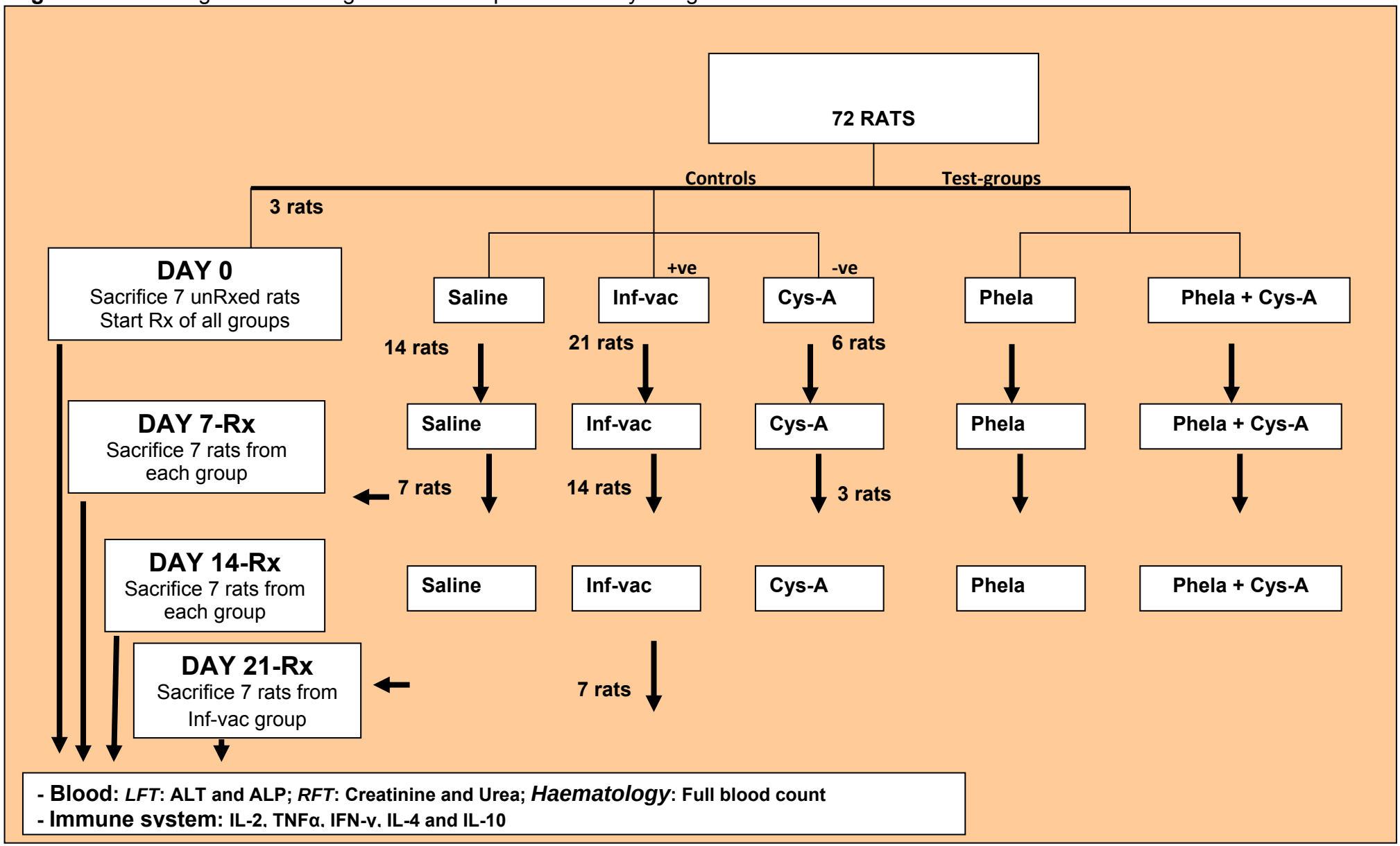
13.4.1. Animal experiment

Experimental animals were bred and obtained from the University of the Free State Animal house. The study was approved by the Animal Ethics committee of the University of the Free State and designated with the following ethics approval number: ETOVS 07/08. The rats were cared for by the University of the Free State Animal house by qualified staff according to international guidelines. The rats had free access to standard rat chow and water, *ad libitum.* Cages were cleaned twice a week and they were housed in an air conditioned environment and kept on a light/dark cycle of 12hr/12hr. The husbandry condition was kept constant throughout the whole study.

13.4.2. Animal experiment dosing schedule and design

Male Sprague Dawley (SD) rats were used. The animals were divided into six groups in which three groups had 14 rats each, while the fourth group had 21 rats and the fifth and sixth groups each had 6 rats (Figure 13.1). The four groups were treated daily with either normal-saline, orally (control); cyclosporine 2 mg/kg in olive oil, subcutaneously (Cys: negative control); Phela 15.4mg/kg, orally (test-group-1), and Phela+cyclosporine (Phela+Cys: test-group-2). Thereafter, 7 animals from each group, with the exception of the cyclosporine group with 3 animals per group, were sacrificed after 7 and 14 days of treatment. The fifth group was inoculated once intramuscularly (laterally on the thigh) with influenza vaccine 0.2 ml (positive control), after which 7 animals were sacrificed after 7, 14 and 21 days. The sixth group was not treated with anything (untreated), hence was used to determine the status of the immune system at base line (i.e., in unmanipulated animals).

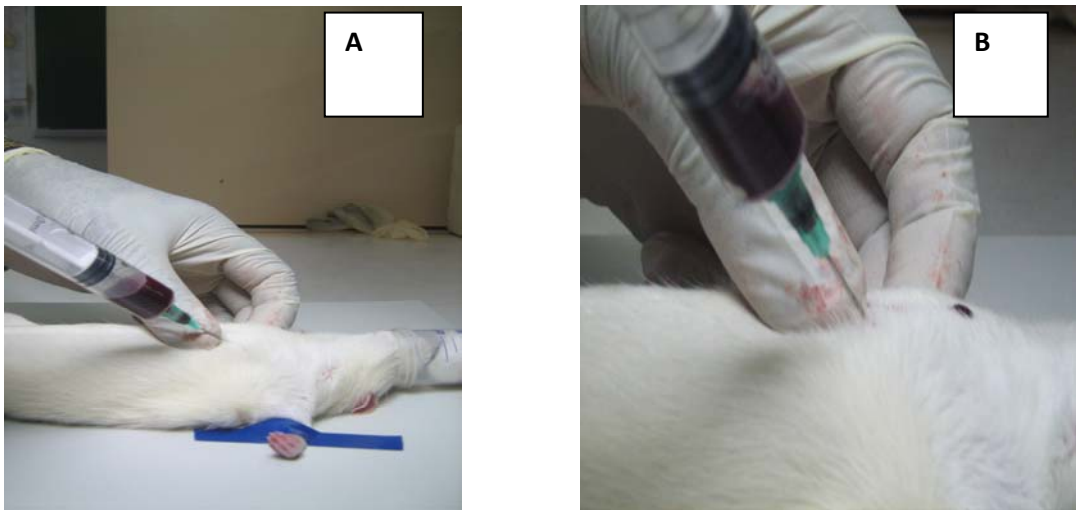
Figure 13.1: A diagram illustrating the animal experiment study design



13.4.3. Standard animal sacrificing procedure

On the day preceding the last day of dosing (day 8, day 15, and day 22), rats were taken to the pharmacology laboratory for blood collection and sacrificing. Under ether anaesthesia, blood (8-10 ml) was drawn via cardiac puncture (figure 13.2A - B) and immediately aliquoted into the appropriate test-tubes. The rat was sacrificed by exsanguination whilst still under anaesthesia. Blood was collected for liver function tests and full blood count, phela concentration, cyclosporine levels and cytokine analysis. Phela concentrations were measured by HPLC using a method developed in our laboratory, and cyclosporine was analysed by fluorescent Polarization Immuno-Assay (FPIA) using a Kit (cyclosporine assay for whole blood) according to the manufacturer's instructions

Figure 13.2: Pictures showing a SD rat on a surgical table under anesthesia (**A**) and blood collection withdrawal through a heart puncture (**B**) [By author]



13.4.4. Blood collection

Blood collection was done in EDTA tubes (for full blood count), Heparin tubes (for liver function tests; renal functions tests), and serum collection in red toped tubes. The serum tubes were allowed to stand, followed by centrifugation at 4000 rpm (3 202 g) for 10 minutes at 4°C. The serum was collected and stored at – 20°C until time of analysis by ELISA (Section 13.5).

13.4.5. Blood tests

EDTA tubes (for the haematology analysis) and ISST tubes (for the biochemistry analysis) were sent to the PathCare laboratories at the Universtas Hospital in Bloemfontein for analysis. Haematology tests carried out were for the full blood count. For biochemistry, Liver function tests done were for alanine transaminase (ALT) and alkaline phosphatase (ALK) levels. Renal function tests carried out were creatinine and urea levels. The PathCare laboratories performed both the biochemistry and hematology analysis results available within 48 hours.

13.5. ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

13.5.1. Cytokine Analysis

Rat serum was analysed using ELISA kits to detect levels of TH_1 cytokines (IL-2, IFN-γ, TNF-α) and TH_2 cytokines (IL-4, IL-10) under investigation. Furthermore, rat serum was randomly selected from all groups (normal-saline; control, cyclosporine; negative control, influenza vaccine; positive control, Phela; test-group 1 and Phela+cyclosporine; test-group 2) resulting in analysis of four rat serum samples per treatment group per seven days. The calibration standards for each cytokine were in the range of 63 – 2000 pg/ml. Table 13.1 (p 191) illustrates how the standards and rat serum per group were loaded on the 96 well micro-plate, with blank (tan),

calibration standards (blue), control (light orange), negative control (red), positive control (light purple), test-group 1 (light pink) and test-group 2 (light green).

Table 13.1 The table illustrates the layout of sample loading on the micro plates, with a letter number combination (i.e A 3) representing group name and rat number.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	A2	A2	C2	C2	J3	J3	F2	F2	H1	H1
B	Std 1	Std 1	A3	A3	C3	C3	J2	J2	F3	F3	H2	H2
C	Std 2	Std 2	A5	A5	C5	C5	K1	K1	F4	F4	H3	H3
D	Std 3	Std 3	B1	B1	D1	D1	K2	K2	F5	F5	H4	H4
E	Std 4	Std 4	B2	B2	D2	D2	E1	E1	G1	G1	I1	I1
F	Std 5	Std 5	B3	B3	D3	D3	E2	E2	G2	G2	I2	I2
G	Std 6	Std 6	B4	B4	D5	D5	E3	E3	G3	G3	I3	I3
H	A1	A1	C1	C1	J1	J1	E4	E4	G4	G4	I4	I4

13.5.2. ELISA procedure

Figure 13.3 (p 192) and figure 13.4 (p 193) illustrate the ELISA procedure for quantitative detection of TH_1 and TH_2 cytokines. The concentrated buffers were diluted to known working concentrations and the experiment was done at room temperature. The micro-plates used were pre-coated with an anti-rat cytokine (i.e. IL-2) and thereafter the standards and rat serum were loaded and incubated for an hour to allow the cytokine to bind to the coating antibody (fig 13.3 step 1; p 192). Excess serum was washed off with a wash buffer (fig 13.3 step 2; p 192).

Furthermore, the biotin conjugated anti-rat cytokine (i.e. anti-IL-2) was added and incubated for two hours to enhance binding to the captured cytokine (fig 13.4; A-2; p 193). Once again the washing step removed excess biotin conjugated anti-rat cytokine.

In fig 13.3 step 3 and 4 (p 192), Streptavidin-Horse radish Peroxidase was added and allowed to bind during another incubation step and the excess was washed off. The substrate solution was added and allowed to develop in the dark. A coloured product was formed in proportion to the amount of rat cytokine present in the test sample or standard. The reaction was stopped with an acid and a yellowish colour formed (fig 13.4 B; p 193). Absorbance was measured by UV spectrophotometer with a micro plate reader set at 450 nm filter (fig 13.3 step 5; p192). A standard curve was plotted from the calibration standards and the rat cytokine (i.e. IL-2) concentrations determined.

Figure 13.3: A schematic representation of the steps followed during cytokine ELISA (http: en// Wikipedia [Accessed on 15 January 2010])

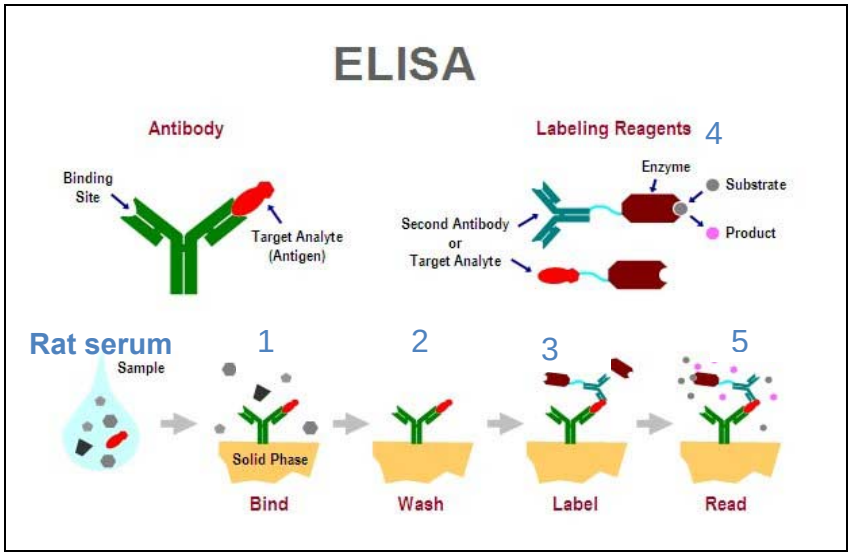
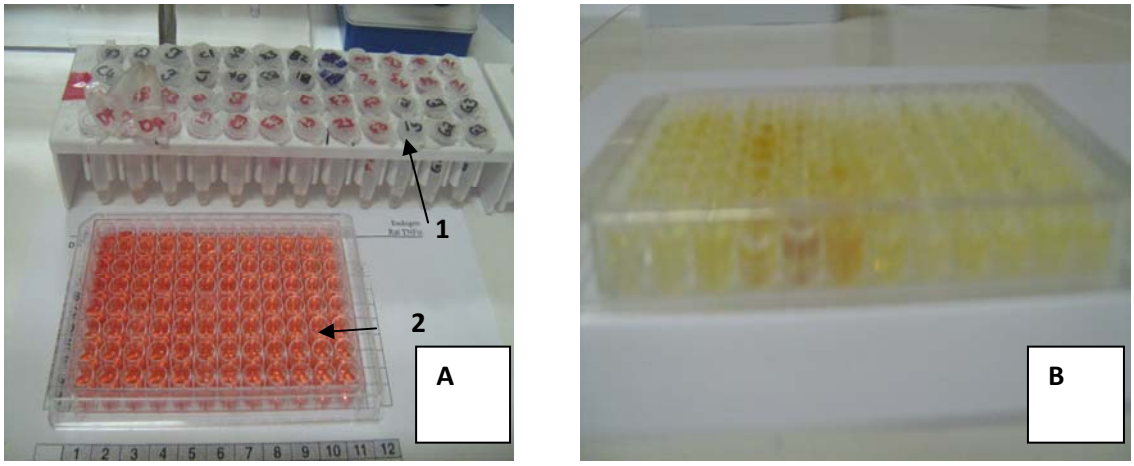


Figure 13.4: Pictures showing steps during ELISA analysis: In (**A**) Rat serum on a sample rack (**1**) and A sample loaded micro plate loaded incubating at room temperature (**2**). In (**B**) a yellow colour in a micro plate after the reaction was stopped with an acid [By author]



13.5.3. Data analysis

The response of cytokines to the treatment with different agents was evaluated by analysing the response pattern in the TH_1 (IL-2, INF-γ and TNF-α) and TH_2 (IL-4 and IL-10) cytokines versus the respective controls.

13.5.4. Statistical analysis

All statistical analysis was done using the Graph Pad Instat program and graphs drawn on an excel program. Data was analysed by non-parametric methods using the GraphPad Instat statistical program. Accordingly, summary data were reported as median and range, and the Mann-Whitney Test was used for data comparison with the level of significance set at $p < 0.05$.

13.6. RESULTS

13.6.1. Physiological parameters

Table 13.2 (p 195) shows the changes in animals' weights. The change in weight was 0 – 1.8% in the first week, 1 – 5.5% in the second week, and 8.5% in the third week. This weight was within the expected range for age and all the changes in the different groups were similar to those of the control group. Moreover, these changes in weight at 7 and 14 days did not warrant a change in drug dosage, while dosing was not applicable at 21 days after influenza vaccination because the vaccine was given once.

Table 13.3 A (p 196) and 13.3 B (p 197) illustrate the full blood count data. In Table 13.3 A (p 196), there were no changes in the WBC on the three occasions. However, Table 13.3 B (p 197) shows that there was significant reduction in the platelet count in the cyclosporine group [91 (74 -1186)] on days 7 and 14, and this was antagonized by Phela in the Phela + cyclosporine group.

Table 13.4 (p 198) shows the liver function tests indicated by the ALT and ALK. All groups had normal ALT levels with the exception of cyclosporine treated animals that had lower levels. The decrease was significant with a p-value of 0.0167 after 7 and 14 days treatment, while the ALK levels were lower in the phela + cyclosporine groups with the p-value of 0.0262 and 0.0070 for both treatment periods.

Table 13.5 (p 199) reflects the renal function tests of creatinine and urea, were found to be normal. However, the creatinine levels were found to be elevated after 14 days of phela + cyclosporine treatment. While urea levels were also raised after 7 days of cyclosporine treatment and returned back to normal after 14 days.

Table13.2: Summary of mean weights and standard deviation of rats before and after treatment.

Drug Administered	BEFORE (g)	AFTER (g)	Change (g)
	7 DAYS TREATMENT		
Saline	396 ± 15	402 ± 17	6
Influenza vaccine	405 ± 11	409 ± 12	4
Cyclosporine	249 ± 6	276 ± 9	27
Phela	377 ± 23	379 ± 23	2
Phela +Cyclosporine	406 ± 17	402 ± 20	- 4
	14 DAYS TREATMENT		
Saline	405 ± 18	422 ± 23	17
Influenza vaccine	396 ± 13	410 ± 19	14
Cyclosporine	246 ± 4	328 ± 2	82
Phela	402 ± 9	423 ± 12	21
Phela + Cyclosporine	387 ± 20	402 ± 16	15
	21 DAYS TREATMENT		
Influenza vaccine	388 ± 19	421 ± 21	33

Table 13.3 A: Summary of full blood test (red blood cells) levels recorded as median (range) after 7 and 14 days treatment.

GROUP		RCC (x10^12/l)	Haemoglobin (g/dl)	Haematocrit (l/l)	MCV (fl)	MCH (pg)	MCHC (g/dl)	Platelet count (x10^9/l)
7 DAYS TREATMENT								
Saline	**(n = 7)**	9.1 (8.4 – 9.2)	16.2 (15.2 – 16.4)	0.70 (0.68 – 0.73)	78 (77 - 81)	18 (18 – 18)	23 (23 – 23)	1051 (322 - 1154)
Influenza vaccine	**(n = 7)**	8.7 (2.2 – 9.1)	16.4 (16.2 – 17.3)	0.70 (0.18 – 0.75)	80 (78 – 82)	19 (18 – 19)	23 (23 – 23)	1105 (213 – 1177)
Cyclosporine	**(n = 3)**	7.7 (6.4 – 8.0)	15.2 (11.8 – 15.4)	0.69 (0.57 – 0.69)	88 (87 – 89)	19 (18 – 20)	22 (21 – 22)	**584 (124 – 1067)**
Phela	**(n = 3)**	8.7 (8.4 – 8.9)	15.8 (15.8 – 16.0)	0.69 (0.69 – 0.69)	79 (78 - 82)	18 (18 - 19)	23 (23 – 23)	1032 (746 – 1164)
Phela + Cyclosporine	**(n = 4)**	8.3 (8.1 - 9.1)	15.6 (15.0 – 16.8)	0.68 (0.66 – 0.73)	81 (80 - 81)	19 (18 - 19)	23 (23 – 23)	1101 (1020 – 1134)
14 DAYS TREATMENT								
Saline	**(n = 7)**	8.7 (8.0 – 8.9)	16.0 (15 .0 – 16.2)	0.69 (0.66 – 0.71)	80 (79 - 82)	19 (18 - 19)	23 (23 - 24)	1023 (212 – 1174)
Influenza vaccine	**(n = 7)**	8.7 (6.9 – 9.9)	16.4 (12.8 – 16.9)	0.71 (0.56 – 0.74)	82 (78 – 82)	19 (18 – 19)	23 (23 – 24)	1057 (126 – 1257)
Cyclosporine	**(n = 3)**	7.6 (7.2 – 8.0)	15.0 (13.6 – 15.3)	0.67 (0.62 – 0.69)	88 (86 – 88)	19 (19 – 20)	22 (22 – 23)	**91 (74 – 1186)**
Phela	**(n = 7)**	8.8 (8.3 – 9.3)	16.0 (15.7 – 16.9)	0.69 (0.68 – 0.72)	80 (77 - 82)	19 (18 - 19)	23 (23 - 24)	1005 (427 – 1264)
Phela + Cyclosporine	**(n = 7)**	8.8 (6.4 – 9.4)	16.3 (15.7 – 18.0)	0.70 (0.48 – 0.75)	78 (75 - 80)	18 (17 - 25)	23 (23 - 34)	1062 (481 – 2392)
21 DAYS TREATMENT								
Influenza vaccine	**(n = 7)**	8.9 (8.3 – 9.3)	16.0 (15.5 – 16.6)	0.71 (0.67 – 0.73)	79 (78 - 81)	18 (18 - 19)	23 (23 – 23)	1023 (136 – 1130)

Abbreviations + reference values **RCC**: Red cell count **x10^12/l)**, Haemoglobin (11.5 – 16.1g/dl), **MCV**: Mean corpuscular volume (48 – 70 fl), **MCH**: Mean corpuscular hemoglobin, **MCHC**: Mean corpuscular hemoglobin concentration (40 g/dl), Platelet count (150 – 460**(x10^9/l)**)

Table 13.3 B: Summary of full blood test (white blood cells) levels recorded as median (range) after 7 and 14 days treatment.

GROUP		WCC (x10^9/l)	Neutrophils (x10^9/l)	Lymphocytes (x10^9/l)	Monocytes (x10^9/l)	Eosinophils (x10^9/l)	Basophils (x10^9/l)
7 DAYS TREATMENT							
Saline	**(n = 7)**	6.5 (3.5 – 8.2)	0.53 (0.63 – 1.37)	5.06 (2.82 – 7.33)	0.06 (0.04 – 0.20)	0.11 (0.02 – 0.14)	0.06 (0.04 – 0.10)
Influenza vaccine	**(n = 7)**	4.2 (3.6 – 6.0)	0.72 (0.58 – 0.91)	3.49 (2.63 – 4.95)	0.02 (0.02 – 0.14)	0.09 (0.07 – 0.11)	0.04 (0.02 – 0.05)
Cyclosporine	**(n = 3)**	6.5 (4.6 – 7.0)	0.64 (0.43 – 0.98)	5.14 (3.66 – 6.30)	0.16 (0.16 – 0.26)	0.06 (0.03 – 0.13	0.03 (0.03 – 0.09)
Phela	**(n = 3)**	4.7(4.1 – 5.9)	0.58 (0.44 – 0.77)	3.59 (3.51 – 4.78)	0.08 (0.04 – 0.18)	0.10 (0.08 – 0.18)	0.05 (0.05 – 0.07)
Phela + Cyclosporine	**(n = 4)**	3.8 (4.3 – 6.7)	0.53 (0.64 – 0.77)	3.05 (3.33 – 5.58)	0.08 (0.09 – 0.15)	0.05 (0.09 – 0.16)	0.07 (0.05 – 0.08)
14 DAYS TREATMENT							
Saline	**(n = 7)**	4.4 (3.2 – 6.5)	0.61 (0.54 – 0.88)	3.38 (2.49 – 5.39)	0.05 (0.02 – 0.16)	0.04 (0.03 – 0.11)	0.06 (0.04 – 0.12)
Influenza vaccine	**(n = 7)**	2.0 (4.3 – 5.0)	0.79 (0.11 – 0.91)	3.20 (1.42 – 4.12)	0.04 (0.04 – 0.08)	0.09 (0.04 – 0.11)	0.04 (0.03 – 0.05)
Cyclosporine	**(n = 3)**	7.5 (3.3 – 9.1)	1.10 (0.46 – 1.35)	5.70 (2.61 – 7.63)	0.19 (0.17 – 0.45)	0.03 (0.03 – 0.07)	0.18 (0.18 – 0.18)
Phela	**(n = 7)**	3.6 (2.8 – 4.9)	0.54 (0.30 – 0.71)	3.11 (2.16 – 3.70)	0.06 (0.02 – 0.16)	0.08 (0.02 – 0.11)	0.05 (0.04 – 0.17)
Phela + cyclosporine	**(n = 7)**	5.3 (4.7 – 7.3)	0.92 (0.61 – 1.48)	4.16 (3.59 – 5.87)	0.17 (0.05 – 0.19)	0.09 (0.06 – 0.11)	0.11 (0.06 – 0.16)
21 DAYS TREATMENT							
Influenza vaccine	**(n = 7)**	5.3 (3.6 – 5.9)	0.65 (0.53 – 0.81)	3.87 (0.58 – 4.84)	0.09 (0.03 – 0.30)	0.09 (0.06 – 0.16)	0.09 (0.06 – 0.11)

Abbreviations + reference values **WCC**: white cell count, Neutrophils (1.77 – 3.38**(x10^9/l)**), Lymphocytes (4.78 – 9.12**(x10^9/l)**), Eosinophils (0.03 – 0.08**(x10^9/l)**), Monocytes (0.01 – 0.04**(x10^9/l)**), Basophils (0.00 – 0.03**(x10^9/l)**)

Table 13.4: Summary of liver function test levels recorded as median (range) after 7 and 14 days treatment.

GROUP		Liver function tests			
		ALT (U/L)	p-value	ALK (U/L)	p-value
			7 DAYS TREATMENT		
Saline	(n = 7)	54 (51 - 60)		272 (211 – 295)	
Influenza vaccine	(n = 7)	57 (47 – 65)	0.6546	254 (199 – 284)	0.5350
Cyclosporine	(n = 3)	47 (47 – 50)	0.0167	327 (233 – 359)	0.2267
Phela	(n = 3)	53 (50 – 91)	0.9490	293 (192 – 361)	0.4060
Phela + cyclosporine	(n = 4)	51 (34 – 61)	0.2002	213 (181 – 242)	0.0262
			14 DAYS TREATMENT		
Saline	(n = 7)	59 (57 – 62)		300 (260 – 327)	
Influenza vaccine	(n = 7)	56 (48 – 84)	0.7104	263 (239 – 278)	0.0963
Cyclosporine	(n = 3)	36 (36 – 51)	0.0167	240 (171 – 292)	0.1167
Phela	(n = 7)	55 (52 – 69)	0.0407	255 (184 – 297)	0.0379
Phela + cyclosporine	(n = 7)	51 (48 – 64)	0.1410	240 (178 – 290)	0.0070
			21 DAYS TREATMENT		
Influenza vaccine	(n = 7)	56 (53 – 68)	0.0407	241 (195 – 261)	0.0012

Abbreviations: **ALT**: alanine transaminase (17.5 – 30.2 U/L), **ALK**: alkaline phosphates (56.8 – 128 U/L)

Table 13.5 Summary of Renal function test levels recorded as median (range) after 7 and 14 days treatment.

GROUP		Renal function tests			
		CREATININE (µmol/L)	p-value	UREA (mmol/L)	p-value
7 DAYS TREATMENT					
Saline	(n = 7)	29 (22 – 59)		7.1 (6.0 – 7.7)	
Influenza vaccine	(n = 7)	41 (27 – 47)	0.1795	7.1 (6.7 – 7.7)	0.4039
Cyclosporine	(n = 3)	31 (25 – 31)	0.9091	9.1 (8.2 – 9.2)	0.0167
Phela	(n = 3)	32 (24 – 56)	0.7981	7.5 (6.8 – 8.7)	0.1248
Phela + cyclosporine	(n = 4)	37 (27 – 42)	0.4428	6.6 (6.2 – 7.6)	0.999
14 DAYS TREATMENT					
Saline	(n = 7)	26 (20 – 34)		7.5 (6.4 – 8.0)	
Influenza vaccine	(n = 7)	32 (19 – 44)	0.2772	7.1 (6.4 – 7.8)	0.2767
Cyclosporine	(n = 3)	20 (15 – 37)	0.6475	7.2 (6.9 – 8.1)	0.9990
Phela	(n = 7)	30 (25 – 40)	0.1101	6.4 (6.0 – 7.0)	0.3546
Phela + cyclosporine	(n = 7)	33 (27 – 46)	0.0476	7.2 (5.7 – 7.8)	0.1795
21 DAYS TREATMENT					
Influenza vaccine	(n = 7)	36 (30 – 44)	0.0070	7.0 (6.2 – 8.3)	0.2246

Abbreviations: Creatinine (0.2 – 0.8 µmol/L l), urea (15 – 21 mmol/L)

13.6.2. Cyclosporine concentration levels

Table 13.6 (p 202) is a table reflecting cyclosporine levels after 7 and 14 days' treatment. After 14 days' treatment cyclosporine levels were high at 88 ng/ml; however phela antagonized cyclosporine hence a lower concentration of 68 ng/ml.

13.6.3. Cytokine profiles

The effects of the different agents on the TH_1 and TH_2 cytokines tended to be more pronounced at 7 days of therapy. Thereafter, the cytokine levels in most cases either returned to baseline or were similar to those in the control group, most probably owing to endogenous immune adjustments. The results are reported as median (range) and mean and standard deviation are in appendix E, table E-5 (p 260) and E-6 (p 261).

13.6.3.1. Influenza vaccine

Figure 13.5 (p 203 – 204) shows that the effect of Influenza vaccine on TH_1 (fig 13.5 A; p 203) cytokines (IL-2, IFN-γ and TNF-α) and TH_2 (fig 13.5 B; p 204) cytokines (IL-4 and IL-10) was not different from that of the control. However, the response to influenza vaccine treatment exhibited a trend whereby there was a raise in the levels of TH_1 cytokines (IL-2, IFN-γ and TNF-α) at 7 days, followed by a progressive drop and return to baseline, while the TH_2 cytokines (IL-4 and IL-10) exhibited a downward trend without return to baseline.

13.6.3.2. Cyclosporine

Figure 13.6 (p 205 – 206) shows that treatment with cyclosporine led to a reduction in the levels of TH_1 (fig 13.6 A; p 205) cytokines (IL-2, IFN-γ and TNF-α), and this was statistically significant for IL-2 at 7 days of treatment ($P = 0.0357$). Regarding TH_2 (fig 13.6 B; p 206) cytokines, cyclosporine led to a sharp rise in IL-10 at 7 days after which it returned to baseline at 14 days of treatment.

13.6.3.3. Phela

Figure 13.7 (p 207 – 208) shows that the effect of treatment with Phela on TH_1 (fig 13.7 A; p 207) cytokines (IL-2, IFN-γ and TNF-α) and TH_2 (fig 13.7 B; p 208) cytokine (IL-4 and IL-10) levels was not different from that of the control. However, the response to Phela exhibited a trend whereby there was a rise in the TH_2 cytokines levels (IL-4 and IL-10) at 7 days followed by a progressive drop and return to baseline, while the TH_1 cytokines exhibited an upward trend without return to baseline.

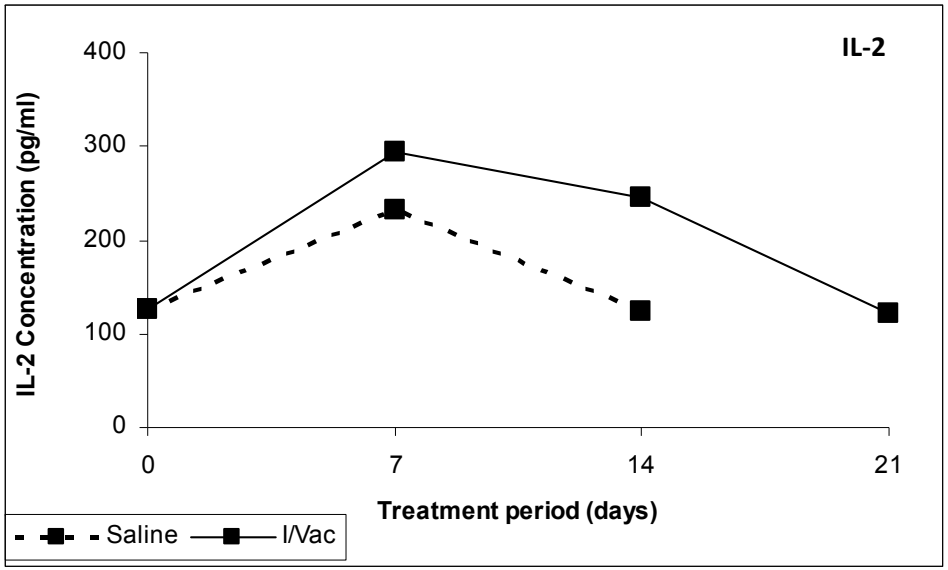
13.6.3.4. Phela + Cyclosporine

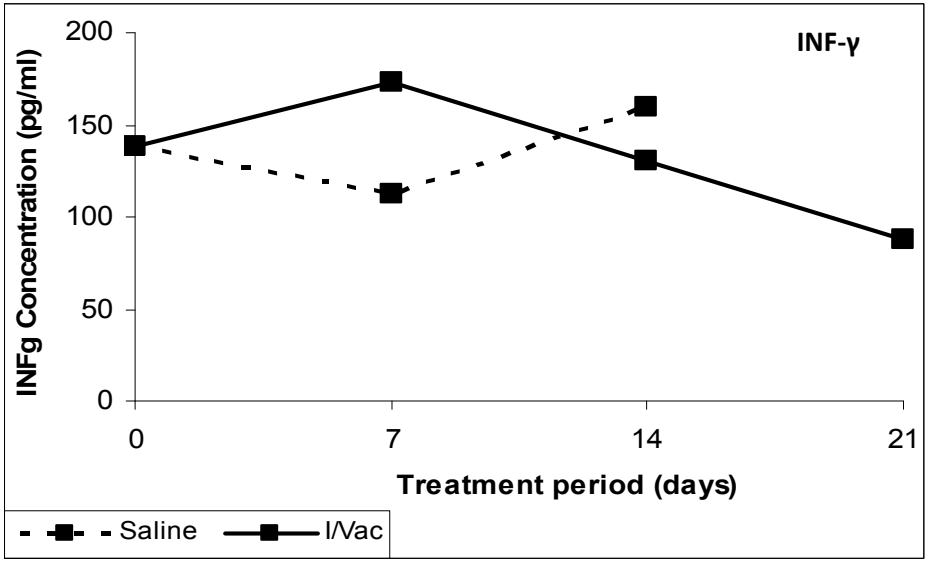
In Figure 13.8 (p 209 – 210), co-administration of phela and cyclosporine led to a reversal of the effects of cyclosporine, i.e., increased levels of TH_1 (fig 13.8 A; p 209) cytokines (IL-2, IFN-γ and TNF-α) versus reduced levels of TH_2 (fig 13.8 B; p 210) cytokines (IL-4 and IL-10) at 7 days. Owing to wide variations, only the raise in IL-2 was statistically significant ($p = 0.0357$). The corresponding concentrations of cyclosporine were 66 (52 – 160) ng/ml after 7 days and 68 (48 – 76) ng.ml after 14 days of treatment. There was a significant difference between the concentrations of the cyclosporine only group versus those with Phela+cyclosporine ($P = 0.0167$), but this was observed after 14 days of treatment ($P = 0.2667$).

Table 13.6: Cyclosporine levels after 7 and 14 days' treatment

Drug administered	concentration (ng/ml)	P-value
7 DAYS' TREATMENT		
Cyclosporine	38 (38 – 42)	
Phela + cyclosporine	66 (52 – 160)	0.0167
14 DAYS' TREATMENT		
Cyclosporine	88 (62 – 116)	
Phela + cyclosporine	68 (48 – 76)	0.2667

Figure 13.5 A: The effect of **Influenza vaccine** on IL-2, IFN-γ, and TNF-α in healthy rats (n = 4)





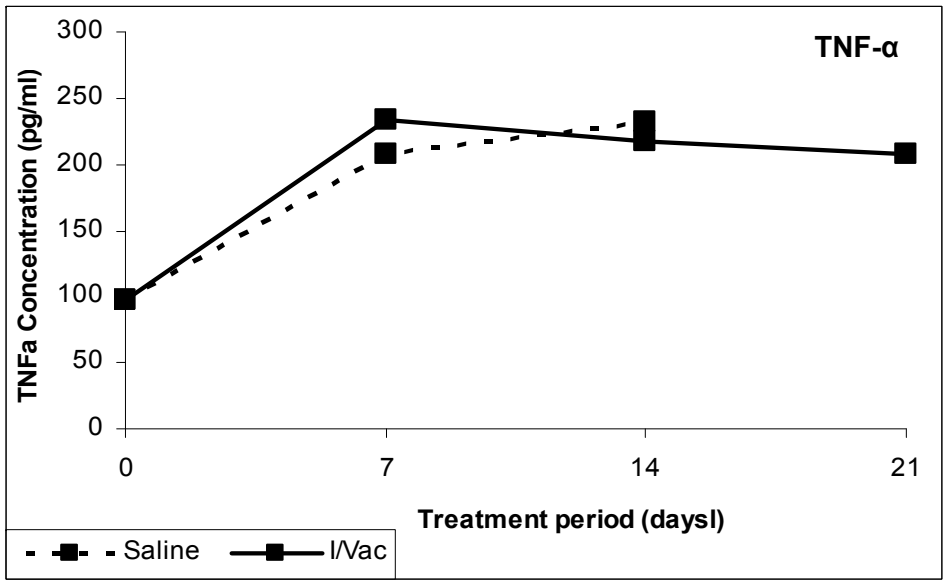
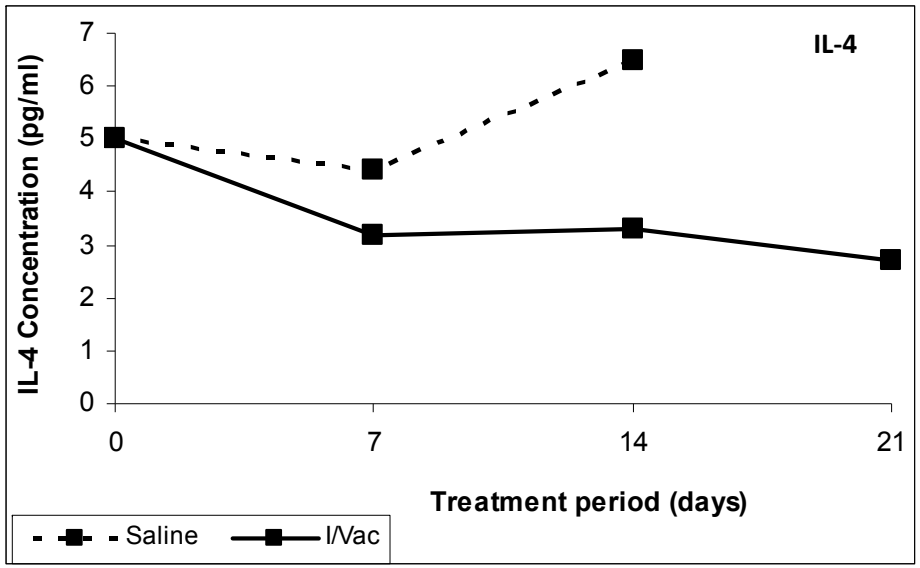


Figure 13.5 B: The effect of **Influenza vaccine** on IL-4 and IL-10 in healthy rats (n = 4)



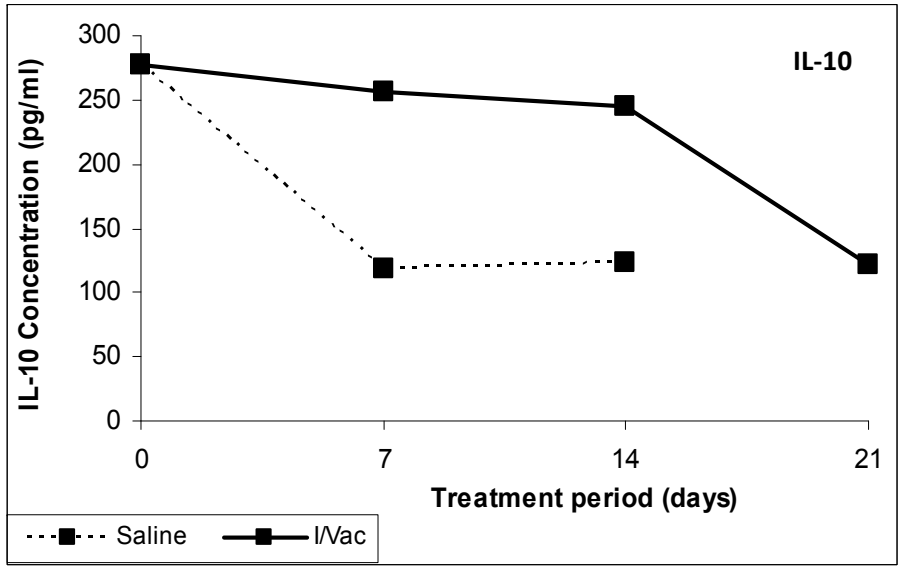
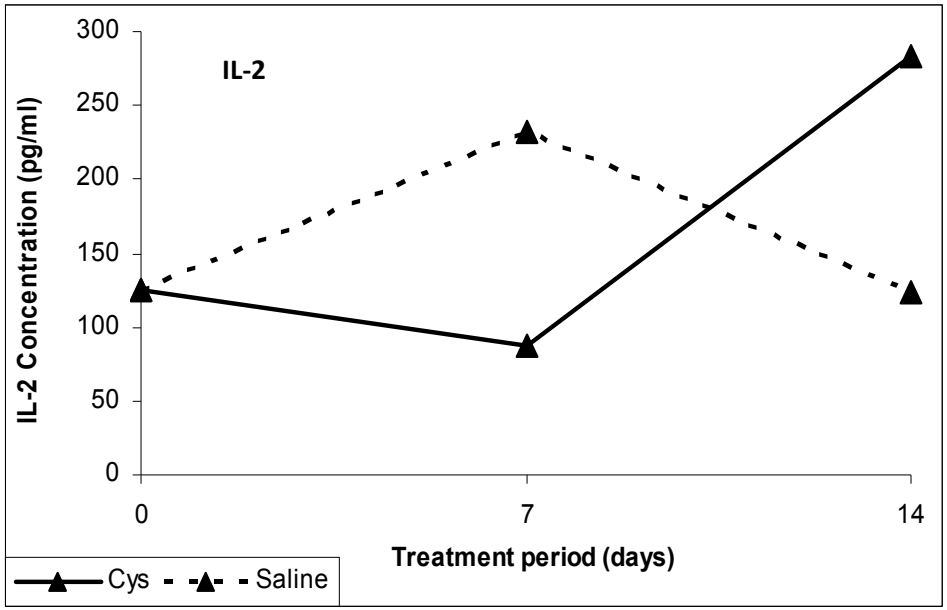
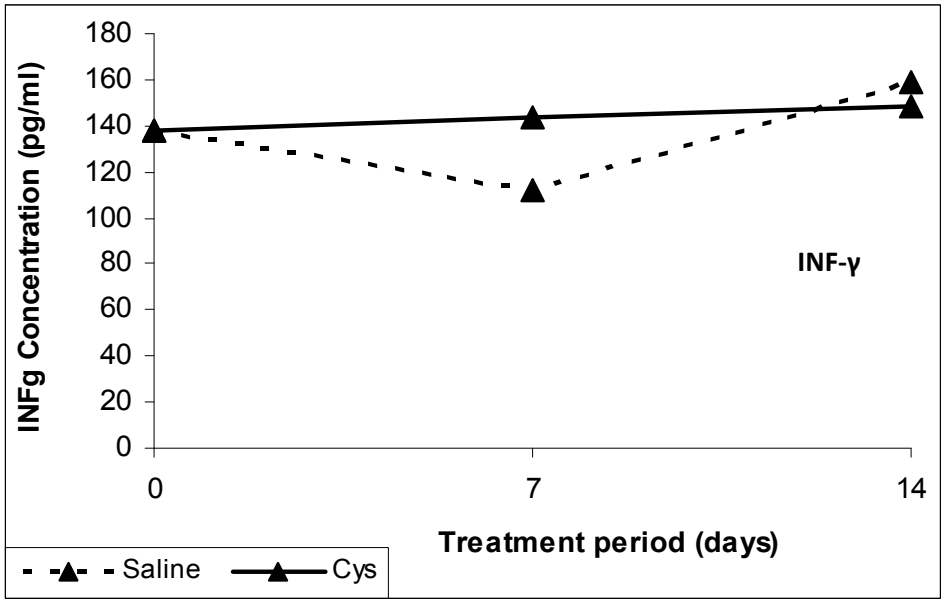


Figure 13.6 A: The effect of **Cyclosporine** on IL-2, IFN-γ, and TNF-α in healthy rats (n = 4).





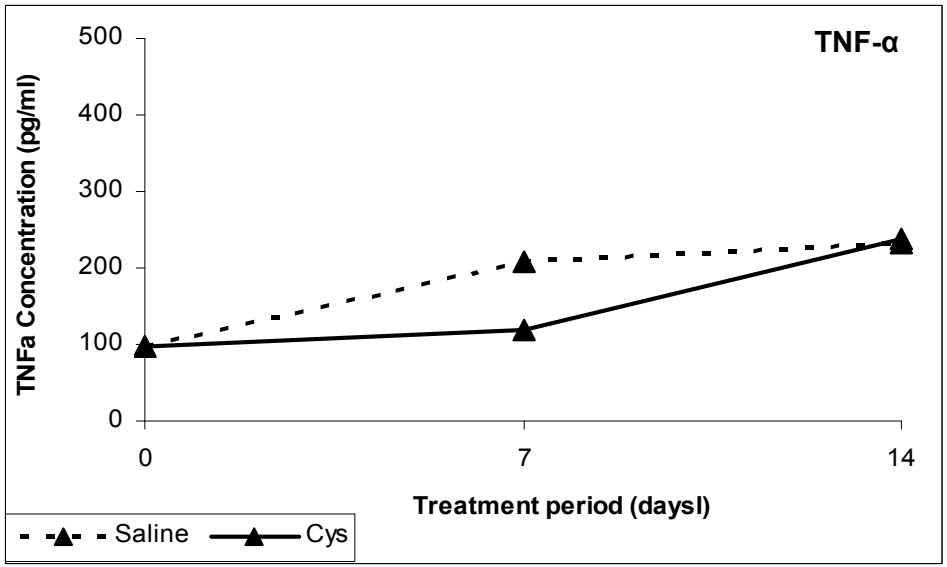
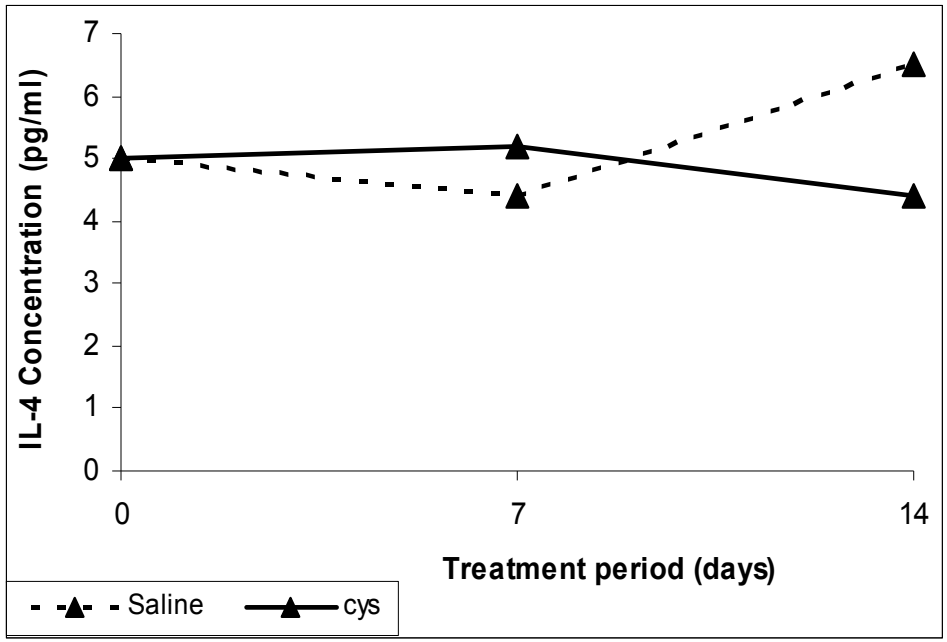


Figure 13.6 B: The effect of **Cyclosporine** on IL-4 and IL-10 in healthy rats (n = 4).



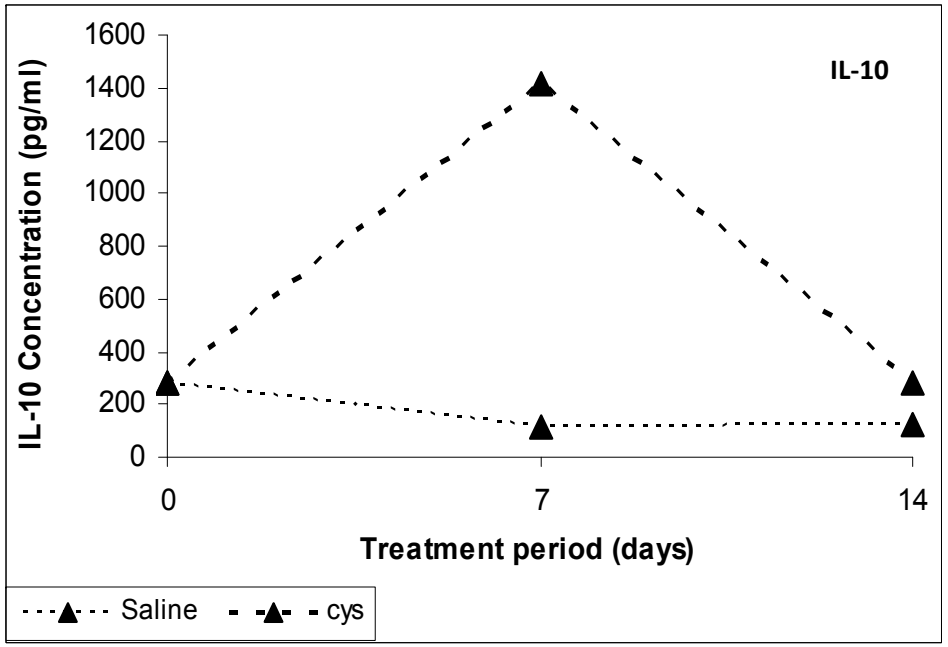
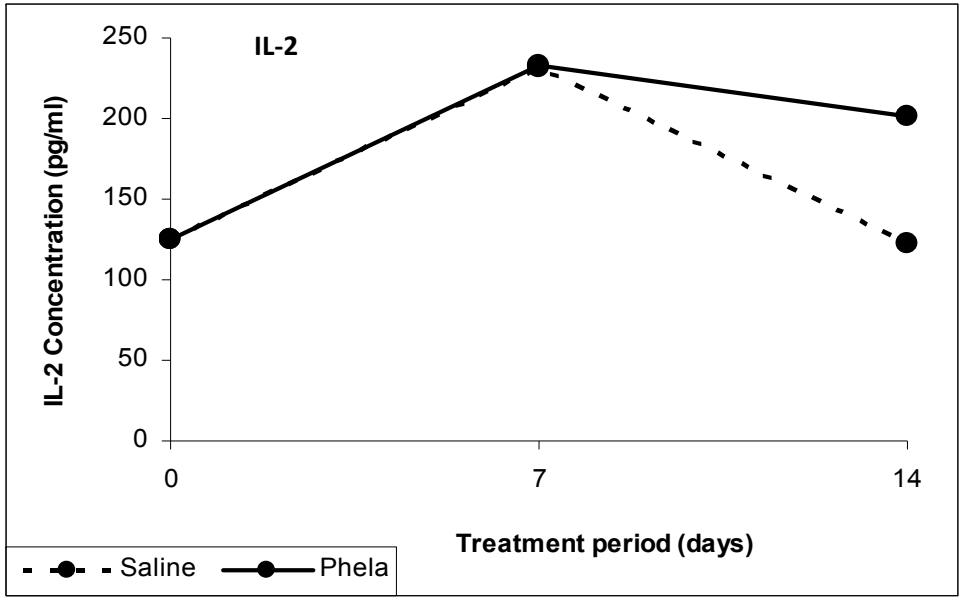
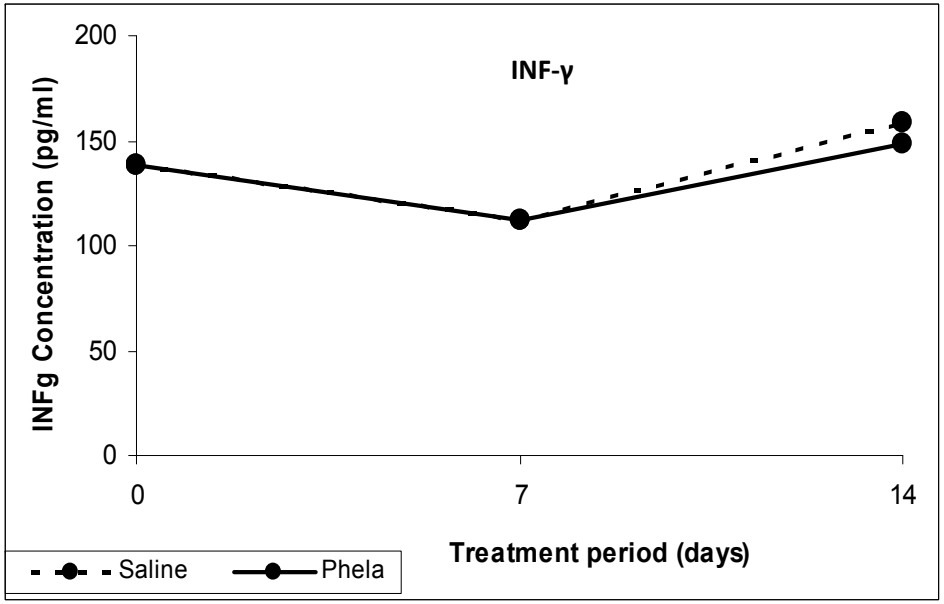


Figure 13.7 A: The effect of **Phela** on IL-2, IFN-γ and TNF-α in healthy rats (n = 4).





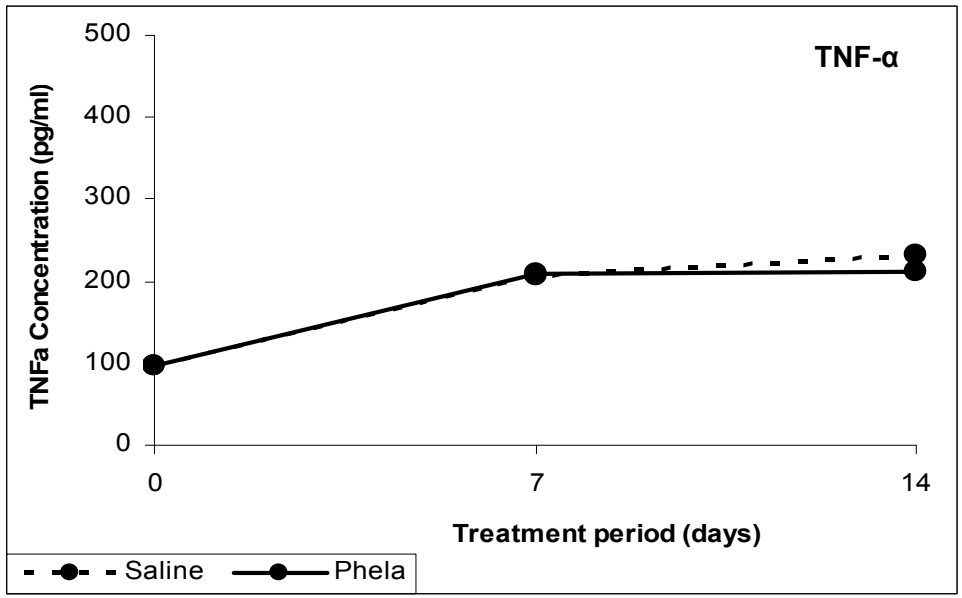
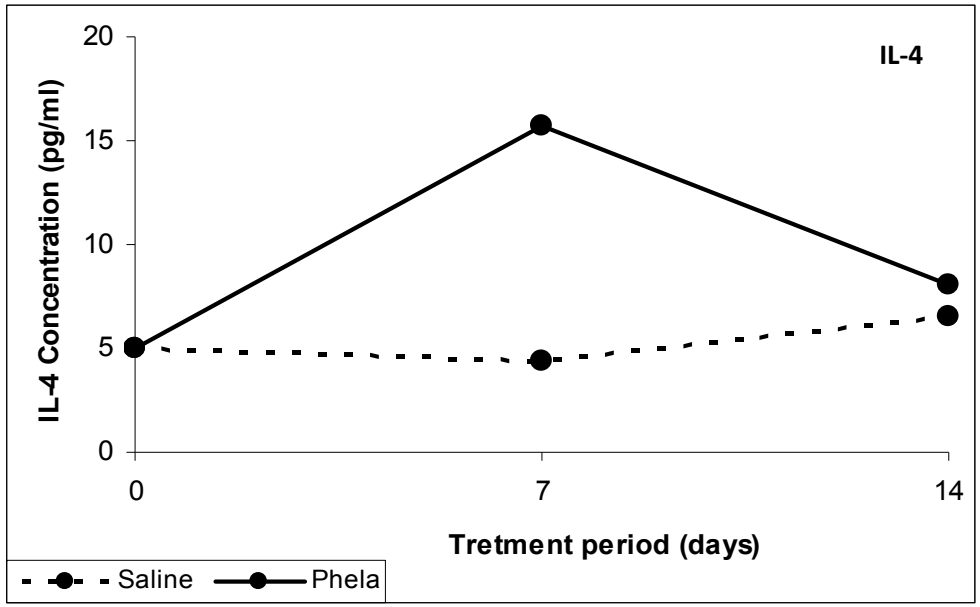


Figure 13.7 B: The effect of **Phela** on IL-4 and IL-10 in healthy rats (n = 4).



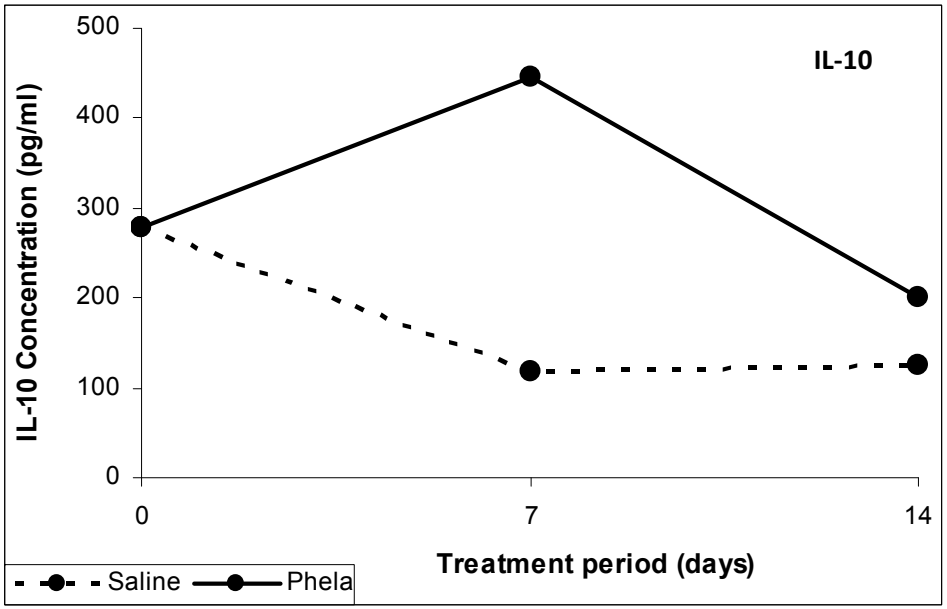
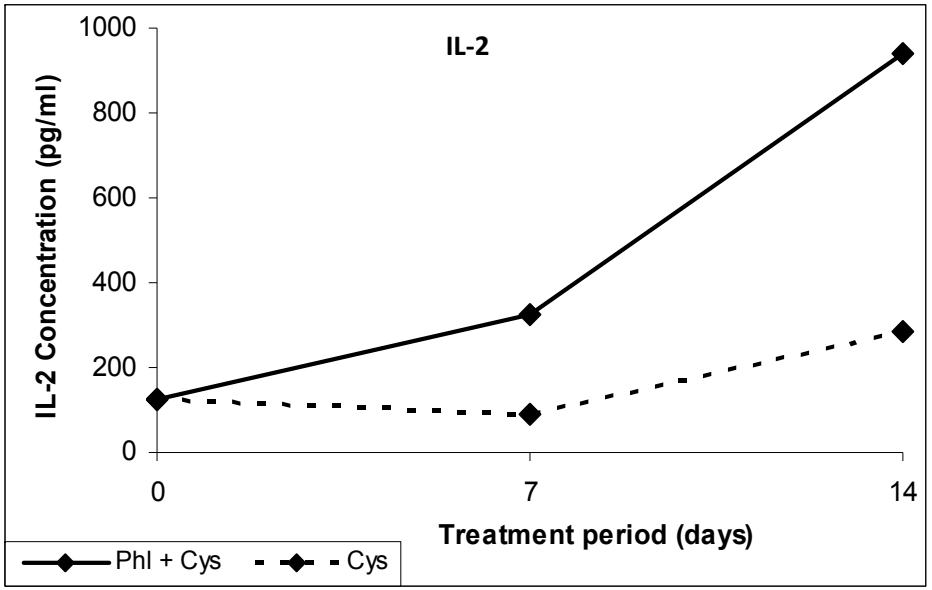
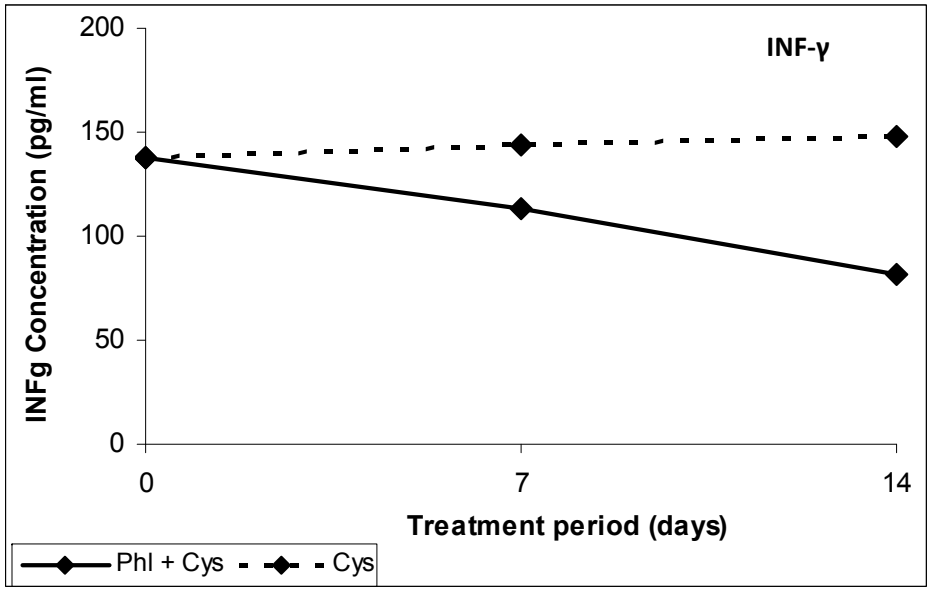


Figure 13.8 A: The effect of **Phela on Cyclosporine** induced effects on IL-2, INF-γ and TNF-α in healthy rats (n = 4).





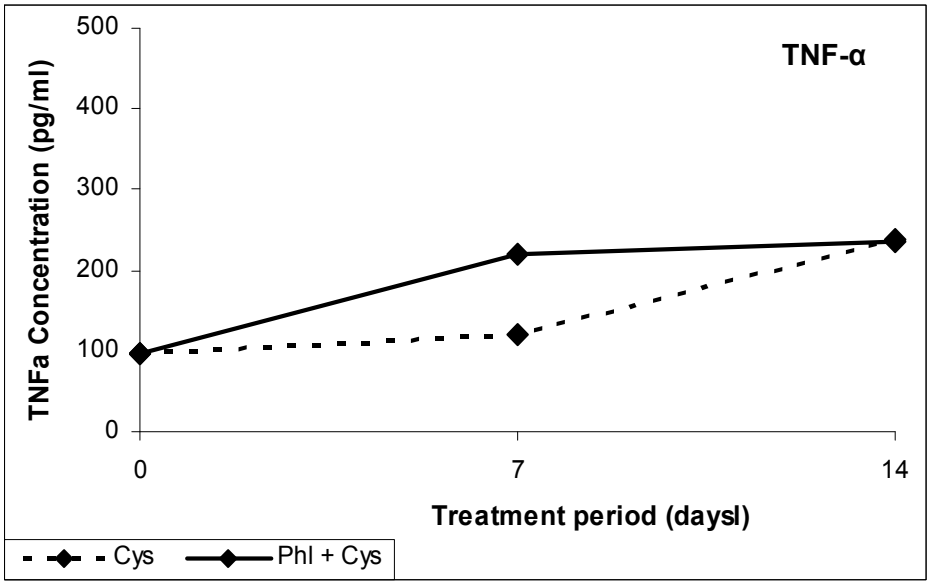


Figure 13.8 B: The effect of **Phela on Cyclosporine** effects on IL-4 and IL-10 in healthy rats (n = 4).

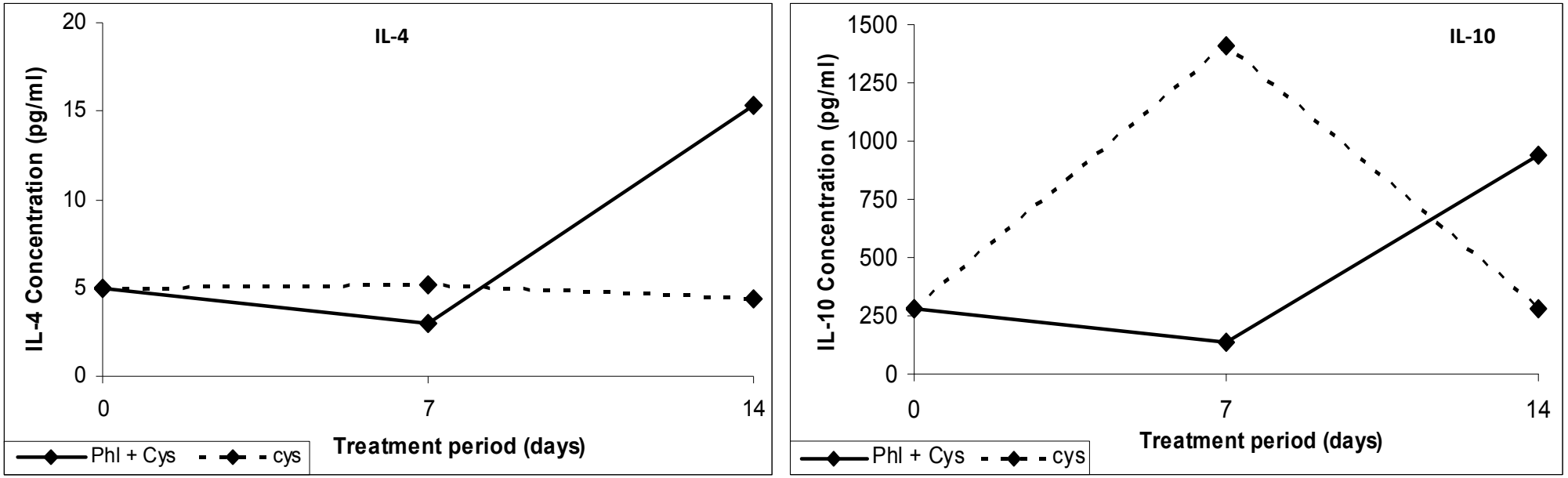


Table 13.7: TH_1cytokine levels recorded as median (range) after treatment with different drugs used during the study

	TH_1 CYTOKINES					
Drug administered	**IL-2**	**P-value**	**IFN-γ**	**P-value**	**TNF-α**	**P-value**
Untreated	125 (11 – 130)	0.036	138 (130 – 146)	0.125	97 (63 – 150)	0.071
	7 DAYS TREATMENT					
Saline	232 (167 – 460)		112 (58 – 159)		207 (203 – 275)	
Influenza vaccine	294 (245 – 373)	0.556	173 (73 – 189)	0.056	233 (194 – 243)	0.905
Cyclosporine	88 (51 – 150)	0.357	144 (149 – 149)	0.125	120 (69 – 397)	0.515
Phela	233 (174 – 355)	0.841	112 (95 – 145)	0.377	210 (194 – 222)	0.548
Phela + cyclosporine	325 (268 – 433)	0.421	113 (63 – 143)	0.421	219 (216 – 274)	0.421
	14 DAYS TREATMENT					
Saline	274 (215 – 328)		159 (120 – 182)		232 (211 – 248)	
Influenza vaccine	214 (183 – 309)	0.343	130 (111 – 291)	0.486	217 (204 – 237)	0.343
Cyclosporine	176 (120 – 217)	0.114	148 (114 – 174)	0.620	238 (78 – 624)	0.857
Phela	224 (217 – 295)	0.056	148 (86 – 179)	0.686	211 (209 – 212)	0.109
Phela + cyclosporine	280 (206 – 518)	0.730	82 (57 – 155)	0.114	235 (216 – 2920)	0.486
	21 DAYS TREATMENT					
Influenza vaccine	283 (199 – 304)	0.999	88 (73 – 98)	0.029	207 (171 – 209)	0.029

Table 13.8: TH_2 cytokine levels recorded as median (range) after treatment with different drugs used during the study

	TH_2 CYTOKINES			
Drug administered	**IL-4**	**P-value**	**IL-10**	**P-value**
Untreated	5.0 (4.6 – 5.4)	0.800	277 (208 – 348)	0.400
	7 DAYS TREATMENT			
Saline	4.4 (3.5 – 6.9)		118 (116 – 133)	
Influenza vaccine	3.2 (2.7 – 6.0)	0.400	257 (177 – 742)	0.057
Cyclosporine	5.2 (4.8 – 6.8)	0.700	1412 (432 – 1665)	0.100
Phela	15.7 (2.7 – 29.6)	0.857	446 (104 – 1359)	0.400
Phela + cyclosporine	3.0 (1.5 – 15.2)	0.629	134 (83 – 1438)	0.629
	14 DAYS TREATMENT			
Saline	6.5 (3.3 – 13.3)		124 (115 – 148)	
Influenza vaccine	3.3 (2.8 – 5.1)	0.400	245 (213 – 330)	0.100
Cyclosporine	4.4 (3.9 – 4.8)	0.700	284 (277 – 340)	0.100
Phela	8.1 (2.4 – 13.3)	0.999	201 (130 – 1048)	0.114
Phela + cyclosporine	15.3 (5.6 – 29.8)	0.229	940 (125 – 1362)	0.114
	21 DAYS TREATMENT			
Influenza vaccine	2.7 (2.3 – 3.3)	0.200	121 (112 – 188)	0.999

13.7. DISCUSSION

13.7.1. Mechanism of action

As indicated earlier, the cytokine profile determines the type of immune response to any antigen. Increased TH_1 cytokines leads to CMI, while increased TH_2 cytokines leads to humoral immunity. In the case of viral infection, the CMI is effective in mediating removal of virus-infected cells, while the TH_2 cytokines mediate production of antibodies to defend against viral invasion. However, the two cytokines must co-exist because they regulate each other regarding their actions in that TH_1 cytokines are pro-inflammatory while the TH_2 are anti-inflammatory, and in production where some TH_1 inhibit/stimulate release of specific TH_2 cytokines and vice versa.

Nevertheless, it suffices to say that traditional medicines that increase TH_1 cytokines would be the ideal candidates for development as immune boosters against established viral infections, while those that stimulate TH_2 cytokines would be ideal for preventing viral infections. However, in this study, it has been demonstrated that Phela acts on the immune system in that it increased, albeit not statistically significantly, the TH_2 cytokines of a normal immune system at 7 days of treatment, and increased or boosted the TH_1 cytokines against cyclosporine induced immune suppression. Boosting in this case refers to the fact that the response to phela+cyclosporine treatment, particularly by IL-2, was higher than in the control and phela-only groups. This implies that phela has a dual action on cytokines in that it stimulates the relevant cytokines, depending on the situation. The mild-moderate stimulation of TH_2 cytokines of a normal immune system was probably to inhibit TH_1 cytokine production and limit inflammation, which was successfully achieved, while stimulation of TH_1 cytokines, when they were suppressed by cyclosporine was a most appropriate action for the situation.

Furthermore, this also implies that phela's actions on TH_1 cytokines are more pronounced on a suppressed than on a normal immune system, which is in line with the clinical observations studies in which phela led to improvement of immune markers in patients with a compromised immune system **(**Matsabisa et al., 2006)

13.7.2. Interaction with cyclosporine

The interaction between Phela and cyclosporine is unlikely to be a pharmacokinetic interaction as the concentrations of cyclosporine were not different in the two groups, i.e., cyclosporine group vs. phela+cyclosporine group. The difference in concentrations of cyclosporine after 7 days of treatment was most probably due to delayed absorption from the subcutaneous injection site in the cyclosporine only group. In addition, preliminary studies in our laboratory in which the effect of Phela on the activity of each the five major human drug metabolizing cytochrome P450 isoforms (CYP2D6, CYP3A4, CYP1A2, CYP2C19, and CYP2C9) were studied, showed that phela had no significant effect on the activity of any of these iso-enzymes, especially CYP3A. CYP3A is the enzyme responsible for cyclosporine metabolism. Furthermore, treatment of rats with phela at the same dose used here for 3 and 7 days, had no effect on the activity of rat CYP3A and other enzymes, and this was confirmed by quantification of CYP3A by the SDS-PAGE and Western Blot methods. Therefore, the mechanism by which phela interacted with cyclosporine is not clear.

Phela also antagonized the effect of cyclosporine on platelets. Cyclosporine is known to cause thrombotic thrombocytopenic purpura due to its activation of platelet aggregation (Lian, 1980; Grace et al, 1987; Phadke et al., 2004) and depletion of the prostacyclin-stimulating factor (Neild et al., 1983; Leithner et al., 1982). Unfortunately, this study did not evaluate for these factors, hence the mechanism by which Phela prevented the cyclosporine induced thrombocytopenia is not known.

On the other hand, since Phela alone had no effect on the TH_1 cytokines, one might say that cyclosporine augmented the action of Phela, but this remains to be confirmed. In the same perspective, the effects of Phela could be considered as not different from a dietary supplement which can aid restoration of a pathological situation with no further

effect on a normal immune system. The latter calls for a review of the dietary recommendations in immune compromised patients because some foods such as Phela would be more relevant than others.

Lastly, the findings of this study are also important regarding the chemotherapy for transplant patients, as they highlighted a potentially adverse interaction between Phela and cyclosporine that can lead to graft rejection. Therefore, transplant patients using cyclosporine should not use Phela products.

13.7.3. Optimum Time to evaluate cytokine response

It was observed that cytokine profiles varied with the time lapse since administration of the drugs. For instance, except in a few cases, the response at 7 days was always reversed or weaker by the 14^{th} day of treatment. This was most probably because the immune system countered the disruption of the respective immune mediators. It could also account for the failure by Phela to stimulate a normal immune system, but it was not clear whether the response to Phela would be more remarkable beyond 14 days. Nevertheless, this study has demonstrated that the time period since administration of the test antigen is important for the interpretation of cytokine response to antigens. It was therefore appropriate in this study to use the response at 7 days as more relevant than that at 14 days even though both occasions were considered in the analysis

13.7.4. Positive and negative controls

Since most studies on cytokines are characterized by wide variations in cytokine response that make not only interpretation of data very difficult, but also cast doubt on the robustness of the experimental methods used, it was decided to include the positive and negative controls using drugs of which the immune response is well characterized, i.e., influenza vaccine and cyclosporine, respectively. The conviction was that if the

experiment can reproduce the expected response by the two drugs, it would confirm the observations in all other groups as robust.

Regarding the response to influenza vaccine, wide variation in cytokine response to the different Influenza vaccines has been reported (Tsai et al., 2005; Krakauer et al., 1995; Mcelhaney et al., 2002; Mcelhaney et al., 1998; Mcelhaney et al., 1992; Tamizifar et al., 1997; Saurwein-Teiss et al., 1998**).** Nevertheless, most of these studies demonstrated either a raise or no effect on TH_1 cytokines (IL-2, IL-12, TNF-ά, and IFN-γ) with minimal effect on TH_2 cytokines (Mcelhaney et al., 1995; Mcelhaney et al., 2002; Mcelhaney et al., 1992; Tamizifar et al., 1997; Saurwein-Teiss et al., 1998**)**, while some demonstrated a raise in TH_2 cytokines as well **(**Virelizier et al., 1974; Mitchell et al., 1983**).**

Furthermore, the response by TH_1 cytokines was stronger with whole virus vaccine than with subunit vaccines (Mcelhaney et al., 1995; Saurwein-Teiss et al., 1998**)**. In the current study, Influvac 2009 is a trivalent subunit vaccine which, as expected, induced a mild increase in the TH_1 cytokines on day 14 and a moderate increase in the TH_2 cytokines at day 7. Even then, our follow-up was for only 21 days, yet it was observed in humans that an increase in cytokines after Influenza vaccination may reach maximum after 3 and 12 weeks post treatment (Mcelhaney et al., 1995). As such, it is likely that TH_1 cytokines could have been higher after 21 days. Unfortunately, since the age corresponding to 12 weeks in rats is not known, monitoring rats for 12 weeks could not be done because the effects of aging may influence response to the influenza vaccine (Krakauer et al., 1995; Mcelhaney et al., 2002; Mcelhaney et al., 1998; Mcelhaney et al., 1992**)**. In conclusion, in this study, the response to Influvac 2009 was as expected: there was a mild effect on TH_1 cytokines and moderate suppression of TH_2 cytokines, and this imparted a CMI (Mcelhaney et al., 1998**)**.

Regarding cyclosporine, the current study, like in other studies elsewhere, demonstrated that cyclosporine inhibits production of TH_1 cytokines (IL-2, TNF-ά, IFN-γ) while increasing production of IL-10 with minimal or no effect on IL-4 (Corsini et al., 2006; Alberu et al., 2001; Cho et al., 2002; Kim et al., 2000; Andersson et al., 1992;

Bonnotte et al., 1996; Kim et al., 2000). Therefore, in spite of the wide variations that might have led to a lack of statistically significant differences, the observations on the effects of Phela on the immune system are accurate. In fact, the effect of cyclosporine on cytokines, especially IL-10, was suggested to be at transcriptional level because cyclosporine increased the IL-10 mRNA **(**Cho et al., 2002**)**. It is therefore most probable that Phela interacts with cyclosporine at transcriptions level, but this too remains to be confirmed.

13.8. CONCLUSION

In conclusion, using a rat model, it has been demonstrated that Phela is an immune stimulant that antagonizes the effects of cyclosporine with minimal effect on a normal immune system in the first 14 days of treatment. This implies that Phela is an ideal candidate for further evaluation and development into an immune booster medication.

CHAPTER 14

CONCLUSION AND FUTURE STUDIES

14.1 CONCLUSIONS

The objectives of the study were achieved as follows:

14.1.1 A comprehensive method for fingerprinting of phela by TLC, HPLC with UV and fluorescence detectors and GC with and mass selective detector was successfully developed and applied in the study.

- This comprehensive method is a novel approach that can be applied to other herbal products under development.

14.1.2 A method for quantifying phela in plasma by high performance liquid chromatography with UV detector was developed and validated, and can be used for fingerprinting phela in plasma.

14.1.3 A high performance liquid chromatography with fluorescence detector was developed and used to monitor phela in rat plasma.

14.1.4 Peak area/unit volume was used to derive the pharmacokinetics of unknown compounds as well as to monitor phela in plasma.

- This is the first time peak-kinetics has been used to monitor a natural product *in vivo* and this opens new avenues for the evaluation of the technique in other products.

14.1.5 Using a rat model, it has been demonstrated that phela is an immune stimulant that antagonizes the effects of cyclosporine, with minimal effect on a normal immune system in the first 14 days.

- This is the first time the mechanism of action of Phela has been evaluated and established.

14.2 FUTURE STUDIES

14.2.1 There is a need to further develop and validate HPLC_FL for monitoring of phela in plasma. This would require enhancing sample extraction to increase sensitivity.

14.2.2 There is a need to determine compounds responsible for the immune boosting effects. This would require undertaking spectroscopic and NMR studies on phela product.

14.2.3 Furthermore, there is a need to analyse phela products manufactured in different geographical areas, using the comprehensive fingerprinting method for quality control purposes.

14.2.4 Regarding animal studies, there is a need to undertake studies in humans to determine whether the same interactions occur in humans and to determine the effective dose. This would entail phase 2 clinical trials.

14.2.5 There is a need to investigate the role of cyclosporine on phela at mRNA level in order to understand the effect of phela on cyclosporine and the immune system.

REFERENCES

Anonymous, May 2001. Guide for industry, Bio-analytical method validation, U.S Department of health and human services Food and Drug administration (FDA).

AIDS http://en.wikipedia.org/wiki/AIDS. [Accessed 24 September 2007]

AIDS epidemic update, 2006. (www.unaids.org/en/HIV_data/2006GlobalReport). [Accessed 10 January 2008].

Agarwal K, Makhija A, 2002. Cytokine profile in patients with HIV infection and opportunistic infections. *14th international AIDS Conference*. (abstract no. A10108).

Alaerts G, Matthijs N, Smeyers-Verbeke J. Vander Heyden Y. 2007. Chromatographic fingerprint development for herbal extracts: a screening and optimization methodology on monolithic columns, *Journal of Chromatography A*, **1172**, 1 - 8.

Alberu J, Richaud-Patin Y, Mancilla E, Dı´liz H, Correa-Rotter R, Chew-Wong A, Uribe N, De Leo C, Llorente L, 2001. Effect of Cyclosporine Withdrawal on IL-10 Production in Kidney Transplant Recipients. *Journal of Transplant Proceedings,* **33:** 2024 - 2027.

Andersson J, Nagy S, Groth G, Andersson U, 1992. Effects of FK 506 and cyclosporine A on cytokine production studied in vitro at single cell level. *Journal of Immunology*, **75:** 136 -142.

Babakhnian R, 1995. Cytokines, HIV and the immune system: An overview of the role of cell messengers in HIV infection. *AIDS treatment update*. [Abstract]

Bahbouhi B, Landay A, 2004. Dynamics of cytokine expression in HIV productively infected primary CD 4+ T cells, *Journal of Blood* **103**, 4581- 4586.

Barouch D, Letvin L, 2004. The role of cytokine DNAs as vaccine adjuvants for optimizing cellular immune responses, *Journal of immunological reviews* **202**, 266 - 274.

Bo T, Liu H, 2004. Separation methods for pharmacologically active xanthones, Journal *of chromatography B* **812**, 165 - 174.

Bonnotte B, Pardoux C, Bourhis H, Caignard A, Burdiles M, Chehimi J, Mami-Chouaib F, Chouaib S, 1996. Inhibition of human allogeneic mixed lymphocyte response by cyclosporine A: relationship with IL-12 pathway. *Journal of Tissue Antigens* **48:** 265 - 270.

Breen E, 2002. Pro and anti-inflammatory cytokines in human immunodeficiency virus infection and acquired immunodeficiency syndrome, *Journal of pharmacology and therapeutic*s **95**, 295 - 304.

Bruce P, Minkkinen P, Riekkola L, 1998. Practical method validation: validation sufficient for an Analytical Method. *Journal of Mikrochimica Acta* **128**: 93 - 106.

Cho L, Kim U, Min Y, Min J, Lee H, Park H, Cho S, Kim Y, 2002. Cyclosporine Differentially Regulates Interleukin-10, Interleukin-15, and Tumor Necrosis Factor-ά Production by Rheumatoid Synoviocytes. *Journal of Arthritis Rheuatism.* **46:** 42 - 51.

Clerici M, Clivio A, 1999. The relationship between cytokines, complement and HIV infection, *Journal of HIV and new viruses* 115 - 131.

Corsini E, Vismara L, Lucchi L, Viviani B, Govoni S, Galli L, Marinovich M, Racchi M, 2006. High interleukin-10 production is associated with low antibody response to influenza vaccination in the elderly. *Journal of Leukocyte Biology* **80:** 376 - 382.

Dadgar D, Burnett E, Choc G, Gallicano K, Hooper W, 1995. Application issues in Bio-analytical validation, Sample analysis and data reporting. *Journal of Pharmaceutical and Biomedical Analysis*. **13:** 89 - 97.

Dadgar D, Burnett E, 1995. Issues in evaluation of Bio-analytical method selectivity and drug stability. *Journal of Pharmaceutical and Biomedical Analysis*. **14:** 23 – 31.

Dinarello C, 2000. Pro-inflammatory cytokines, *chest journal* **118**, 503 - 508.

Drago F, Floriddia L, Cro M, Giuffrida S, 2002. Pharmacokinetics and bioavailability of a *Ginkgo biloba* extract. *Journal of Ocular Pharmacology and Therapeutics*. **18**, 197 - 202.

Eilers C, 2004. Parametric Time Warping, *Journal of Analytical Chemistry*. **76**, 404-411.

Fakoya A, Matear M, 1997. HIV infection alters both type 1 and 2 cytokines but does not induce a polarized type 1 or 2 state. *Journal of AIDS* **11**, 1445 - 1452

Fan X, Cheng Y, Ye Z, 2006. Multiple chromatographic fingerprinting and its application to the quality control of herbal medicines, *Journal of Analytical chimica Acta.* **555**, 217 - 224

Fauci A, Lane H, 2007. HIV disease: *AIDS related disorders, Harrison's internal medicine.* Chp 173, (www.accessmedicine.com/popup.aspx?aID=75462). [Accessed 15 January 2008].

Ganong F, 2001. Review of medical physiology 17th edition. Lange Medical Publishers. 473 - 497.

Gibaldi M, 1984. Non-compartmental pharmacokinetics. In: M. Gibaldi, ed. Biopharmaceutics and Clinical Pharmacokinetics. Philadelphia: Lea and Febiger, 17 - 28.

Gokhale R, 2007. Cytokine response to strenuous exercises in athletes and non-athletes an adaptive response, *Journal of cytokines.* **40**: 123 - 127.

Government Gazette, 2008. African Traditional Medicine Draft Policy for South Africa Effective on 25 Jul 2008. URL source:

http://www.pmg.org.za/files/docs/080725health-traditionalmedicinepolicy.pdf [Accessed on 23rd December 2009]

Grace A, Barradas A, Mikhailidi P, Jeremy Y, Moorhead F, Sweny P, Dandona P, 1987. Cyclosporine A enhances platelet aggregation. *Journal of Kidney International*, **32,** 889 - 895

Green J, 1996. A practical guide to analytical method validation. *Journal of Analytical Chemistry.* **68**: 305A - 309A.

Hayney S, Muller D, 2003. Effect of Influenza Immunization on CYP3A4 Activity *In Vivo*. *Journal of Clinical Pharmacology*, **43:** 1377 - 1381

Hess G, Rossol S, 1989. Modification of the immune response against hepatitis B virus by the human immunodeficiency virus, *Journal of Rheumatology international* **9**, 175 - 179.

HIV http://en.wikipedia.org/wiki/HIV. [Accessed 24 September 2007]

HIV/AIDS Symptoms www.mayoclinic.com/health/hiv-aids/DS00005/DSECTION=7. [Accessed 24 August 2007]

Hoai N, Dejaegher B, Tistaert C, Thi Hong N, Rivière C, Chataigné G, Phan Van K, Chau Van M, Quetin-Leclerc J, Vander-Heyden Y, 2009. Development of HPLC fingerprints for *Mallotus* species extracts and evaluation of the peaks

responsible for their antioxidant activity. *Journal of Pharmaceutical and Biomedical Analysis*, **50**, 753 - 763

Horn T, 1999. Immune-Based therapies www.thebody.com/content/art14513.html [[Accessed 24 August 2007]

Human immunodeficiency Virus (HIV) pathogenesis www.manbir-online.com/std/hiv.1.htm. [Accessed 24 August 2007]

Immune booster helps HIV patients, Study says, Reuters news media Oct 2005.

Immune response to HIV, 2007. Harrison's Internal medicine: Mcgraw-Hill Companies, chapter 173 [www.acessmedicine.com]

Immune System www.wikipedia.com/immune system. [Accessed 31st August 2007].

Impi, immune booster and anti-viral www.herbalafrica.co.za/products/impi.htm. [Accessed 16 November 2007]

Interleukin http://en.wikipedia.org/wiki/interleukin [Accessed 24 August 2007].

Jacobson M, Feinman L, Liebes L, 2001. Pharmacokinetics, safety, and antiviral effects of hypericin, a derivative of St. John's wort plant, in patients with chronic hepatitis C virus infection .*Journal of Antimicrobial Agents Chemotherapy*. **45**, 517 - 524.

Ji B, Xu S, Hu Z, Vander-Heyden Y, 2005. Development, optimization and validation of a fingerprint of *Ginkgo biloba* extracts by high-performance liquid chromatography, *Journal of Chromatography A,* **1066**, 97 - 104

Jimene R, 2005. Cytometric bead array for measurement of cytokines in urine and plasma of patients undergoing renal rejection, *Journal of cytokines* **32**, 45 - 50.

Karnes T, Shiu G, Shah P, 1991. Validation of bio-analytical methods. *Journal of Pharmaceutical Research* **40**: 221 - 225.

Kassidas A, MacGregor F, Taylor A, 1998. Synchronization of batch trajectories using dynamic time warping. *The American Institute of Chemical Engineers Journal.* **44**: 864 - 875

Kaur D, Ravichandran V, 2008. High performance thin layer chromatography method for estimation of conessine in herbal extract and pharmaceutical dosage formulations, *Journal of pharmaceutical and Biomedical analysis* **46**: 391 - 394

Kedzierska K, Crowe S, 2001. Cytokines and HIV-1: interactions and clinical implications, *Journal of antiviral chemotherapy* 12, 133 - 150.

Kenneth M, Kulmatycki K, 2005. Drug disease interactions: role of inflammatory mediators in disease and variability in drug response, *Journal of pharmaceutical science **8***: 602 - 625.

Gafner S, Sudberg S, Sudberg M, Vilinski J, Gauthier R, Bergeron R, 2006. Chromatographic Fingerprinting as a Means of quality control: distinction

between *Actaea racemosa* and four different *Actaea* species. *IV International conference on Quality and safety in Botanicals*.

Kim U, Cho L, Kim L, Yoo H, Lee S, Joo S, Min K, Hong S, Lee H, Park H, Cho S, Kim Y, 2000. Divergent effect of cyclosporine on TH_1/TH_2 type cytokines in patients with severe, refractory rheumatoid arthritis. *Journal of Rheumatology*, **27,** 324 - 331.

Krakauer T, Russo C, 2001. Serum Cytokine Levels and Antibody Response to Influenza Vaccine in the Elderly. *Journal of Immuno-pharmacology Immuno-toxicology*. **23:** 35 - 41

Kuge T, Shibata T, Willett S, 2003. Wood creosote, the principal active ingredient of seirogan, an herbal antidiarrheal medicine: a single-dose, dose-escalation safety and pharmacokinetic study. *Journal of Pharmacotherapy*. **23,** 1391-1400.

Lappin B, Campbell M, 2000. The TH_1 & TH_2 classification of cellular immune responses: concepts, current thinking and applications in haemotological malignancy, *Journal of blood reviews* **14** : 228 - 229.

Lein B, 1998. HIV and Immune boosters. *HIV Plus newsletter*. (www.aidsinfonyc.org/hivplus/issue2/soc/immune.html) [Accessed 24 September 2007]

Leithner C, Sinzinger H, Pohanka E, Schwarz M, Kretschmer G, Syne G, 1982. Recurrence of haemolytic uraemic syndrome triggered by cyclosporin A after renal transplantation (letter). *Lancet*. 1470.

Levy J, 2006. HIV pathogenesis: virology and immunologic features with attention (wwwimedscape.com/view article/487744). [Accessed 24 September 2008]

Lian C, 1980. The role of increased platelet aggregation in TTP. *Journal of Semin Thromb Hemost*, **6:** 401 - 415.

Liang Z, Xie P, Chan K, 2004. Quality control of herbal medicines. *Journal of Chromatography B*, **812**, 53 - 70.

Lin G, Li P, Li S, 2001. Chromatographic analysis of Fritillaria isosteroidal alkaloids, the active ingredients of Beimu, the antitussive traditional Chinese medicinal herb, *Journal of Chromatography A* **935**, 321 – 338

Lv H, Sun H, Liu L, 2008. Pharmacokinetic studies of Chinese triple herbal drug formula, *Journal of phyto-medicine*.

Malmquist G, Danielsson R, 1994. Alignment of chromatographic profiles for principal component analysis: a prerequisite for fingerprinting methods, *Journal of Chromatography A*. **687**, 71 - 88

Matsabisa G, Ebrahim O, 2006. The preclinical and clinical evaluation of a traditional medicinal product, PHELA, an immune modulator used in patients with HIV and AIDS (Appendix F).

Mcelhaney E, Gravenstein S, Hooton W, Arora A, 2002. The Effect of a Booster Vaccination on T-Cell Responses to Influenza Vaccination in Older Adults. *Journal of Interscience Conference of Antimicrobial Agents Chemotherapy*, *2002 Sep 27-30*. **42:** abstract no. G-145.

McElhaney E, Meneilly S, Beattie L, Helgason D, Lee F, Devine O, Bleackley C, 1992. The Effect of Influenza Vaccination on IL-2 Production in Healthy Elderly: Implications for Current Vaccination Practices. J*ournal of Gerontology.* **47,** M3 - M8

McElhaney E, Meneilly S, Pinkoski J, Lechelt E, Bleackley C, 1995. Vaccine-related determinants of the interleukin-2 response to influenza vaccination in healthy young and elderly adults. *Journal of Vaccine.* **13:** 6 - 10.

Mcelhaney E, Gravenstein S, Upshaw M, Hooton W, Krause P, Drinka P, 1998. Immune response to influenza vaccination in institutionalized elderly: effect on different T-cell subsets**.** *Journal of Vaccine*. **16:** 403 - 409.

Mitchell M, Calla E, 1983. Fine specificity of the *in vitro* antibody response to influenza virus by human blood lymphocytes. *Journal of Immunology*. **13:** 1229 - 1233.

Montaga W, Billingham E, 1984. *Immunology and the skin* XI edition. Ney York Appleton-Century-Crofts.

Neild H, Rocchi G, Imberti L, Fumagalli F, Brown Z, Remuzzi G, Williams D, 1983. Effect of cyclosporin A on prostacyclin synthesis by vascular tissue. *Journal of Thrombosis Research*, **32:** 373 - 379

Nielsen P, Carstensen M, Smedsgaard J, 1998. Aligning of single and multiple wavelength chromatographic profiles for chemometric data analysis using correlation optimized warping, *Journal of Chromatography A*. **805**: 17 - 35.

Opal S, De Palo V, 2000. Anti-inflammatory cytokines, *Chest journal.* **117**: 1162-1172.

Peschel W, 2007. The Traditional Herbal Medicine Directive within the European regulatory framework for Herbal Products. Latinoam. Caribe *Plant. Medicine. Aromaticas.* **6**: 102 – 111.

Phadke K, Bagirath A, 2004. Mycophenolate mofetil as an alternative to cyclosporine in post renal transplant thrombocytopenia. Journal of *Pediatric Nephrology*, **19** (2004)**:** 822 - 823

Picker L, 2006. Immunopathogenesis of acute AIDS virus infection, *Current opinion in immunology* **18**: 339 - 405.

Pinto L, Shearer G, 2000. Immune based approaches for control of HIV infection and viral induced immunopathogenesis, *Journal of clinical immunology.* **97**: 1 - 8.

Pravdova V, Walczak B, Massart L, 2002. A comparison of two algorithms for warping of analytical signals. *Journal of Analytica Chimica Acta*. **456**: 77 - 83.

Priimagi L, Tefanova V, 2005. The role of serum TH_1 and TH_2 cytokines in patients with chronic hepatitis B and hepatitis C, *ACTA medica lituanica.* **12**: 28 - 31.

Pujol D, 2004. *CM 4*, A herbal supplement with clinical data support, *www.thebody.com [Accessed 5 October 2007].*

Reeves G, Todd I, 2000. *Lecture notes on immunology* 2nd edition. Blackwell Science inc.

Romagnani S, 1991. Human TH_1 and TH_2 subsets: doubt no more. *Journal of Immunology Today*. **12:** 256 - 257.

Rosenberg Z, Fauci A, 1991. Immuno pathogenesis of HIV infection, *FASEB journal*. **5**: 2382 - 2388.

Saurwein-Teiss M, Zisterer K, Schmitt T, Gluck R, Cryz S, Grubeck-Loebenstein B, 1998. Whole virus influenza vaccine activates dendritic cells (DC) and stimulates cytokine production by peripheral blood mononuclear cells (PBMC) while subunit vaccines support T cell proliferation. *Journal of Clinical Experimental Immunolology.* **114:** 271 - 276

Schlaak J, Tully G, 1999. HBV –specific immune defect in chronic hepatitis B (CHB) is correlated with a dysregulation of pro and anti inflammatory cytokines, *Journal of clinical experimental immunology* .**115**: 508 - 514.

Sherwood L, 2001 *Human physiology "from cells to systems"* 4th edition. Thomson-Brooks-Cole 377 - 379.

Shah V, Midha K, Dighes S, Mcgilverg I, Skelly J, Yacobi A, Layloff T, Viswanathan C, Cook C, Mcdowall R, 1992. Analytical method validation: Bio-availability, Bio-equivalence and pharmacokinetics studies. *Journal of Pharmaceutical Sciences*. **81**: 309 – 312.

Shah V, Midha K, Dighes S, Mcgilverg I, Skelly J, Yacobi A, Layloff T, Viswanathan C, Cook C, Mcdowall R, 2002. Bio-analytical method validation – A revisit with a decade of progress . *Journal of Pharmaceutical Sciences*. **17**: 1551 - 1557.

Smilie T, Khan I, 2009. A comprehensive approach to identifying and authenticating botanical products. Journal of Clinical Pharmacology and Therapeutics **87**: 175 - 186

Springfield P, Eagles F, Scott G, 2005. Quality assessment of South African herbal medicines by means of HPLC fingerprinting*, Journal of Ethno-pharmacology.* **101**: 75 – 83

Tamizifar H, Jennings R, Ali S, Potter W, 1997. Induction of IL-2 and IFN-7 in BALB/c mice immunised with subunit influenza A vaccine in combination with whole cell or acellular DTP vaccine*. Journal of Medical Microbiology*. **46:** 61-66.

TH_1 vs TH_2 http://sprojects.mmi.mcgill.ca/immunology/Th1&Th2.htm [Accessed 6 November 2007]

The Pathogenesis of AIDS www.mcb.uct.ac.za/cann/335/AIDSI.html [Accessed 24 August 2007]

Tsai Y, Hanson Q, Straka J, Hoke R, Ordovas M, Peacock M, Arends L, Arnett K, 2005. Effect of influenza vaccine on markers of inflammation and lipid profile. *Journal of Laboratory and Clinical Medicine*. **145:** 323 - 327.

UNAIDS 2008. Report on the global AIDS epidemic. Geneva, UNAIDS

UNAIDS, 2009. Report on the global AIDS epidemic. Geneva, UNAIDS

Vicenzi E, Biswas P, 1997. Role of pro-inflammatory cytokines and β-chemokines in controlling HIV replication, *Journal of Leukocyte Biology.* **62** : 34 - 40.

Virelizier L, Postlethwaite R, Schild C, Allison C, 1974. Antibody responses to antigenic determinants of influenza virus hemagglutinin. *Journal of Experimental Medicine* **140 :** 1559-1570

Walczak B, Wu W, 2005. Fuzzy warping of chromatograms, Chemometrics and Intelligent Laboratory Systems. **77:** 173.

WHO, 2009. General Guidelines for Methodologies on Research and Evaluation of Traditional Medicines, Geneva, 2009, 1.

WHO, UNICEF and UNAIDS, 2009. Towards universal access: scaling up priority HIV/AIDS interventions in the health sector. Progress report, 2009. Geneva, World Health Organization.

Worldwide HIV & AIDS statistics, 2007. www.avert.org/worldstats.htm [Accessed 1 September 2007]

Xie P, Chen S, Liang Y, 2006. Chromatographic fingerprint analysis: rational approach for quality assessment of traditional Chinese herbal medicine, *Journal of chromatography A.* **1112**: 171 – 180

Xu J, Liang Z, Chau T, Vander-Heyden Y, 2006. Pretreatments of chromatographic fingerprints for quality control of herbal medicines, *Journal of Chromatography A.* **1134**: 253 – 259

Yarnell E, Absacal K, 2001. Herbal immune modulators as potential therapy for HIV patients, *Journal of the American botanical council.* **55**: 16.

Ye G, Li Y, 2003. SPE-HPLC method for the determination and pharmacokinetic studies on paeoniflorin in rat serum after oral administration of traditional Chinese medicinal preparation Guan-Xin-Er-Hao decoction, *Journal of pharmaceutical and biomedical analysis.* **33**: 521 - 527

APPENDICES

Ref: Chapter 8, methanol extracts with 20 % acetonitrile in water as mobile phase on HPLC_UV.

Figure A-1: A chromatogram of blank methanol after elution with 20 % acetonitrile in water mobile on HPLC_UV

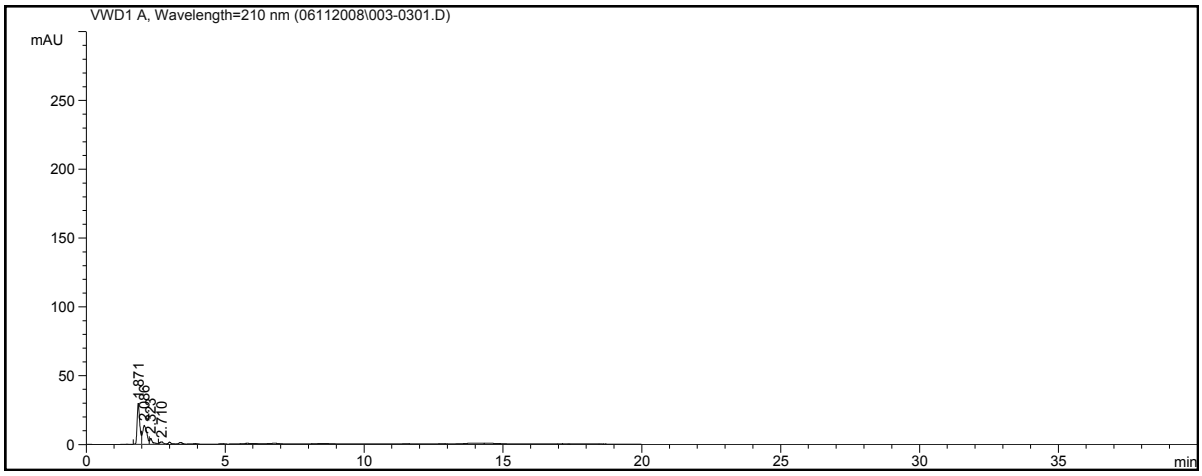
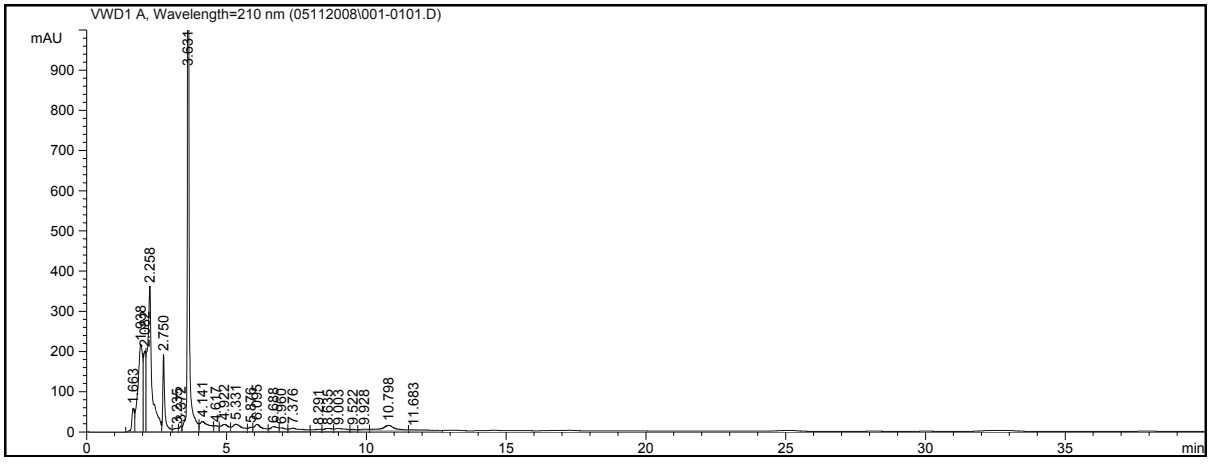


Figure A-2: A chromatogram of 20 % methanol extract after elution with 20 % acetonitrile in water mobile on HPLC_UV



Ref: Chapter 8, methanol extracts with 20 % acetonitrile on HPLC_UV.

Figure A-3: A chromatogram of 60 % methanol extract after elution with 20 % acetonitrile in water mobile HPLC_UV

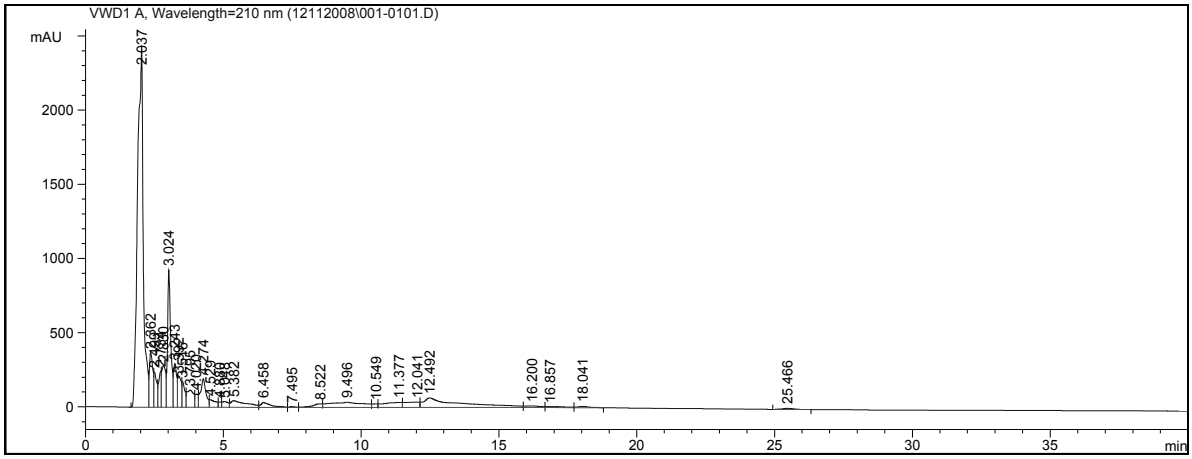
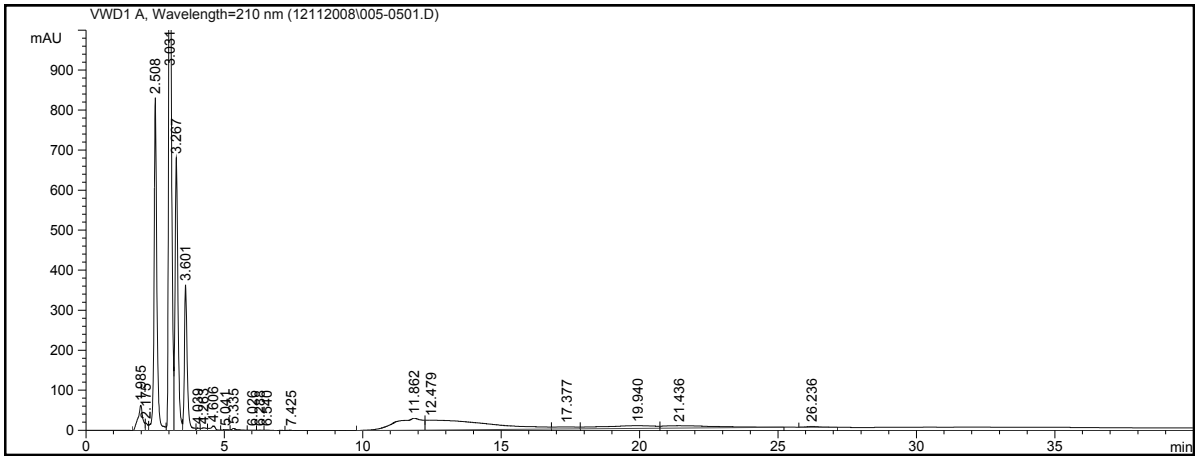


Figure A-4: A chromatogram of methanol extract after elution with 50 % acetonitrile in water mobile HPLC_UV



Ref: Chapter 8, phela extracts with 50 % acetonitrile on HPLC_UV.

Figure A-5: A chromatogram of a water extract after elution with 50 % acetonitrile in water mobile on HPLC_UV

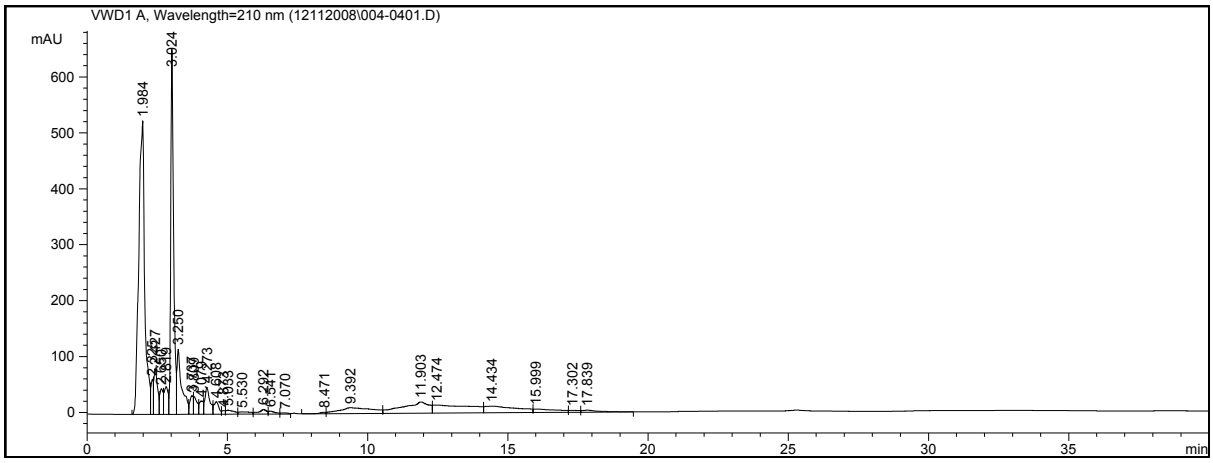
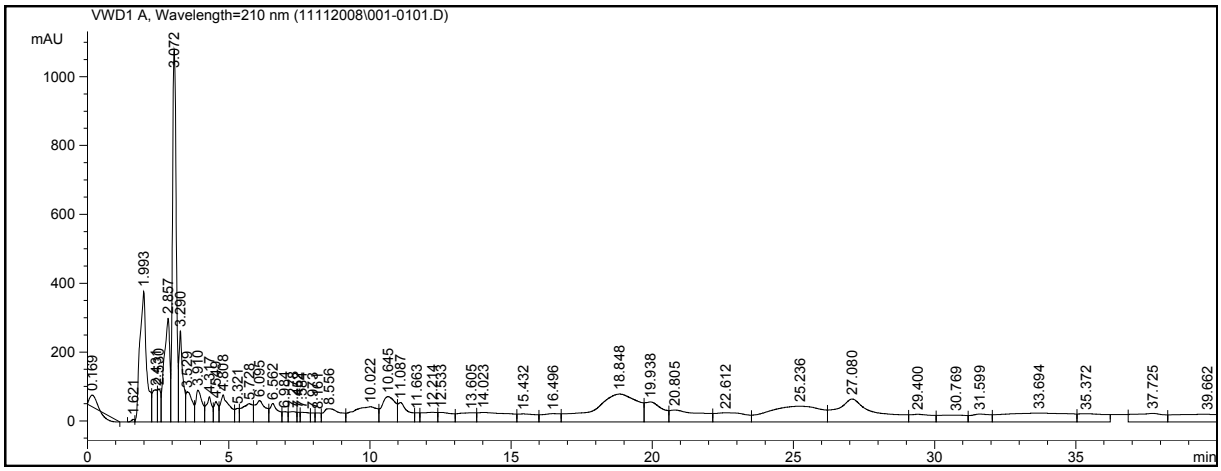


Figure A-6: A chromatogram of ether extract after elution with 50 % acetonitrile in water mobile on HPLC_UV



Ref: Chapter 8, phela extracts with 50 % acetonitrile on HPLC_UV

Figure A-7: A chromatogram of blank hexane after elution with 50 % acetonitrile in water mobile on HPLC_UV

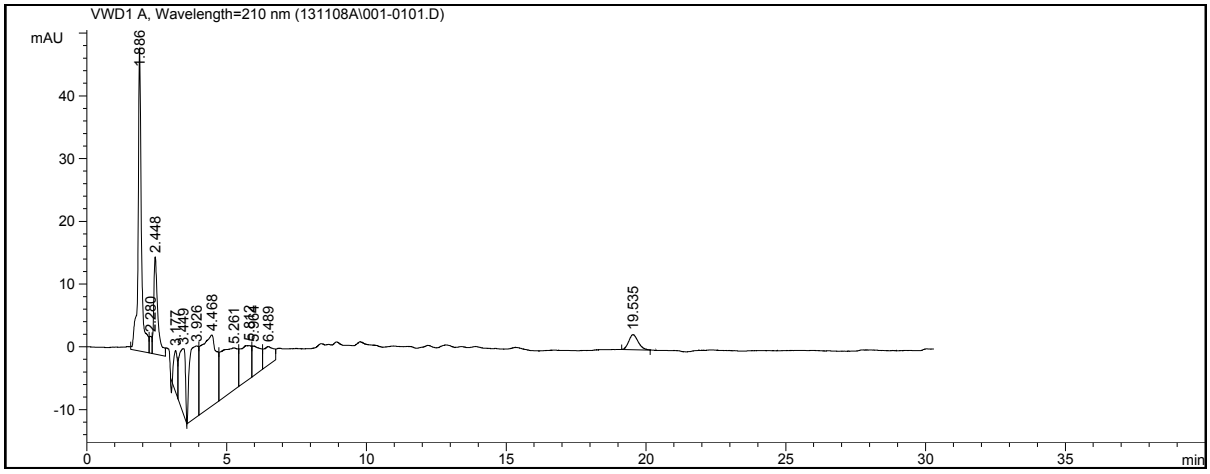
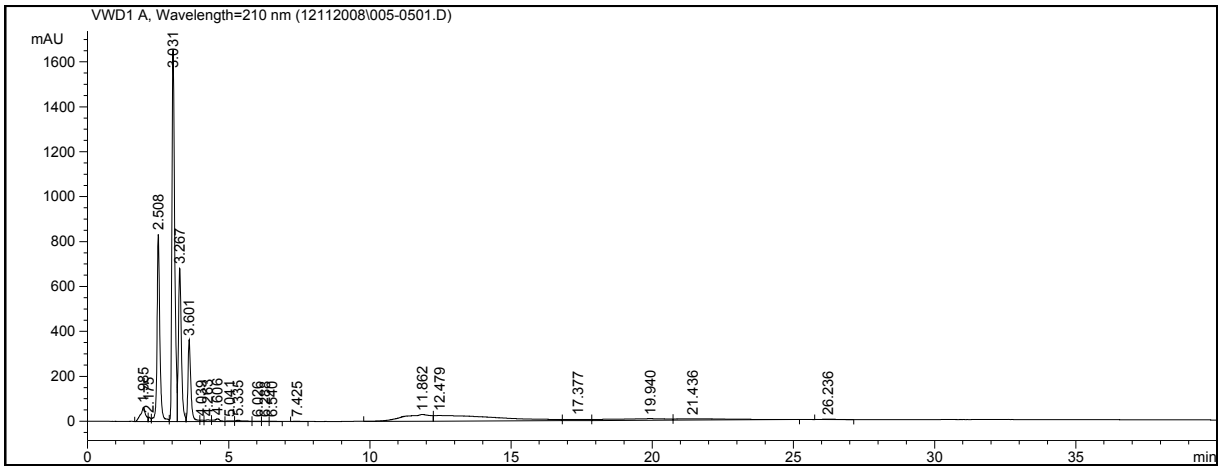
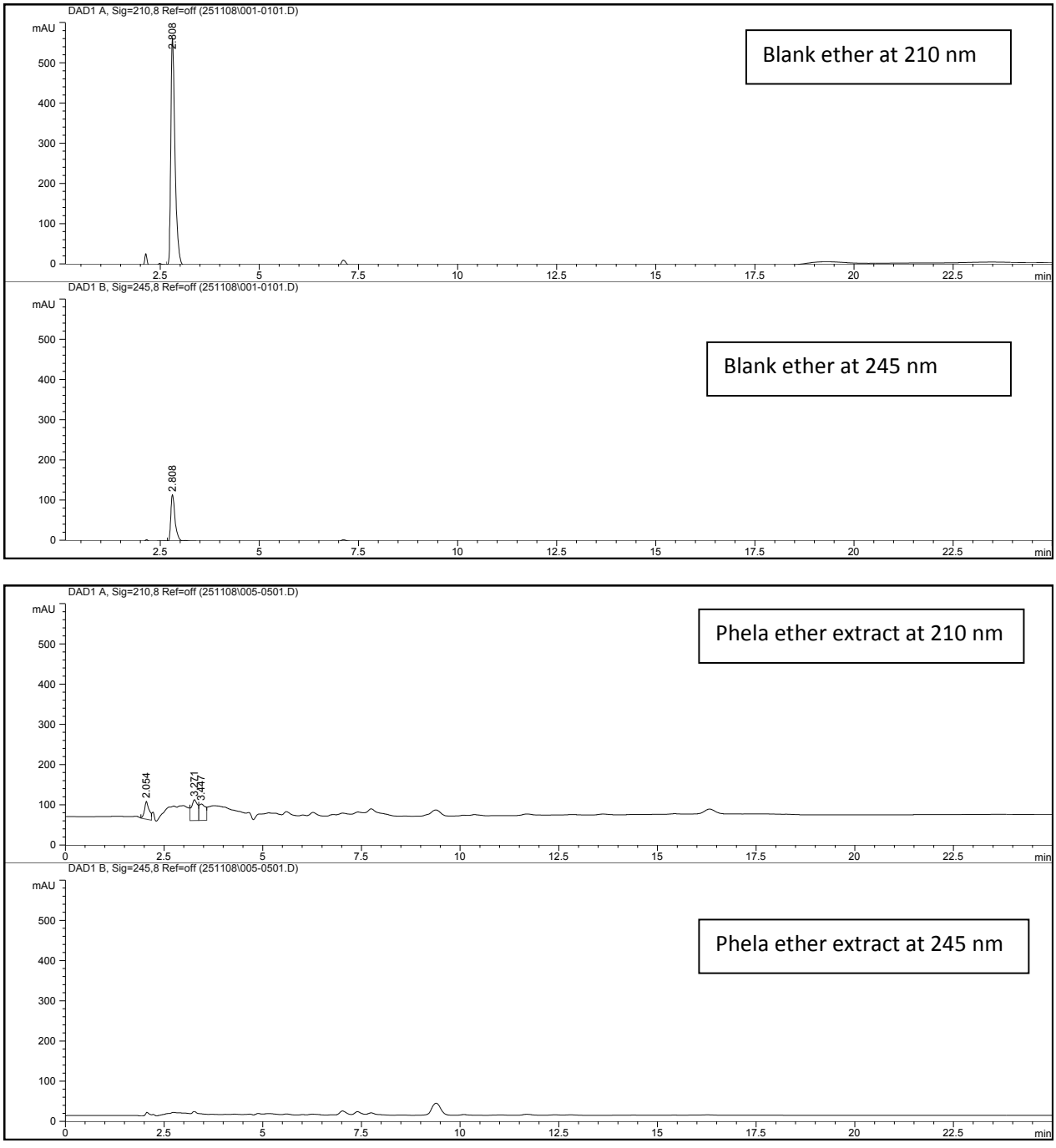


Figure A-8: A chromatogram of hexane extract after elution with 50 % acetonitrile in water mobile on HPLC_UV



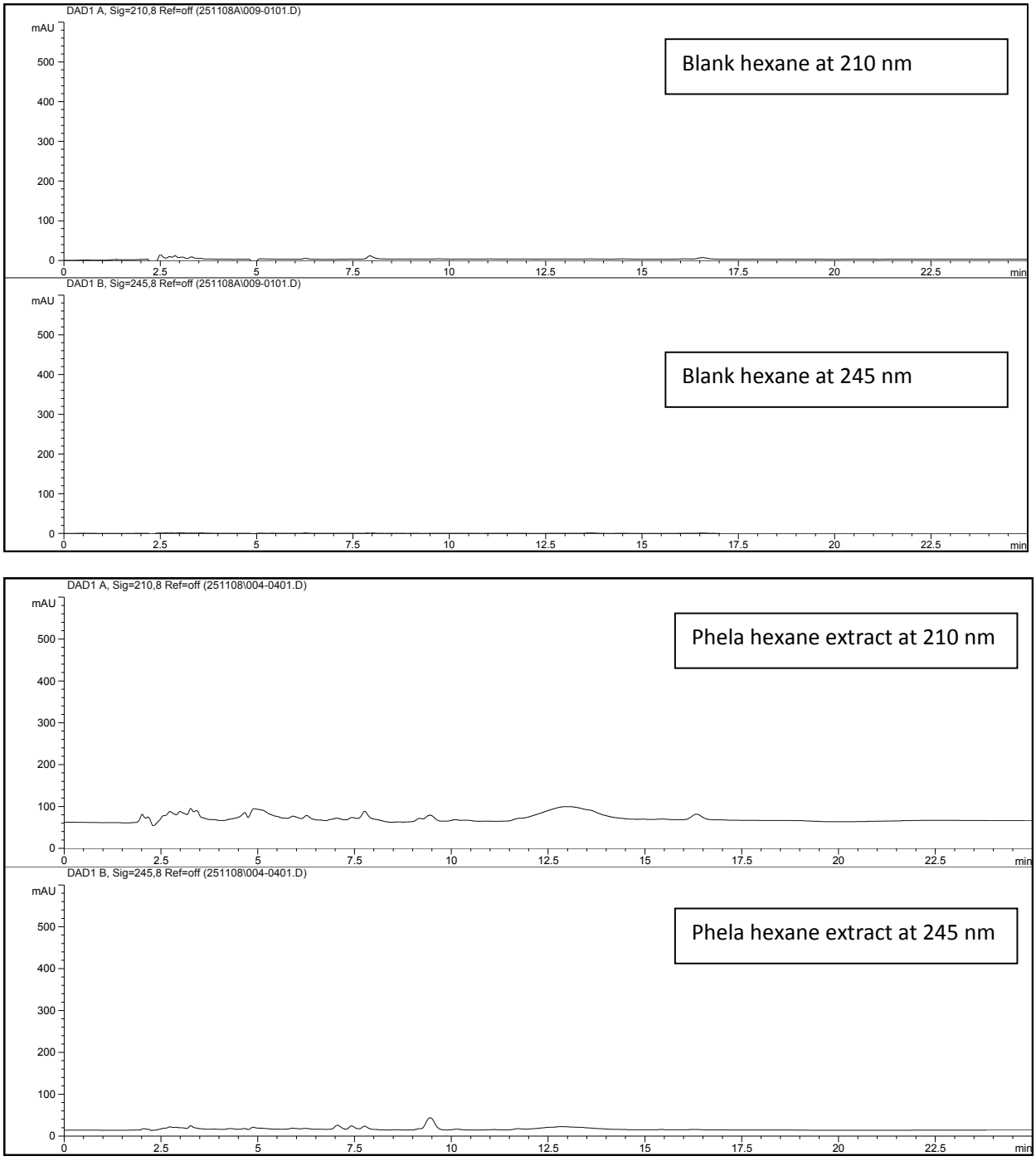
Ref: Chapter 8, pH based extraction

Figure A-9: HPLC_UV chromatogram of blank ether and phela basic ether extract detected at 210 and 245 nm



Ref: Chapter 8, pH based extraction

Figure A-10: HPLC_UV chromatogram of blank hexane and phela basic hexane extract detected at 210 and 245 nm



Ref: Chapter 8, phela acidic extract with 70 % acetonitrile on HPLC_UV at different flow rates

Figure A-11: A chromatogram of an acidic phela extract after elution with 70 % acetonitrile in water mobile on HPLC_UV at a flow rate of **1.0 ml/min.** Peak 1 and 2 eluting at 4.7 and 11.5 minutes respectively, while IS is at 5.2 minutes

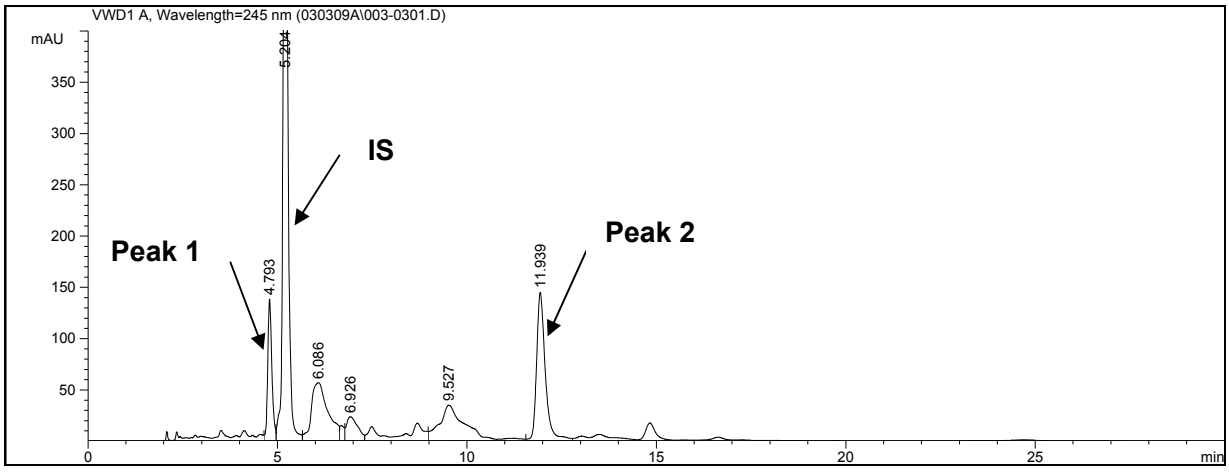
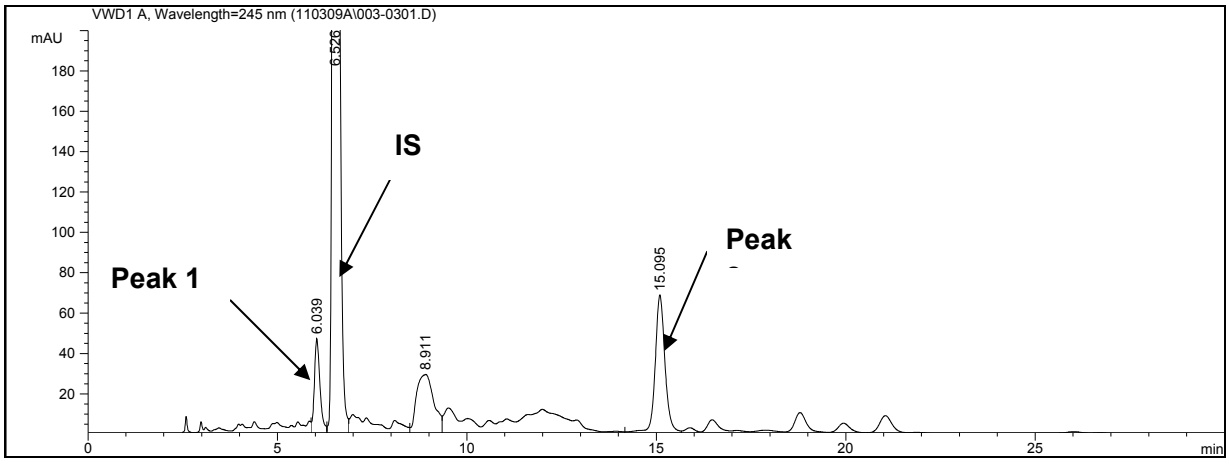


Figure A-12: A chromatogram of an acidic phela extract after elution with 70 % acetonitrile in water mobile on HPLC_UV at a flow rate of **0.8 ml/min.** Peak 1 and 2 eluting at 6.0 and 15.0 minutes respectively, while IS elutes at 6.5 minutes



Ref: Chapter 9, blank chromatograms,

Figure B-1: Blank HPLC-UV chromatogram for Phela's TLC-Band 2 (Chap. 10, Fig. 10.3a) and band 3 (Chap. 10, Fig. 10.4a)

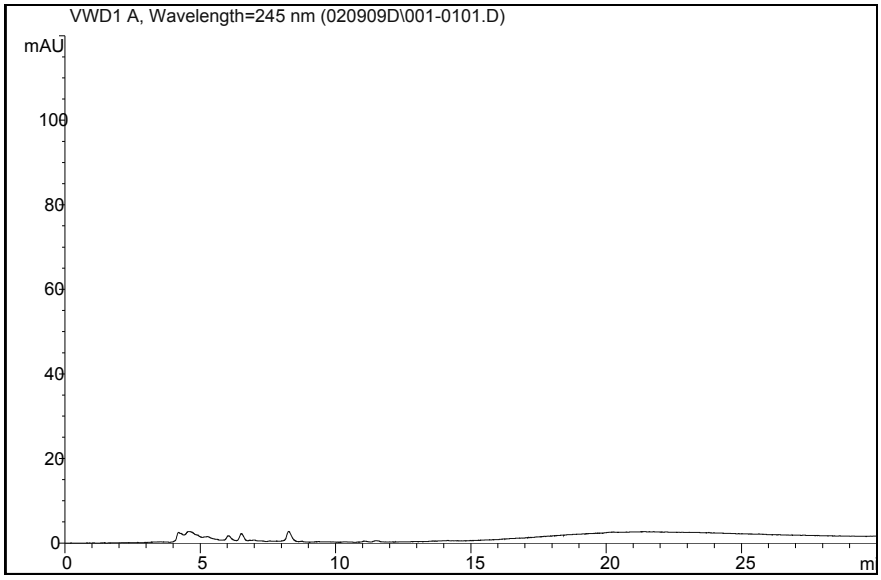
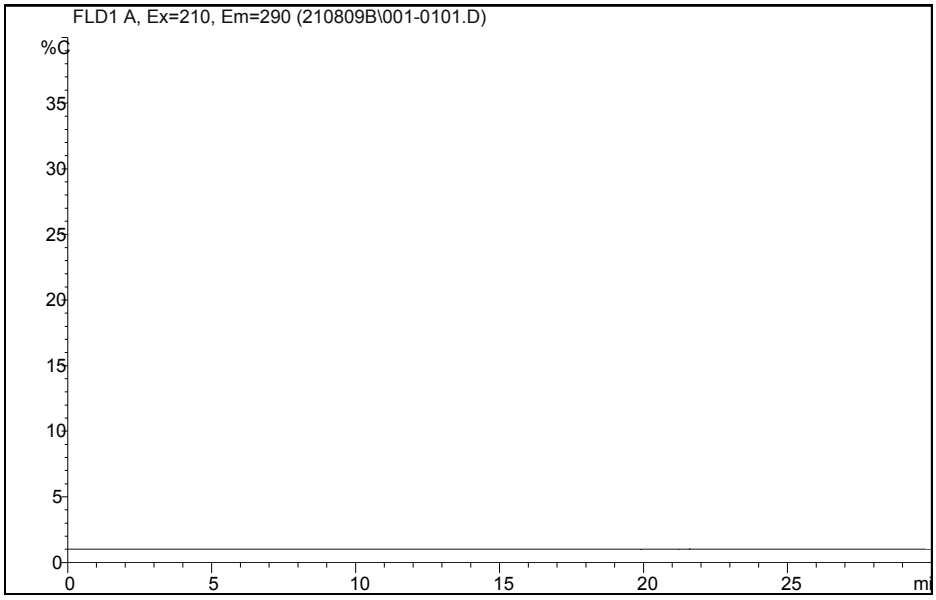


Figure B-2: Blank HPLC_FL chromatogram for Phela's TLC-Band 2 (Chap. 10, Fig. 10.3b) and band 3 (Chap. 10, Fig. 10.4b)



Ref: Chapter 10, blank chromatograms,

Figure B-3: Blank HPLC_UV chromatogram for Phela's column chromatography (CC) fraction (FR) 17 (CC-FR-17) (Chap. 10, Fig. 10.7b) and other CC-fractions (Appendix B; fig B-6)

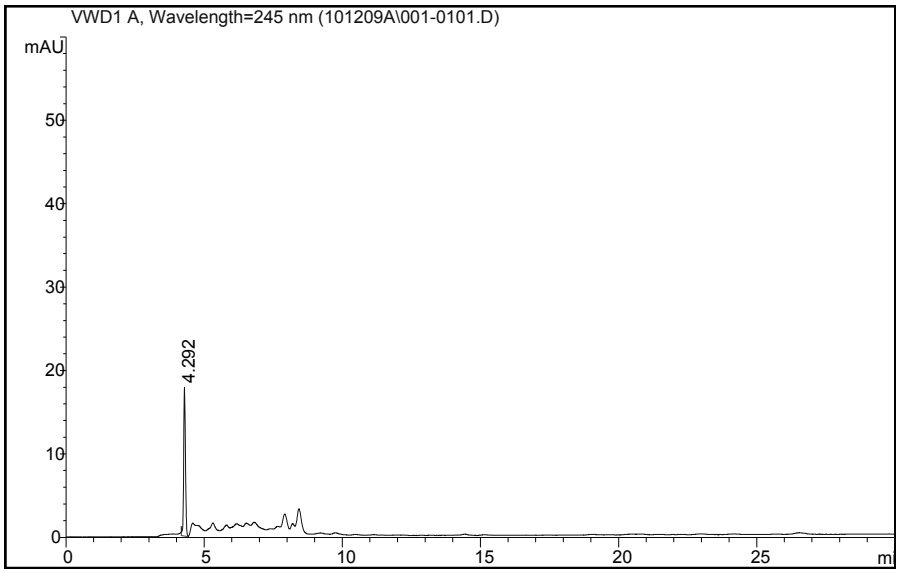
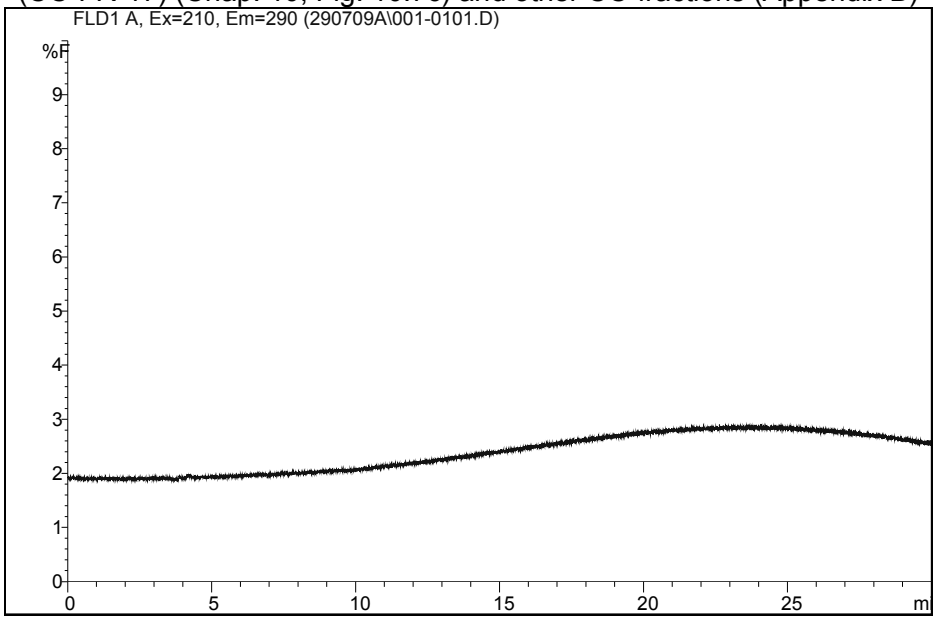
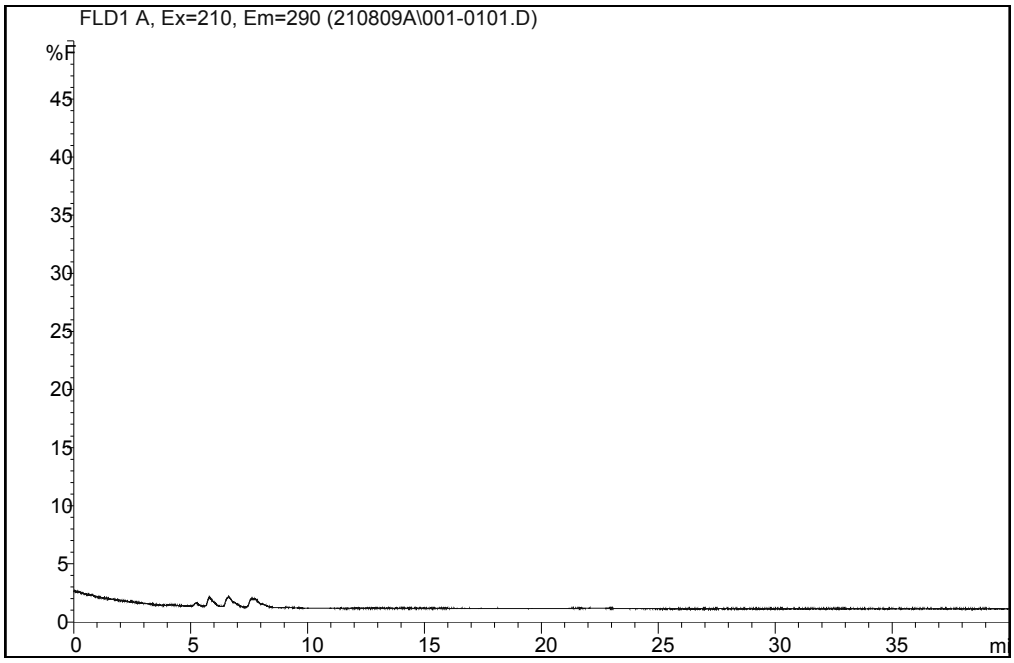


Figure B-4: Blank HPLC_FL chromatogram for Phela's column chromatography (CC) fraction (FR) 17 (CC-FR-17) (Chap. 10, Fig. 10.7c) and other CC-fractions (Appendix B)

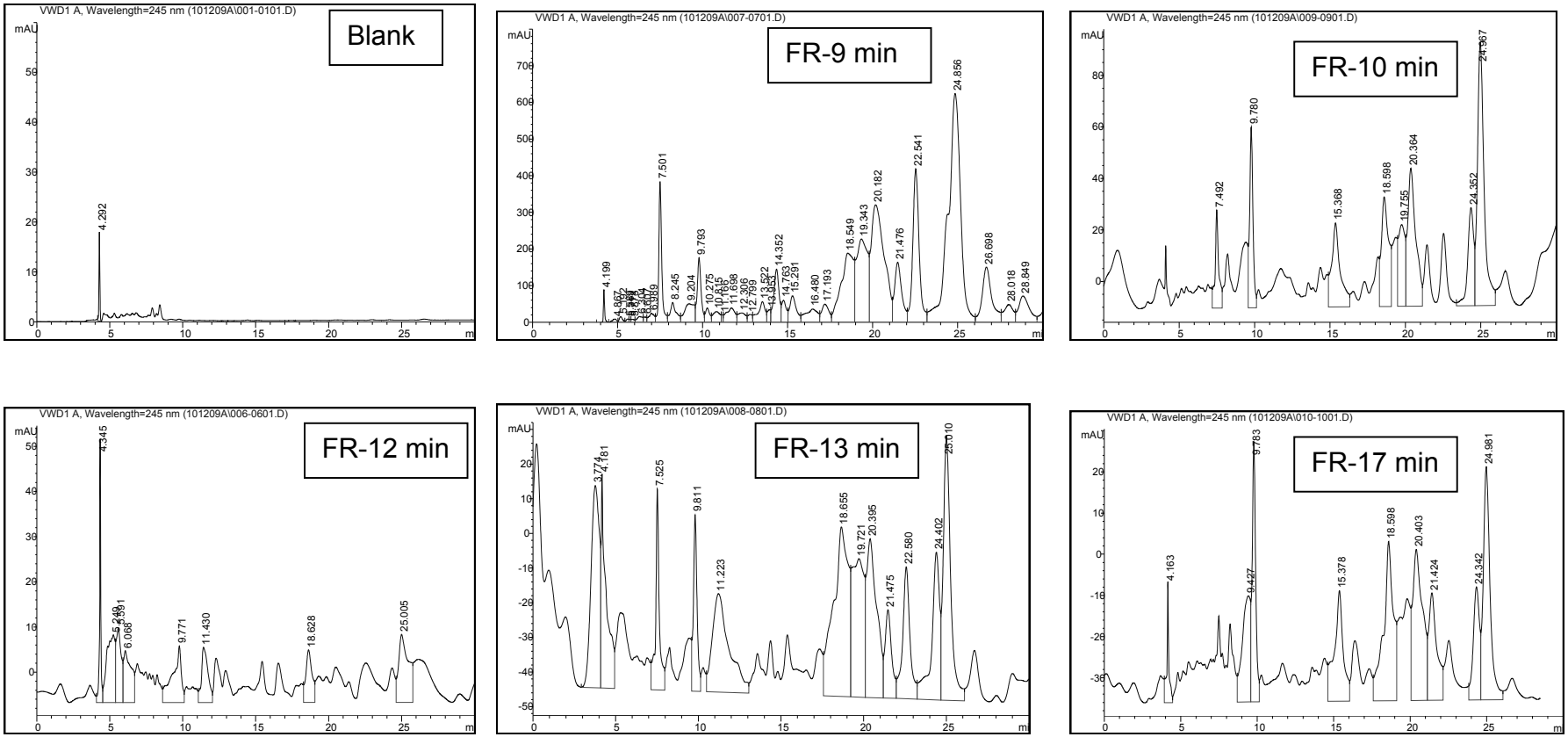


Ref: Chapter 10, blank chromatograms,

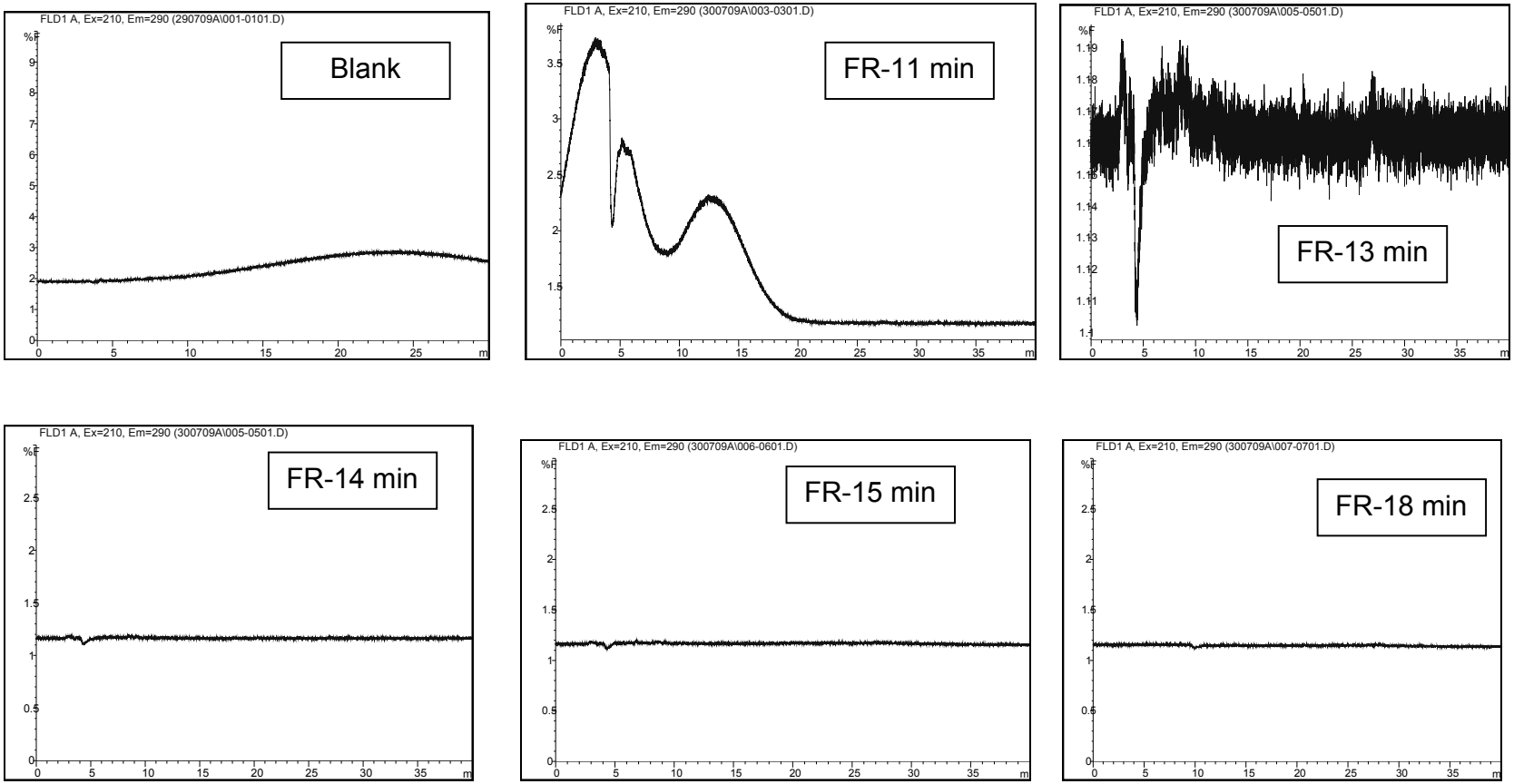
Figure B-5: Blank HPLC_FL chromatogram for Phela's TLC-Band 3 (Chap. 10, Fig. 10.11a) and SJW's TLC- band B (Chap. 10, Fig. 10.11b)



Ref: Chapter 10, Other Column Chromatography (CC) Fractions analyzed by HPLC-UV
Figure B-6: Blank HPLC_UV chromatograms of a blank along with CC fractions



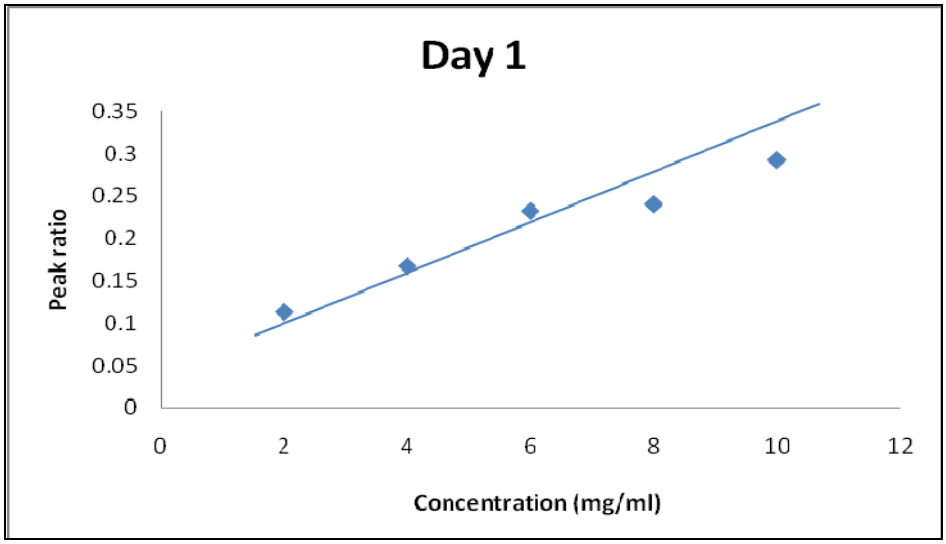
Ref: Chapter 10, Other Column Chromatography (CC) Fractions analyzed by HPLC_FL
Figure B-7: HPLC_FL chromatograms of a blank along with some CC fractions

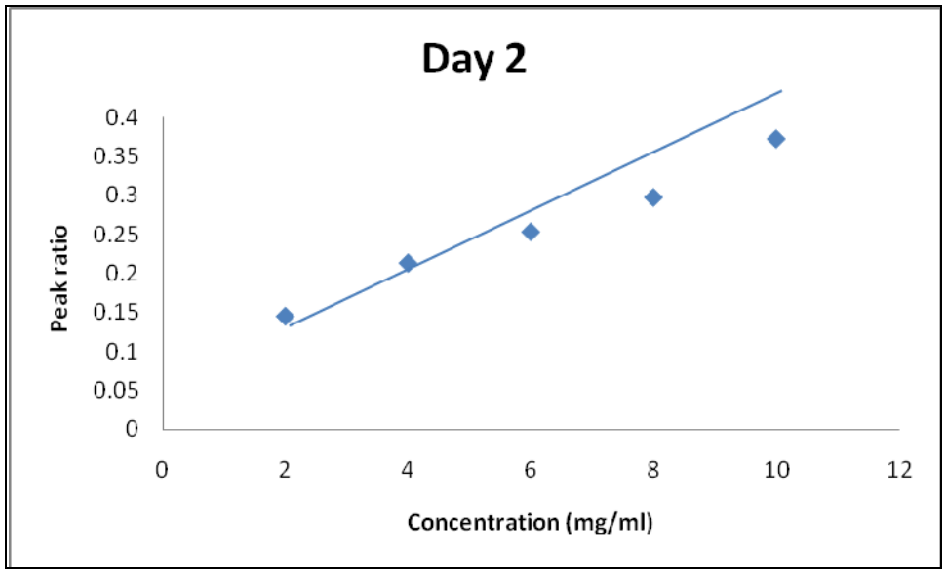


Ref: Chapter 11, Daily ratios used for plotting a calibration curve (n = 5) during validation
Table C-1: Table with marker peak 2 ratios of calibration curve over 5 days (Chapter 11)

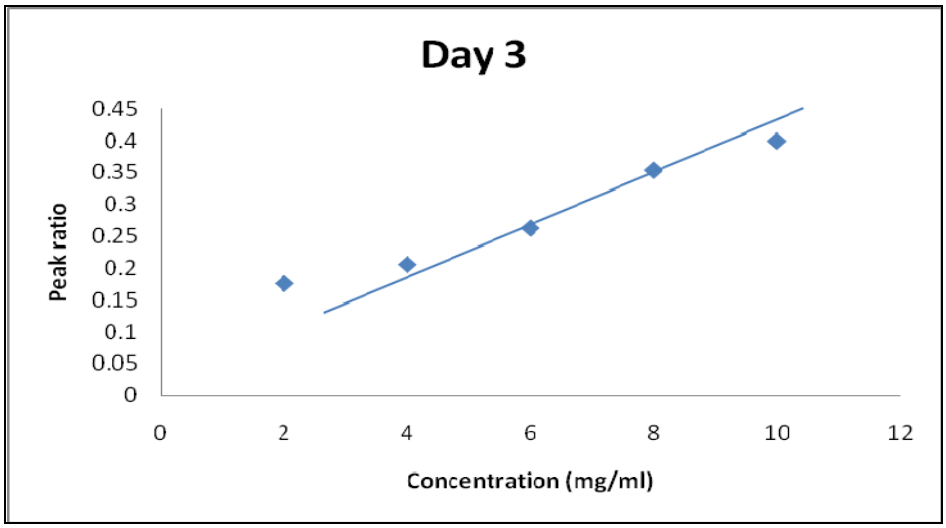
	Ratios over 5 days							
Concentration	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Mean**	**SD**	**CV %**
2	0.113	0.145	0.176	0.151	0.166	**0.150**	0.024	16.06
4	0.167	0.213	0.206	0.168	0.197	**0.190**	0.021	11.30
6	0.232	0.253	0.263	0.189	0.239	**0.235**	0.028	12.11
8	0.240	0.297	0.354	0.205	0.294	**0.278**	0.057	20.64
10	0.292	0.372	0.400	0.235	0.379	**0.336**	0.070	20.74

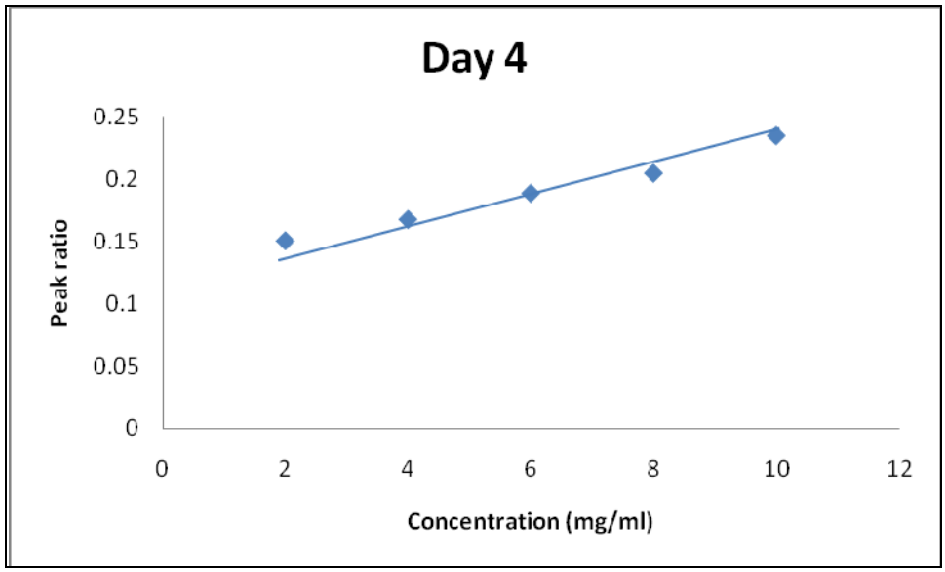
Ref: Chapter 11, Calibration curves over five days (day 1 and day 2) with concentration (mg/ml) versus peak ratio



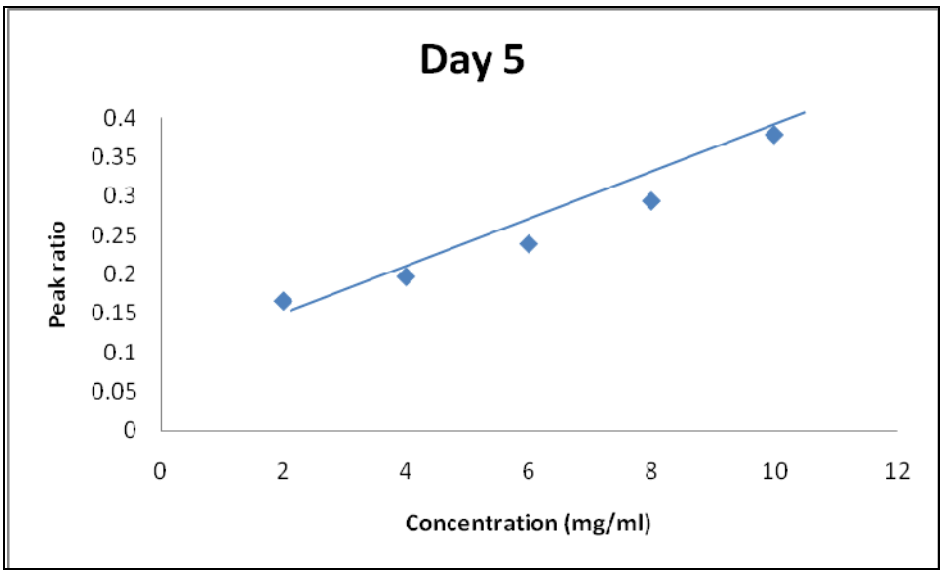


Ref: Chapter 11, Calibration curves over five days (Day 3 and day 4) with concentration (mg/ml) versus peak ratio





Ref: Chapter 11, Calibration curves over five days (day 5) with concentration (mg/ml) versus peak ratio



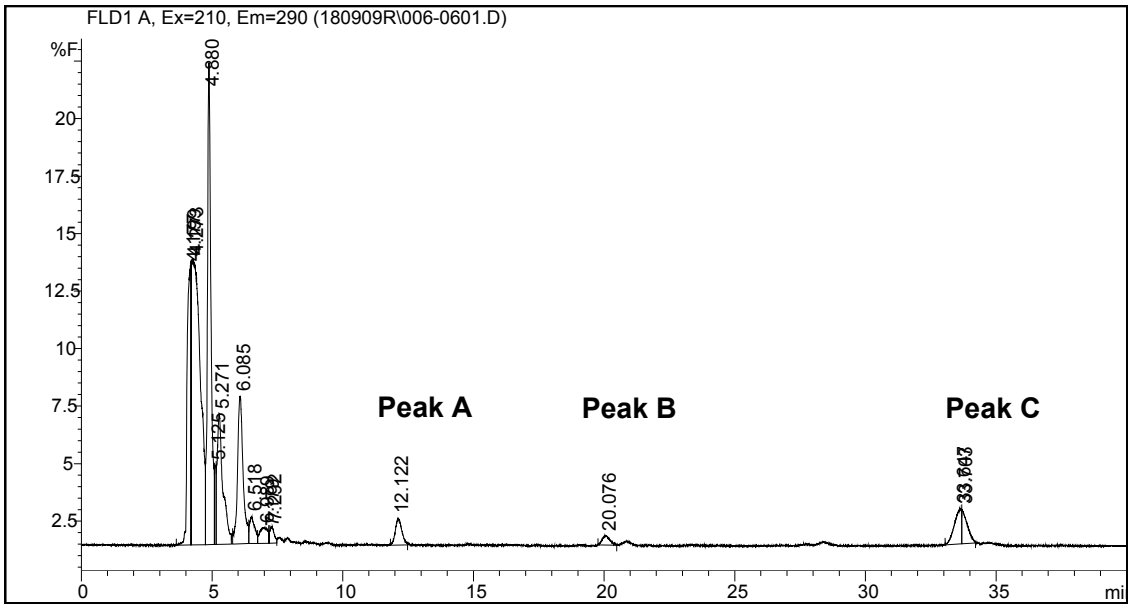
Ref: Chapter 11, Long-term stability testing (4 months)

Table C-2: Stability concentrations (mg/ml) and percentages of 2, 6 and 10 mg/ml after storage at -20°C for 4 months.

Known Concentration (mg/ml)	Derived Concentration (mg/ml)	Derived percentage (%)
2	3.4	170
6	2.1	35
10	3.0	30

Ref: Chapter 11

Figure C-1: HPLC_FL chromatogram of 100mg/ml phela sample used a quality control sample during rat plasma analysis

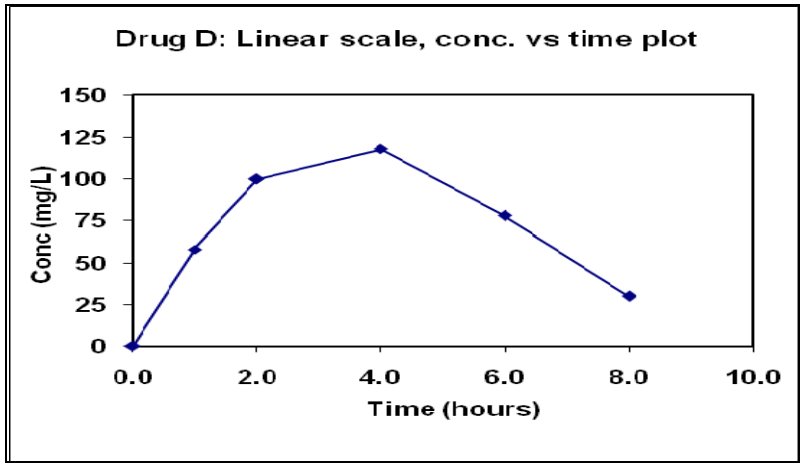


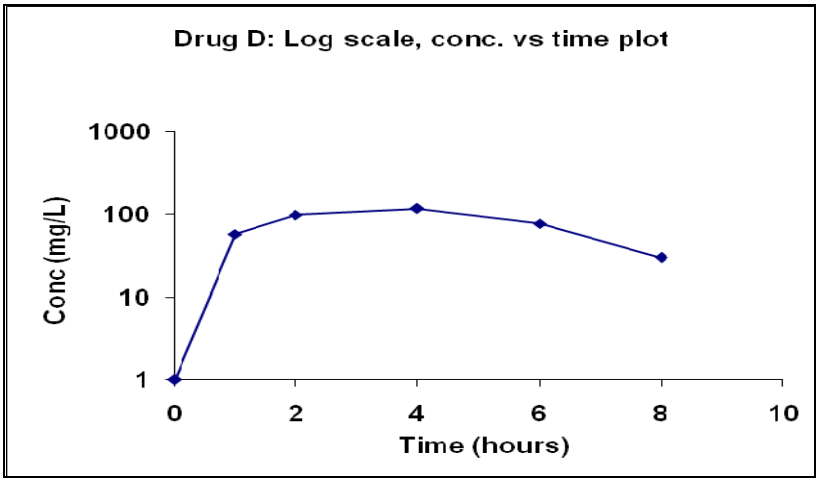
Ref: Chapter 12, Concentration (peak-area/L) range per test group (Test group 1)

Table D-1: Concentration (peak-area/L) of group 1 after 1, 2, 4, 6 and 8 hours of phela oral administration

Time (hours)	Concentration (peak-area/L)
1	58
2	100
4	118
6	78
8	30

Figure D-1: Linear and log curves of group 1, concentration versus time plots

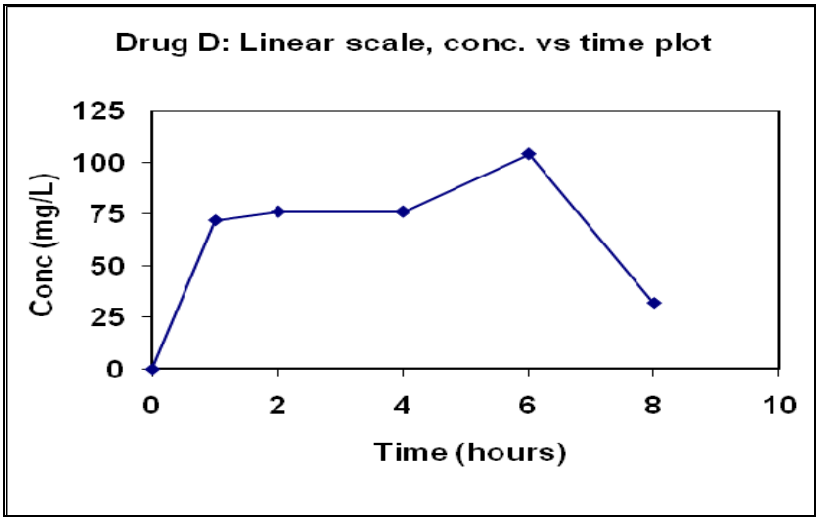


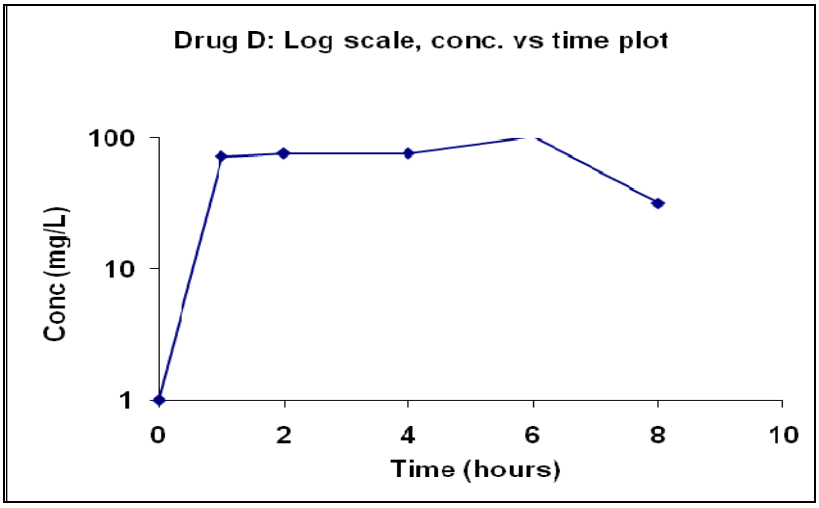


Ref: Chapter 12, Concentration (peak-area/L) range per test group (Test group 2)
Table D-2: Concentration (peak-area/L) of group 2 after 1, 2, 4, 6 and 8 hours of phela oral administration

Time (hours)	Concentration (peak-area/L)
1	72
2	76
4	76
6	104
8	32

Figure D-2: Linear and log curves of group 2, concentration versus time plots



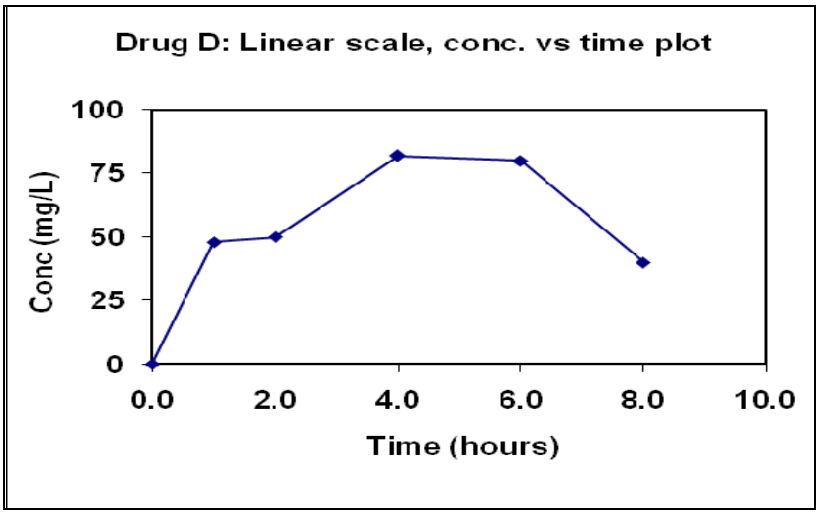


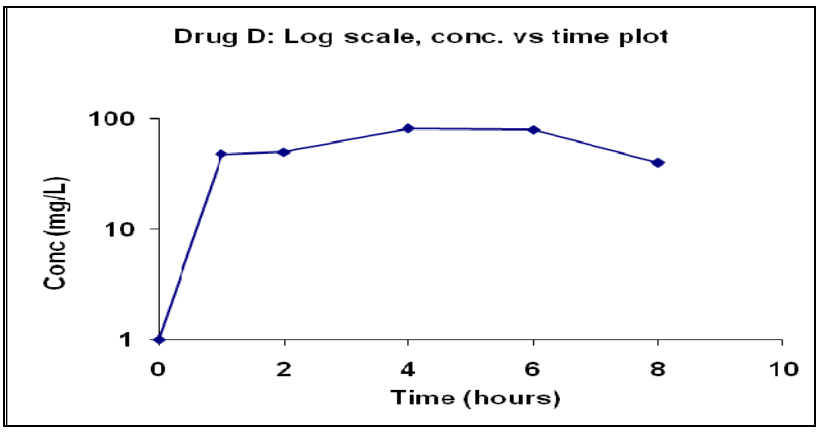
Ref: Chapter 12, Concentration (peak-area/L) range per test group (Test group 3)

Table D-3: Concentration (peak-area/L) of group 3 after 1, 2, 4, 6 and 8 hours of phela oral administration

Time (hours)	Concentration (peak-area/L)
1	48
2	50
4	82
6	80
8	40

Figure D-3: Linear and log curves of group 3, concentration versus time plots





Ref: Chapter 13, Haematology test (Full Blood tests)

Table E-1: Summary of full blood tests (red blood cells) levels recorded as mean ± SD after 7 and 14 days' treatment.

GROUP		RCC (x10^12/l)	Haemoglobin (g/dl)	Haematocrit (l/l)	MCV (fl)	MCH (pg)	MCHC (g/dl)	Platelet count (x10^9/l)
7 DAYS TREATMENT								
Saline	**(n = 7)**	8.9 ± 0.28	16.0 ± 0.44	0.71 ± 0.02	79 ±1.4	18.0 ± 0.00	22.9 ± 0.38	858 ± 360
Influenza vaccine	**(n = 7)**	7.7 ± 0.31	16.5 ± 0.36	**0.64 ± 0.20**	80 ±1.7	18.7 ± 0.52	23.0 ± 0.00	992 ± 347
Cyclosporine	**(n = 3)**	7.4 ± 0.85	14.1 ± 2.02	0.65 ± 0.07	88 ± 1.0	19 ± 1.0	21.7 ± 0.58	592 ± 472
Phela	**(n = 3)**	8.7 ± 0.24	15.9 ± 0.12	0.69 ± 0.0	80 ± 2.1	18.3 ± 0.58	23.0 ± 0.00	981 ± 214
Phela + Cyclosporine	**(n = 4)**	8.5 ± 0.42	15.8 ± 0.76	0.69 ± 0.03	81 ± 0.5	18.8 ± 0.50	23.0 ± 0.00	1089 ± 50
14 DAYS TREATMENT								
Saline	**(n = 7)**	8.6 ± 0.31	15.9 ± 0.42	0.69 ± 0.02	80 ± 1.2	18.6 ± 0.53	23.1 ± 0.38	909 ± 322
Influenza vaccine	**(n = 7)**	8.6 ± 0.93	15.7 ± 1.50	0.69 ± 0.07	81 ± 1.5	18.7 ± 0.49	23.1 ± 0.38	947 ± 416
Cyclosporine	**(n = 3)**	7.6 ± 0.40	14.6 ± 0.91	0.66 ± 0.04	87 ± 1.2	19.3 ± 0.58	22.3 ± 0.58	450 ± 637
Phela	**(n = 7)**	8.8 ± 0.31	16.1 ± 0.39	0.70 ± 0.02	79 ± 1.6	18.6 ± 0.53	23.1 ± 0.38	979 ± 273
Phela + Cyclosporine	**(n = 7)**	8.7 ± 1.0	16.4 ± 0.78	0.68 ± 0.09	78 ± 1.7	19.0 ± 2.71	24.7 ± 4.11	**1040 ± 652**
21 DAYS TREATMENT								
Influenza vaccine	**(n = 7)**	8.9 ± 0.31	16.1 ± 0.39	0.70 ± 0.02	79 ± 1.3	18.3 ± 0.49	23.0 ± 0.00	856 ±349

Abbreviations + reference values **RCC**: Red cell count **x10^12/l)**, Haemoglobin (11.5 – 16.1g/dl), **MCV**: Mean corpuscular volume (48 – 70 fl), **MCH**: Mean corpuscular haemoglobin, **MCHC**: Mean corpuscular haemoglobin concentration (40 g/dl), Platelet count (150 – 460**(x10^9/l)**)

Ref: Chapter 13, Haematology test (Full Blood tests)

Table E-2: Summary of Full blood test (white blood cells) levels recorded as mean ± SD after 7 and 14 days' treatment.

GROUP		WCC (x10^9/l)	Neutrophils (x10^9/l)	Lymphocytes (x10^9/l)	Monocytes (x10^9/l)	Eosinophils (x10^9/l)	Basophils (x10^9/l)
7 DAYS TREATMENT							
Saline	**(n = 7)**	5.8 ± 1.7	0.77 ± 0.32	4.76 ± 1.59	0.08 ± 0.06	0.09 ± 0.04	0.08 ± 0.03
Influenza vaccine	**(n = 7)**	4.4 ± 0.8	0.70 ± 0.12	3.58 ± 0.73	0.04 ± 0.04	0.09 ± 0.01	0.04 ± 0.01
Cyclosporine	**(n = 3)**	6.0 ± 1.3	0.68 ± 0.28	5.03 ± 1.32	0.19 ± 0.06	0.07 ± 0.05	0.05 ± 0.03
Phela	**(n = 3)**	4.9 ± 0.9	0.60 ± 0.17	3.96 ± 0.71	0.10 ± 0.07	0.12 ± 0.05	0.06 ± 0.01
Phela + Cyclosporine	**(n = 4)**	4.8 ± 1.3	0.64 ± 0.13	3.82 ± 1.18	0.10 ± 0.03	0.09 ± 0.02	0.07 ± 0.05
14 DAYS TREATMENT							
Saline	**(n = 7)**	4.5 ± 1.2	0.66 ± 0.12	3.69 ± 10.6	0.07 ± 0.05	0.05 ± 0.03	0.07 ± 0.03
Influenza vaccine	**(n = 7)**	3.8 ± 1.2	0.66 ± 0.31	3.01 ± 1.00	0.05 ± 0.02	0.08 ± 0.02	0.04 ± 0.01
Cyclosporine	**(n = 3)**	6.6 ± 3.0	0.97 ± 0.46	5.31 ± 2.53	0.27 ± 0.16	0.04 ± 0.02	0.18 ± 0.0
Phela	**(n = 7)**	3.8 ± 0.7	0.51 ± 0.13	3.01 ± 0.50	0.07 ± 0.05	0.07 ± 0.03	0.07 ± 0.05
Phela + cyclosporine	**(n = 7)**	5.9 ± 1.0	0.97 ± 0.29	4.53 ± 0.97	0.15 ± 0.05	0.09 ± 0.02	0.11 ± 0.04
21 DAYS TREATMENT							
Influenza vaccine	**(n = 7)**	5.0 ± 0.8	0.66 ± 0.11	3.54 ± 1.48	0.11 ± 0.09	0.09 ± 0.04	0.09 ± 0.02

Abbreviations + reference values **WCC**: white cell count, Neutrophils (1.77 – 3.38**(x10^9/l)**), Lymphocytes (4.78 – 9.12**(x10^9/l)**), Eosinophils (0.03 – 0.08**(x10^9/l)**), Monocytes (0.01 – 0.04**(x10^9/l)**), Basophils (0.00 – 0.03**(x10^9/l)**)

Ref: Chapter 13, Liver functions test

Table E-3: Summary of liver function tests levels recorded as mean ± SD after 7 and 14 days' treatment.

GROUP		Liver function tests	
		ALT (U/L)	**ALK (U/L)**
7 DAYS TREATMENT			
Saline	**(n = 7)**	55 ± 4	258 ± 34
Influenza vaccine	**(n = 7)**	57 ± 6	248 ± 34
Cyclosporine	**(n = 3)**	48 ± 2	306 ± 65
Phela	**(n = 3)**	62 ± 15	276 ± 58
Phela + cyclosporine	**(n = 4)**	50 ± 8	209 ± 22
14 DAYS TREATMENT			
Saline	**(n = 7)**	59 ± 2	292 ± 25
Influenza vaccine	**(n = 7)**	61 ± 12	265 ± 14
Cyclosporine	**(n = 3)**	41 ± 9	234 ± 61
Phela	**(n = 7)**	56 ± 6	251 ± 43
Phela + cyclosporine	**(n = 7)**	54 ± 6	235 ± 39
21 DAYS TREATMENT			
Influenza vaccine	**(n = 7)**	57 ± 5	241 (195 – 261)

Abbreviations: **ALT**: alanine transaminase (17.5 – 30.2 U/L), **ALK**: alkaline phosphates (56.8 – 128 U/L)

Ref: Chapter 13, Renal functions test

Table E-4: Summary of Renal function tests levels recorded as mean ± SD after 7 and 14 days treatment.

GROUP		Renal function tests	
		CREATININE (µmol/L)	**UREA (mmol/L)**
7 DAYS TREATMENT			
Saline	**(n = 7)**	34 ± 13	6.9 ± 0.57
Influenza vaccine	**(n = 7)**	40 ± 7	7.1 ± 0.37
Cyclosporine	**(n = 3)**	29 ± 4	8.8 ± 0.55
Phela	**(n = 3)**	34 ± 10	7.6 ± 0.71
Phela + cyclosporine	**(n = 4)**	34 ± 6	6.9 ± 0.69
14 DAYS TREATMENT			
Saline	**(n = 7)**	26 ± 5	7.4 ± 0.56
Influenza vaccine	**(n = 7)**	32 ± 9	7.1 ± 0.51
Cyclosporine	**(n = 3)**	24 ± 12	7.4 ± 0.62
Phela	**(n = 7)**	32 ± 5	6.5 ± 0.37
Phela + cyclosporine	**(n = 7)**	33 ± 6	7.0 ± 0.69
21 DAYS TREATMENT			
Influenza vaccine	**(n = 7)**	37 ± 6	7.0 ± 0.78

Abbreviations: Creatinine (0.2 – 0.8 µmol/L l), urea (15 – 21 mmol/L)

Ref: Chapter 13, TH_1 cytokines levels

Table E-5: Summaries of TH_1 cytokines (IL-2, IFN-γ and TNF-α) levels recorded as mean ± SD (pg/ml)

	TH_1 CYTOKINES		
Drug administered	**IL-2**	**IFN-γ**	**TNF-α**
Untreated	122 ± 9	138 ± 8	103 ± 44
	7 DAYS TREATMENT		
Saline	291 ± 121	103 ± 411	226 ± 31
Influenza vaccine	301 ± 55	152 ± 53	226 ± 23
Cyclosporine	96 ± 50	148 ± 3	196 ± 176
Phela	248 ± 68	116 ± 20	208 ± 11
Phela + cyclosporine	348 ± 77	106 ± 33	233 ± 25
	14 DAYS TREATMENT		
Saline	273 ± 61	155 ± 26	231 ± 16
Influenza vaccine	230 ± 56	166 ± 85	216 ± 15
Cyclosporine	171 ± 49	145 ± 30	313 ± 280
Phela	243 ± 34	140 ± 40	211 ± 1
Phela + cyclosporine	316 ± 125	94 ± 47	245 ± 32
	21 DAYS TREATMENT		
Influenza vaccine	267 ± 48	87 ± 13	199 ± 18

Ref: Chapter 13, TH_2 cytokines levels

Table E-6: Summaries of TH_2 cytokines (IL-4 and IL-10) levels recorded as mean ± SD (pg/ml)

	TH_2 CYTOKINES	
Drug administered	**IL-4**	**IL-10**
Untreated	5.0 ± 0.5	278 ± 100
	7 DAYS TREATMENT	
Saline	4.9 ± 1.8	122 ± 9
Influenza vaccine	3.8 ± 1.5	358 ± 262
Cyclosporine	5.6 ± 1	1170 ± 652
Phela	15.9 ± 14.6	588 ± 590
Phela + cyclosporine	5.7 ± 6.4	448 ± 661
	14 DAYS TREATMENT	
Saline	7.7 ± 5.1	129 ± 17
Influenza vaccine	3.7 ± 1.2	263 ± 61
Cyclosporine	4.4 ± 0.4	290 ± 17
Phela	12.5 ± 12.9	395 ± 438
Phela + cyclosporine	16.5 ± 10	842 ± 546
	21 DAYS TREATMENT	
Influenza vaccine	2.8 ± 0.5	140 ± 42

PRELIMINARY RESEARCH OF PHELA, PRESENTED IN OTHER CONFERENCES

The Preclinical and Clinical Evaluation of a Traditional Medicinal Product, PHELA, an Immune Modulator used in Patients with HIV and AIDS

Motlalepula G. Matsabisa[1Ŧ], Osman Ebrahim[2], Ebrahim Yusoof[2], Mohammed Dhansay[1a], Jurgen Seier[1b], Monghezi Mdhluli[1b] Amaboo Dhai[4] and Andrew Walubo[3]

[1]Indigenous Knowledge Systems, Medical Research Council (MRC), [1a]Nutrition Intervention, Medical Research Council (MRC), [1b]Primate Unit, Medical Research Council (MRC), [2]Brenthurst Clinic [3]Department of Pharmacology, University of the Free State and [4]Bioethics Unit, WITS

[Ŧ] Corresponding author

There is increasing use of traditional medicines in the management of HIV and AIDS. The reasons for this are many, but are mainly because there is no cure for HIV and AIDS and there are no simple and satisfactory programmes for managing this pandemic. On the other hand, because most traditional medicines have been used for decades, they have been assumed to posses some form of safety. Unfortunately, their efficacy in serious diseases such as HIV/AIDS has not been evaluated in controlled clinical trials, and their concomitant use with prescription drugs has not been well-studied.

Here, a product called Phela[4], which is a combination of a number of medicinal plants and which has been widely used in African traditional medicines, has been evaluated for its possible toxicological effects, its safety in controlled randomised phase I clinical trials, and for any interaction with the cytochrome-P450 major drug metabolizing enzymes.

The presentation will highlight both preclinical and clinical methodologies developed to evaluate African medicinal products used in HIV and AIDS and present the outcomes of these studies. These results will enable us to develop controlled Phase II clinical studies for the evaluation of the efficacy of African traditional medicines used as immune boosters for HIV and AIDS patients.

[4] The product was initially submitted, under a contractual agreement, for evaluation by Traditional Doctor Anthony Imbwana and Dr Phumelele Yako. The name PHELA arbitrarily has been given for referral purposes only

PUBLICATIONS FROM THE THESIS

CONFERENCE ABSTRACTS

I. Presented

II. 'Makhotso Lekhooa, Andrew Walubo, Jan JB DU Plessis and Gilbert M Matsabisa. Fingerprinting of '*Phela',* a traditional medicine, in plasma by High Performance Liquid Chromatography (HPLC). The 2nd Annual Conference of the South African Society for Basic and Clinical Pharmacology, Potchefstroom, 23 -26th September 2008.

III. Accepted for presentation

IV. M.R Lekhooa, A. Walubo, JB du Plessis and M.G. Matsabisa. A Comprehensive Method for Identification of *'Phela'*, a Traditional medicine herbal Medicine, Using Different Chromatographic Techniques. The 16th World Congress Of Basic and Clinical Pharmacologists (Worldpharma 2010). Copenhagen, Denmark, 17 – 23 July 2010

V. M.R Lekhooa, A. Walubo, JB du Plessis and M.G. Matsabisa. The Use of Metabolite Peakokinetics to monitor *'Phela'*, a Herbal Medicine, in Plasma after Oral Administration in Rat model. The 16th World Congress Of Basic and Clinical Pharmacologists (Worldpharma 2010). Copenhagen, Denmark, 17 – 23 July 2010

VI. M.R Lekhooa, A. Walubo, JB du Plessis and M.G. Matsabisa. The effect of *'Phela'*, a traditional medicine extract, on interleukin-2 in a rat model. The 16th World Congress Of Basic and Clinical Pharmacologists (Worldpharma 2010). Copenhagen, Denmark, 17 – 23 July 2010

Fingerprinting of Phela, a traditional medicine, in plasma by High Perfomance Liquid Chromatography (HPLC)

'Makhotso Lekhooa*, Andrew Walubo, Jan JB Du Plessis and Gilbert M. Matsabisa#

University of the Free State, Department of Pharmacology and #The Indigenous Knowledge System Program, Medical Research Council, Cape Town.

*Correspondence: LekhooaMR.md@ufs.ac.za

Purpose: *Phela* is a traditional medicine, prepared using well defined parts of four African medicinal plants. In a phase 1 clinical study [PLWA] of *Phela* as an immune booster in HIV positive and AIDS patients, *Phela* led to an increase in appetite, weight gain and 200 % increase in CD 4 cell count with an overall increase in quality of life from as low as 30 % to 100 %. Currently *Phela* is being studied in rat model to understand its mechanism of action. For the purpose of this study a chromatographic fingerprinting method was developed to monitor *Phela* in plasma.

Methods: Preliminary extractions and HPLC runs of standard solutions of *Phela* led to identification a marker peak by which to analyze the product.

Sample Preparation: To 1ml of plasma, IS was added after which it was acidified with 0.5M HCL and extracted with 5ml of hexane. The organic phase was evaporated to dryness under nitrogen and the residue was reconstituted with 100µl of mobile phase of which 50µl were injected in the HPLC.

HPLC Analysis: The sample was analyzed on HP 1100 series with an isocratic pump and a UV detector set at 245nm. Separation was done on C_{18} column (250mm x 4.6 mm x 5microns) coupled to a C_{18} guard column. The mobile phase was Acetonitrile: water (70/30: v/v), and the rate was 0.5ml/min with a runtime of 30 minutes.

Method Validation: Calibration curves (2 mg/ml – 10 mg/ml) were run over 5 days and the linear regression and correlation coefficient (r) were calculated using GraphPad® statistical program. Accuracy was tested a**t** 2, 6 and 10 mg/ml, while stability was tested at room temperature, 4°C and -20°C at 24 hours, 48 hours and 1week.

Results: A marker peak for *Phela* was identified at a retention time of 25 minutes at which there was no interference in plasma. The average 5 days calibration curve was linear (y = 0.02x – 0.59; r = 0.9983) with a CV% of ±20%, while accuracy at 2, 6, 10 mg/ml was 103%, 85% and 102%, respectively. Stability was at -20°C was 97%, 77% and 124% at 24 hrs, 48 hrs and 1 week, respectively. The method was used to monitor *Phela* in rat plasma. In conclusion, an HPLC method for fingerprinting of *Phela* and monitoring its time profile in plasma was successfully developed.

A Comprehensive Method for Identification of 'Phela', a traditional Herbal medicine using different Chromatographic Techniques

M.R. Lekhooa, A. Walubo, JB du Plessis and *M.G. Matsabisa

Department of Pharmacology, University of the Free State, Bloemfontein, South Africa, and *Indigenous Knowledge Systems Lead Programme, Medical Research Council, Cape Town, South Africa.

Phela is a herbal mixture of four African traditional medicinal plants that has been used for decades in wasting conditions and, after successful observation studies in humans, is now being developed by the Medical Research Council as an immune booster for patients with compromised immune system. Therefore, a chromatographic fingerprint of Phela was needed for quality control purposes. Here, a comprehensive method for fingerprinting of *Phela* is described. It involved extraction of the product by either acidic extraction or a simple 'salting-out' method, followed by Thin Layer Chromatography (TLC) and/or preparative Column Chromatography (CC) that were supported by High Performance Liquid Chromatography with UV-detector (HPLC-UV), HPLC with fluorescence-detector (HPLC-FL), HPLC with Photo Diode Array detector (HPLC-PDA) and Gas-Chromatography-Mass Selective Detector (GC-MSD) spectrometry. An internal standard was used where appropriate. Peak area and retention times were used for validation of the methods and fingerprints, respectively. The fingerprints were successfully used to differentiate Phela from another herbal product made from Hypericum perforatum (St. John's Wort) thereby illustrating their high potential for use in differentiating *Phela* from other herbal medicines. By generating fingerprints of phela using different chromatographic techniques based on a standardized extraction process, this approach provides a variety of methods from which laboratories can select and use according to availability of expertise and/or instruments and cost-benefit analysis. Furthermore, it will enable wide application in quality control of the product using validated methods, thereby promoting effective monitoring of the finished product in all countries that will use it.

The Use of Metabolite Peakokinetics to Monitor '*Phela*', a Herbal Medicine, in Plasma after Oral Administration in a Rat Model

M.R. Lekhooa, A. Walubo, JB du Plessis and *M.G. Matsabisa

Department of Pharmacology, University of the Free State, Bloemfontein, South Africa, and *Indigenous Knowledge Systems Lead Programme, Medical Research Council, Cape Town, South Africa.

Phela is a herbal traditional medicine that is under development for use as an immune booster in immune compromised individuals. Therefore, before major *in vivo* investigations could be done, there was a need to establish a plasma marker for concentration monitoring of Phela. Chromatographic separation was achieved on a reverse phase column with 70 % acetonitrile at 0.5 ml/min.. Emission and excitation wavelengths were 210 and 290 nm, respectively. Sprague Dawley rats were used and approval from the animal ethics committee was obtained. Three groups of 5 rats each were administered with *Phela* (15.4 mg/kg) and 1 rat from each group was sacrificed at 1, 2, 4, 6 and 8 hours. On the HPLC analysis, a new peak in samples from treated animals was observed at 9.2 minutes, which implied that it was a metabolite of Phela. Plasma concentration was expressed as peak area per unit plasma volume (PK-area/L) and this was used to derive the relevant pharmacokinetic parameters. The metabolite's half-life was 3.47±0.35 hours and reached maximum concentration at 4.67±1.15 hours. It was estimated that the concentration at steady state (Css) would be 47.52 ± 5.94 PK-area/L with no drug accumulation (Acc index =.009 ± 0.004) on chronic dosing. The use of peak area per unit volume to derive pharmacokinetics of unknown compounds (peakokinetics) as well as to confirm ingestion of *Phela* were demonstrated with a hope that they may appeal to those experiencing similar problems with monitoring of herbal products of which little is known.

The effect of Phela, a traditional medicine extract, on interleukin-2 in a rat model

M.R. Lekhooa, A. Walubo, JB du Plessis and *M.G. Matsabisa

Department of Pharmacology, University of the Free State, Bloemfontein, South Africa, and *Indigenous Knowledge Systems Lead Programme, Medical Research Council, Cape Town, South Africa.

Phela is a traditional medicine extract that is being investigated for immune boosting properties in patients infected with the human immune deficiency virus (HIV). Therefore, the aim of this study was to determine the effect of *Phela* on interleukin 2 (IL-2), a major stimulant of cell mediated immunity, in a rat model. Sprague Dawley rats were used and approval from the animal ethics committee was obtained. Three groups of 14 rats each were treated daily with either normal-saline (control), Phela-only (test-group), *Phela*+cyclosporine-A *(Phela*-CycA: negative control), and a fourth group was inoculated once with influenza vaccine (positive control). Thereafter, 7 animals from each group were sacrificed after 7 and 14 days of treatment. Serum IL-2 was measured by ELISA. On days 7 and 14, IL-2 concentrations (pg/ml: median [range]) in the control, (232 [167-460] and 274 [215-328]) and *Phela*-only treated group (233 [174-355] and 224 [217-295]) were similar. However, although not statistically significant, IL-2 levels (pg/ml: median[range]) were higher in the Phela CycA treated group (325 [268-433] and 280 [206-518]) compared to the Phela-only-treated group. The response to influenza stimuli was moderate at 7 days (294 [245-373]) and this returned to 'normal' by 14 days (214 [183-309]. These results show that *Phela* did not stimulate IL-2 of the normal immune system but stimulated it when the immune system was suppressed by cyclosporine-A. This implies that *Phela* may stimulate IL-2 in patients with a compromised immune system such as in HIV positive patients. In conclusion, *Phela* is an immune stimulant.

PUBLICATIONS FROM THE THESIS

I. MANUSCRIPTS IN PREPARATION

II. Lekhooa M.R., Walubo A., du Plessis J.B.D and Matsabisa M.G: A comprehensive method for identification of *Phela,* traditional herbal medicine, using different chromatographic techniques. J. Chromatography A. Submitted Feb 2010.

III. Lekhooa M.R., Walubo A., du Plessis J.B.D and Matsabisa M.G: The use of metabolite peakokinetics to monitor *Phela* a herbal medicine, in plasma after oral administration in a rat model. J. Therapeutic Drug Monitoring. Submitted Feb 2010.

IV. Lekhooa M.R., Walubo A., du Plessis J.B.D and Matsabisa M.G: *Phela* stimulated the immune system during cyclosporine-A antagonism. J. Phytotherapy Research. Submitted Feb 2010.

SUMMARY

KEY: *Phela, chromatographic-fingerprinting, Pharmacokinetics, immune-stimulant, quality-control, Traditional-herbal-medicine-products, peak-kinetics, cyclosporine-A-antagonist, cytokines.*

Phela is a herbal traditional medicine product prepared using well defined parts of four African medicinal plants. The aim of the study was to determine the mechanism of action by which phela boosted the immune system of a rat model.

Unfortunately, subsequent literature research revealed that very little is known about phela. As such chromatographic methods were developed by which to identify phela and for quality control purposes. Of note, the developed methods complemented each other; they were compiled into a comprehensive method for phela fingerprinting. It involved sample extraction of the phela by either acidic extraction or a simple "salting-out" method, followed by Thin Layer Chromatography (TLC), and/or preparative Column Chromatography (CC) that were supported by High Performance Liquid Chromatography with UV-detector (HPLC_UV), HPLC with fluorescence detector (HPLC_FL), HPLC with photo diode array detector (HPLC_PDA) and Gas Chromatography-Mass selective detector (GC_MSD) spectrometry. The method was successfully used to differentiate phela from another herbal product made from *Hypericum perforatum* (St John's wort) illustrating its high potential for application to other herbal medicines in development.

Thereafter a HPLC_UV method for monitoring phela in plasma was developed and validated. However, due to its pitfalls, a HPLC_FL method was developed as well. The HPLC_UV method had a linear regression equation of $y = 0.02x – 0.59$, correlation coefficient of $r^2 = 0.9983$ and accuracy within 15 %. For HPLC_FL three marker peaks were selected and standardized by the retention time. The developed HPLC_UV and HPLC_FL methods were tested by analysing blood collected from rats treated with phela after 1, 2, 4, 6 and 8 hours respectively. Unfortunately, analysis by HPLC_UV

method had no peaks whereas, during HPLC_FL method analysis, a new peak was observed at 9.2 minutes.

The peak was depicted as a metabolite for phela and was then utilized as the plasma marker. Since the metabolite is unknown and therefore no standard exists by which to express its plasma concentration in conventional units, the changes in peak area per unit volume (L) of plasma (peak-area/L) were used to derive the appropriate pharmacokinetic parameters hence peak-kinetics. The predictive parameters show that concentration of the metabolite at steady state would be 48 peak-area/ml, hence no accumulation of the drug.

The final part of the study was undertaken to understand the mechanism of action of phela using a rat model by observing for changes in the levels of TH_1 and TH_2 cytokines. Rats were divided into six groups. The first group was not treated with anything. The four groups were treated daily with either normal-saline, orally (control); cyclosporine in olive oil, subcutaneously (negative control); Phela, orally (test-group 1) and phela+cyclosporine (test-group 2). The last group was inoculated once with influenza vaccine. The treatment period was 14 days and in each group rats were sacrificed after 7 and 14 days. Haematology tests, biochemistry tests, and cyclosporine level analysis were done. Serum cytokine analysis for TH_1 (IL-2, IFN-γ and TNF-α) and TH_2 (IL-4 and IL-10) cytokines was measured by ELISA.

On days 7 and 14, the concentrations of TH_1 cytokines in the Phela-only treated group were similar to the control. However, the TH_1 cytokines were higher in the Phela+cyclosporine-A treated group than in the cyclosporine-A group, and cyclosporine-A concentrations were similar in both groups. These results show that Phela did not affect TH_1 cytokines of a normal immune system but stimulated them when the immune system was suppressed by cyclosporine-A. This implies that Phela is a cyclosporine-A antagonist that may stimulate a compromised immune system. In conclusion, Phela is an immune stimulant.

OPSOMMING

SLEUTEL: *Phela, chromatografiese vingerafdruk, farmakokinetika, immuunstimulant, kwaliteitsbeheer, Tradisionele-kruie-medisinale-produkte, piek-kinetika, siklosporien-A-antagonis, sitokiene.*

Phela is ‘n kruie tradisionele medisinale produk voorberei uit goed-gedefineerde dele van vier Afrika medisinale plante. Die doel van die studie was om die meganisme van werking te bepaal waardeur phela die immuunsisteem versterk in ‘n rotmodel.

Ongelukkig toon ‘n literatuur soektog dat daar baie min bekend is omtrent phela. As sulks is chromatografiese metodes ontwikkel vir die identifikasie van phela en vir kwaliteitsbeheer-doeleindes. Van belang is die feit dat die ontwikkelde metodes mekaar komplementeer; hulle is saamgevoeg in ‘n omvattende metode vir die bepaling van ‘n phela vingerafdruk. Dit behels monster ekstraksie van phela deur middel van ‘n suurekstraksie of ‘n eenvoudige “uit-soutings” metode, gevolg deur Dunlaag Chromatografie (TLC), en/of voorbereidende kolom chromatografie (CC) ondersteun deur Hoë verrigting Vloeistof Chromatografie met UV-detektor (HPLC_UV); HPLC met fluoresensie detektor (HPLC_FL); HPLC met foto-diode-rangskikking detektor (HPLC_PDA), en Gas Chromatografie-Massa selektiewe detektor (GC_MSD) spektrometrie. Die metode is suksesvol gebruik om phela te onderskei van ‘n ander kruieproduk gemaak van *Hypericum perforatum* (St John’s wort). Dit illustreer die hoë potensiaal vir applikasie in ander kruiemedisynes in ontwikkeling.

Daarna is ‘n HPLC_UV metode ontwikkel en gevalideer vir die monitering van phela in plasma. Weens die inherente probleme van die metode is ‘n HPLC_FL metode ook ontwikkel. Die HPLC_UV metode het ‘n liniêre regressievergelyking van $y = 0.02x – 0.59$, korrelasiekoëffisiënt van $r^2 = 0.9983$ en akkuraatheid binne 15 %. Vir HPLC_FL is drie merker pieke geselekteer en gestandardiseer volgens die retensietyd. Die ontwikkelde HPLC_UV en HPLC_FL metodes is getoets deur die analise van bloed versamel vanaf rotte behandel met phela na onderskeidelik 1, 2, 4, 6 en 8 ure.

Ongelukkig was daar geen pieke tydens analise met die HPLC_UV metode nie, terwyl ‘n nuwe piek waargeneem is teen 9.2 minute met die HPLC_FL metode.

Die piek is geïnterpreteer as ‘n metaboliet vir phela en is toe gebruik as die plasmamerker. Aangesien die metaboliet onbekend is en daar dus geen standaard is waarvolgens die plasma konsentrasie in konvensionele eenhede uitgedruk kan word nie, is die veranderinge in piekarea per eenheid volume (L) van plasma (piek-area/L) gebruik om die toepaslike farmakokinetiese parameters te bepaal, dus piek-kinetika. Die voorspellende parameters wys dat die konsentrasie van die metaboliet by gelykvlak 48 piek-area/ml behoort te wees en dat daar, dus geen ophoping van die middle is nie.

Die finale deel van die studie is onderneem om die meganisme van werking van phela te verstaan. ‘n Rotmodel is gebruik om die verandering in die vlakke van TH_1 en TH_2 sitokiene waar te neem. Die rotte is in ses groepe verdeel. Die eerste groep is nie behandel nie. Vier groepe is daagliks behandel met òf normale soutoplossing, oraal (kontrole); siklosporien in olyfolie subkutaan (negatiewe kontrole), Phela, oraal (toetsgroep 1) en phela+siklosporien-A (toetsgroep 2). Die laaste groep is een maal ingeënt met influenza vaksien. Die behandelingsperiode was 14 dae en in elke groep is rotte geslag na 7 en na 14 dae. Hematologie-toetse, biochemie-toetse, en siklosporienvlak-bepalings is gedoen. Serum sitokien analise vir TH_1 (IL-2, IFN-γ en TNF-α) en TH_2 (IL-4 en IL-10) sitokiene is gemeet met ELISA.

Op dag 7 en dag 14 was die konsentrasies van TH_1 sitokiene in die groep war slegs met Phela behandel is soortgelyk aan die kontrole. Die TH_1 sitokiene was egter hoër in die Phela+siklosporien-A behandelde groep as in met die siklosporien-A groep, en siklosporien-A konsentrasies was soortgelyk in beide groepe. Hierdie resultate wys dat phela nie TH_1 sitokiene in ‘n normale immuunsisteem affekteer nie, maar die immuunsisteem stimuleer wanneer dit onderdruk word deur siklosporien-A. Dit impliseer dat phela ‘n siklosporien-A antagonis is wat ‘n ingekorte immuunsisteem stimuleer. Ter opsomming kan dus aanvaar word dat phela ‘n immuunstimulant is.