DEVELOPMENT OF A MODEL TO CHARACTERIZE THE EFFECT OF *PHELA* ON SELECTED IMMUNE MARKERS IN IMMUNE-SUPPRESSED RATS

By

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ABSTRACT

The therapeutic potential of several plant species and necessity for scientific validation of the use of plant derived medicines has prompted interest in field of traditional medicines. According to the WHO, in Africa alone, up to 80% of the population use herbal medicines to meet their primary health care needs and most of them have not been scientifically tested. Understanding the mechanism of action of herbal medicines is necessary for their proper use with regard to indications and limitations. One of South African traditional herbal medicines, *Phela* is currently being developed for use in immune compromised patients; hence there is a need to establish its mechanism of immunomodulation.

Unfortunately, there is no appropriate animal model for the testing of immune-boosters. The current models involve either *in vitro* or *ex vivo* models. Furthermore, an ideal model would be a disease specific model, but this would not tell much about the mechanism of action, and would call for testing of every product in each disease model. As such, based on the understanding of the model of immune response in particular diseases, an *in vivo* model in which the cell mediated, humoral or non-specific immune response can be studied is more appropriate. Hence an animal model by which to evaluate purported immune boosters and traditional medicine to understand their mechanism of action on the immune system is essential. Here, it was proposed to undertake a study to develop a rat model by which to characterize the effect of *Phela* on selected immune markers in immune-suppressed rats. The above mentioned aim was achieved through six objectives outlined below.

Firstly, an HPLC method with two detectors was applied to ensure consistency of all batches of *Phela* that were used throughout the study before undertaking an *in vivo* study. Two mark peaks were observed after analysis of *Phela* by HPLC-DAD. *Phela* fingerprint was confirmed by comparing the current results from both methods with those obtained previously. Secondly, an HPLC-UV assay was developed, validated and applied for the simultaneous determination of cyclophosphamide and dexamethasone concentration in rat plasma. The retention time was at 4.2, 5.7 and 8.1 minutes for cyclophosphamide, dexamethasone and internal standard, respectively. The method was linear with regression and correlation coefficients of y =

0.04x+0.11 and 0.999 for cyclophosphamide, and y = 0.32x–1.52 and 0.998 for dexamethasone, and their respective recoveries of 102 – 108 % and 99 – 107 %. The drugs were stable at -20 °C up to a month. Thereafter, the slide-a-lyzer technique was used to rule out potential interactions of *Phela* with the immunesuppresants [cyclosporine, cyclophosphamide and dexamethasone] before co-administration in rats experiment. Despite wide variations, the results indicated that there was no significant difference between the free fractions of drug-only group when compared with drug+*Phela* group. As thus, the above mentioned drugs could be co-administered with *Phela* without interference.

In order to develop an animal model, three rat experiments were undertaken. For the first experiment, rats were treated with three escalading doses of *Phela* for three weeks, along with levamisole a known immune stimulant and a control group. Five rats were sacrificed once weekly per group. Physiological function tests and immune markers (CD_4, CD_8, IgG, IgM, IL-2 and IL-10) concentration was determined. *Phela* caused increase in white cell count, which correlates with elevated lymphocyte sub-sets (i.e. CD_4 and CD_8 count) after treatment with all three doses and this observation peaked by day 14 of treatment. Moreover, *Phela* led to ample stimulation of the immune system as indicated by increased CD_4 cell count and IL-2 at doses of 5 and 15.4 mg/kg. This selective effect implies that *Phela* can be indicated in diseases that interfere with CD_4 and IL-2 count, but this needed to be confirmed in a diseased model. The 15.4 mg/kg dose was selected to be used in subsequent studies.

For the second experiment, the aim was to determine the optimum dose and time it takes to achieve optimum immune suppression by known immune suppressants; cyclosporine, cyclophosphamide and dexamethasone. Different groups of twelve rats each were treated with cyclosporine, cyclophosphamide and dexamethasone only, along with a control group in each case. Physiological and immune tests described in the first experiment were also done. As expected, the animals exhibited abnormal physiological function tests in association with progressive immunesuppression. Cyclosporine inhibited the cell mediated immunity, while cyclophosphamide suppressed the humoral immunity and the suppressive effect of dexamethasone was multi-systemic. In all cases, the immunesuppression continued up to the end of the study period. The optimum dose and time of each drug was established. This

implies that a rat model of drug induced immune suppression was successfully developed. This rat model was to be validated when the immune suppressed rat model was co-administered with a test drug, in this case *Phela,* to understand its mechanism of immune modulation which is described in the experiment that follows.

The aim of the third experiment was to apply the rat model to establish the mechanism of action of a purported immune booster *Phela* on the immune system. Different groups of fifteen rats each were pre-treated with cyclosporine, cyclophosphamide or dexamethasone only to induce immunesuppression. Thereafter, the control-groups continued on the immunesuppresant only and the test groups we co-treated with an immunesuppresants (CsA/CP/Dex) and *Phela* for 21 days. Tests described in the first experiment were similarly done. *Phela* stopped the progression of immunesuppression in rats treated with cyclosporine as indicated by the reversal and/or resistance to CsA induced changes in the WCC, neutrophils, lymphocytes, CD_4, CD_8 cells and IL-2 count. Furthermore, *Phela* prevented progression of CP-induced body and thymus weight loss, suppression of IgG and IgM, and minimal effect on CD_4 and CD_8 cell count. Observations from the results indicated that the mechanism of immunomodulation of Phela in rats is cell mediated. Therefore, *Phela* would be candidate for testing against diseases or disorders associated with suppressed CMI, such as HIV/AIDS and Tuberculosis.

In conclusion, a rat immunesuppression model has been successfully developed and applied to establish the mechanism of immunomodulation of *Phela* in rats. Characterizing the mechanism of *Phela* in rats has indicated the scope of its application for use during diseases with a loss of cell mediated immunity. This model is a necessity in South Africa and across the world at large where many traditional herbal medicines and their products are purported as immune stimulants but lack proof of indication and a scope of application. Furthermore, this model is a tool and/approach that can be used to scientifically validate any immune stimulant and/or traditional medicine to establish its mechanism of action on the immune system, describing its limitations and contra-indications thereof. Lastly, the rat model was applied using *Phela* a known South African immune stimulant to establish its mechanism of.

DEDICATION

This work is dedicated to my mother

MALEKHOOA G. LEKHOOA

Your love, support, guidance and never-ending faith in me

You are my pinnacle of strength

For a gracious and loving heart

I am forever grateful

DECLARATION OF INDEPENDENT WORK

I, '*Makhotso Rose Lekhooa* declare that the doctoral research thesis or publishable, interrelated articles for the PHILOSOPHIAE DOCTOR in Pharmacology that I herewith submit at the University of the Free State, is my independent work and that I have not previously submitted it for a qualification at another institution of higher education.

I, '*Makhotso Rose Lekhooa* hereby declares that I am aware that the copyright is vested in the University of the Free State.

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SUPERVISOR'S DECLARATION

I, Prof. A. Walubo, the promoter of this thesis entitled: ***development of a model to characterize the effect of Phela on selected immune markers in immune-suppressed rats*** hereby certify that the work in this project was done by 'Makhotso Rose Lekhooa at the department of Pharmacology, University of the Free State.

I hereby declare that submission of this thesis and also affirm that it has not been submitted previously to this or any other institution for admission to a degree or any other qualification.

_____ _____

Signature Date

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5. SIMULTANEOUS DETERMINATION OF CYCLOPHOSPHAMIDE AND DEXAMETHASONE PLASMA CONCENTRATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH UV WAVELENGTH SWITCHING

LIST OF ABBREVIATIONS

%	Percentage
µg	Micrograms
µm	Micrometers
ADME	Absorption, distribution, metabolism and elimination
AIDS	Acquired Immuno Deficiency Syndrome
ALP	Alkaline phosphatase
ALT	Alanine transaminase
APC	Antigen-Presenting Cells
ARV	Anti-Retroviral
AST	Aspartate transaminase
ATM	African traditional medicines
AUC	Area under the curve
BUN	Blood urea nitrogen
CC	Column chromatography
CD_4	Helper T-cells
CD_8	Cytotoxic Killer T-cell
Cm	Centimeters
CMI	Cell-Mediated Immunity
CMIA	Chemiluminescent micro particle Immuno assay
Conc	Concentration
CP	Cyclophosphamide
CsA	Cyclosporine
CV %	Coefficient of Variance percentage
CYP	Cytochrome P
DAD	Diode array detector
DC	Dendtric Cells
Dex	Dexamethasone
DIPN	Diisopropylnaphtalene
DNA	Deoxyribonucleic acid

EDTA	Ethylene diaminete traacetic acid
ELISA	Enzyme linked immunoassay
EPA	Environmental protection agency
ET	Equilibrium time
FBC	Full blood count
FDA	Food and drug administration
FI	Fusion inhibitors
FLD	Fluorescence detector
FPIA	Fluorescent Polarization Immuno-Assay
GC-MSD	Gas Chromatography Mass Selective Detector
GMP	Good Manufacturing Practice
Gp	Glycol-Protein
HAART	Highly Active Anti-Retrovial therapy
HCl	Hydrochloric acid
HIR	Humoral Immune Response
HIV	Human Immunodeficiency Virus
HP	Hewlett Packard
HPLC	High performance Liquid chromatography
HPLC-DAD detector	High Performance Liquid Chromatography with a diode-array
HPLC-FLD detector	High Performance Liquid Chromatography with a fluorescence
HPLC-UV	High Performance Liquid Chromatography with a UV detector
HRP	Horseradish peroxidase
ICH	International conference on harmonization
IFN-γ	Interferon Gamma
Ig	Immunoglobin
IgA	Immunoglobin A
IgD	Immunoglobin D
IgE	Immunoglobin E
IgG	Immunoglobin G

IgM	Immunoglobin M
IKS	Indigenous Knowledge System
IL-2	Interleukin 2
IL- 4	Interleukin 4
IL-10	Interleukin 10
IS	Internal Standard
LC	Liquid chromatography
LEV	Levamisole
LFT	Liver function tests
LOQ	Limit of quantification
Mab	Monoclonal antibody
Mg	Milligrams
Ml	Millimeters
MP	Mobile phase
MRC	Medical research council
MS	Mass spectrometry
Mtb	Mycobacterium tuberculosis
MWCO	Molecular weight cut-off
NaOH	Sodium Hydroxide
NEPAD	New partnership for Africa's development
NFatc	Nuclear factor of activated T-cell
NGO	Non-Government Organization
NHLS	National health laboratory services
NK	Natural Killer Cells
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
NO	Nitric Oxide
NRTI	Nucleoside Analogue Reverse Transcriptase Inhibitor
NtRTI	Nucleotide Reverse Transcriptase Inhibitor
PCP	Pneucystis carinin Pneunomia
PDA	Photo diode array
Pg	Picograms

PHL	*Phela*
PI	Protease Inhibitor
R^2	Correlation coefficient
RCC	Red cell count
RFT	Renal function tests
RNA	Ribonucleic Acid
RT	Retention Time
SADC	Southern African development community
SAL	Saline
SD	Sprague Dawley
Sd	Standard Deviation
SJW	St John's wort
TB	Tuberculosis
TCR	T-cell receptor
TGFβ	Transforming Growth Factor Beta
TH	T-helper
TH_1	T-helper cells 1
TH_2	T-helper cells 2
THM	Traditional Herbal Medicines
TLC	Thin Layer Chromatography
TM	Traditional medicines
TNF-α	Tumor Necrosis Factor Alpha
UFS	University of the Free State
UNAIDS	United Nations Acquired Immuno Deficiency Syndrome
Untx	Untreated
USA	United States of America
UV	Ultra Violet
WCC	White cell count
WHO	World Health Organization

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1.

GENERAL INTRODUCTION

A growing interest to search for naturally occurring products that have been used traditionally is getting worldwide attention. According to Alamgir (2010), immunomodulation using herbal medicines can provide an alternative therapy to conventional chemotherapy for a variety of diseases, especially when the host defense mechanism has to be activated under impaired immune response. However, a gap remains from traditional usage to potential clinical use, owing to insufficient information on efficacy, specifically, the lack of evidence of action against the purported indications and scope of application. Characteristically, most immune stimulants are tried on every illness without scientific basis.

It was envisaged here that understanding the mechanism of action of immune stimulants will enable determination of the most appropriate indications for each product, appropriate time or stage of intervention, and to set specific parameters by which to monitor the response. Huang (2002) states that immunostimulatory effects of a drug, nutritional supplements and/or traditional medicine are difficult to evaluate in healthy people or animals. Specifically, because the response of the immune system from infection is either by cell mediated humoral or non-specific immune response.

Unfortunately, there is no test or animal model by which to determine all these prepositions and the testing of drugs for immune-modulation is not standardized. Current immunology tests do not predict clinical response, while disease specific animal models are not available or easy to develop. On the same note, observational studies lack independent variable which can create a bias and/or mask cause and effect relationships or alternatively suggest correlations where there is none. Even then, for products that have been tested in the clinical studies, the difficulties in standardizing (or defining) the immune status (or stage of the disease) at which the product is effective, have made these clinical evaluations inconclusive. Furthermore, with the knowledge

that the most appropriate immune booster should not affect a normal immune system, immunology tests using normal physiological systems (cells or animals) would also not be appropriate.

In the same perspective, both *in vitro* and *ex vivo* tests using isolated systems or cells would also not be appropriate because the immune system is a complex system that exhibits activation, inhibition, or regulatory responses which does not happen in isolates systems/cells. Also, because of their crude nature and plurality effect on the immune system, testing of herbal medicines for immune modulation cannot be done on isolated systems. Therefore, understanding which of these responses' area affected by a new drug/product and/or traditional herbal medicines is vital to revealing the mechanism of action but most important, the best indication for the use of the drug.

An animal model would not be complete without application, as such for evaluation; it would be a traditional herbal medicine that has immune boosting properties, yet unknown mechanism of action on the immune system. For centuries *Phela* has been used in sub-Saharan Africa in wasting conditions to strengthen and alleviate symptoms of patients with wasting conditions as per anecdotal reports. During the previous decade *Phela* has been studied in order to scientifically validate using both *in vitro* and *in vivo* techniques.

It has been proven that the extracts of *Phela* did not influence CYP450 activity or expression in the liver, hence they should be considered as safe to use with drugs that are metabolized by the CYP450 isoforms (Walubo *et al.*, 2007). Furthermore, *Phela* was not associated with toxicity after it was orally given (up to 12x the recommended dose) to ververt monkeys. Results from our previous studies have indicated that *Phela* stimulates or restore Cyclosporine induced immune suppression indicating possible IL-2 activation. Hence *Phela* is an ideal candidate to evaluate the animal model to be developed. Even though research reports indicate the safety of *Phela*, its mechanism of action remains unknown.

Since the first cases of acquired immunodeficiency syndrome (AIDS) were reported in 1981, the infection has grown to pandemic proportions, resulting in an estimated 78 million infections and 39 million deaths globally. In 2013, about 35 million people were living with HIV, of whom 2.1 million more people were newly infected with HIV and 1.5 million died of AIDS (UNAIDS, 2014). Although HIV and AIDS infections 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 2013, there were 6.3 million people living with HIV in South Africa (UNAIDS, 2014).

During HIV infection, the immune suppression sets in over a cause of time and activates a cascade of events that leads to an incompetent immune system to fight infections. Some of the causes include but not limited to, HIV induced T-lymphocyte loss and dysfunction, altered cytokine network proliferation and a shift from a TH_1 immune response towards a TH_2 humoral immunity which is associated with exacerbation of HIV infection contributing to the progression to AIDS (Clerici and Shearer, 1994; Reuter, 2012; Macallan, 2013).

A search for an immune stimulant that can modulate the immune system to prevent disease progression during HIV infection is on-going. 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 and improving the quality of life of these patients (Vicenzi and Biswas, 1997). Currently, *Phela* is being developed for patients with a compromised immune system (i.e. HIV positive patients) but its mechanism of action is unknown. Unfortunately, there is no test or animal model by which to determine immune boosting prepositions and standardize the testing of immune stimulants such as *Phela* for immune-modulation.

Therefore, a living system or animal model remains the best test-system for immune modulation testing. Furthermore, an ideal model would be a disease specific model, but this would not tell much about the mechanism of action, and would call for testing of every product in each disease model. As such, based on the understanding of the model of immune response in particular diseases, an *in vivo* model in which the cell mediated, humoral or non-specific immune response can be studied is more appropriate. Hence, a necessity to develop a rat model by which to characterise the mechanism of action of purported immune boosters of herbal origin.

The scope of the thesis:

Chapter 1 is the general introduction, while the literature review is presented in chapter 2. It is extensively covered in seven subdivisions labeled as part I to VI. Part I, is about traditional herbal medicines and *Phela*, Part II is the pharmacology of all drugs used in the animal experiment, Part III is an overview on immunology and the relationship of the $TH_{1/2}$ paradigm with onset of some disease, Part IV is a brief study of the immunopathology of HIV/AIDS, Part V is the challenges with lack of animal models, Part VI elaborates on the problem statement and Part VII is a review of analytical methods necessary for this study. Furthermore, Chapter 3 is a summary of observations from the review and study aims and objectives.

The experimental chapters comprise of *in vitro* studies (chapter 4, 5 and 6) and *in vivo* studies (chapter 7, 8 and 9). Fingerprinting batches of *Phela* was covered in chapter 4; thereafter a High Performance Liquid Chromatography with UV detector for the detection of cyclophosphamide and dexamethasone in plasma is in chapter 5 is explained. Chapter 6 is a study of possible drug interactions of *Phela* with the immune suppressants using a dialysis equalizer technique. The dose of *Phela* for immunomodulation in healthy rats was established in chapter 7. A rat model of induced immuno suppression was established in chapter 8. Thereafter, the mechanism of immunomodulation of *Phela* was established in chapter 9. Lastly, chapter 10 draws the conclusion and suggests themes for future studies.

2.

LITERATURE REVIEW

2.1. AN OVERVIEW ON HERBAL MEDICINES AND PHELA A TRADITIONAL HERBAL MEDICINE

2.1.1. AFRICAN HERBAL MEDICINES BACKGROUND

Phenomenal interest is growing indicating traditional medicines (African, ayurveda, Japanese, Chinese etc.) as a possible solution for healthcare challenges. According to the WHO traditional medicine strategy 2014 – 2023, herbal medicines are widely used and are of rapidly growing health system, economic importance and they stand out as a way of coping with relentless rise of chronic non-communicable diseases.

In Africa alone, up to 80 % of the population uses herbal medicines to help meet their primary health care needs, while in China they account for around 40 % of all health care delivered. According to the draft policy on African traditional medicine (ATM) for South Africa, there is a reality that the majority of South Africans still uses and continues to use ATM for their primary healthcare needs. The term traditional medicines is used interchangeably with herbal medicines, which include herbs, herbal materials, herbal preparations and finished herbal products that contain parts of plants or other plant materials as active ingredients (WHO traditional medicine strategy, 2002 – 2005).

Africa is endowed with many plants that can be used for medicinal purposes to which they have taken full advantage. In fact, out of the approximated 6400 plant species used in tropical Africa, more than 4000 are used as medicinal plants. In South Africa, approximately 3000 plant species are used as medicines, of which as many as 700 species are traded in large quantities of informal medicinal plant markets (Abdillahi *et al*., 2009; Street *et al*., 2013). According to the South African draft policy (2008) marginalization of ATM there is a dearth of research on the subject, with only 25 of the 3000 South African plants fully biomedically characterized in terms of their medicinal properties of which *Phela* is one of them.

2.1.2. *PHELA* AN IMMUNE BOOSTER

Phela is an evaluated traditional medicine and is the code name for the herbal mixture of four South 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. The consumption of *Phela* has been calculated to equate to an adult human dose of 15.4 mg/kg (Lekhooa *et al*., 2012a).

2.1.2.1. Preparation and on-going research

At the Medical Research Council, *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. Of date, the Indigenous Knowledge Systems (IKS) Lead Programme of the Medical Research Council (MRC) and the Department of Health of South Africa, embark on investigating these claims in scientifically controlled phase II clinical trials in HIV positive patients.

2.1.2.2. Previous studies

Phela's evidence of its efficacy was first obtained from anecdotal reports by both patients and traditional healers. These reports were supported by the subsequent findings in observational studies involving medical doctors in the Western Cape and Gauteng provinces (Matsabisa *et al., 2006*). Each of the four plants, has a wide therapeutic spectrum. *Clerodendrum glabrum* has anti-inflammatory and anti-pyretic effects (wahba *et al*., 2011). Moreover, the plant has been used to treat of snake bites, intestinal parasite, coughs, fever and diabetes (Ndlovu et al., 2013; Adamu et al., 2014). According to Nidiry (2005) *Polianthes tuberosa* is used as an anti-fungal. *Rotheca myricoides* has anti-malaria properties (Muregi et al., 2007). *Senna occidentails* has hepatoprotective and it's used for the treatment of tuberculosis, gonorrhea, dysmenorrheal, anemia, flu and liver and urinary tract diseases (Silva *et al*., 2011)

During the controlled observation clinical studies, conducted on 500 HIV positive and AIDS patients, *Phela* was used as an immune booster. The results showed an

increase in the patients' appetite, 23 % increase in weight gain, 80 % decrease in viral load 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).

The *in vitro* studies of *Phela* extract indicated that it has no effect on the activity and significant interaction with the different isoforms of CYP450 enzymes, making it safe to use with other drugs (Walubo *et al.*, 2007). On the same note, *Phela* was found to have no cytotoxic effect on cells *in vitro*. [*Medical Research Council, Indigenous Knowledge Systems Lead Programme Internal report, 2011*].

In another study by the MRC, a sub-chronic toxicology study of *Phela* in *vervet* monkeys over 3 months was done. The study involved using 12 times the traditionally recommended adult dose (15.4 mg/kg), which showed that *Phela* did not have toxic effects on the test animals. Furthermore, results from phase I clinical trials in healthy human participants, indicated that no toxicity was exhibited when using *Phela.* The results of the different studies have been presented at national and numerous international conferences. No toxicity was exhibited in Phase 1 clinical trials of Phela in healthy participants [*Medical Research Council, Indigenous Knowledge Systems Lead Programme Internal report, 2009*].

Previously in our laboratory, a chromatographic fingerprint for *Phela* was established using different chromatography techniques to identify and authenticate it in its crude form and to monitor it in plasma (Lekhooa *et al*., 2012b). Furthermore, rats' studies were used to establish *Phela* metabolite kinetics after single oral dose and its effect on the immune system. The metabolite had a half-life of 3.47±0.35 hours and reached maximum concentration at 4.67±1.15 hours. Furthermore, it was estimated the concentration at steady state would be 47.52±5.94 PK-area/L with no drug accumulation (Acc index = 0.009±0.04) after a once-off daily dose (Lekhooa et al., 2012c)

Rats were treated with *Phela* 15.4 mg/Kg once off and kinetic parameters determined over eight hours. The peak values indicated that the *Phela* wouldn't accumulate. Thereafter, *Phela* was given orally (15.4 mg/Kg) over 14 days in SD

rats, to screen for its effect on the immune system. The results showed that *Phela* antagonized cyclosporine's immune suppressive effects by increasing interleukin-2 serum levels (Lekhooa *et al*., 2012a).

2.1.2.3. *C*hallenges with ATM research

Despite a widespread usage of herbal medicine in both developed and developing countries, concerns still arise regarding the health care policies, safety, efficacy, quality, rational use, access, availability, preservation and further development. The WHO has outlined its strategy to address all the concerns, and it is from this framework that various organizations (i.e. African Union, New partnership for African development (NEPAD), Southern African development community (SADC) etc. adopt and modify the guidelines to create policies that will promote integration of traditional medicines in the health care system.

Medicinal plants are used in the treatment of many diseases and illnesses, and their usage and efficacy have a growing interest to western societies. Many medicinal plants have been purported to be immune boosters or stimulants, and some of these have been used or tested for boosting the immune system in viral illnesses, cancer etc. Even though medicinal plants have been reported to serve as biological response modifiers by activating, increasing and/or restoring the reactivity of immunological responses, the exact mechanism of action remains unknown (Chen *et al*., 2006). Unfortunately, most of the traditional medicines and their products have not attracted wide clinical application owing to insufficient information on efficacy, specifically, and the lack of evidence of action against the purported indications (Ganju *et al*., 2003; Plaeger, 2003).

Various products have been claimed as immune boosters for HIV treatment. The products include nutritional supplements (i.e. beet-root, garlic and lemon juice), traditional herbal medicines (i.e. *Phela*, Curcumin, procydin, *Hypoxis Hemerocallidea, Sutherlandia*) and other products marketed under various trade names (Mills, 2005; Ojikutu *et al*., 2007). *Phela*, *Hypoxis Hemerocallidea and Sutherlandia* have been scientifically evaluated for their purported indications and are commonly used in South Africa.

2.1.2.4. *Phela* as an ideal candidate for testing an animal model for immune suppression

To date, research reports have proven that *Phela* is well evaluated, however there is still need for further studies to develop and scientifically validate it for clinical use. Even though *Phela* stimulated the immune system, its exact mechanism of immunomodulation remains unknown. These findings potentiate *Phela* as an ideal test candidate for evaluating the immune-suppression rat model, as it is under development as an immune booster for immune-compromised patients. Knowledge of the mechanism of immunomodulation of *Phela* will give inside on how it can be appropriately prescribed, its contra-indications and potential interactions.

2.1.3. OVERVIEW ON MECHANISMS OF DRUG-DRUG INTERACTIONS

Drug interactions are an ongoing concern in treatment of 20 - 30 % of all adverse drug reactions are caused by interactions between drugs and/or herbal medicines and these reactions are clinically relevant in up to 80 % of elderly patients. Furthermore, these drug interactions can be categorised as pharmaceutical, pharmacokinetic, or pharmacodynamic (Beljen et al., 2004). Pharmaceutical interactions occur when two compounds interact because of they are incompatible either physically or chemically. Pharmacodynamic interactions alter the pharmacologic response to a drug and may result in additive synergistic or antagonistic effects. On the same note, pharmacokinetic drug interactions include changes in absorption, distribution, metabolism and elimination. Here, metabolising enzymes or drug transporters are often involved in these processes.

Concerns over interactions between complementary medicines and alternatives medicines such as herbal medicines and conventional medicines (drugs) has substantially increased, especially as there is a paucity of data regarding such interactions, and particularly with chemotherapy drugs, which have a narrow therapeutic index (Alsanad et al., 2014). It is also a well known concept that drug interactions my cause clinical problems. It is therefore important to continue applying *in vitro* and *in vivo* techniques to predict possible interactions amongst drugs and/ or herbs. For the purpose of this study it was necessary to screen for potential herb-drug interaction between *Phela* and immunesuppresants prior to in vivo studies.

2.2. PHARMACOLOGY OF LEVAMISOLE, CYCLOSPORINE, CYCLOPHOSPHAMIDE AND DEXAMETHASONE

2.2.1. IMMUNE STIMULANT DRUGS

Immunostimulants are substances (e.g. drugs, traditional herbal medicines, endogenic agents and/or nutrients) that stimulate the immune system by inducing or increasing activity of any of its components. Immunostimulants are categorized into either specific immunostimulant; providing antigenic specificity in immune response or non-specific immunostimulant; augment immune response irrespective of antigenic specificity. Many endogenous substances such as some hormones are non-specific immunostimulant, activating both innate and adaptive immune responses. For the purpose of this study only levamisole (LEV) a known immune stimulant was reviewed and used as a control during the animal experiments.

2.2.1.1. Levamisole (LEV)

2.2.1.1.1. Indications and mechanism of action

LEV (**Figure 2.1**) is an anthelminthnic and immunomodulator belonging to a class of synthetic imidazothiazole derivates. LEV was first released in the 1960's and has been marketed under various brands. LEV is a white crystalline powder, highly soluble and stable in acid aqueous media and supplied as either hydrochloride or levamisole phosphate. LEV is considered to be safe and has been used extensively to eradicate worms in animals and humans.

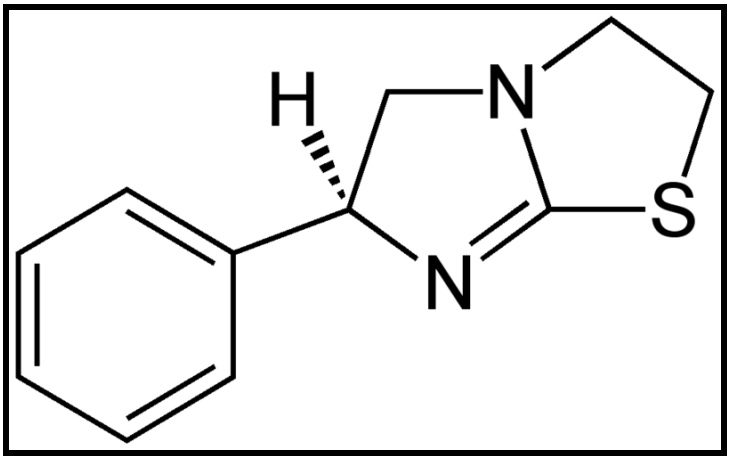


Figure 2.1: A chemical structure of levamisole (Bilandzic *et al.*, 2010)

The effect of LEV on the immune system is complex, and a specific mechanism of action is not fully understood. LEV can stimulate formation of antibodies to various antigens; enhance T-cell responses by stimulating T-cell activation and proliferation, potentiatiate monocytes and macrophage functions including phagocytosis and chemotaxis and increase in neutrophil mobility, adherence, and chemotaxis.

LEV immunostimulation is demonstrated in hosts with impaired immunity, augmenting the immune response to normal levels (Stogaus, 1995). Various research outputs have shown that LEV exerts its immune stimulatory effect by boosting the cell mediated immune response (Renoux *et al.*, 1976; Faanes *et al.*, 1977; Kimball, 1996; Bilandzic *et al.*, 2010).

2.2.1.1.2. Pharmacokinetics

LEV is a fast and short-acting drug, with a half-life of 4 hours. Elimination is completed within 2 hours occurring due to break-down in the liver, but small amounts of LEV are excreted by the kidneys and still smaller amounts are passed in the faeces (Stogaus, 1995).

2.2.1.1.3. Adverse effects and contra-indications

LEV exhibits side effects such as nausea, vomiting, diarrhea, fatigue, muscle cramps and flu-like symptoms. Alcohol may aggravate the symptoms. It is contra-indicated in pregnant women (Stogaus, 1995).

2.2.2. IMMUNE SUPPRESSANT DRUGS

Immunosuppressant drugs are agents that inhibit or prevent the activity of the immune system. They are used in immunotherapy for treatment of autoimmune diseases and to prevent rejection of transplanted organs (Braun *et al*., 1997). These drugs are classified into five groups defined by their mechanism of action: inhibition of cytokine production and/or action (glucocorticoid), disruption of cell metabolism, prevention of lymphocyte proliferation (cytostatic), antibodies that block T-cell surface molecules (monoclonal and polyclonal), drugs acting on immunophilins and other drugs. Another classification of the immunosuppressive drugs is based on how they interact with T-cell activation cascade and the classification. In treatment

regimens, immunosuppressive drugs always consist of at least two or more agents with a different mechanism of action, that disrupt the different levels of T-cell activation (Harvey, 2009; Fernandez *et al*., 2002; Gummert *et al*., 1999; Mattos *et al*., 1996).

2.2.2.1. Cyclophosphamide (CP)

2.2.2.1.1. Indications and mechanism of action

CP (**Figure 2.2**) is an inactive pro-drug that requires enzymatic bio-activation to manifest cytostatic activity. It has a broad spectrum effect, and can be used singly or as part of a regimen in the treatment of a wide variety of neoplastic diseases. It is frequently used as both an anti-cancer drug for treatment of leukemias, lymphomas and solid tumors, and also as an immunosuppressive drug (Nakahara *et al.*, 2010). Following activation and formation of a mustard agent, it attaches the alkyl group to the guanine base of DNA at number 7 nitrogen atoms of the imidiazole ring and acts as a bi-functional alkylating agent. It forms both intra- and interstrand DNA cross-links and DNA-protein cross links which all lead to inhibition of DNA replication during the resting phase of the cell cycle (De Jonge, 2005).

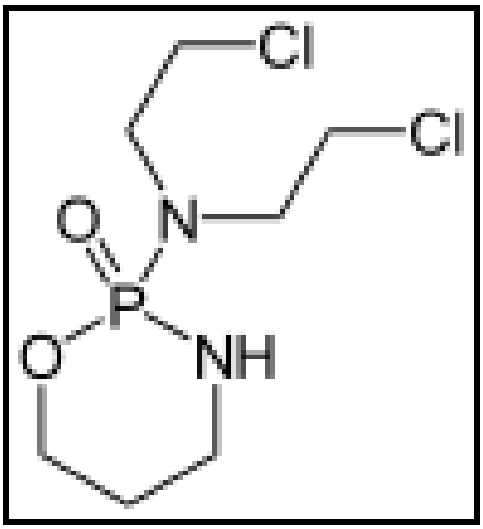


Figure 2.2: A chemical structure of Cyclophosphamide (Nakahara *et al.*, 2010)

2.2.2.1.2. Pharmacokinetics

CP is administered both oral/parenteral and over a wide dosage range. The dose regimen is often designed according to the underlying disorder. To achieve immunosuppression it is often given orally. CP is extensively metabolized to both

active and inactive metabolites. It has a half-life of 5 – 9 hours. CP is activated into an active metabolite; 4 hydroxy-cyclophosphamide by various CYP450 isoforms, with CYP2B6 having the highest yield of the metabolite. This metabolite is de-activated into 4-keto-cyclosphosphamide and is excreted as such in the urine. The parent drug is minimally excreted into faeces, after biliary transport or into urine by glomerular filtration (De Jonge, 2005).

2.2.2.1.3. Adverse effects and contra-indications

The most prominent adverse effects are bone marrow depression, alopecia, gastrointestinal effects (i.e. nausea, vomiting and diarrhea) and hypersensitivity (Harvey, 2009). CP is known to be mutagenic, carcinogenic, teratogenic, and fetotoxic in humans. CP is contra-indicated in patients with hypersensitivity, and in women who are either pregnant or breast feeding.

2.2.2.2. Cyclosporine (CsA)

2.2.2.2.1. Indications and mechanism of action

CsA (**Figure 2.3**) is a lipophilic cyclic polypeptide composed of 11 amino acids and it's extracted from a soil fungus. It is used for prophylaxis of organ rejection in kidney, liver and cardiac allergenic transplants. It is often used for acute rejection of transplanted organs when combined with other drugs (Ciesielski *et al*., 1996).

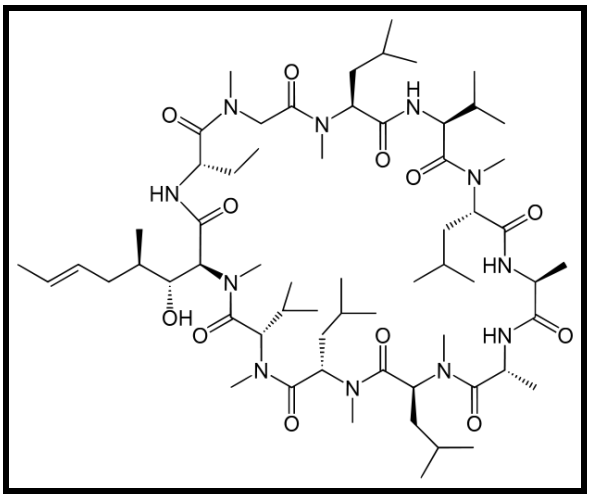


Figure 2.3: A chemical structure of Cyclosporine (Harvey, 2009)

In a multi-drug regimen, CsA suppresses cell-mediated immunity and affects the humoral immunity to a lesser extent. CsA diffuses into a T-cell, and it binds to calcineurin to form a complex. The latter is responsible for dephosphorylating of cytosolic Nuclear factor of activated T cells (NFATc) and inhibition of NFATc blocks the synthesis of a number of cytokines especially IL-2 (Harvey, 2009).

2.2.2.2.2. Pharmacokinetics

CsA may be given either orally or intravenously. CsA has a half-life in the range of 10 – 27 hours. The blood distribution is concentration dependant and has a variable oral bio-availability. It is hepaticly metabolized by CYP3A4 and results in 15 metabolites, of which 9 have designated structures. The metabolites are excreted through the biliary route, while a small fraction of the drug is through the urine (Novartis, 2009).

2.2.2.2.3. Adverse effects and contra-indications

The adverse reactions are dose dependent and in some cases, CsA dosage adjustment can result in reversal of adverse effects. Nephrotoxicity, hypertension, hyperlipidemia, hyperkalemia, tremors, hirsutism, glucose intolerance and gum hyperplasia are prevalent adverse reactions. Anaphylactic reactions occur more frequently with parental administrations (Harvey, 2009; Novartis, 2009).

2.2.2.3. Dexamethasone (Dex)

2.2.2.3.1. Indications and mechanism of action

Dex (**Figure 2.4**) is a synthetic glucocorticoid, used for the treatment of allograft rejection, as well as autoimmune, allergic and malignant diseases, largely based on its potent effects on inflammatory and immune responses. It inhibits the production of IL-2, IL-4 and IFN-γ and the proliferation and function of T-cells. It suppresses both humoral and cell mediated immunity (Czock, 2005).

2.2.2.3.2. Pharmacokinetics

Dex binds to albumin. It has a half-life of 36 – 54 hours after intravenous administration (Czock, 2005).

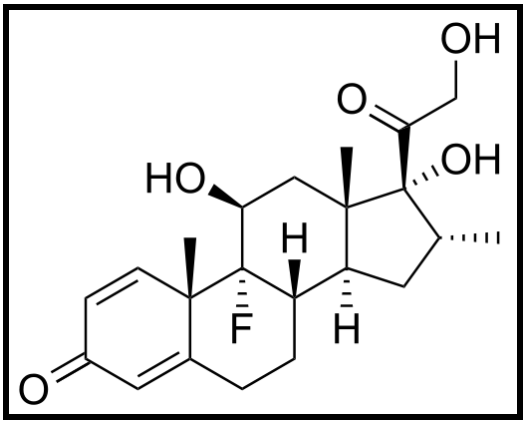


Figure 2.4: A chemical structure of Dexamethasone (Czock, 2005)

2.2.2.3.3. Adverse effects and contra-indications

Possible side effects include increased appetite, immune suppression, fluid retention, convulsions and glaucoma Czock, 2005).

2.3. AN OVERVIEW ON IMMUNOLOGY

2.3.1. INTRODUCTION

The immune system is a collection of mechanism and responses in an organism against infection. The immune system has three layers, primarily the skin which is the physical barrier, the non-specific innate system (rapid response) and pathogen specific adaptive (slow response) immune system. **Figure 2.5** illustrates constituents of the innate and adaptive immune response to invading pathogens. The immune system 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 direction of the immune response is defined by the pathogen; hence a viral infection would elucidate a cell mediated response whereas a bacterial infection would be humoral response.

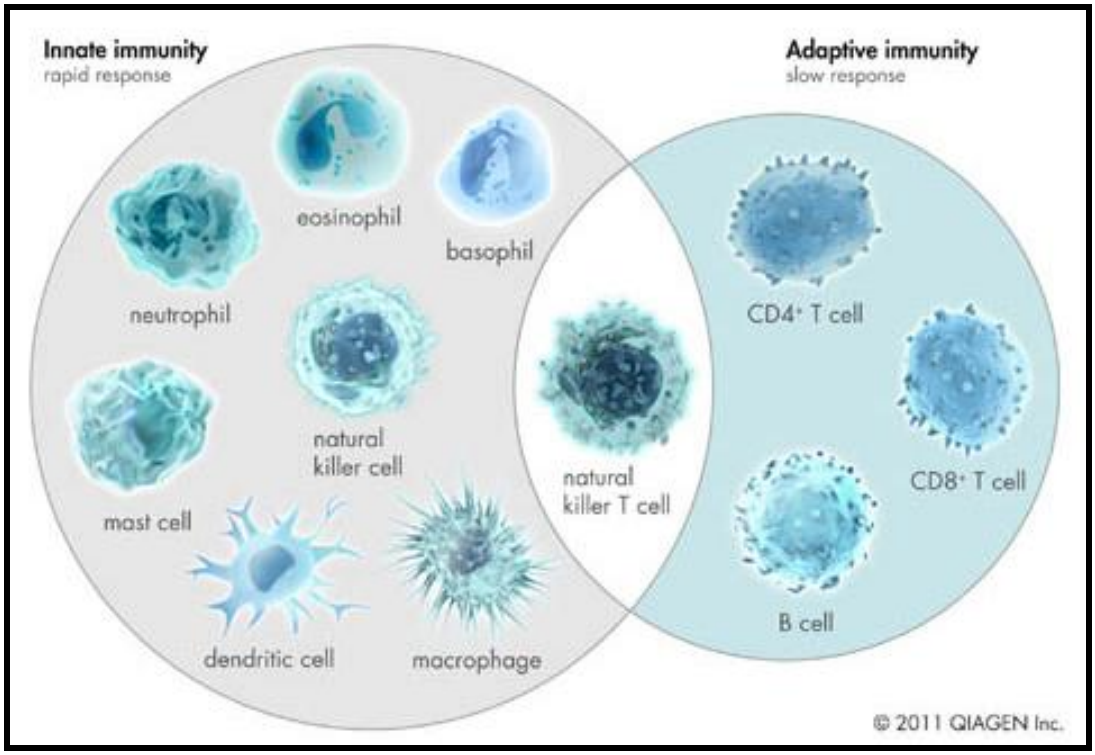


Figure 2.5: Diagram of the immune cells and function http://www.sabiosciences.com/pathwaymagazine/minireview/innateadaptiveimmunity.php (Accessed on 25 August 2014)

2.3.2. IMMUNOMODULATION

Immunomodulation denotes to any changes in the immune response that can involve induction, expression, amplification or inhibition of any part or phase of the immune response. Furthermore, a drug can be classified as either an immune stimulant, immune suppressant and/or both depending on the receptors being activated at a certain time. In phytotherapy, immunomodulators may be defined as botanicals that alter the activities of the immune system via cytokines, hormones, neurotransmitters and other peptides (Spelman *et al.,* 2006; Leckeweg *et al.,* 2007). Due to their crude nature, herbal extracts have a plurality effect on the immune system and immunomodulatory capabilities (Alamgir *et al.,* 2010).

2.3.3. INNATE IMMUNITY

Innate immunity (**Figure 2.6 A**) refers to a non-specific immune response characterized by inflammation, interferons, natural killer cells, activation of the complement system and up regulation of cytokines involved during inflammation

(Sherwood, 2001). Tissue damage and invading pathogens trigger inflammation response through proliferation of phagocytic specialist, activation of interferons and platelets to repair the damaged tissue (Berridge *et al.,* 2012).

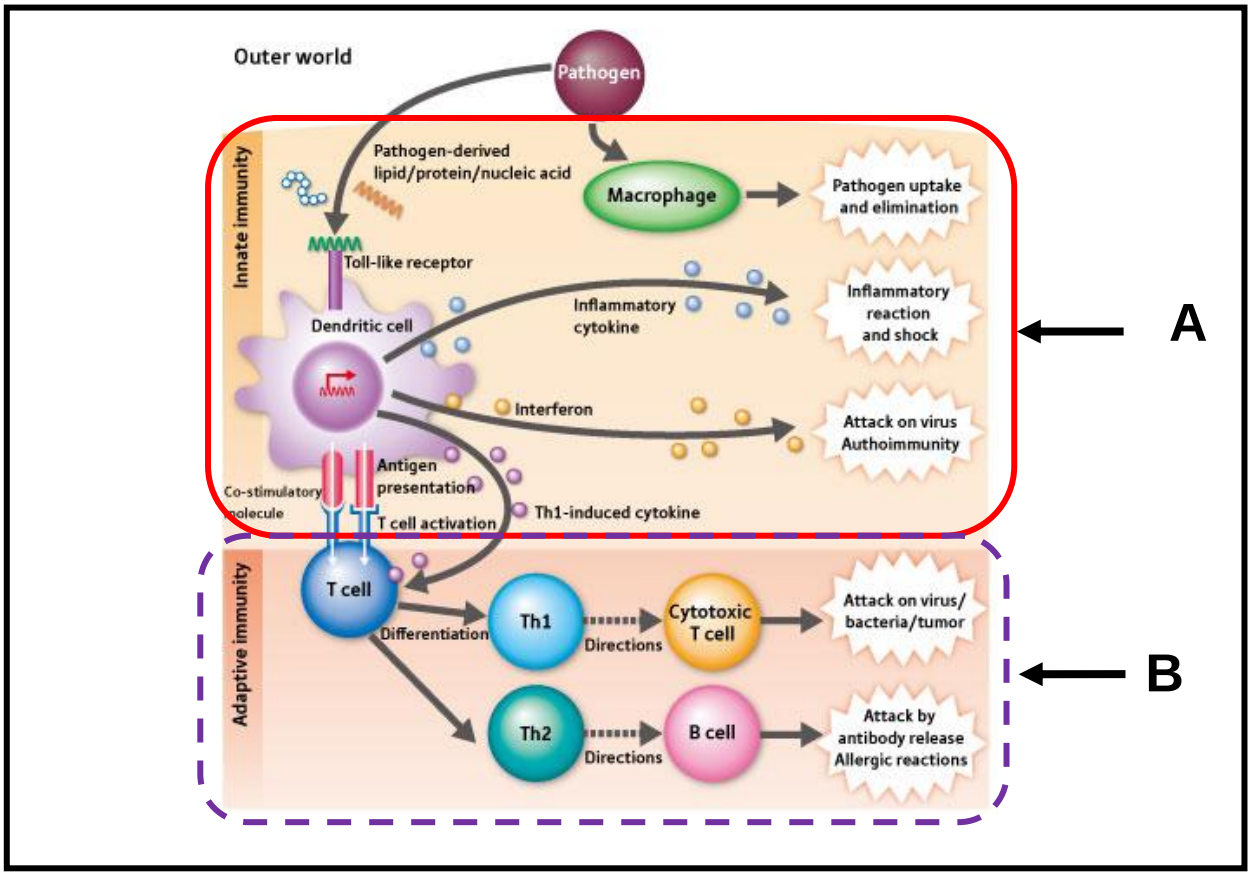


Figure 2.6: Diagram of the functioning of an innate and adaptive immunity. http://www.rikenresearch.riken.jp/eng/frontline/5028 (Accessed 28 Feb 2014)

A: *Innate immunity response against a pathogen activates macrophages for elimination and inflammation action.* ***B****: T-lymphocytes differentiate into either cell mediated promoting* TH_1 *cells promoting cell mediated immunity or* TH_2 *cells that induce a humoral immunity response*.

Inflammation is the first response of the immune system to infection which leads to increased phagocytes and plasma proteins to the injured area, to either isolate, destroy, inactivate the invaders, remove debris and/or prepare for subsequent healing and repair. An acute inflammation is an initial short-term response of the body to harmful stimuli, which is characterized by redness, swelling, pain, heat, and loss of function. Whereas, chronic inflammation is a pathological condition

characterized by concurrent active inflammation, tissue destruction, and failed attempts at repair.

Interferons defend against viral infections, while natural killer cells lyse and destroy virus-infected host's cells.

Leukocytes are white blood cells forming the secondary arm of an innate immunity, comprising of phagocytes (i.e. macrophages, neutrophils, and dendritic cells), mast cells, eosinophils, basophils and natural killer cells. Also, these cells have an associated role in activating the adaptive immune system. Dendritic cells serve as a link between innate and adaptive immune systems, because they present antigens to T-lymphocytes cells, which is one of the key cell types of an adaptive immune system. Leukocytes can be classified based on the shape and number of their nuclei, granulocytes and agranulocytes (Sherwood, 2001; Lappin *et al.*, 2000).

Granulocytes are made up of neutrophils, basophils and eosinophils. Neutrophils facilitate chemo-taxis during inflammation. While, basophils and eosinophils secrete chemical mediators that are involved in defense against parasites and play a role in allergic reactions, such as asthma.

Agranulocytes comprise of lymphocytes, monocytes and macrophages. Macrophages act as scavengers, ridding the body of worn-out cells and other debris, and as antigen-presenting cells that activate the adaptive immune system. Lymphocytes are the effective cells of the adaptive immune response and will further be described in the sections that follow.

2.3.4. ADAPTIVE IMMUNITY

An adaptive immunity (**Figure 2.6 B**) is mediated by B- and T-lymphocytes cells and enhances pathogen eradication by adding antigen specificity and memory onto pre-existing innate immunity. An adaptive immunity is either antibody mediated (humoral immunity) or cell-proliferating (cell-mediated immunity) directly attacking infected cells. Furthermore, the adaptive immunity is regulated by cytokines at any specified time (Wan and Flavell, 2009).

2.3.5. TH_1/TH_2 RESPONSE

The $TH_{1/2}$ paradigm is built on the phenomenon that naive T-helper cells (TH_0) can differentiate into functional subsets of TH-cells depending on the stimuli of the micro-environment of the cell (Amsen *et al*., 2009; Saito *et al*., 2010). TH_1 and TH_2 response is a self-regulatory immune mechanism; increase in the TH_1 micro-environment promotes cell mediated immunity and simultaneously leads to a decreased TH_2 immune response and vice versa (**Figure 2.7**). The shift in the $TH_{1/2}$ balance correlates with the type of pathogen invading, causing proliferation of specific cytokines to evoke an immune response that is relevant to combat the infection (D'Elios *et al*., 1998). To better elaborate this concept, here the antigen presenting cells (APC) secrete auto-regulatory cytokine interleukin 12 (IL-12), which activates naïve TH_0 cells to differentiate into interleukin 2 (IL-2) and interferon-γ (IFN-γ) secreting TH_1 cells.

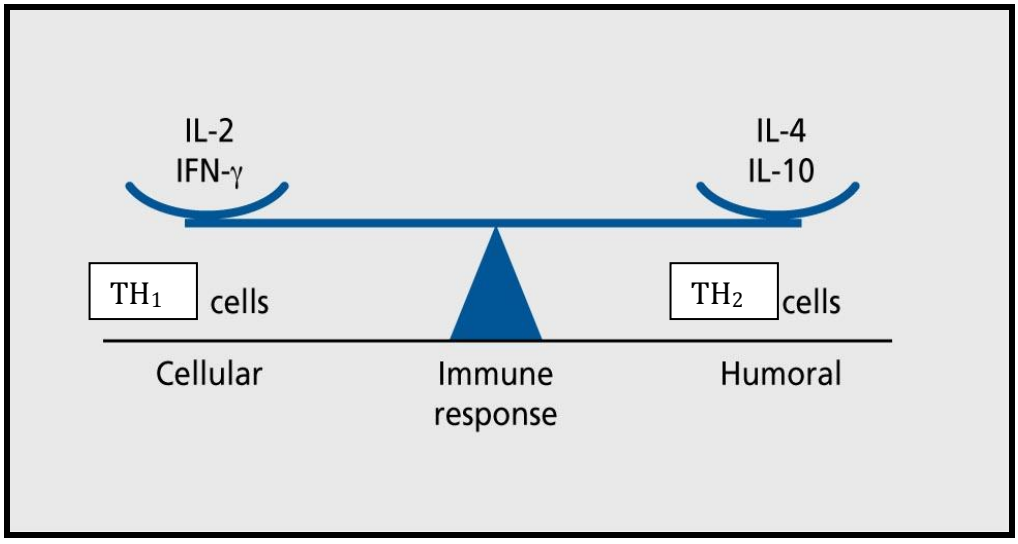


Figure 2.7: is a diagram to illustrate the TH_1 and TH_2 immune response. *IL-2 and IFN-γ promote cell mediate immunity, while IL-4 and IL-10 enhance humoral immune response*. http://www.dialogues-cns.com/publication/cytokines-neurophysiology-neuropsychology-and-psychiatric-symptoms) (Accessed 28 Feb 2014).

The above mentioned cytokines trio (IL-2, IL-12 and IFN-γ) are considered the major pro-inflammatory and TH_1 cytokines. They promote cell mediated immune response by stimulating natural killer cells, cytotoxic T-cells (CD_8) and macrophages. On the other hand, up-regulation of interleukin 4 (IL-4) and interleukin 10 (IL-10), leads to humoral immunity. These cytokine duo (IL-4 and IL-10) are champions for anti-

inflammatory and TH_2 immune response. This is evident through mast cell and eisonophils proliferation. Furthermore, plasma cells differentiate into antibody producing B-cells, inducing immunoglobulin switching to IgE (Elenkov *et al*., 1999; Wan and Flavell, 2009). This paradigm has received lots of consideration in recent years in understanding of processes during immune disease pathology and it has been incorporated in developing therapies thereof.

2.3.6. CELL MEDIATED IMMUNE RESPONSE

Cell-mediated immunity (**Figure 2.8**) is directed primarily at microbes that survive phagocytes and microbes that infect non-phagocytic cells and is referred to as TH_1 response. An antigen presenting cell (APC) presents the antigen to T-lymphocytes and secrets IL-1 which promotes IL-2 proliferation.

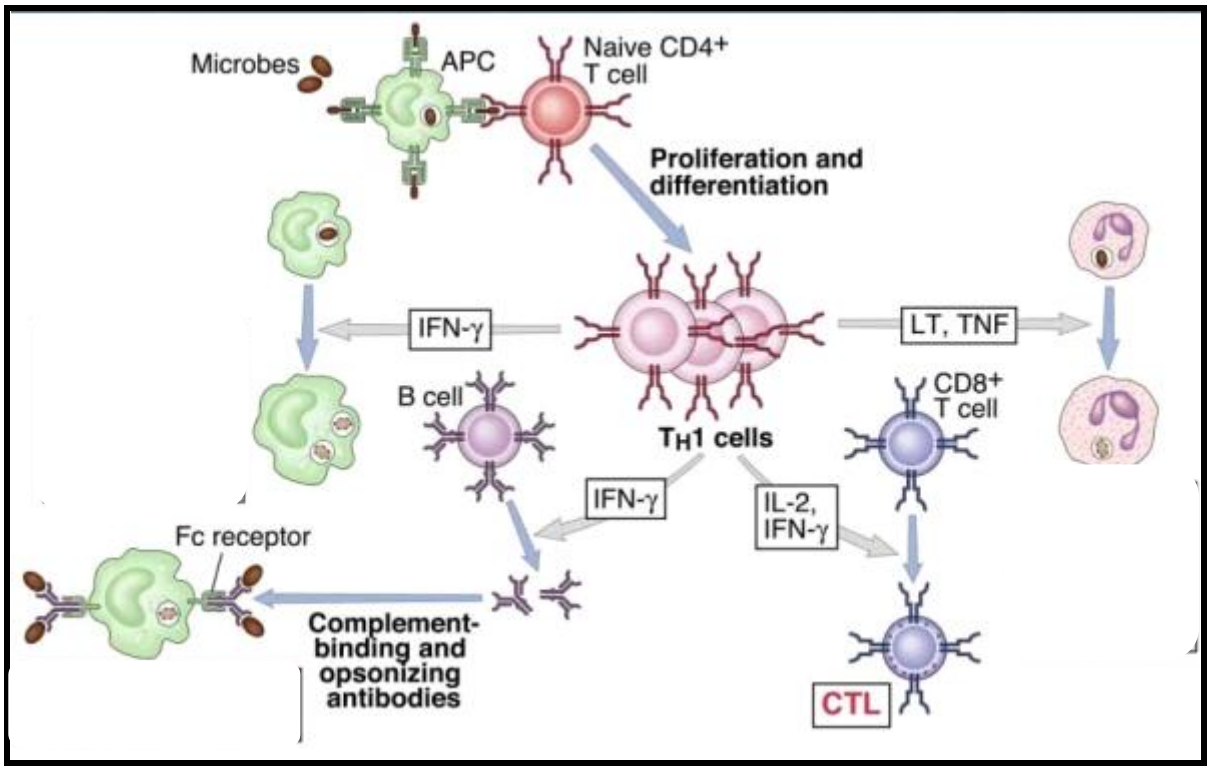


Figure 2.8: An illustration of cell mediated immunity response. APC activates a naïve CD_4 T-cell into an IL-2 and IFN-γ secreting TH_1 cell that activate the complement system binding and antibodies opsonizing to overcome the pathogen. http://wenliang.myweb.uga.edu/mystudy/immunology/ScienceOfImmunology/Cytokines.htm (Accessed 28 Feb 2014).

T-Lymphocytes differentiate into TH_1, secreting IFN-γ and TNF-β. These cytokines initiate cell mediated immunity by activating macrophages, natural killer cells and cytotoxic T-cells (CD_8), which kill bacteria and virus infected cells respectively. It is most effective in removing virus-infected cells and participating in defending against fungi, protozoans, cancers and intracellular bacteria. Another mechanism to achieve cellular immunity is activation of the antigen-specific cytotoxic T-lymphocytes, 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).

2.3.6.1. T-Lymphocytes

The CD_4 T-cells are activated in the lymph nodes and are the central part of the adaptive immune response. T-lymphocytes are classified according to their function as either helper, cytotoxic cells, suppressor or γδ cells. For the purpose of this study we will focus on the two main subsets. Cytotoxic T-cells (CD_8) kill and destroy virus and/or pathogen-infected cells. T-helper cells also known as CD_4 cells regulate the innate and adaptive immune response when activated by cytokines from APC immunity (Wan and Flavell, 2009).

Naive CD_4 are bi-potential and they serve as precursors for functional cells of the immune system. TH_0 cells differentiate into different subsets: TH_1, TH_2, recently discovered TH_{17} and regulatory T-cells depending on the antigen and cytokine environment encountered during activation. Presence of cytokines influence the differentiation of the above mentioned T-cell subsets. TH_1 cells secrete IFN-γ, IL-2 and TNF-β which promote cell mediated immunity whereas TH_2 cells primarily secrete IL-4, IL-10 and IL-13 promoting humoral immunity (Elenkov, 1999; Wan and Flavell, 2009; Cope *et al*., 2011).

2.3.6.2. Cytokines promoting cell mediated response

Cytokines are a group of proteins and peptides that are used in organisms as signaling compounds. They are particularly important in immunological, inflammatory

and infectious diseases. TH_1 cytokines activate macrophages and promote cell-mediated immune responses against invasive intracellular pathogens, enhancing fever and tissue destruction (Dinarello, 2000; Priimagi *et al*., 2005). The main trio is IL-2, IL-12 and interferon-γ.

Interleukin 2 (IL-2) has pro-inflammatory properties and is an effector cytokine secreted by TH_1, CD_8 and dendritic cells for the proliferation of a cell mediated immune response. Also, it is regarded as the major switch for immune system through promotion of rapid clonal expansion and activated effector cells. Antigenic stimulation leads to IL-2 proliferation which in turn activates the immune system. Furthermore IL-2 down regulates the immune response following pathogen clearance (Sirskyj, 2008; Wan and Flavell, 2009)

Interleukin 12 (IL-12) is a regulatory protein produced by activated macrophages. It stimulates the growth of an activated CD_4 and CD_8 cells and natural killer cells. Moreover, it promotes inflammation and TH_1 response. It activates production of IFN-*γ (*Griffin, 2010; Hamza *et al*., 2010*).*

Interferon-γ (IFN-γ) is a signature differentiation cytokine for TH_1 cells and natural killer, CD_4 and CD_8 cells secrete it. It has antiviral properties by inhibiting viral replication and is activated by IL-12 polarization (Wan and Flavell, 2009).

2.3.6.3. Cell mediated immunity and Tuberculosis (TB)

Loss of cell mediated immune (CMI) response is often related to a setting in of some chronic diseases such as HIV and TB infection (Pawlowski *et al.,* 2012). Clinical TB along with HIV and malaria are leading global health problems with higher infectious mortality in the world (Berrington and Hawn, 2007). *Mycobacterium tuberculosis (Mtb)* is an intracellular pathogen that causes TB infection, and can only be effectively eliminated from the host when there is an efficient interaction between infected macrophages and antigen-specific T-cells. During, TB infection crucial contribution of CMI is underlined by the observation that patients with impaired T-cell function (e.g. patients receiving immunosuppressive therapy, AIDS patients, or elderly) is more susceptible to the development of clinical diseases. Whereas

patients with defective humoral immunity are not as prone (e.g. sickle cell disease, measles and multiple myeloma) (Stenger and Mondlin, 1999).

CMI responses in TB are orchestrated to T-cells contributing to protective immunity in TB include natural killer cell, macrophages, CD_4, CD_8 and dendritric cells, each with a distinct mechanism. Moreover, up regulation of TH_1 cytokines (IL-2, IL 12 and IFN-*γ)* combats TB infection by recruitment and activation of innate cells such as monocytes and granulocytes to clear the Mtb pathogen (Cooper and Khader, 2008; Prezzemolo *et al.,* 2014). Recent studies have demonstrated that lysis of the infected targets as well as direct killing of intracellular bacteria by CD_8^+ are involved in the T-cell response to mycobacterium infection (Stenger and Mondlin, 1999). Also, an induction of classical class I-restricted CD_8 T-cells by immunization conferred protection against re-challenge, and implicated these cells in protective immunity against TB which involves several phenotypic T-cells subsets, multiple mechanisms of antigen recognition and distinct effector functions. Anti-TB therapy uses an increase in dual IFN-γ/IL-2 cytokine proliferation (Mcshane, 2002).

2.3.7. HUMORAL IMMUNE RESPONSE

Humoral immunity (**Figure 2.9**) involves substances found in the humors, or body fluids and it's also known as the TH_2 response. Naïve TH_0 cells that have differentiated into TH_2 cells activating plasma cells mature into antibody producing B-lymphocytes cells. IL-4 and IL-10 activate macrophages and promote switching to IgE. Humoral Immune Response (HIR) is the aspect of immunity that is mediated by antibodies, which are produced by B-cells that have matured into plasma cells. Secreted antibodies bind to antigens on the surfaces of invading microbes which flag them for destruction.

Moreover, other accessory processes accompany it, including TH_2 cell activation and cytokine production, germinal center formation and isotope switching, affinity maturation and memory cell generation. Its use 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). The following cytokines IL-4, IL-5 and IL-10 drive the humoral immune response and are secreted by the TH_2 cells.

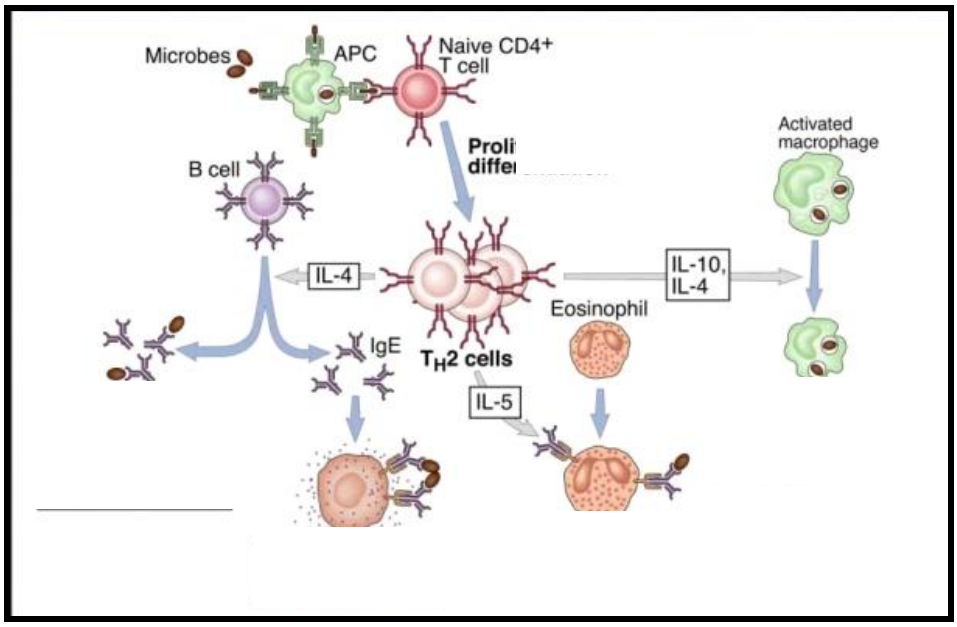


Figure 2.9: An illustration of humoral immune response.

Diagram illustrates humoral immune response. Naïve TH_0 cells differentiate into TH_2 cells cause plasma cells to mature into Antibody producing B-cells. IL-4 and IL-10 activate macrophages.

http://wenliang.myweb.uga.edu/mystudy/immunology/ScienceOfImmunology/Cytokines.htm (Accessed 28 Feb 2014)

2.3.7.1. B-lymphocyte and Immunoglobulins

An immunoglobulin is a large Y-shaped protein produced by plasma cells and used by the immune system to identify and neutralize pathogens such as bacteria and viruses. Immunoglobulins are divided into five isotypes IgA, IgD, IgE, IgG, IgM based on their function. For the purpose of this study, the focus is on the functions of IgG and IgM.

IgG is the most abundant of all isotypes, constituting about 75 % in the serum. IgG has various mechanism of action that include: agglutination, opsonization, activation of the complement system via the classic pathway, neutralization of toxins and also the antibody derived cell mediated cytotoxicity. This antibody crosses the placenta to provide the fetus with immunity. IgM appears early during a pathogen invasion indicating infection. Furthermore, it can agglutinate red blood cells.

2.3.7.2. Cytokines promoting humoral immune response

Interleukin 4 (IL-4) is produced by CD_4 T-cells, basophils and mast cells. Upon antigen stimulation it directs naïve T cells to differentiate into TH_2 cytokine secreting cells thus inhibit a TH_1 immune response. Also IL-4 is crucial for the IgE switching. (Sirskyl, 2008; Kedzierska, 2001).

Interleukin 10 (IL-10) is a potent anti-inflammatory cytokine that inhibits cell-mediated immunity response and is associated with increased B-cell activity and antibody switching all characterizing human immune response (Opal *et al.*, 2000).

2.3.7.3. Humoral immune response and measles virus

In order to understand the role of HIR in disease pathogenesis, we look at measles immune suppression. Measles is a highly contagious viral disease characterized by a prodromal illness of fever, coryza, cough and conjunctivitis followed by the appearance of a diffuse maculopapular rash. The resulting immune suppression increases susceptibility to infection leading to secondary infections. Some of the potential mechanisms of immune suppression following measles virus infection include lymphocyte apoptosis, impaired lymph proliferative responses, induction of immunomodulatory cytokines, including IL-10 and IL-4, down-regulation of IL-12 production and impaired terminal differentiation and antigen presentation by dendritic cells (Carsillo *et al.*, 2009; Griffin *et al.*, 2012).

The reduction of interleukin 12 (IL-12) secretions during acute measles results in a TH_2 response. TH_2 cytokine predominance after resolution of the rash produces an environment favoring B-cell maturation that facilitates the establishment of humoral memory important for lifelong protection from re-infection while depressing macrophage activation and induction of TH_1 response that may be required for combating new pathogens. Measles vaccine offers both cellular and humoral immunity, with the latter being the most effective. There is evidence to suggest that the induction of specific cell-mediated immunity may be as important as antibody production for long-lasting immunity against measles (Meroni, 1994; Ovysyannikova

et al., 2003). The attenuated measles vaccines have been effective in prevention of infection in children world wide (Moss *et al*., 2004).

2.4. AN OVERVIEW ON HIV IMMUNOPATHOLOGY

2.4.1. HIV PATHOGENESIS

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). According to the United Nations 2014 AIDS regional report, an estimated 35 million people are infected with HIV worldwide. In 2013, an estimated 24.7 million people in the sub Saharan were HIV positive and of this 6.3 million were South Africans. These observations have led to increased research globally and nationally towards finding other options to improve the quality of life of HIV positive patients.

2.4.1.1. Human immunodeficiency virus clinical stages

HIV is a retrovirus that leads to an acquired immune deficiency syndrome (AIDS). 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 feeding. The most commonly described HIV types are HIV_1 and HIV_2. HIV_1 is more virulent, easily transmittable and is the cause of the majority of HIV infections globally whereas, HIV_2 is less transmittable and is largely confined to West Africa.

Figure 2.10 illustrates four clinical stages occurring during HIV infection. 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 (Kedzierska, 2001). *Stage I* occurs during HIV primary infection and has 10 years median on-set duration. It is the asymptomatic phase and most of the patients maintain normal health and/or are unaware of the disease. The peripheral blood CD_4 T-lymphocyte cell is above 500 cells/mm^3 (Levy, 2003). Even though the viral load level the peripheral blood drops significantly, people remain infectious and HIV antibodies are detectable in the blood. Research reports indicate that HIV is very active in the lymph nodes. (Fauci, 2007).

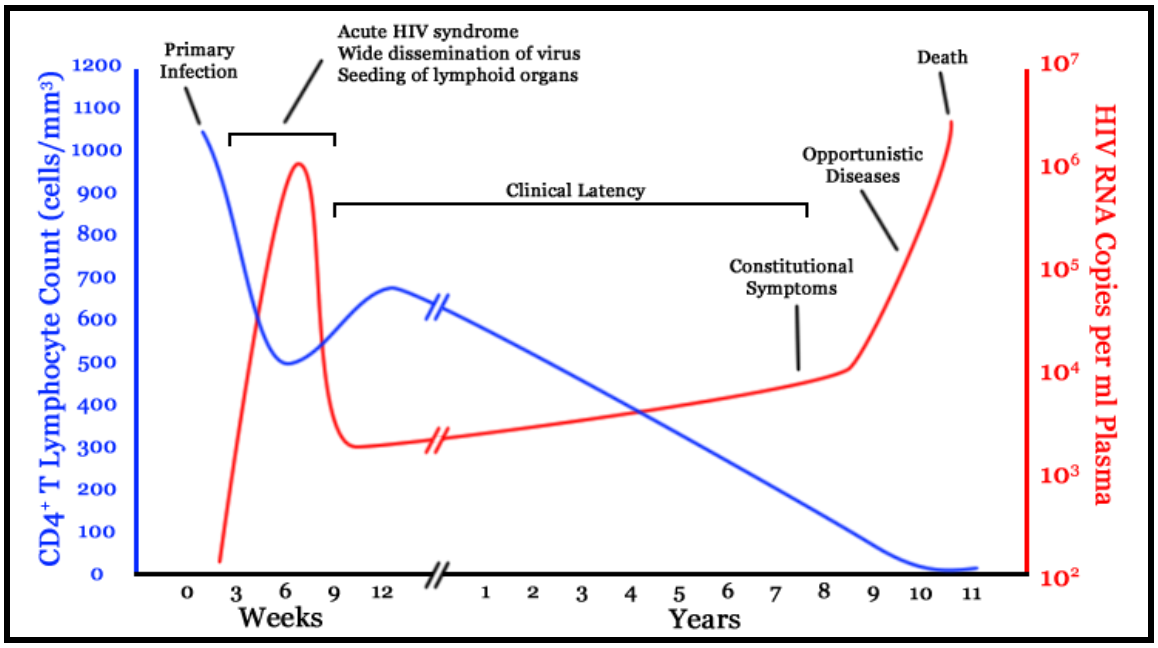


Figure 2.10: A generalized graph of the relationship between HIV copies (viral load) and CD_4 counts over the time course of HIV with stages I - IV.
[http://en.wikipedia.org/wiki/HIV; Accessed 24 September 2013]

Stage II is an acute HIV syndrome and is marked by increased viral load, early symptoms and a declining CD_4 count ranging from 350 – 499 cell/mm^3 (Levy, 2003; Primagi, 2005). 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 III is the clinical latency characterized by the late symptomatic phase and is marked by a CD_4 count lower than 200 cell/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 IV is marked by the loss of cellular immunity and marked by an increase of opportunistic infections and malignancies, leading to acquired immunodeficiency syndrome (AIDS) (Tasca *et al*., 2012; Girei and Fatima, 2013). The CD_4 cell count is less than 50 cells/mm^3.

2.4.2. HIV induced immunopathology

HIV infection is characterized by the progressive deterioration of the cellular immune system that leads to immune suppression and renders the individual more susceptible to opportunistic diseases (Jimere *et al.*, 2005; Gokhale, 2007; Tasca *et al.*, 2012). The principal damage caused by the human immunodeficiency virus is the progressive deterioration of the cellular immune system that leads to severe immuno depression and renders the individual more susceptible to opportunistic disease and/or certain types or neoplasm (Li and Ling, 2012; Tasca, 2012; Fabbiani, 2013; Macallan, 2013).

The immune suppression sets in over a course of time and activates a cascade of events that leads to an incompetent immune system to fight infections. Some of the causes include but not limited to, HIV induced T-lymphocyte loss and dysfunction, altered cytokine network proliferation and a shift from a TH_1 immune response towards a TH_2 humoral immunity which is associated with exacerbation of HIV infection contributing to the progression to AIDS (Clerici and Shearer, 1993; Bal *et al*., 2005; Reuter, 2012).

Since 1984, impairment responsiveness of IL-2 in the peripheral blood mononuclear cells (PBMCs) has been studied at the different stages of HIV infection and in AIDS patients. (Lane *et al*., 1984; Li *et al.,* 2012; Reuter, 2012; Catalfamo, 2012). Reports from Barcellini (1993) and Maggi (1994) indicate that IL-2 deficiency is one of the first immunological impairments observed in HIV positive patients. Furthermore, Kedzierska (2001) confirmed that IL-4 and IL-10 were up-regulated in HIV patients at some various diseases stages before starting the highly active anti-retroviral therapy (HAART) regimen.

In 2012, Li and Ling indicated that HIV_1 has a selective tropism for CD_4 T-cells and causes both quantitative and qualitative defects on these cells. As the CD_4 T-cells lost their ability to serve as helper cells to other white blood cells of the immune system, B-lymphocytes were hyperactive in persons with AIDS as indicated by elevated levels of immunoglobulin and increased number of activated B-cells in circulation. (Breen, 2002; Fraternale *et al*., 2011).

In literature, observed immune change in HIV positive patients can be related to a shift from TH_1 towards TH_2 immune response contributes significantly towards disease progression (Clerici and Shearer,1994; Shearer, 1998). During the HIV asymptomatic phase TH_1 response is dominant and combats infection though not eliminating the virus (Breen, 2002). Whereas during HIV clinical latency stage a loss of cell mediated immunity, mediates increased levels of viral replication, extensive damage to the immune system and cascade of events initiating progression to AIDS. Also cytokine secretion was compromised in AIDS progression with IL-2 and IFN-γ losses (Breen, 2002; Li and Ling 2012; Fabbiani, 2013). Another observation implication in AIDS patients is that an inhibited IL-2 and IFN-γ, further confirming CMI loss and shift to a TH_2 based immunity.

2.4.3. Current HIV therapies and challenges

HAART is a combination therapy of anti-retroviral drugs with different mechanisms of action to prevent resistance in patients and to lower risk of clinical progression of the disease and/or opportunistic malignancies (Levy, 2003). Anti-retroviral drugs (ARVs) prevent multiplication of the virus using different mechanisms of action and are classified into either reverse transcriptase (NtRTI's), nucleoside analogue reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTI's), protease inhibitors (PIs) and fusion inhibitors (FI). 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. HAART has been very effective to increase the CD_4 T-cell count and decrease the viral load, thus enhancing the survival of the majority of infected patients (Reuter, 2012; Kang *et al*., 2012).

The current HAART regimens are insufficient to clear HIV from the body and have debilitating side effects on a long-term (Sirskyl *et al*., 2008). Moreover, it is associated with persistent drug resistance. Hence, a need to look into possible immune boosters that can promote and/or enhance cell mediated immunity. As thus, emphasis has been placed on finding mechanisms that promote immunological control of viral replication over the long-term. One of this is to support and wherever possible, to restore TH_1 pathway (Kidd, 2003).

2.5. AN OVERVIEW ON ANIMAL MODEL DEVELOPMENT AND CHALLENGES

Animal models are used during drug development process for the identification of targets for therapeutic intervention and to provide proof of therapeutic principle of drugs. The most widely used animal models use inbred strains of mice and rats (Hart, 2004). Preclinical testing for immunomodulatory drugs is performed on rodents (mice and rats), and non-human primates, primarily *cynomolgus* monkeys and to a lesser extend *rhesus* monkeys and baboons (House, 2000).

Using non-human animal models has an advantage over humans due to ethical and practical reasons. (i.e. statistical evaluation of results, access to variety of lymphoid tissues and ability to test immunosuppressive drugs). Guidelines have been developed for immunotoxicity testing with the most recent being an International Conference on Harmonization (ICH) S8-guideline, yet no guidelines have been developed for immunomodulation testing of drugs (Kawabata, 2011).

During immunotoxicity studies analysis of functional and non-functional parameters reveal immunosuppressive effects of a test substance. The non-functional parameters are clinical signs of toxicities; body weight, hematology, relative weights of lymphoid organs, histopathology of lymphoid organ as described in the environmental protection agency (EPA) guidelines (Hou *et al*., 2007). The ICH-S8 scope focused only on non-clinical testing for unintended immunesuppression and immuno enhancement, but did not include immunomodulation.

The current practice for immunomodulatory drugs is addressed on a case-by-case basis. This approach is recommendable since the type of testing should be based on the mechanism of action of the immunomodulatory drugs. The indication, use (acute vs chronic) and intended patient population provides flexibility needed to do the best science appropriate for the specific drug being developed (Kawabata, 2011).

Currently, the approach is either a disease specific model, or tests that involve treatment of animals or humans with the drug or herbal product and then isolate the specific cells (i.e. lymphocytes, macrophages, natural killer cells etc.) and testing them *in vitro*. These findings from this approach of research are limited as they cannot be translated for usage in clinical situations (Nakahara, 2010; Manikannan *et al*., 2011; Smith *et al*., 2003).

Isolation of cells after treatment (*ex vivo*) indicates a predominate emphasis on reductionist science, in which the focus is on individual components in isolation from the system as a whole. Rarely is an immune or infectious disease process caused by a single modality or failure, but rather by multiple factors acting on many components, either in sequence or simultaneously, to bring about changes in system-wide behavior. This is evident for most chronic diseases (Bray, 2008). It is for this reasons that such isolated systems cannot apply to a clinical situation, because the chronic diseases are due to multi-factorial and counter-regulatory responses of the immune system that are not present *in vitro*.

Assessment of immunomodulation in rodents may be performed in two basic models, an initial assessment (screening) assay to establish whether a drug or herbal product has an effect on the immune system, and a more detailed assessment (mechanistic) assay, to establish the drug's mechanism of action on the immune system (House, 2000). Initial screening is a preliminary step to establish immune competence of the drug under study, and its efficacy on the immune system if the drug is found to have immunomodulation; a mechanistic assay will have to follow. The mechanistic assay tests include specific immune cells, cell mediated immunity markers, production of cytokines or other bioactive molecules (House, 2000). There remains a need to develop an *in vivo* model, to study the mechanism of immune modulation of drugs and/or herbal products.

An ideal model would be a disease specific model, but this would not tell much about the mechanism of action and would require testing every product in each disease model. A gap remains for a model that can help establish or define the mechanism of immunomodulation on any agent under development for clinical use.

2.6. PROBLEM STATEMENT

The use of immune boosters (both conventional or of traditional medicine origin) is empirical or irrational with regard to indications and therapeutic response due to limited knowledge on their mechanism of immune modulation. Also, they are purported to have immune boosting properties yet there is no scientific evidence to support such claims. Furthermore, the current HAART regimens are insufficient to clear HIV from the body, have debilitating side effects on a long-term and are associated with persistent drug–resistance (Sirskyl, 2008). Hence, a need to look into possible immune boosters that can promote and/or enhance cell mediated immunity. As such, emphasis has been placed on finding mechanisms that promote immunological control of viral replication over the long-term. One of these is to support and wherever possible, to restore TH_1 pathway (Kidd, 2003) necessitating an agent that can either reverse and/or prevent the CMI loss in HIV positive patients.

Extensive literature study revealed that the TH_1/TH_2 model provides a useful paradigm to understand immunomodulation of the immune system and has been essential in developing/designing therapies and/or vaccines to combat disease. Change in Immune markers (i.e. cytokines, immunoglobulins and leukocytes) give insight into immune function and patho physiological processes that lead to disease. Thus elucidating the changes in some of the above mentioned immune markers concentration during immune activation (suppression/stimulation) can be used to develop an animal model by which to establish mechanism of immunomodulation of any purported immune booster.

Phela is a traditional medicine, with purported immune boosting properties, and an ideal candidate for further analysis to study its mechanism of action on the immune system which is necessary for clinical use. Unfortunately, there is no animal model by which to evaluate immune boosters (including *Phela*) for the mechanism of action on the immune system. It is for this reason that the aim was to develop an animal model by which to establish the mechanism of immunomodulation of purported immune boosters.

2.7. REVIEW OF ANALYTICAL METHODS

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. The assumption for use of an internal standard is for it to serve as qualitative marker, to monitor or detect stability and to correct errors in dilution and pipetting (Karnes *et al.*, 1991).

2.7.1. Reviewed HPLC methods for cyclophosphamide and dexamethasone analysis in plasma

Various HPLC methods for the determination of immunesuppresants in biological fluids have been described; so far they differ in chromatography type (reverse phase or ion-pair) or detection system (UV, fluorescence or mass spectrometry). Reverse phase and UV detection have been the most recommended (Malothu *et al.*, 2009). A thorough study of various publications was carried out in order to develop and validate a desirable HPLC method to simultaneously detect cyclophosphamide and dexamethasone in plasma. The method ought to be quick and inexpensive to be used for therapeutic drug monitoring and be applicable on the equipment available in our laboratory.

In literature review, HPLC methods with varying detectors are mainly used to quantify dexamethasone derivatives in pharmaceutical preparations. These various reports gave insight into HPLC conditions (i.e. detection wavelength, mobile phase, column) and extractions procedure guidelines to quantify dexamethasone in plasma. The most common mobile phase was a combination of varying percentage of acetonitrile to either water or buffer. While wavelength was set at 254 nm and column varied in length either 150 mm or 250 mm (Kumar *et al.*, 2006; Song *et al*., 2004; Chen *et al*., 2008; Urban *et al*., 2009).

Similarly, literature survey of cyclophosphamide detection revealed HPLC methods with either UV or mass spectrometry detection (Jonge *et al*., 2004; Malothu *et al*., 2009; Rosu *et al*., 2003; Worry *et al*., 2011; Dhakane *et al,* 2013). The HPLC

conditions and extraction procedure gave insight on how to approach the methodology development. Furthermore, findings in the literature showed methods that simultaneously detect antineoplastic agents used for cancer therapy (Larson *et al*., 2003; Ahmad *et al*., 2011; Buchwald *et al*., 2012).

2.7.1.1. Reviewed validation parameters

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. For the purpose of this study, the validation parameters that were evaluated the calibration curve, accuracy and stability testing.

2.7.1.2. Calibration curve and precision

A calibration line is a curve showing the relation between the concentration of the analyte in the sample and the detected response. Five to eight concentrations (excluding blank values) are sufficient to define the standard curve and have to be within the therapeutic range of the drug under investigation and used to derive unknown concentration. A correlation coefficient of > 0.999 is generally considered as evidence of an acceptable fit of the data to the regression line (Green, 1996).

2.7.1.3. Accuracy testing

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 within the range of therapeutic concentration range is recommended. The mean value should be within 15 % of the actual value except at lower limit of quantification, where it should not deviate by more than 20 %. The deviation of the mean from the true value serves as the measure of accuracy.

2.7.1.4. Stability testing

Stability defines the storage conditions necessary for the analyte when immediate sample analysis is not possible. 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 the 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.

2.7.2. Methods reviewed for cyclosporine analysis in plasma

Various clinical laboratory methods for analysis of CsA have been developed. HPLC, immunoassay based methods and HPLC mass spectrometry (LC/MS) represents the most frequently used methods (Yang *et al*., 2007). Furthermore, these methods are necessary during therapeutic drug monitoring of CsA in patients due to its narrow therapeutic index (Butch *et al*., 2004). HPLC with UV detection is not as sensitive as it may be compared to an MS detector, causing a limitation for laboratories such ours where the latter detector is not available and making immunoassays an option. Three main approaches of whole blood CsA immunoassay are CEDIA, (Enzyme multiplied immunoassay technique) EMIT and fluorescence polarization immunoassay (mFPIA) (Schutz *et al*., 1998).

2.7.3. QUALITY CONTROL OF TRADITIONAL HERBAL MEDICINES (THM)

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). 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 (Khan *et al.*, 2006; Smillie, 2010). Two major approaches applied for quality control are analytical fingerprinting and marker compounds.

2.7.3.1. Chromatographic fingerprinting of traditional medicines

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 *et al*., 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.

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. 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).

2.7.3.2. HPLC methods reviewed for *Phela* fingerprinting

A comprehensive approach was previously developed in our laboratory and was applied to fingerprint *Phela* for quality control purpose (Lekhooa *et al*., 2012b). The approach consisted of the following methods: thin layer chromatography (TLC), column chromatography (CC), high performance liquid chromatography with either

photo diode array (HPLC-PDA) or fluorescence (HPLC-FLD) detector and gas chromatography with mass spectrometry (GC-MSD).

2.7.4. IMMUNO ASSAY TECHNIQUES

Immunoassays are biochemical tests used to quantify concentration or the presence of a protein in a biological sample using an antibody. They are used in clinical laboratories, pharmaceutical analysis and basic scientific investigation. Immunoassay methods are based on competitive binding reaction between fixed amounts of unlabelled sample analyte for a limited amount of binding sites on a highly specific anti-analyte antibody. The importance and widespread use of immunoassay methods is attributed to their specificity, high throughput and high sensitivity for the analysis of wide range of analytes in biological samples (Darwish, 2006).

Immunoassays are categorized based on the type of labeling they have and also on the analyte of interest at a specific time. The label can either be a radio isotope (radio immunoassay), enzymatic (enzyme immunoassay), fluorophore (fluoro immunoassay), chemiluminescent (chemiluminescence immunoassay), and liposome-encapsulate marker (liposome immunoassay). Furthermore the newer generation methods use DNA technology (cloned enzyme donor immunoassay), more sensitive instrument systems (Flow-injection and capillary electrophoresis immunoassay) for drug therapeutic monitoring. Availability of instruments, analyte under investigation and cost are some of the defining parameters in choosing an immuno assay method.

2.7.4.1. Enzyme linked immunosorbent assay (ELISA)

ELISA is a bioanalytical tool used for the rapid detection and quantification of antigens and/or antibodies in sample (Gan *et al.*, 2013; Osuchowski *et al*., 2006). The technique can be applied directly or as a sandwich to detect very low concentrations of the antigen as illustrated in **figure 2.11** ELISA is frequently used in studies on cytokine production in response to treatment of cell lines or laboratory animals (Maltzan *et al.*, 2011).

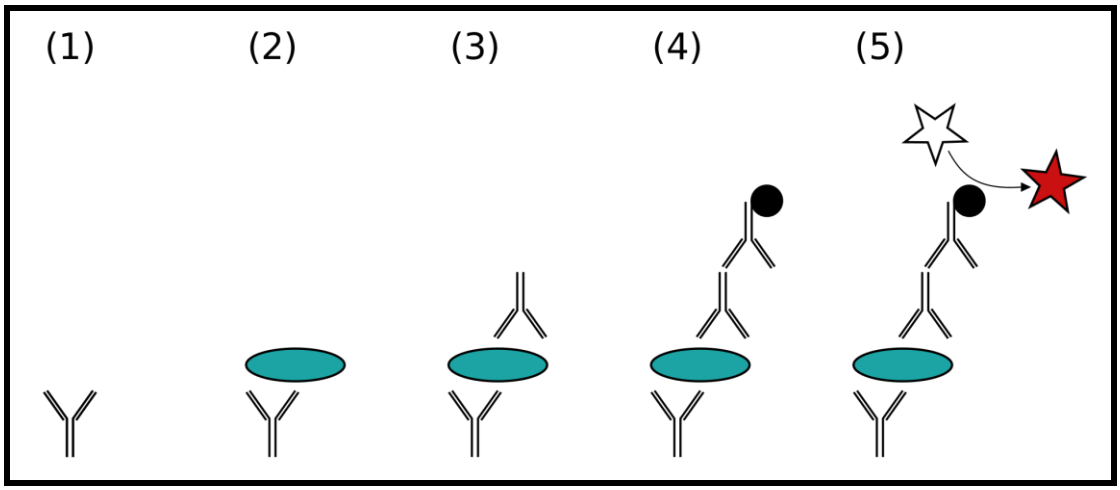


Figure 2.11: ELISA procedure illustrations. http://en.wikipedia.org/wiki/ELISA (Accessed 4 November 2014)

Figure 2.11 is an illustration of ELISA procedure. This is a 96 well microtiter plate pre-coated with an anti-antigen. Thereafter, the biological sample and primary antibody is added and the excess is washed off in step 3. A secondary antibody conjugated with an enzyme is added in step 4, thereafter washing off excess secondary antibody in step 5. In step 6 a chromogen which an enzyme substrate is added and incubated in the dark to allow color change to develop. The microplate reader is used to measure the Intensity of the color which correlates with the concentration of the antigen. A calibration curve is used to convert response to concentration of antigen.

3.

OBSERVATIONS FROM THE REVIEW, AIMS AND OBJECTIVES

3.1. OBSERVATIONS FROM THE REVIEW

- The mechanism of action of most immune boosters, which include conventional drugs and traditional medicines remain unknown and this makes it difficult to evaluate response as well as establish guidelines for their proper use i.e. specific indications and appropriate dose.
- *Phela* is a traditional medicine extract that was reported to modulate the immune system by increasing plasma IL-2 level after suppression by cyclosporine. Unfortunately the mechanism of action is unknown.
- Understanding the specific mechanism of action of the drug (i.e. the level at which the drug acts within the cascade of CMI or HIR) is vital for the rational use of the drug (i.e., establishing guidelines for their proper use).
- The immune system is a complex system whereby immune modulators may act at different levels, which may relate to different diseases differently. For instance, cell mediated immunity (CMI) is important for TB and HIV, while humoral immune response (HIR) is important for small pox and measles.
- Results from current animal models involving either *in vitro* or *ex vivo* (isolation of specific cells from animals or human such as lymphocytes to analyze in vitro) studies cannot be translated clinically.
- According to the literature, there are no guidelines for immunomodulation studies; the approach is done on a case-by-case approach depending on the drug under development.
- The immune response is even more complex owing to auto-regulation whereby, for instance, anti-inflammatory cytokines regulate the action and release of the pro-inflammatory cytokines to optimise the scale of inflammatory response. Therefore, *in vitro* observations cannot be extended to *in vivo*, indicating that only *in vivo* studies are the best for investigating/confirming mechanisms of immune modulation.
- Unfortunately, there is no *in vivo* model by which to screen drug candidates (pure or mixtures (i.e. traditional herbal medicines), for mechanism of immune modulation.

- There is a need to develop an animal model by which to evaluate the mechanism by which any drug and/or traditional medicines modulates the immune system.

3.2. AIM

- To develop an animal model by which to characterise the effect of *Phela* on selected immune markers in immune suppressed rats.

3.3. OBJECTIVES

- To fingerprint *Phela* using chromatographic techniques for identification and quality control purposes.
- To develop methods to analyze immunesuppresants (cyclophosphamide, cyclosporine and dexamethasone) in plasma.
- To test for potential interaction of *Phela* with immunesuppresants (cyclophosphamide, cyclosporine and dexamethasone) *in vitro*.
- To establish an appropriate dose of *Phela* for immune stimulation in rats.
- To develop an animal model to study the mechanism of immunomodulation in rats.
- To study the effect of *Phela* on immune suppressed rats.

3.4. EXPECTED OUTCOME

1. *Phela*'s chromatographic fingerprint for identification.
2. Analytical methods to analyze cyclophosphamide, cyclosporine and dexamethasone and the application of this assay to measure their concentrations in plasma.
3. Understanding the interaction of *Phela* with cyclophosphamide, cyclosporine and dexamethasone *in vitro*.
4. An appropriate dose of *Phela* for immune stimulation in rats.
5. An animal model to study the mechanism of immunomodulation of drugs and/or herbal products on selected immune markers in immune suppressed rats.
6. Knowledge and understanding of *Phela*'s mechanism of immunomodulation, in order to define its appropriate indications and contra-indication and/or potential drug interaction.

4.

PHELA FINGERPRINTING BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH A DIODE ARRAY DETECTOR AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTOR

4.1 SUMMARY

Introduction: Fingerprinting of *Phela* was necessary to ensure consistency in the formulation of the product to be used. It involved using two methods that were previously developed in our laboratory.

Method I: The first method was a high performance liquid chromatography with a diode array detector (HPLC-DAD). *Phela* powder was dissolved in 0.1 M hydrochloric acid after which it was extracted with hexane and evaporated under nitrogen. The residue was reconstituted with mobile phase and 50 µl was injected onto the HPLC where it was eluted with 70 % acetonitrile at a flow rate of 0.5 ml/min for 40 minutes. Separation was done on a C_{18} (250 mm x 4.6 mm x 5 µm) column coupled to a C_{18} guard column and detected at λ245 nm wavelength.

Results: Mark peaks were observed at 10.4±2.2 and 23.4±1.0 minutes and time difference of 13.0±1.2 minutes.

Method II: The second method was a high performance liquid chromatography with fluorescence detector (HPLC-FLD). Alternatively, *Phela* powder was treated with ammonia sulphate and extracted with isopropanol. Thereafter, the sample preparation and HPLC conditions were set as described above. The detector was set at a wavelength of 210 nm for emission and 290 nm for excitation.

Method II results: The four mark peaks (A, B, C and D) were observed at the following retention times (in minutes), 13.0±0.8 for A, 23.0±2.4 for B, 31.2±0.7 for C, 39.2±0.6 for D and the respective time difference of 10.1±3, 18.2±1.0, and 26.2±1.1.

Conclusion: *Phela* fingerprint was confirmed by comparing the current results from both methods with those obtained previously and were similar.

4.2 INTRODUCTION

The aim of this chapter was to apply the methods previously published by Lekhooa (2012b), to fingerprint batches of *Phela* to be used throughout the project. The HPLC methods with a diode array detector (HPLC-DAD) and Fluorescence detector (HPLC-FLD) will be used.

METHOD I: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH A DIODE ARRAY DETECTOR (HPLC-DAD).

4.3 MATERIALS

4.3.1 APPARATUSES

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 vortex mixer (Scientific Industries Inc, U.S.A) was used for mixing. A nitrogen evaporator with a heating block was used for evaporating samples. Finn pipettes from Thermo Lab systems (Canada) were used for spiking volumes and used for adding extraction solvents. A shaker was used for horizontally shaking the samples.

4.3.2 CHEMICALS AND REAGENTS

Phela was manufactured and supplied by the Indigenous Knowledge Systems Lead Programme of the South African Medical Research Council. HPLC graded: hexane, methanol, acetonitrile, (Burdick and Jackson, U.S.A) and hydrochloric acid, (Merck, Germany) were evaluated as extraction solvents. De-ionised water was prepared in our laboratory by Millipore water system (Milli –Q^{Tm}).

4.4 EXPERIMENTAL PROCEDURES

4.4.1 CHROMATOGRAPHIC AND SYSTEM CONDITIONS OF HPLC-DAD METHOD

Chromatographic system: This is an HPLC system consisting of a Hewlett-Packard 1100 series system that comprised of a gradient pump, an auto-sampler, degasser and a built in diode array detector. Data was recorded by 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 the mobile phase, the flow rate was 0.5 ml/min and the run time was 40 minutes. Diode array detector was set at 245 nm.

4.4.2 SAMPLE PREPARATION OF HPLC-DAD METHOD

Phela powder (200 mg) was mixed with 0.1 M hydrochloric acid (100 µl) in 5 replicates, thereafter extracted with 5 ml of hexane, and horizontally shaken for 15 minutes. Thereafter, centrifugation followed at 4 °C and 2 500 rpm (1 251 g) 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-dad for analysis.

4.4.3 DATA ANALYSIS OF HPLC-DAD METHOD

Each mark peak's retention time was noted and the time difference between mark peak 1 and 2 was derived. The mean and standard deviation of each mark peak was calculated using the excel program. Graph Pad© Instat program was used for statistical comparison of test mark peaks to the previous ones.

4.5 RESULTS

4.5.1 CHROMATOGRAPHIC PERFORMANCE OF HPLC-DAD METHOD

Figure 4.1 shows a chromatogram of blank (A) and spiked *Phela* extract (B) with the retention time of mark peak 1 at 12.2, mark peak 2 at 24.4 minutes and time difference of 11.2 minutes. *Phela* extract had sharp and symmetrical peaks indicating, the robustness of the HPLC-DAD method to separate various compounds within the extract.

Figure 4.2 shows a UV spectra of mark peak 1 (A) and 2 (B). The UV spectrum of peak 1 had the highest intensity of 310 mau at 260 nm, whereas peak 2 was 380 mau at 220 nm respectively further confirming the differences of the compounds present within the extract.

Table 4.1 is a summary of mark peaks' retention time shifts recorded as mean and standard deviation. Mark peak 1 at 10.4±2.2, mark peak 2 at 23.4 ±1.0 and mark peak time difference of 13.0±1.2 minutes. The mean retention time was calculated to correct the shift that is commonly observed in traditional medicines fingerprinting. This observation could be due to various reasons such as time of collection and/or different geographical areas.

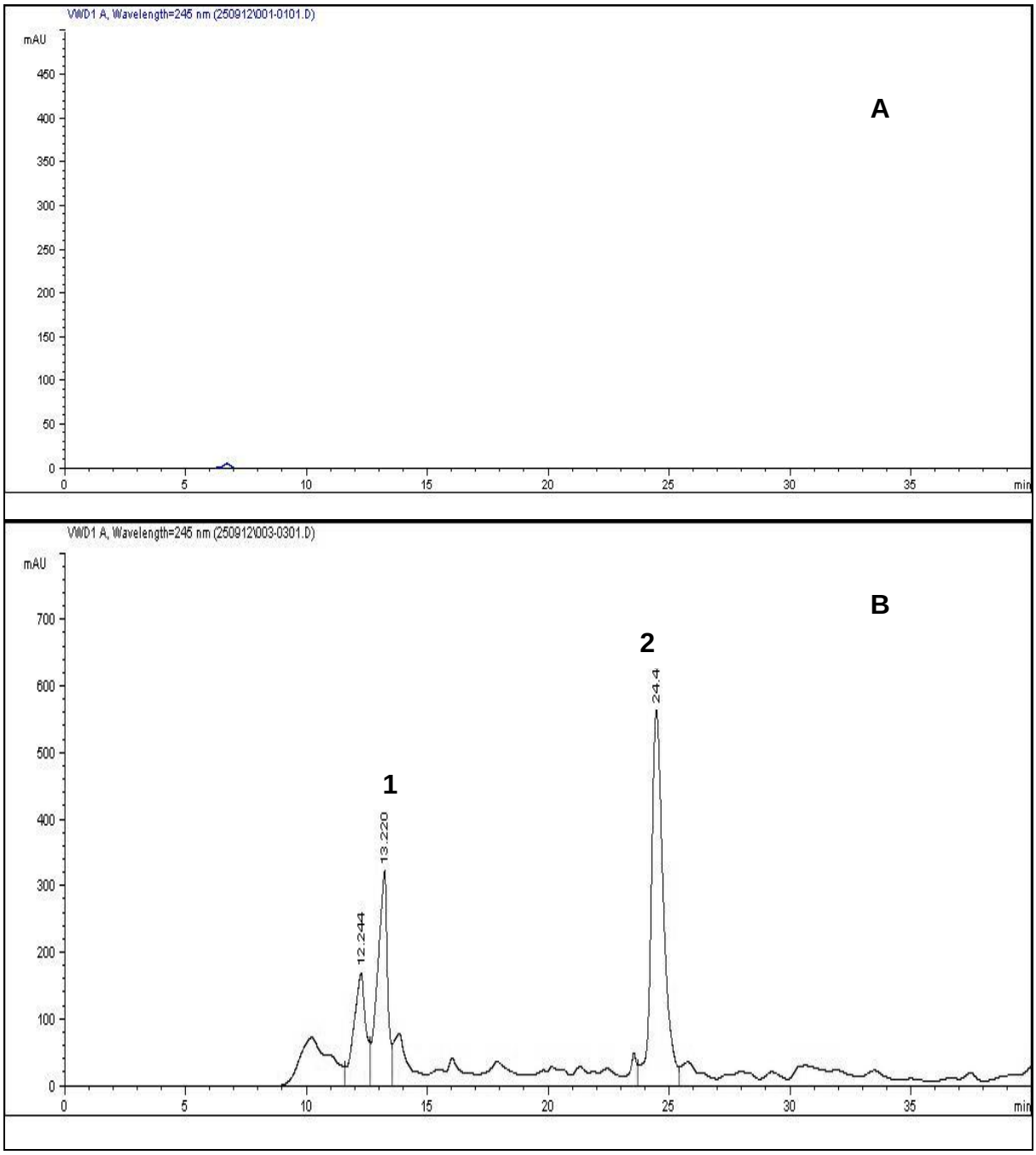


Figure 4.1: Chromatogram of blank (A) and spiked *Phela* extract (B) with mark peak 1 at 13.2 minutes and mark peak 2 at 24.4 minutes.

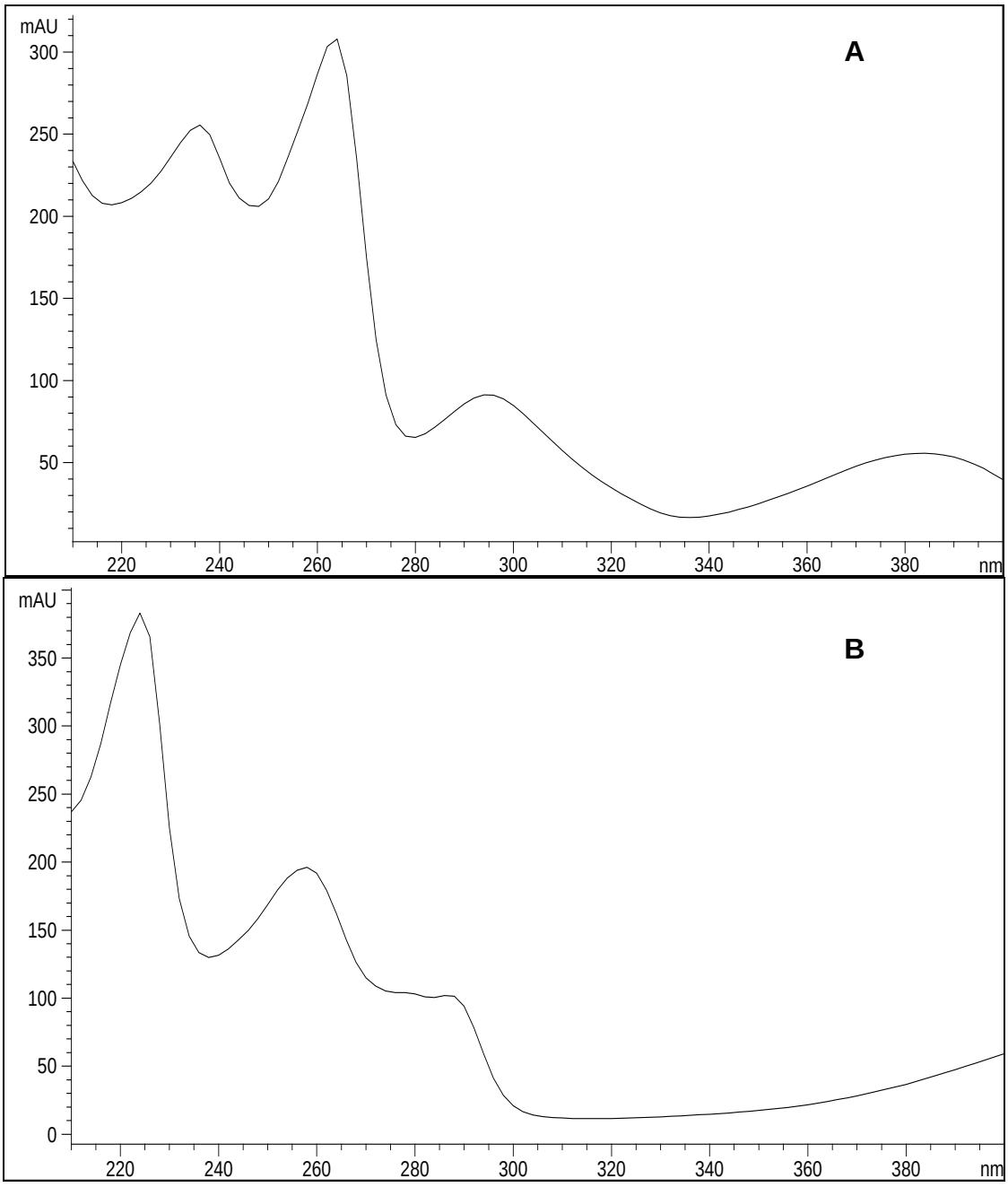


Figure 4.2: UV spectra of marker peak 1 at 13.2 minutes (A) and mark peak at 24.4 minutes (B).

Table 4.1: A summary of statistical analysis of marker peak retention time shifts of spiked *Phela* extract after HPLC-DAD analysis.

Sample	Peak 1	Peak 2	Peak RT difference
1	8.6	22.5	13.9
2	8.9	22.7	13.8
3	13.2	24.5	11.3
4	9.0	22.8	13.8
5	12.2	24.4	12.2
Mean	**10.4**	**23.4**	**13.0**
SD	**2.2**	**1.0**	**1.2**

Abbreviations: Peak RT (Retention time) difference *= Mark peak 2 retention time (-) mark peak 1 retention*

4.5.2 COMPARISON OF HPLC-DAD TEST MARK PEAKS TO PREVIOUS ONES

The results (**Figure 4.3,** chromatogram performance; **Figure 4.4,** UV spectra) described below are comparable with those previously published by Lekhooa *et al.*, 2012b.

Figure 4.3 shows a chromatogram of blank (A) and spiked *Phela* (B) with the retention time of mark peak 1 at 9.8, mark peak 2 at 24.9 minutes and time difference of 15.1 minutes. The two marker peaks were sharp and symmetrical and the retention time is similar to the previously published results, summarized in **Table 4.1.**

Figure 4.4 shows a UV spectra of mark peak 1 (A) and 2 (B) respectively. Peak 1 UV spectrum had the highest intensity of 310 mau at 260 nm whereas peak 2 intensity was 380 mau at 220 nm respectively. Moreover, confirming the similarity to the current UV spectra in **Figure 4.2**.

Furthermore, the mark peak and retention time were also not different. Mark peak 1 at 9.35±0.71 (P = 0.394), and mark peak 2 at 24.5±0.71 (P = 0.098) minutes (Lekhooa *et al*.,

2012b). This observation confirms similarity between the current chromatograms (**Figure 4.1**) and previous ones (**Figure 4.3**).

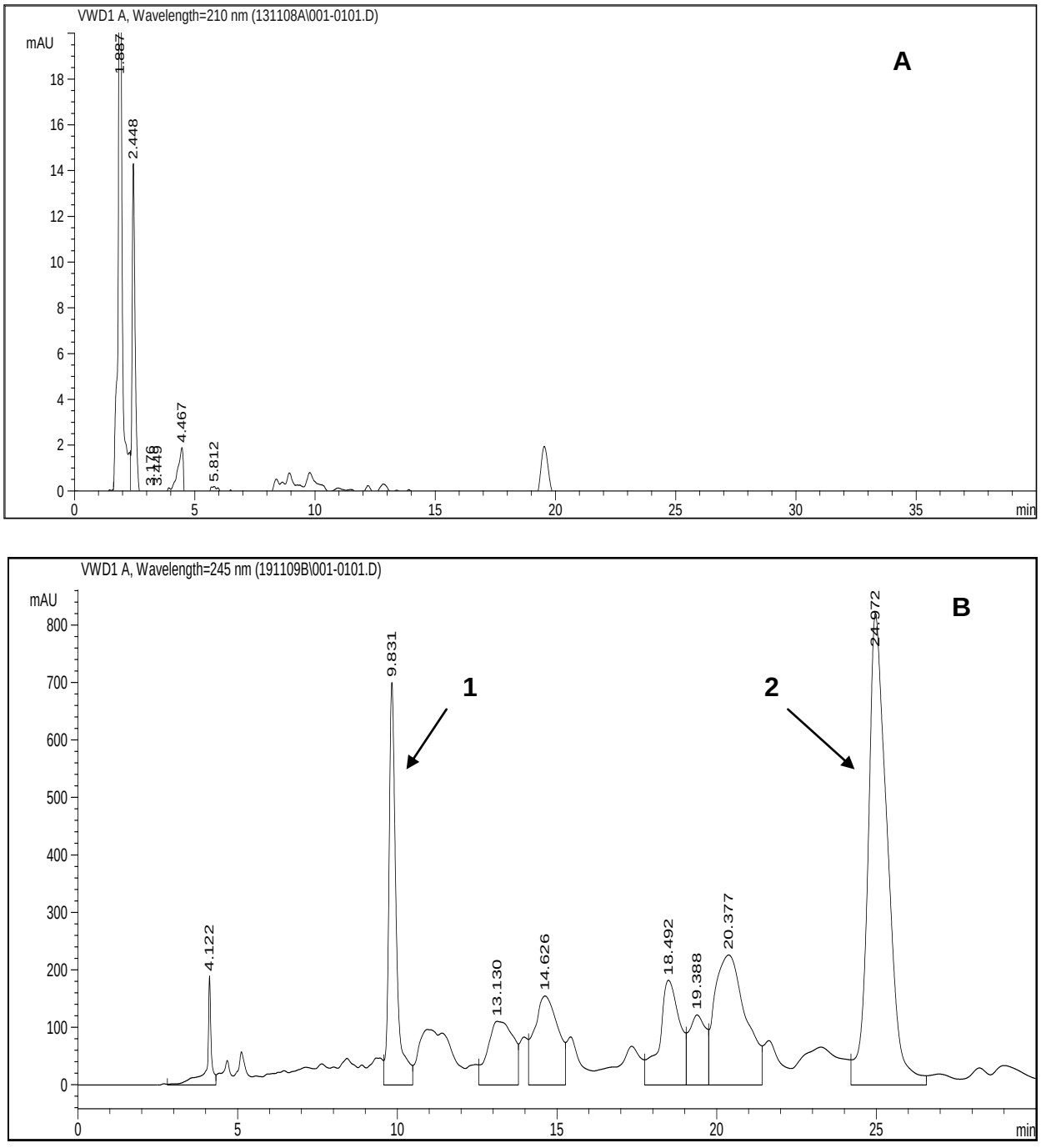


Figure 4.3: Chromatogram of blank (A) and spiked *Phela* (B) with mark peak 1 at 9.8 minutes and mark peak 2 at 24.9 minutes of spiked *Phela.*

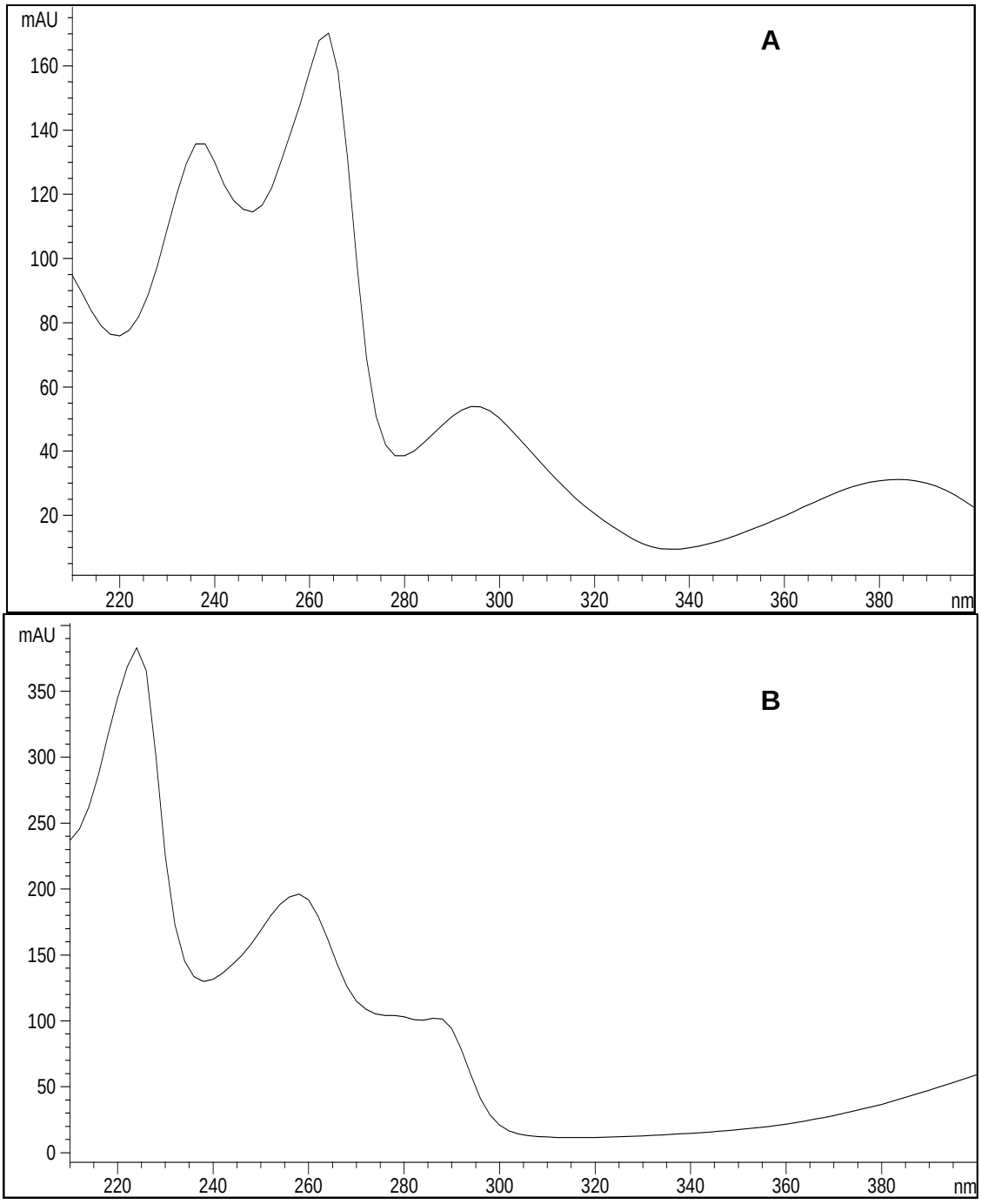


Figure 4.4: UV spectra of marker peak 1 at 9.8 minutes (A) and mark peak at 24.9 minutes (B).

METHOD II: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH A FLUORESCENCE DETECTOR (HPLC-FLD)

4.6 MATERIALS

The apparatuses and reagents used in this part of the study were the same as those used in **Sections 4.2.1 and 4.2.2**. Ammonia sulphate was purchased from Sigma–Aldrich (Germany). HPLC graded Isopropanol was purchased from Merck in Germany.

4.7 EXPERIMENTAL PROCEDURES

4.7.1 CHROMATOGRAPHIC AND SYSTEM CONDITIONS OF HPLC-FLD METHOD

The HPLC system and conditions remained the same as those described in **Section 4.3.1**, and a fluorescence detector was used instead of a diode array detector. Fluorescence detector wavelength was set at 210 nm for emission and excitation set at 290 nm.

4.7.2 SAMPLE PREPARATION OF HPLC-FLD METHOD

Phela powder (100 mg) was treated with 1 g ammonia sulphate and extracted with 3 ml of Isopropanol , then left to shake for 15 minutes, and centrifuged at 4 °C and 2 500 rpm (1 251 g) for 10 minutes. The sample preparation was continued as described in **Section 4.3.2**.

4.7.3 DATA ANALYSIS OF HPLC-FLD METHOD

Data analysis was as described in **Section 4.3.3**. The retention time difference was calculated between mark peaks A+B, A+C, and A+D.

4.8 RESULTS

4.8.1 CHROMATOGRAPHIC PERFORMANCE OF HPLC-FLD METHOD

Figure 4.5 shows a chromatogram of blank (A) and spiked *Phela* (B) with the retention time of mark peak A at 11.9, mark peak B at 25.7, mark peak C at 30.5, mark peak D at 39.0 minutes.

The respective time difference between mark peaks A+B, A+C, A+D was 13.8, 18.9 and 27.1 minutes.

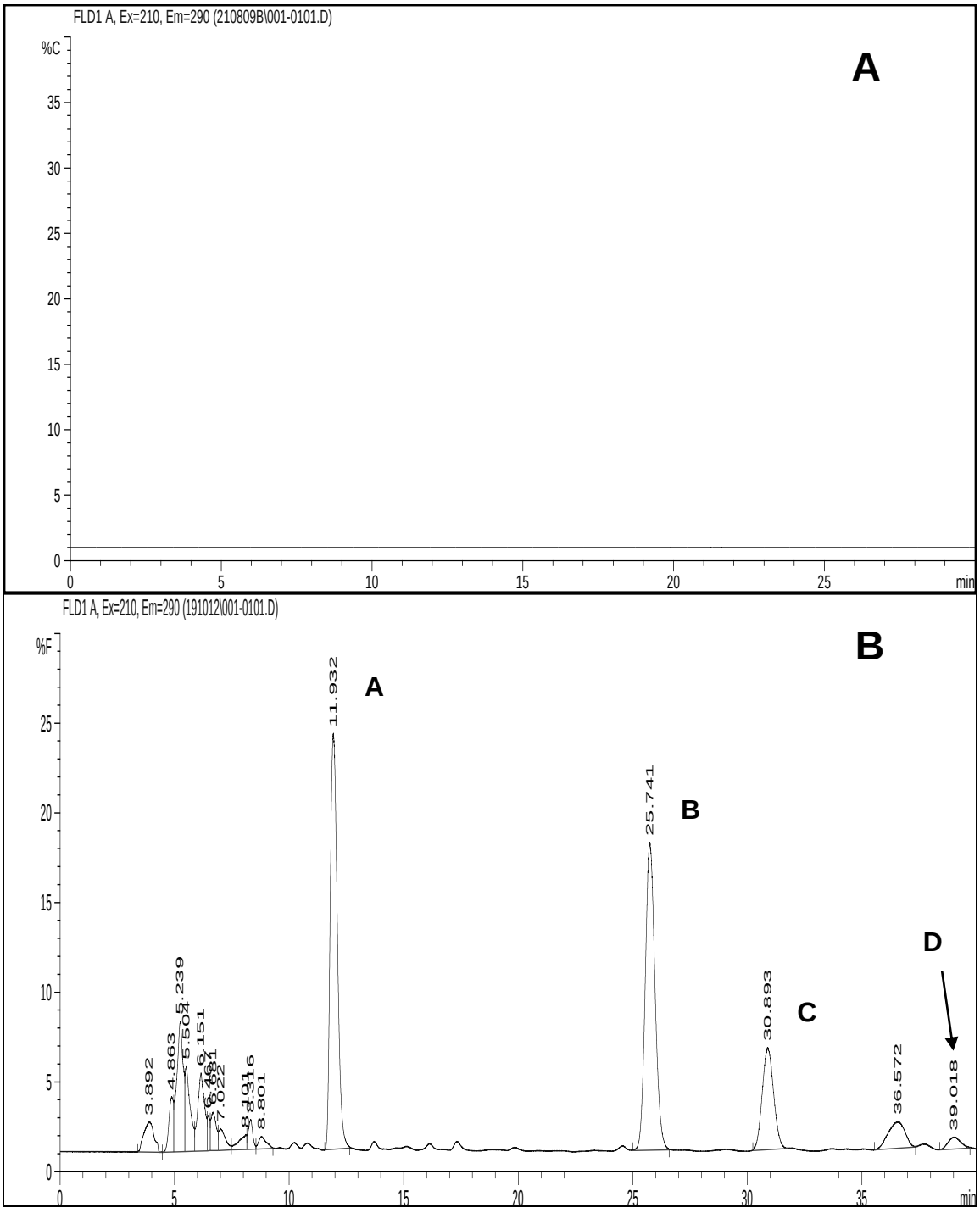


Figure 4.5: Chromatogram of Blank (A) and spiked *Phela* (B) with mark peak A at 11.9, mark peak B at 25.7, marker peak C at 30.8 and mark peak D at 39.0 minutes.

Table 4.2 is a summary of mark peaks' retention time shifts recorded as mean and standard deviation. Mark peak A at 13.0±0.8, mark peak B at 23.0±2.4, mark peak C at 31.2±0.7, mark peak D at 39.2±0.6 minutes. The respective time difference between mark peaks A+B, A+C, A+D were 10.1±3.0, 18.2±1.0, 26.2±1.1 minutes.

Table 4.2: A summary of statistical analysis of marker peak retention time shifts after HPLC-FLD analysis.

Runs	**Marker peaks retention time (min)**				**Peaks RT difference (min)**		
	A	**B**	**C**	**D**	**A & B**	**A & C**	**A & D**
1	11.9	25.7	30.8	39.0	13.8	18.9	27.1
2	13.7	19.6	31.2	38.5	5.9	17.5	24.8
3	13.3	24.5	32.0	39.5	11.2	18.7	26.2
4	12.5	23.4	31.8	40.1	10.9	19.3	27.6
5	13.5	22.0	30.3	39.0	8.5	16.8	25.5
Mean	**13.0**	**23.0**	**31.2**	**39.2**	**10.1**	**18.2**	**26.2**
SD	**0.8**	**2.4**	**0.7**	**0.6**	**3.0**	**1.0**	**1.1**

Abbreviations: Peak RT (Retention time) difference *= Mark peak b retention time - mark peak a retention time.* ***Min*** *= Minutes,* ***SD*** *= Standard deviation*.

4.8.2 COMPARISON OF HPLC-FLD TEST MARK PEAKS WITH PREVIOUS ONES

The results (**Figure 4.6**, chromatogram performance) described below are comparable with those previously published by Lekhooa *et al.*, 2012b.

Figure 4.6 is a chromatogram of blank (A) and spiked *Phela* (B) with the retention time of mark peak A at 12.7, mark peak B at 22.8, mark peak C at 32.3, mark peak D at 38.0 minutes. The respective time difference between mark peaks A+B, A+C, A+D was 13.8, 18.6 and 24.2 minutes.

Furthermore, the mark peaks and retention time were also not different. Mark peak A at 12.34 ± 0.36 (P = 0.122), mark peak B at 22.3 ± 1.18 (P = 0.569), mark peak C at 32.96 ± 0.88 (P = 0.027), and mark peak D at 38.32 ± 0.88 (P = 0.121) minutes. Mark peak C's retention time (P = 0.027) was statistically significant when compared with the previous *Phela* fingerprint (Lekhooa *et al*., 2012b).

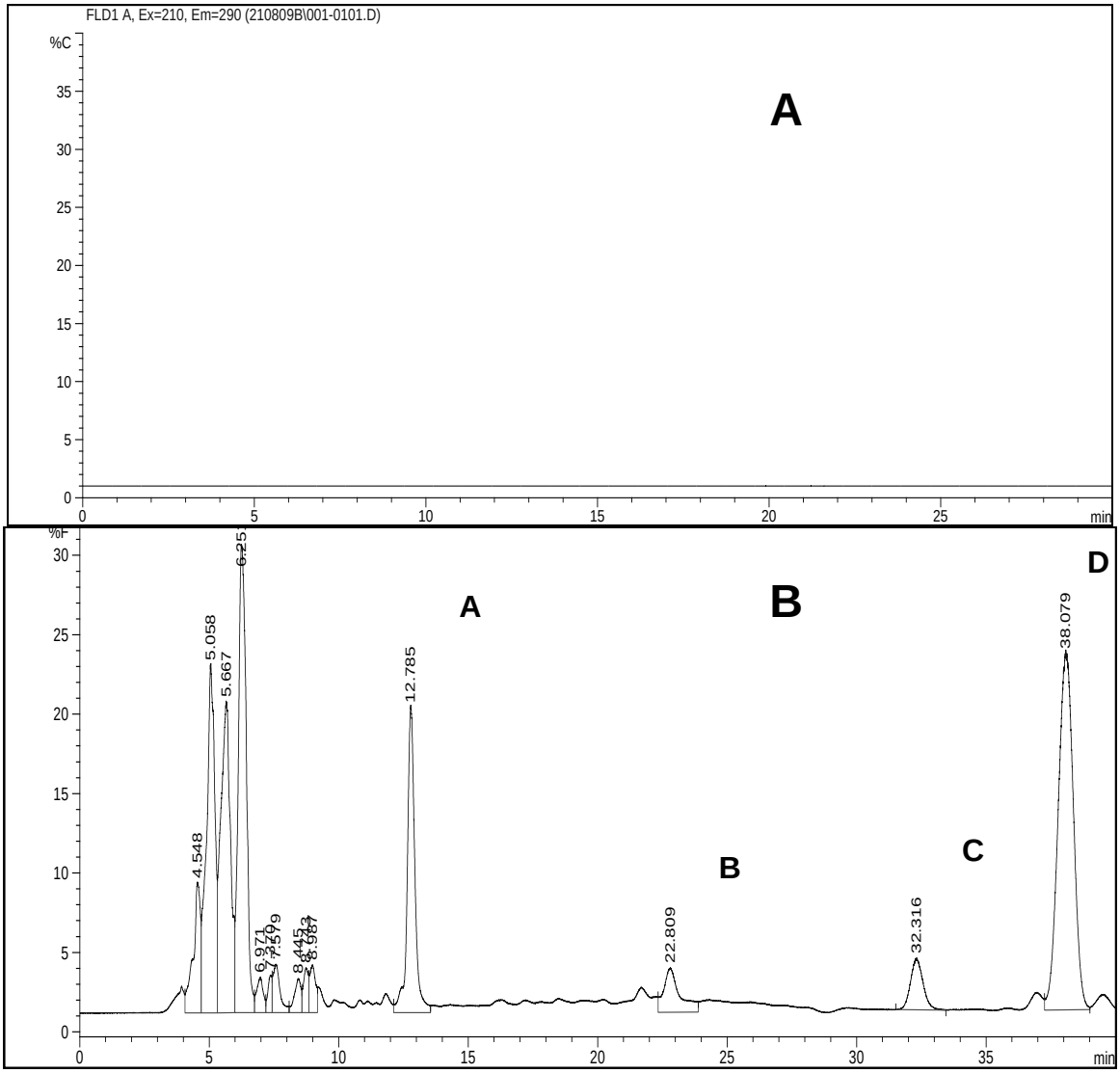


Figure 4.6: Chromatogram of Blank (A) and spiked *Phela* (B) with mark peak A at 12.7, mark peak B at 22.8, marker peak C at 32.3 and mark peak D at 38.0 minutes.

4.9 DISCUSSION

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; Fan *et al*., 2006). Moreover, 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. It is the most reliable and applicable authentication and identification method based on chemical and chromatographic techniques (Smillie, 2010).

In this study, two previously developed HPLC-DAD and HPLC-FLD methods were applied to the batches of *Phela* powder for quality control purposes (Lekhooa *et al*., 2012b). 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. 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 *et al*., 2005).

The results indicated a shift in mark peaks of spiked *Phela* chromatograms in comparison with previous fingerprints, though it was not statistically significant. The observation confirmed a statement by Liang (2010) and his colleagues that retention time shifts are common during traditional medicine analysis. Furthermore, they are caused by various factors during plant collection such as the season, geographic location and/or some instrumental complex factors such as deterioration of stationary phase. Fingerprinting of *Phela* for identification and quality control purposes was necessary to ensure consistency in the formulation before an *in vivo* experiment.

4.10 CONCLUSION

HPLC-DAD and HPLC-FLD methods were successfully applied to fingerprint crude *Phela*.

5.

SIMULTANEOUS DETERMINATION OF CYCLOPHOSPHAMIDE AND DEXAMETHASONE PLASMA CONCENTRATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH UV WAVELENGTH SWITCHING

Presented at the 10th International society of the study of xenobiotics conference (2013)

5.1. SUMMARY

Introduction: A high performance liquid chromatography (HPLC) with a UV detector assay for the simultaneous determination of cyclophosphamide and dexamethasone in rat plasma was developed.

Methodology: Plasma spiked with cyclophosphamide, dexamethasone and surprofen (internal standard), was extracted with ethyl acetate, centrifuged and the supernatant evaporated under nitrogen. The residue was reconstituted with mobile phase and 50 µl was injected into HPLC system. The analytes were eluted with 45 % of acetonitrile in 0.05 M sodium phosphate buffer (pH 3.7; containing 0.5 ml triethylamine) at a flow rate of 1 ml/min for 10 minutes. Separation was achieved on a C_{18} (250 mm x 4.6 mm x 5 µm) column coupled to a guard column and UV detection was at λ190 nm for cyclophosphamide and λ 240 nm for dexamethasone and internal standard. Linearity, accuracy and stability were evaluated.

Results: Retention time for cyclophosphamide, dexamethasone and internal standard were at 4.2, 5.7 and 8.1 minutes respectively. Linear regression and correlation coefficient were $y = 0.04x + 0.11$ and 0.999 for cyclophosphamide, and $y = 0.32x – 1.52$ and 0.998 for dexamethasone, with their respective recoveries of 102 – 108 % and 99 – 107 %. The method was successfully used to analyse cyclophosphamide (24 µg/ml) and dexamethasone (15 µg/ml) concentrations in plasma from treated rats after 24 hours of treatment.

Conclusion: A simple HPLC-UV method for simultaneous analysis of cyclophosphamide and dexamethasone was developed and successfully applied.

5.2. INTRODUCTION

The aim of this study was to develop a HPLC-UV method for the simultaneous determination of cyclophosphamide and dexamethasone.

Decision: In the objectives (**Chapter 3**) was to include cyclosporine during the above mentioned method development, however the UV detector was not sensitive enough for CsA concentration analysis. Hence, the toxicology laboratory that does therapeutic monitoring of CsA in whole blood using chemiluminescent immuno assay (CMIA) kits was used for CsA analysis.

5.3. METHODS

5.3.1. MATERIALS

5.3.1.1. Apparatuses

A vortex mixer (Scientific industries Inc, U.S.A) was used to mix the samples. A 5810R centrifuge and mini spin (Eppendorf, Germany) was used for separation. Precision balances, SBC 31 and SBP 52 (Scaltec Instruments, Germany) were used to weigh standard drugs and chemicals in milligram and gram quantities, respectively. The supernatant was dried with a nitrogen evaporator with a build-in heating block. Columns used were sphereclone C_{18} (250mm x 4.6µm x 5µm), Luna C_{18} (250 mm x 4.6µm x 5 µm) and Luna C_{18} (150 mm x 4.6 µm x 5 µm) purchased from Phenomenex.

5.3.1.2. Chemicals and reagents

Cyclophosphamide and dexamethasone standards were purchased from Sigma Aldrich® (Germany). HPLC graded acetonitrile, dichloromethane, diethyl ether, ethyl acetate, triethylamine, methanol from Burdick and Jackson (U.S.A) and hydrochloric acid (Merck, Germany) were used for sample extraction. Pure substances donated by the toxicology unit in the department of pharmacology were evaluated as internal standards: paracetamol, flurbiprofen, sulfinpyrazone, sulphamethizole, sudenox, surprofen, sulfafurazole, sulfamoxole, pyrazinamide, sulfapyridine, rifampicin, sulfadoxine and tolmedine. Deionised and distilled water was prepared in our laboratory by the millipore water system. (Milli-Q^{Tm}). Drug-free plasma from healthy volunteers was a generous donation from the toxicology laboratory.

5.3.2. CHROMATOGRAPHIC SYSTEM

The HPLC system used in this research consists of a Hewlett-Packard 1100 series with a gradient pump, auto-sampler, de-gasser and a UV detector set at various wavelengths. A computer with LC-Chemstation software was used to record the data.

5.3.3. STOCK SOLUTIONS PREPARATION

A stock solution of 1 mg/ml in methanol of cyclophosphamide and dexamethasone was prepared. From the stock solution, working solutions were prepared by further diluting with mobile phase to 100 µg/ml of each drug. The working solutions were used for spiking plasma.

5.3.4. UV-SPECTRA DETERMINATION

Cyclophosphamide and dexamethasone were prepared in methanol. Methanol was used as blank during UV-vis spectra analysis. A UV-vis spectra for cyclophosphamide and dexamethasone in methanol was run from 200 – 900 nm.

Observation: **Figure 5.1** is a blank UV-spectrum of methanol. **Figure 5.2** is a UV-spectra of cyclophosphamide and dexamethasone respectively. Cyclophosphamide wasn't detectable, while dexamethasone's response peaked at 240 nm.

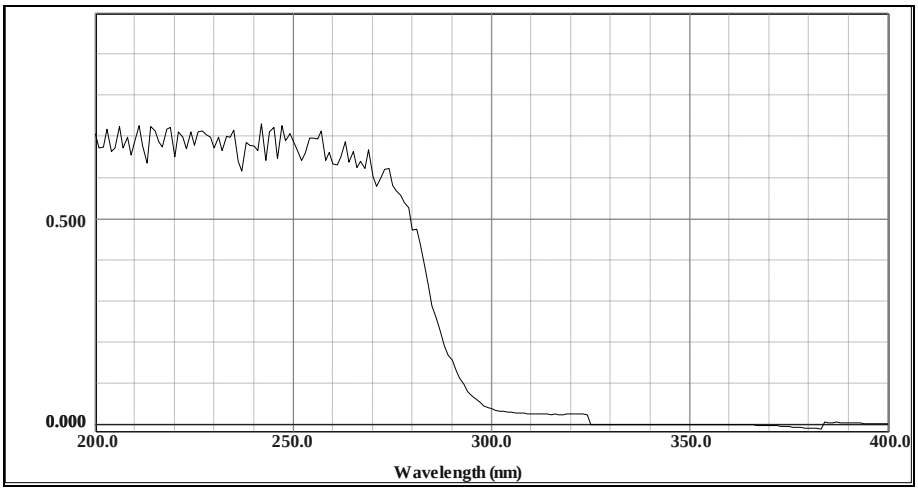
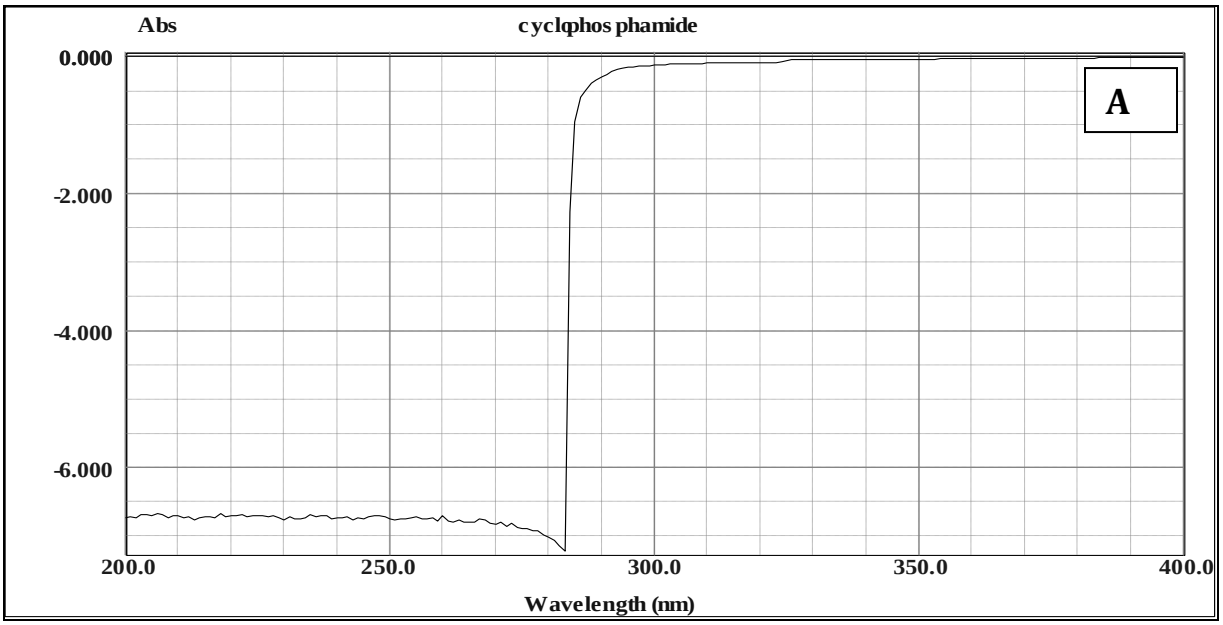


Figure 5.1: A blank methanol UV-spectrum



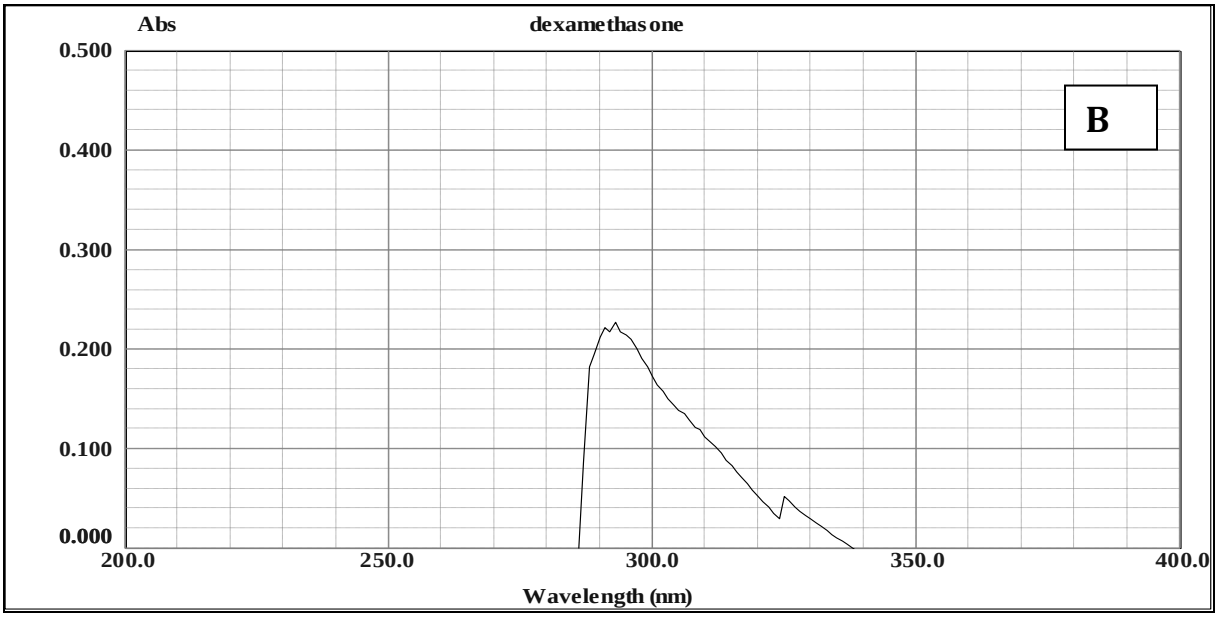


Figure 5.2: A UV spectra of Cyclophosphamide (A) and Dexamethasone (B).

5.3.5. PRELIMINARY EXPERIMENTS

Chromatographic conditions and sample extraction procedure were determined in preliminary experiments as indicated below.

5.3.5.1. Preliminary HPLC conditions

The initial chromatographic conditions were a reverse phase C_{18} (250 mm x 4.6 µm x 5 µm) column with a guard column. Mobile phase was 35 % acetonitrile in 65 % sodium phosphate buffer with 0.5 ml/L of triethylamine at pH of 3.7. The flow rate was 0.65 ml/min and wavelength was set at 198 nm (Ahmad *et al.,* 2011).

Observation: Dexamethasone did not elute at this wavelength only cyclophosphamide did.

Decision: This observation propelled a further investigation on the appropriate wavelength.

5.3.5.2. Wavelength selection

Each drug was run on the HPLC-UV for 15 minutes set at one of the wavelengths (i.e. 190, 197, 198, 210, 240 and 293 nm) at a time. Thereafter, the wavelength switch schedule was set at:

- 240 nm (0 – 3.0 min), 197 nm (3.1 – 5.4 min) and 240 nm (5.3 – 15.0 min).
- 240 nm (0 – 4.5 min), 197 nm (4.6 – 8.0 min) and 240 nm (8.5 – 15.0 min).

Observation: The highest peak response for cyclophosphamide and dexamethasone was observed at 190 and 240 nm respectively (**Appendix 1.2 – 1.5**). Wavelength selection was based on comparison of peak area response versus specific wavelength.

Decision: The following wavelength switch setting: 240 nm (0 – 4.5 min), 197 nm (4.6 – 8.0 min) and 240 nm (8.5 – 15.0 min) was selected for further analysis.

5.3.5.3. Column selection

Three reverse phase columns attached to a guard column were evaluated for optimum analyte separation:

- sphereclone C_{18} (250 mm x 4.6 µm x 5 µm),
- Luna C_{18} (250 mm x 4.6 µm x 5 µm)
- Luna C_{18} (150 mm x 4.6 µm x 5 µm).

Observation: Analytes eluted early after separation on sphereclone C_{18} (250 mm x 4.6 µm x 5 µm) and Luna C_{18} (150 mm x 4.6 µm x 5 µm) columns.

Decision: Luna C_{18} (250 mm x 4.6 µm x 5 µm) achieved optimum analyte separation confirmed by sharp symmetrical peaks and it was selected for further analysis.

5.3.5.4. Flow rate selection

The flow rate was started at 0.65 ml/min and increased to 1 ml/min.

Observation: Analytes eluted early at 0.65 ml/min flow rate. **(Appendix 1.6)**

Decision: The flow rate of 1ml/min was selected for further analysis.

5.3.5.5. Mobile phase selection

The initial mobile phase was 35 % acetonitrile in 65 % sodium phosphate buffer with 0.5 ml/L of triethylamine at pH of 3.7. Acetonitrile percentage was increased to 40 and 45 % to improve peaks' shape, and retention times.

Observation: Peaks overlapped with 35 and 40 % acetonitrile in the mobile phase **(Appendix 1.6).**

Decision: The final mobile phase was 45 % acetonitrile in 55 % sodium phosphate buffer with 0.5 ml/L of triethylamine at pH of 3.7.

5.3.5.6. Sample extraction

Plasma was spiked with either cyclophosphamide or dexamethasone working solution. For cyclophosphamide the final concentration was 20 µg/ml. For dexamethasone the final concentration was 10 µg/ml. Spiked plasma (200 µl) was aliquoted into test tubes, prepared in duplicate and extracted under different conditions (protein precipitation,

liquid liquid extraction, pH-based extraction) explained below. Centrifugation followed extraction at 14 300 rpm (7 156 g) for 15 min and supernatant was evaporated under nitrogen at 47°C. The residue was reconstituted with 150 µl of mobile phase, and 50 µl was injected on the HPLC.

Protein precipitation procedure: Spiked plasma was mixed with 0.1 M perchloric acid (100 µl) to precipitate proteins and further analysed as described above.

Liquid liquid extraction (LLE): Five organic solvents were tested as extraction solvents. To spiked plasma, 1 ml of solvents listed below was added and further analysed as described above.

- Ethyl acetate
- Diethyl ether
- Dichloromethane (DCM)
- DCM: diethyl ether (2:1)
- Ethyl acetate: diethyl ether (3:2).

PH-based extraction: Addition of either 0.1 M hydrochloric acid (20 µl) or 1 M sodium hydroxide (20 µl) to test samples tested pH effect on analyte extraction.

Observation: Chromatograms obtained from protein precipitation, some LLE (diethyl ether, DCM: diethyl ether) and pH-based extractions had multiple peaks making it impossible to identify the drugs of interest. No peaks were observed with dichloromethane extraction **(Appendix 1.7 -1.9).**
Decision: Ethyl acetate was the best solvent and was used further.

5.3.5.7. Internal standard selection

A search for an appropriate internal standard (IS) was done by evaluating the following pure drug standards: acetanilide, paracetamol, flurbiprofen, sulfinpyrazone, sulphamethizole, sudenox, surprofen, sulfafurazole, sulfamoxole, pyrazinamide, sulfapyridine, rifampicin, sulfadoxine and tolmedine. Each pure standard was dissolved in methanol to a concentration of 100 µg/ml. Furthermore, each standard was diluted to 25 µg/ml in mobile phase, and analysed under the same conditions described above in **Chapter 5**, **Section 5.3.5.6.**

Observation: The retention times of standard evaluated as internal standards were as follows: (minutes); sulphamethizole (3.4), sulfamoxole (3.3), sulfapyridine (3.1) and sulfadoxine (4.0) eluted before 4 minutes and interfered with plasma peaks.

Decision: Surprofen had a retention time of 9.7 minutes and was an ideal internal standard.

5.3.5.8. Concentration range and total sample volume determination

Ten calibration standards ranging from 1 – 50 µg/ml per drug were evaluated to establish a calibration range. Furthermore, three volumes 100, 200 and 1000 µl were evaluated for total sample volume.

Observation: The sample volume of 200 and 1000 µl yielded similar results.

Decision: Thus, 200 µl was selected as the total sample volume. The calibration range for cyclophosphamide was 10 – 35 µg/ml and dexamethasone was 5 - 30 µg/ml.

5.3.6. FINAL METHOD CONDITIONS

5.3.6.1. Sample extraction

Plasma (200 µl) spiked with cyclophosphamide and dexamethasone and surprofen as the internal standard was extracted with ethyl acetate. Centrifugation was 10 min long at 6 705 g and the supernatant was evaporated under nitrogen at 47 °C. The residue was reconstituted with 100 µl of mobile phase and 50 µl was injected on the HPLC.

5.3.6.2. HPLC conditions

The analytes were eluted with 45 % of acetonitrile in 0.05 M sodium phosphate buffer (pH 3.7; containing 0.5 ml triethylamine) at a flow rate of 1 ml/min and 10 minutes runtime. Separation was achieved on a RP-Luna C_{18} (250 mm x 4.6 mm x 5 µm) column coupled to a guard column. The UV wavelength was set at 190 nm for cyclophosphamide and switched to 240 nm for dexamethasone and internal standard detection.

5.3.7. METHOD VALIDATION

The finalized conditions in **Section 5.3.6** were validated by linearity, accuracy and stability evaluation of cyclophosphamide and dexamethasone.

5.3.7.1. Linearity

Linearity was evaluated daily over five days with a 6 point calibration curve for each drug. Cyclophosphamide calibration working range was 10 - 35 µg/ml while dexamethasone calibration working range was 5 – 30 µg/ml. The ratio of each calibration standard was calculated by using the height of each drug peak over the height of the internal standard peak. A calibration curve was plotted with peak ratio on the y-axis versus concentration on the x-axis. The mean calibration standards' ratio, 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 ($y = mx + c$).

5.3.7.2. Accuracy/recovery

Low, medium and high concentration of each drug (CP: 10, 25, 35 µg/ml; Dex: 5, 20, 30 µg/ml) were selected and prepared as described **Chapter 5**, **Section 5.3.6** for accuracy evaluation. Blank plasma samples (n = 5) were similarly prepared and used as quality control standards. The calibration curve was used to derive concentration from observed ratios. The derived concentration was converted to recovery percentage. Recovery percentage (%) was calculated as derived concentration divided by known

concentration multiplied by 100 %. The mean (concentration and recovery percentage), standard deviation (SD) and CV % were also calculated for all concentrations.

5.3.7.3. Stability

Different temperature settings were used to evaluate stability of each drug over the short-term and long–term. Three calibration standards, low, medium and high concentration (CP: 10, 25, 35 µg/ml; Dex: 5, 20, 30 µg/ml) were analysed per drug. Duplicate samples per standard were left at either ±25 °C (room temperature), or 4 °C (fridge), and analysed after 24 and 48 hours respectively for short term stability. Similarly, samples were left at -20 °C (freezer) and -80°C (ultra freezer) were analysed after 1 and 4 weeks for long term stability evaluation.

5.3.8. DETERMINATION OF CYCLOPHOSPHAMIDE AND DEXAMETHASONE CONCENTRATION IN PLASMA FROM TREATED RATS

Rat plasma from the animal study (see **chapter 9**) was analysed with this method. The study was approved (ETOVS NR 11/2012) by the Animals Ethics Committee of the University of the Free State. Animals were administered separately, either cyclophosphamide (100 mg/kg) or dexamethasone (1.5 mg/kg), intraperitoneally, and blood was drawn after 24 hours.

5.4. RESULTS

5.4.1. GENERAL

5.4.1.1. Chromatographic performance

Figure 5.3 shows chromatograms analyzed by the final method of blank plasma (A), and spiked plasma (B) with cyclophosphamide and dexamethasone at a retention time of 4.2 and 5.7 minutes and internal standard at 8.1 minutes. The peaks were sharp and symmetrical.

5.4.2. METHOD VALIDATION

5.4.2.1. Cyclophosphamide

5.4.2.1.1. Linearity

Table 5.1 Indicates summarized data of the five days calibration curves of cyclophosphamide. Coefficient of variance percentage (CV %) was less than 15 %.

Figure 5.4 is a plot of the average 5-day calibration curve of cyclophosphamide. Cyclophosphamide linear regression and correlation coefficient was y = 0.0478x + 0.0108 and 0.9969 respectively.

5.4.2.1.2. Accuracy

Table 5.2 provides a summarized data of accuracy for all three cyclophosphamide concentrations. Mean cyclophosphamide accuracy percentages observed were 102±11 (CV % = 10.55), 104±9 (CV % = 9.08) and 108±17 (CV % =15.47) for the 10, 25 and 35 µg/ml, respectively.

5.4.2.1.3. Stability

Figure 5.5 are plots of cyclophosphamide recovery percentage vs time evaluating short term stability of 10, 25 and 35 µg/ml after 24 and 48 hours at ±25 °C (room temperature) and at 4 °C (fridge). The lower concentration (10 µg/ml) was stable at 4°C after 24 (118 %) and 48 (109 %) hours. Other concentrations were stable at ±25 °C and 4 °C for 24 and 48 hours.

Figure 5.6 is plots of cyclophosphamide stability recovery percentage vs time in a fridge (4 °C), freezer (-20 °C) and ultra freezer (-80 °C) after 1 and 4 weeks respectively. Cyclophosphamide was unstable in all temperature settings after 1 and 4 weeks with recovery percentages ranging from 30 - 77 %.

5.4.2.2. Cyclophosphamide concentration in rat plasma

Figure 5.7 is a chromatogram of plasma from cyclophosphamide treated rats after 24 hours. The peak was sharp and symmetrical. Cyclophosphamide concentration was 20 µg/ml.

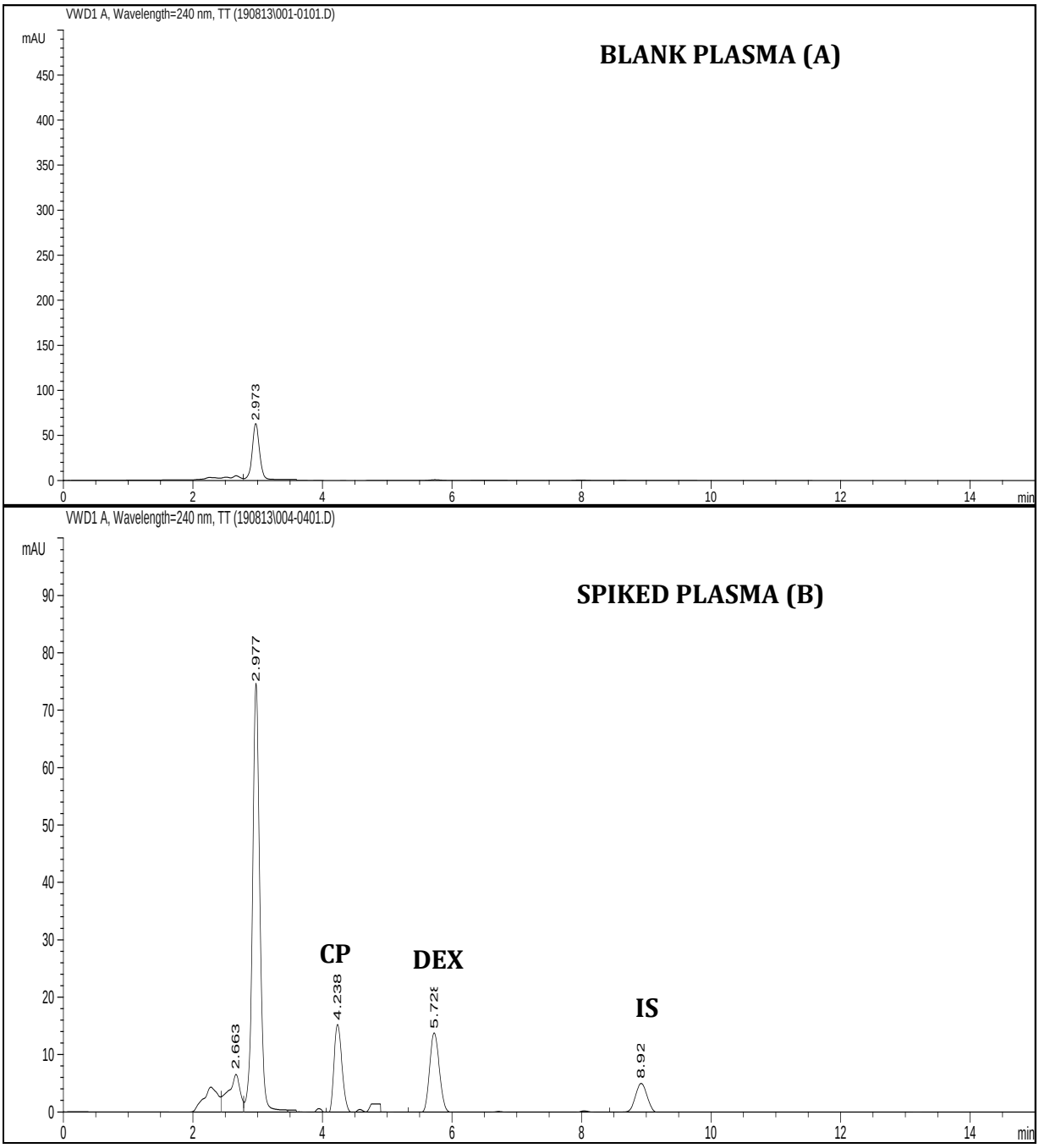


Figure 5.3: A chromatogram of blank plasma (A) and spiked plasma (B) with CP at 4.2 min, Dex at 5.7 min and IS at 8.9 min.
Abrreviations*: CP = cyclophosphamide, Dex = dexamethasone, IS = Internal standard*

Table 5.1: Peak ratios of cyclophosphamide calibration standards over 5 days, with mean, standard deviation and coefficient of variance.

Conc (ug/ml)	DAY1	DAY2	DAY3	DAY4	DAY5	MEAN ±SD	CV%
10	0.57	0.45	0.47	0.55	0.60	**0.53±0.06**	**11.91**
15	0.79	0.68	0.63	0.67	0.75	**0.70±0.07**	**9.28**
20	1.13	0.84	0.84	0.99	1.05	**0.97±0.13**	**13.22**
25	1.32	1.04	1.14	1.09	1.24	**1.17±0.11**	**9.73**
30	1.65	1.33	1.42	1.51	1.53	**1.49±0.12**	**8.07**
35	1.76	1.64	1.53	1.89	1.52	**1.67±0.16**	**9.52**

Abbreviations: CP = cyclophosphamide **Mean =** mean value of values, **SD** = standard deviation, **CV %** = coefficient of variance, **Conc =** concentration

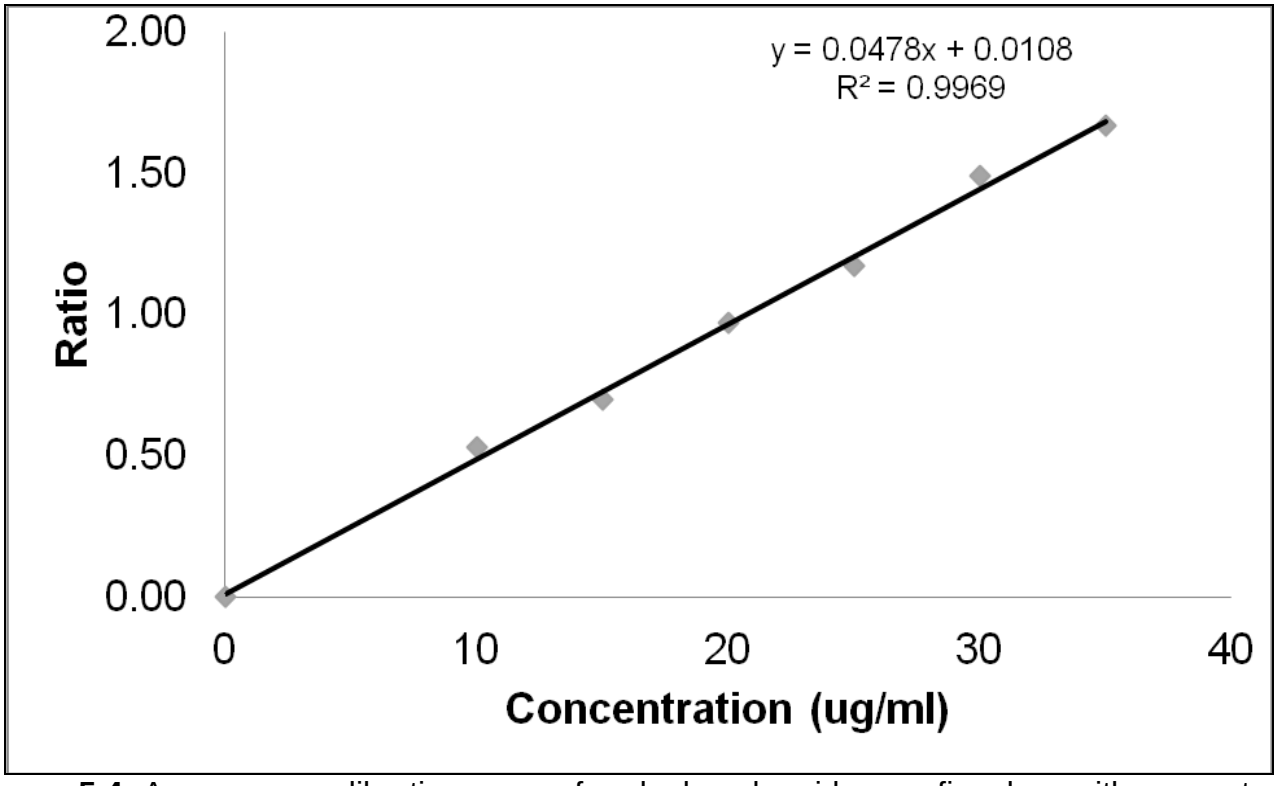
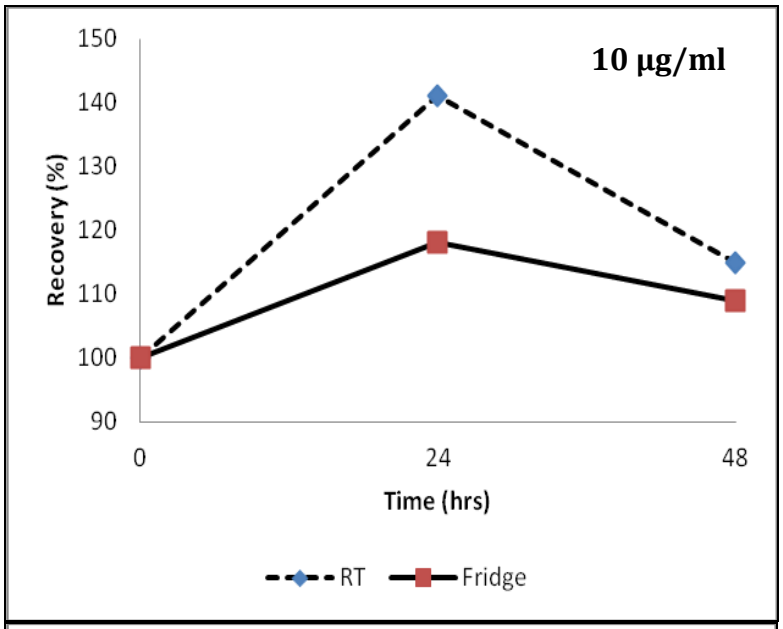


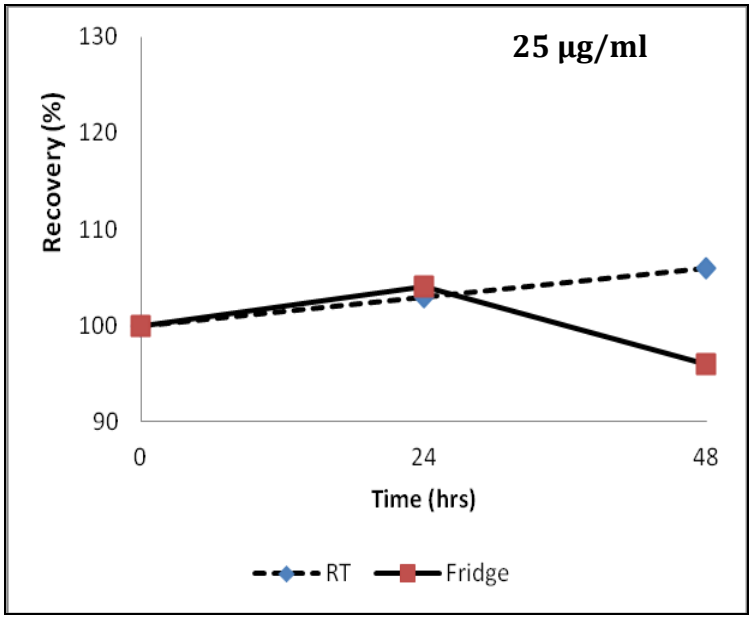
Figure 5.4: An average calibration curve of cyclophosphamide over five days with concentration versus peak ratio and correlation line of y = 0.0478 x + 0.0108 and r^2 = 0.9969

Table 5.2: Summary of cyclophosphamide accuracy testing results; recovered concentration (µg/ml) and percentages (%) of 10, 25 and 35 µg/ml are reported. The mean, standard deviation and CV% are also shown.

Concentration	**10 µg/ml**		**25 µg/ml**		**35 µg/ml**	
Sample	**Conc (µg/ml)**	**Recovery %**	**Conc (µg/ml)**	**Recovery %**	**Conc (µg/ml)**	**Recovery %**
A	10.09	100	24.86	99	40.79	117
B	9.33	93	22.92	92	41.18	118
C	10.60	106	25.25	101	40.79	117
D	9.14	91	28.23	113	27.58	79
E	11.79	118	28.36	113	38.46	110
Mean±SD	**10.2±1.1**	**102±11**	**25.9±2.3**	**104±9**	**37.8±6.0**	**108±17**
CV%	**10.55**		**9.08**		**15.47**	

Abbreviations: Conc = concentration, **CV %** = coefficient of variance.





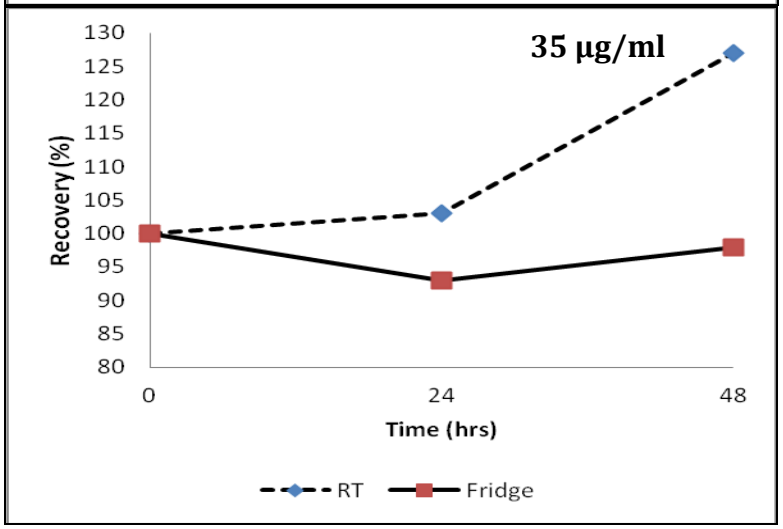


Figure 5.5: Plots showing cyclophosphamide (10, 25 and 35 µg/ml) short-term stability at room temperature (±25 °C) and fridge (4 °C) for 24 and 48 hours

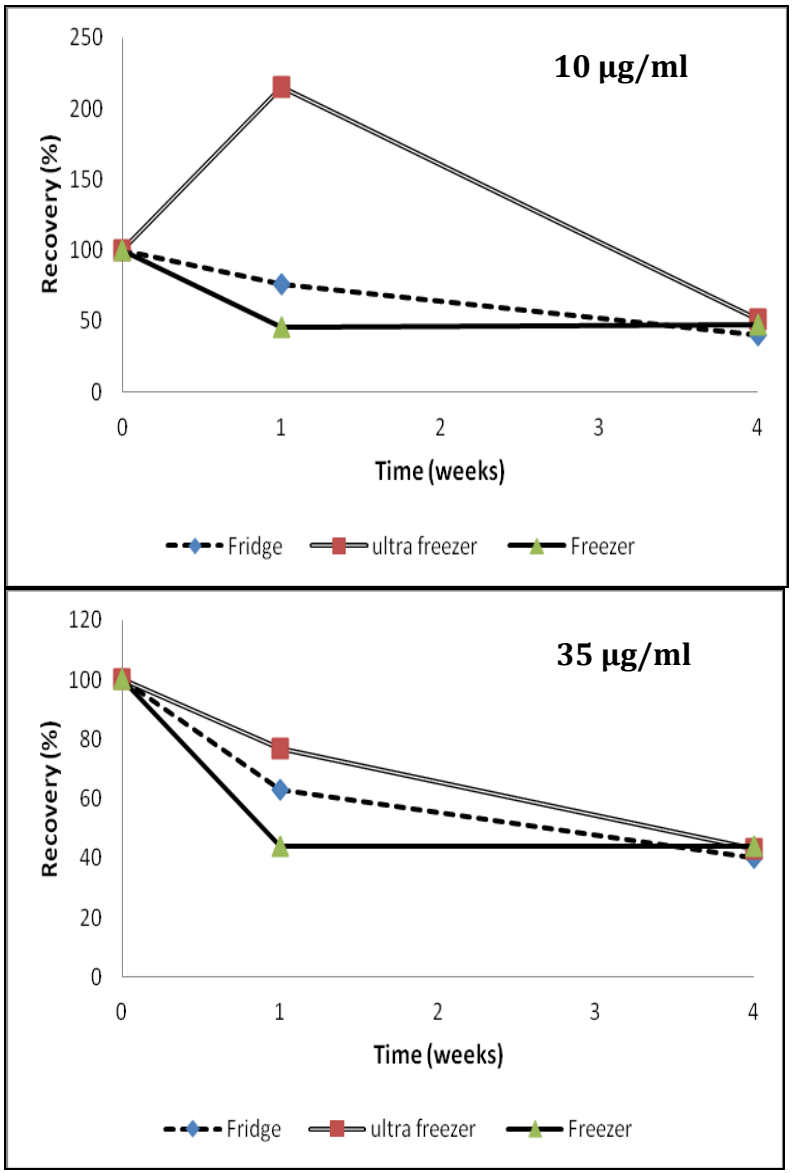


Figure 5.6: Plots showing cyclophosphamide (10, 25, and 35 µg/ml) long-term stability left in the fridge (4 °C), freezer (-20 °C) and ultra freezer (- 80 °C) for 1 and 4 weeks respectively.

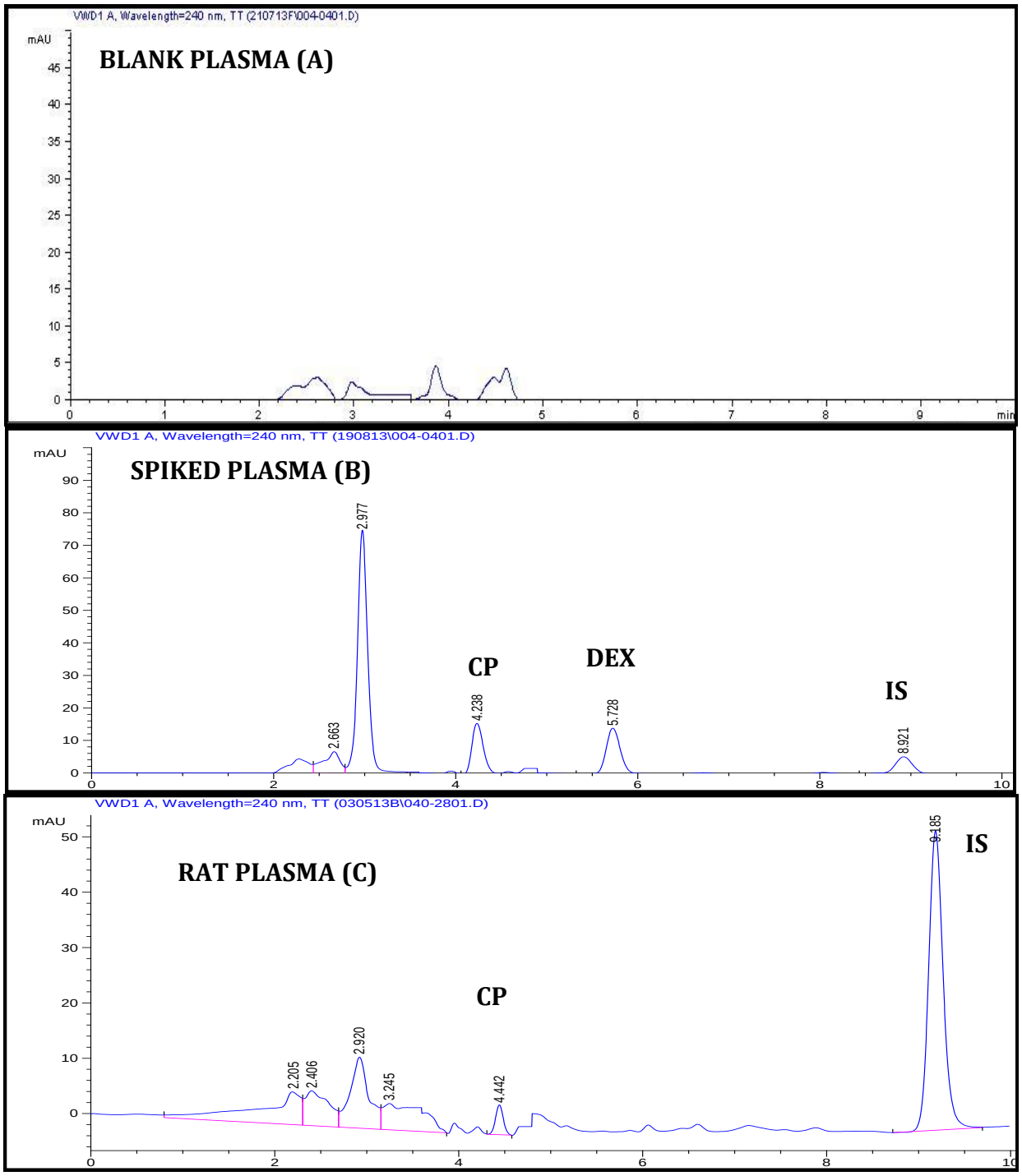


Figure 5.7: Chromatogram blank, (A) spiked (B) and rat plasma (C) after 24 hours treatment with cyclophosphamide. Retention time was at 4.2, 5.7 and 8.1 minutes for CP, Dex and IS. CP eluted at 4.4 minutes in rat plasma.

Abrreviations*: CP = cyclophosphamide, Dex = dexamethasone, IS = Internal standard*

5.4.2.3. Dexamethasone

5.4.2.3.1. Linearity

Table 5.3 Indicates summarized data of dexamethasone five days' calibration curves. Coefficient of variance percentage (CV %) was less than 15 %. **Figure 5.8** is the plot of the average five day calibration curve of dexamethasone. Dexamethasone linear regression was y = 0.261x + 0.0107 and correlation coefficient (r^2) was 0.9979.

5.4.2.3.2. Accuracy/recovery

Table 5.4 provides a summarized data of accuracy for all three dexamethasone concentrations. Mean dexamethasone accuracy percentages were 107±13 (CV % = 12.58), 100±6 (CV % = 5.57) and 99±14 (CV % = 13.80) for the 5, 20 and 30 µg/ml, respectively.

5.4.2.3.3. Stability

Figure 5.9 are diagrams of dexamethasone recovery percentage vs time evaluating short term stability of 5, 20 and 30 µg/ml after 24 and 48 hours at ±25 °C (room temperature) and at 4 °C (fridge). After 24 hours dexamethasone was stable at both temperature settings. After 48 hours dexamethasone was stable at 4 °C for all three concentrations.

Figure 5.10 is plots of dexamethasone stability in the fridge (4 °C), freezer (-20 °C) and ultra freezer (-80 °C) after 1 and 4 weeks respectively. Dexamethasone was stable for 5, 20 and 30 µg/ml after 1 (100, 91 and 105 %) and 4 weeks (101, 102 and 110 %) at -20 °C.

5.4.2.4. Dexamethasone concentration in rat plasma

Figure 5.11 is a chromatogram of plasma in dexamethasone treated rats after 24 hours. The peak was sharp and symmetrical. Dexamethasone concentration was 15 µg/ml.

Table 5.3: Peak ratios of dexamethasone calibration standards over 5 days, with mean, standard deviation and coefficient of variance.

Conc (µg/ml)	DAY1	DAY2	DAY3	DAY4	DAY5	MEAN ±SD	CV%
5	1.35	1.55	1.28	1.07	1.45	**1.34±0.18**	**13.59**
10	2.94	3.22	2.74	2.51	2.47	**2.78±0.31**	**11.34**
15	3.37	4.31	3.76	3.82	3.87	**3.83±0.34**	**8.80**
20	4.37	5.21	5.34	4.98	5.51	**5.08±0.44**	**8.75**
25	4.95	6.75	6.80	6.36	7.31	**6.43±0.90**	**13.91**
30	6.31	7.33	8.87	8.62	8.99	**8.02±1.16**	**14.49**

Abbreviations: Dex = dexamethasone **Mean =** mean value of values, **SD** = standard deviation, **CV %** = coefficient of variance, **Conc =** concentration

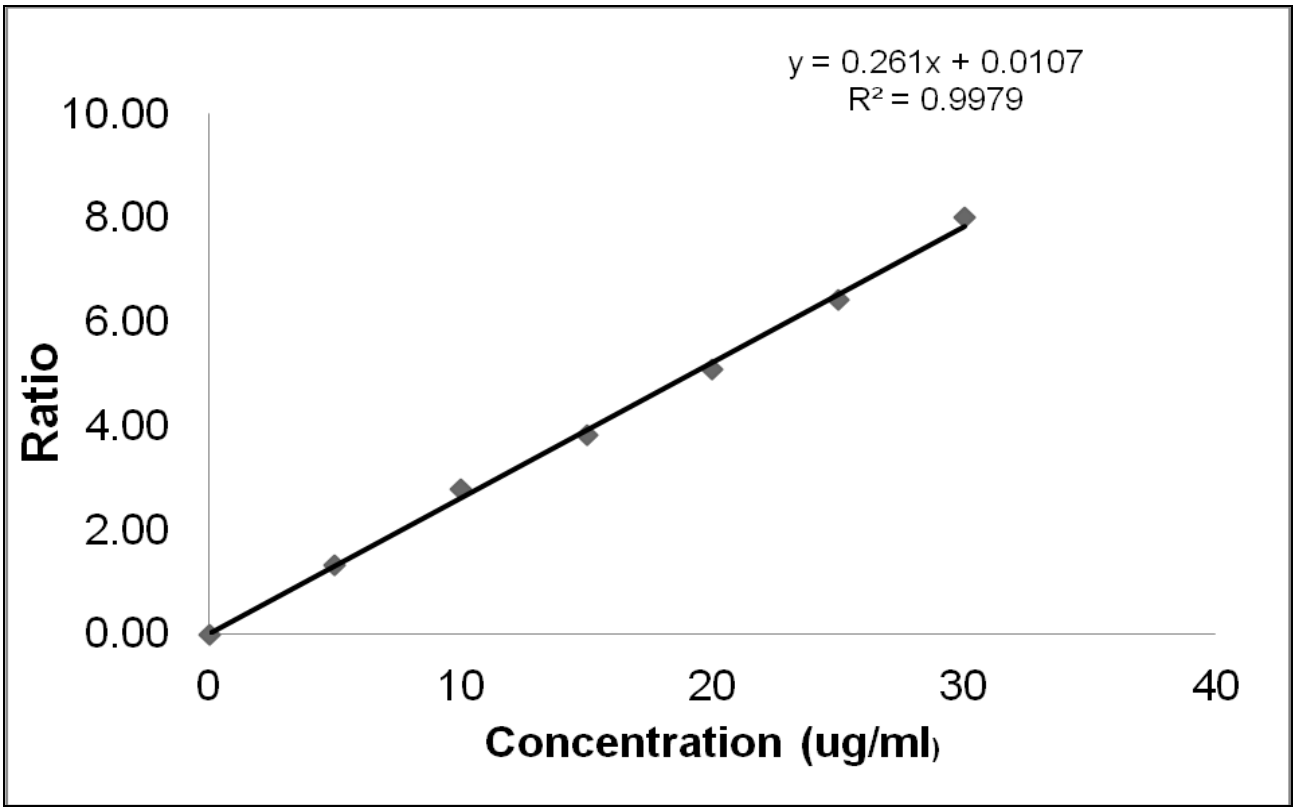
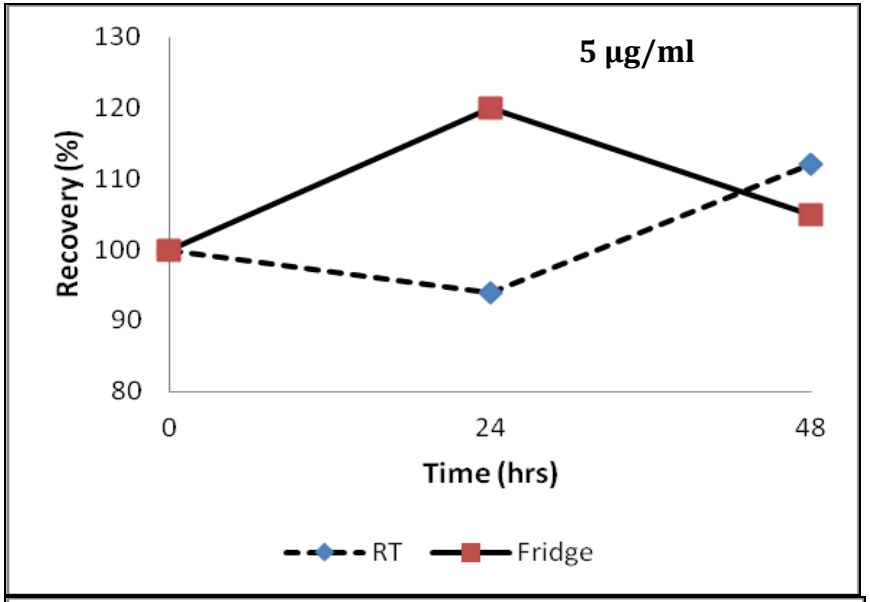


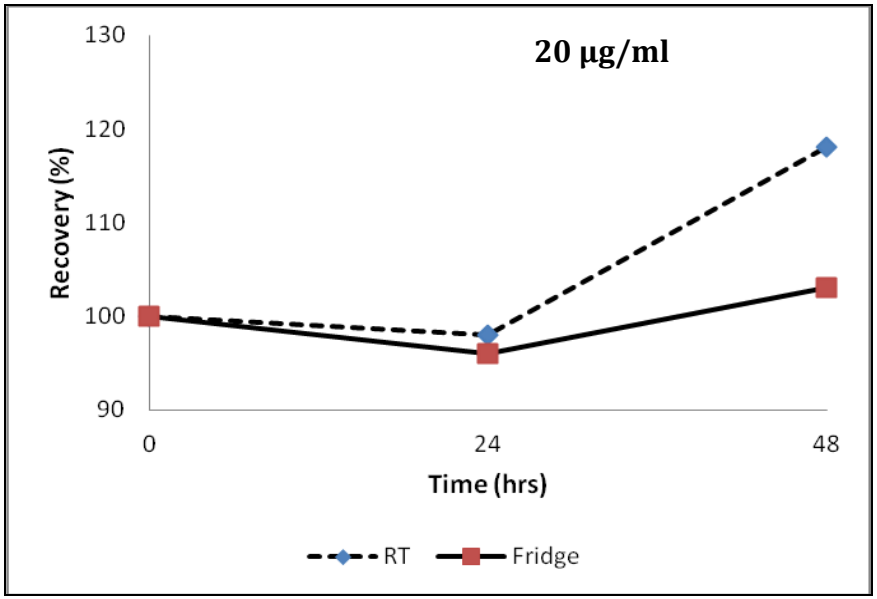
Figure 5.8: An average calibration curve of dexamethasone over five days with concentration versus peak ratio and correlation line of y = 0.261x + 0.0107 and r^2 = 0.9979

Table 5.4: Summary of dexamethasone accuracy testing results; recovered concentration (µg/ml) and percentages (%) of 5, 20 and 30 µg/ml are reported. The mean, standard deviation and CV % are also shown.

	5 µg/ml		20 µg/ml		30 µg/ml	
Sample	**Conc (µg/ml)**	**Recovery %**	**Conc (µg/ml)**	**Recovery %**	**Conc (µg/ml)**	**Recovery %**
A	5.40	108.00	19.96	99.80	32.59	108.00
B	19.34	96.70	4.65	93.00	34.86	116.00
C	19.67	98.35	5.30	106.00	29.77	99.00
D	21.95	109.75	5.98	120.00	24.91	83.00
E	19.21	96.05	5.33	107.00	26.66	89.00
Mean±SD	**20.0±1.1**	**100±6**	**5.3±0.7**	**107±13**	**29.8±4.1**	**99±14**
CV %	**12.58**		**5.57**		**13.80**	

Abbreviations: Conc = concentration, **CV %** = coefficient of variance





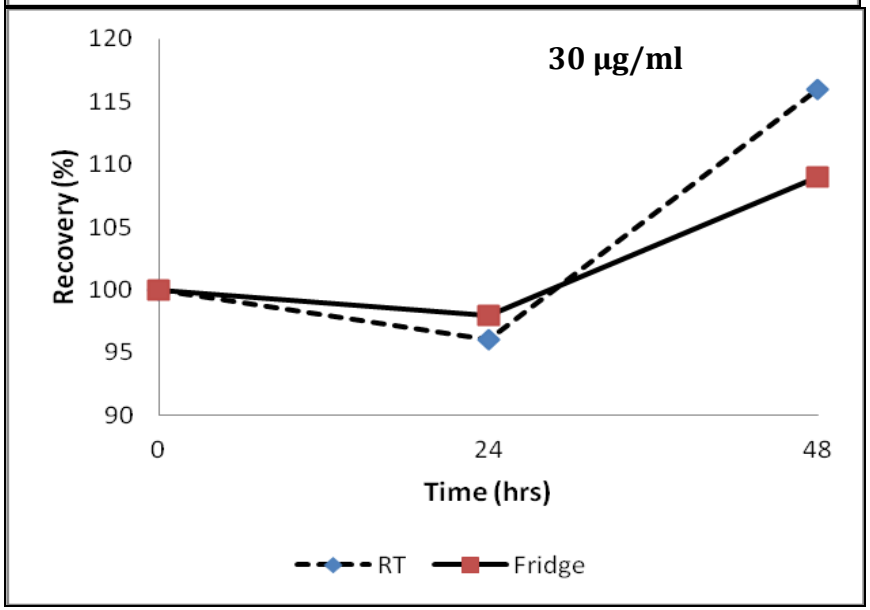


Figure 5.9: Plots showing dexamethasone (5, 20 and 30 µg/ml) short-term stability at room temperature (±25 °C) and fridge (4 °C) for 24 and 48 hours

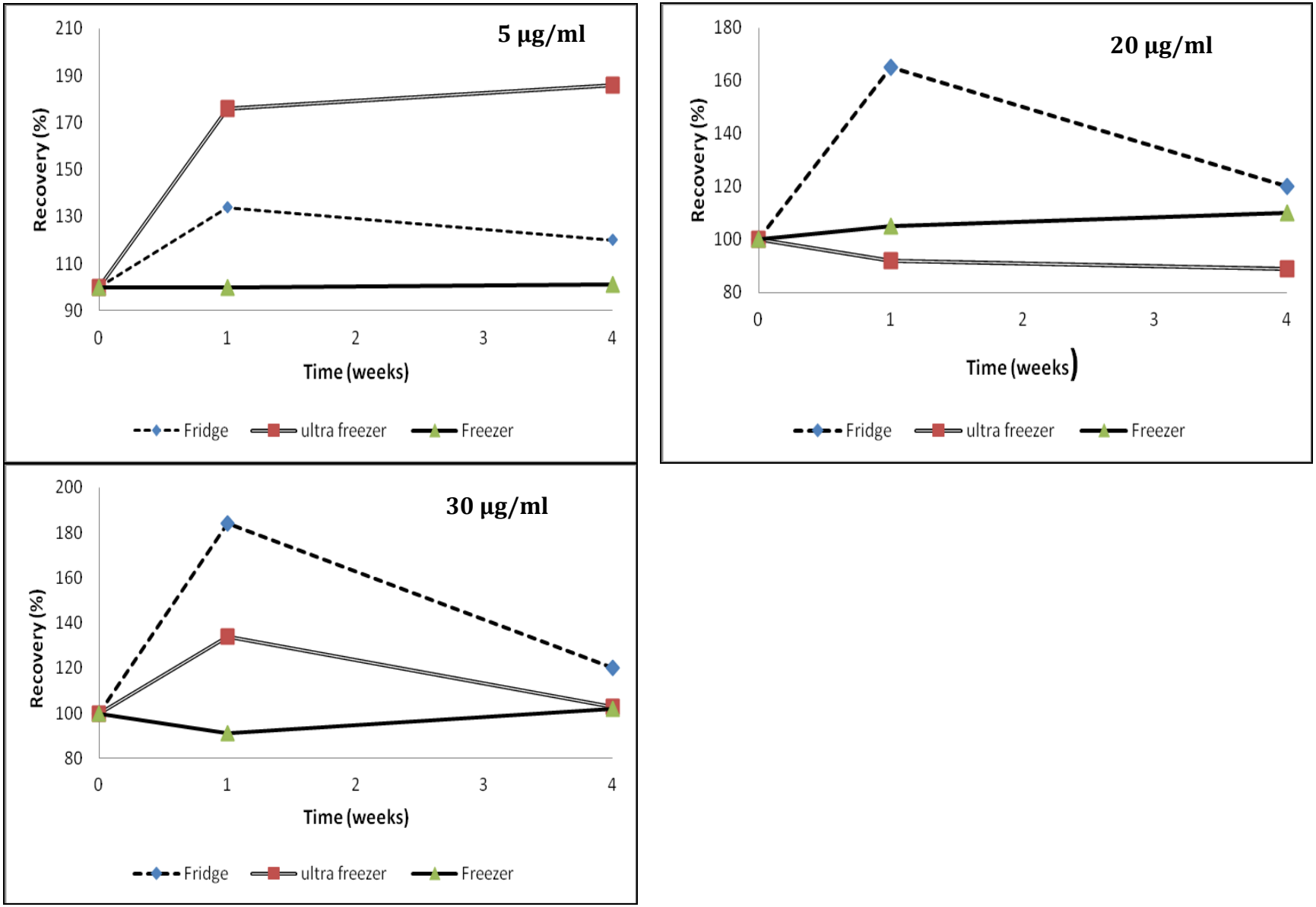


Figure 5.10: Plots showing dexamethasone (5, 20, and 30 µg/ml) long-term stability left in the fridge (4 °C), freezer (-20 °C) and ultra freezer (-80 °C) for 1 and 4 weeks respectively.

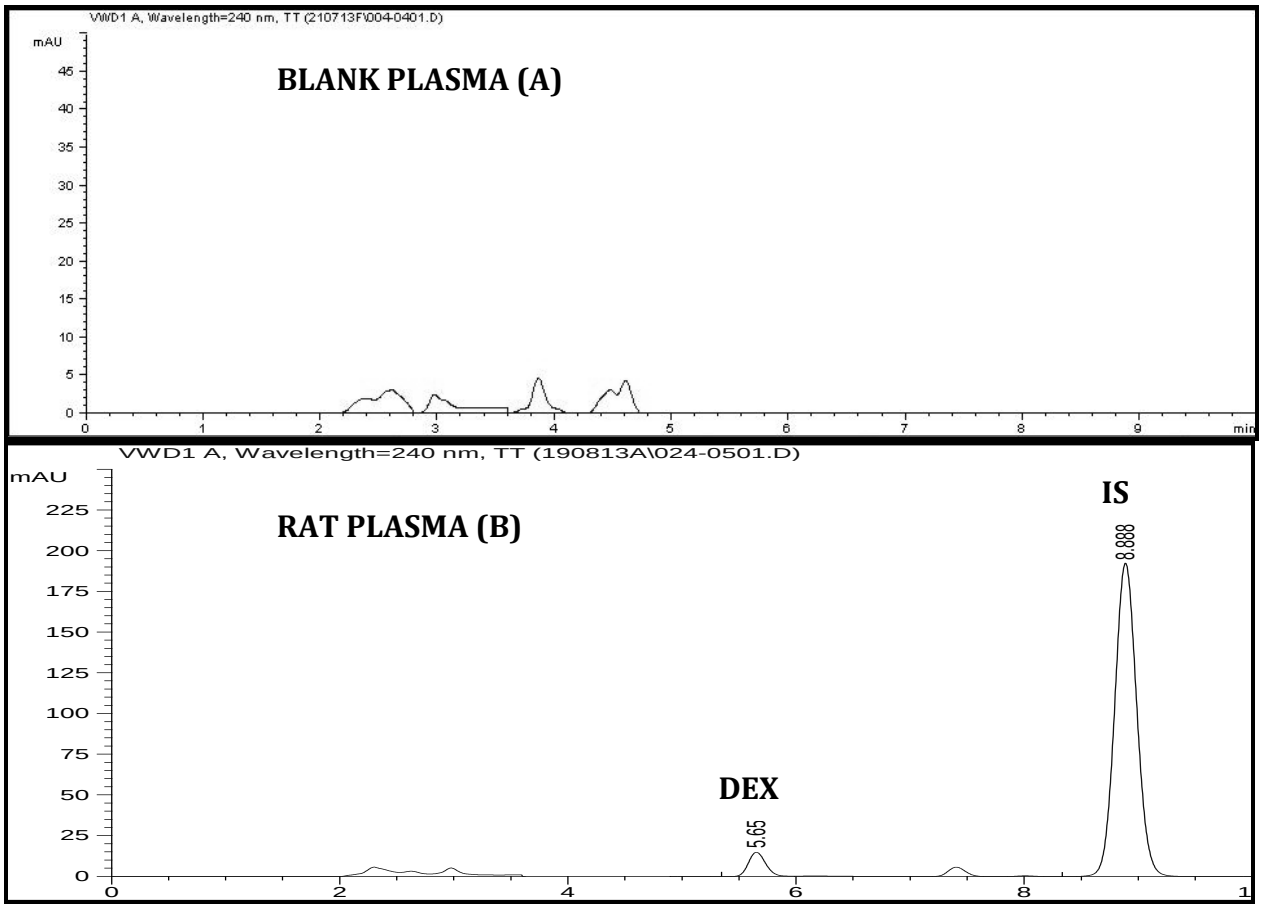


Figure 5.11: Chromatogram of blank (A) and rat plasma (B) after 24 hours treatment with dexamethasone. Dex eluted at 5.6 minutes in rat plasma, similar to spiked plasma in **figure 5.7**.
Abrreviations*: CP = cyclophosphamide, Dex = dexamethasone, IS = Internal standard*

5.5. DISCUSSION

HPLC is a convenient economical, universal, easily accessible and reliable technology for the analysis of number of chemical agents in various mediums (Ahmad et al., 2011). Preliminary experiments included evaluating UV-spectra, column lengths, flow rate, mobile phase, extraction solvents and wavelength switching to determine optimum condition for detecting and monitoring cyclophosphamide and dexamethasone in plasma. Taken into account the instability of cyclophosphamide in strong acidic and basic media a mobile phase with water and acetonitrile in different combination was preferred (Dhakale and Ubale, 2013). Also mobile phase pH or ionic strength should not affect the retention and/or separation of dexamethasone under RP-HPLC (Xiao *et al*., 2008).

The findings indicated that a high performance liquid chromatography method can be used to simultaneously detect and quantify cyclophosphamide and dexamethasone in plasma. The method has been validated. The peaks from the finalized method were sharp and symmetrical. The method was linear and accurate. The linear regression and correlation coefficient is $y = 0.0478x + 0.0108$; $r^2 = 0.9969$ for cyclophosphamide and $y = 261x + 0.0107$; $r^2 = 0.9979$ for dexamethasone. The CV % was less than 15 % for calibrations standards of both drugs. It is rapid, simple and sensitive as proven by the short retention time and a run time of 10 minutes, sharp peaks and simple mobile phase. Moreover, mean accuracy percentages were within 15 % set limit.

The method has been successfully applied to determine plasma cyclophosphamide and dexamethasone concentration from treated rats. This observation confirmed the efficiency of the analysis and reduced laboratory supply costs associated with testing individual drugs.

5.6. CONCLUSION

A fast, accurate, short, simple HPLC-UV switching method for simultaneous analysis of cyclophosphamide and dexamethasone was developed and successfully applied.

6.

TESTING FOR POTENTIAL INTERACTION BETWEEN *PHELA* AND CYCLOSPORINE, CYCLOPHOSPHAMIDE AND DEXAMETHASONE

Presented at the Japanese society of the study of xenobiotics conference (2014)

6.1. SUMMARY

Introduction: Since the subsequent animal experiment would involve co-administration of drugs (cyclosporine, cyclophosphamide and dexamethasone) with *Phela*, there was a need to test for potential interactions with *Phela*. Thus, the aim of the experiment was to screen for direct interactions with any of the three drugs using an equilibrium dialysis by slide-a-lyzer technique.

Methodology: Plasma (3 ml) spiked with the drugs separately was dialyzed against 0.1 M of sodium phosphate buffer (pH 7.4) until equilibrium protein binding for each drug was reached, after which *Phela* was added and dialysis was continued for six more hours. Aliquots (200 µl) were taken every 2 hours from plasma in the chamber and buffer, and were analysed for the respective drug concentrations. Thereafter, the free and bound concentrations were determined.

Results: CP and Dex equilibrated at 4 hours and CsA at 22 hours. Free fraction (%) was (mean±SD): [CP-only, 75.83±22.26 % vs CP+*Phela,* 37.90±9.57 %, P = 0.054; Dex-only, 91.21±5.14 % vs Dex+*Phela,* 87.75±1.38 %, P = 0.085; and CsA-only, 55.95±12.92 % vs CsA+*Phela*, 79.34±12.18 %, P = 0.323] Despite wide variations, there was no significant difference between the free fractions of drug-only when combined with drug+*Phela* group.

Conclusion: There was no interaction between *Phela* and cyclosporine, cyclophosphamide and dexamethasone. This implies that the afore mentioned drugs can be co-administered with *Phela* without interference.

6.2. INTRODUCTION

The aim of this study was to test for potential interaction of *Phela* with cyclosporine, cyclophosphamide and dexamethasone using slide-a-lyzer equilibrium dialysis.

6.3. METHODS

6.3.1. MATERIALS

6.3.1.1. Apparatuses

An agilent HP 1100 series with gradient pump and a built-in degasser was used. A vortex mixer (Scientific industries Inc, U.S.A) was used to mix the samples. A 5810R centrifuge and mini spin (Eppendorf, Germany) was used for separation. Precision balance SBC 31 and SBP 52 (Scaltec Instruments, Germany) were used to weigh milligram and gram quantities, respectively. The supernatant was dried with a nitrogen evaporator with a heating block. Water bath was used for shaking at 37°C provided by the department of pharmacology. Slide-A-Lyzer® dialysis cassette with 10 K molecular weight cut-off (MWCO) was purchased from Pierce Biotechnology (U.S.A). Needles and syringes were purchased from Lasec (South Africa) and used to fill the cassette and sample from it. Money bags were obtained from Absa and standard banks.

6.3.1.2. Chemical and reagents

Cyclophosphamide, cyclosporine and dexamethasone standards were purchased from Sigma Aldrich© (Germany). *Phela* was manufactured and supplied by the Indigenous Knowledge Systems Lead Programme of the South African Medical Research Council. HPLC graded acetonitrile, ethyl acetate, Triethylamine and methanol from Burdick and Jackson, U.S.A and hydrochloric acid (Merck, Germany) were used. Deionised and distilled water was prepared by the student in our laboratory with the Millipore water system. (Milli-Q^{Tm}). Drug-free plasma from healthy volunteers was a generous donation from the toxicology laboratory.

6.3.2. EXPERIMENTAL PROCEDURES

6.3.2.1. Plasma stock solution preparation

Drug-free plasma (3 ml) was spiked with either CsA (450 µg), CP (1725 µg) or Dex (230 µg). The above-mentioned groups were classified as control groups (control-1: CP; control-2: CsA; control-3: Dex). *Phela* (3850 µg) was dissolved in water and left overnight at 4°C. Thereafter, it was centrifuged at 4 °C and 4 000 rpm (2 002 g) for 20 minutes. The supernatant was added to immune-suppressant spiked plasma. Moreover, addition of *Phela* to plasma containing immune suppressant, denoted respective test groups (Test-1: CP+*Phela*; Test-2: CsA+*Phela*; Test-3: Dex+*Phela*).

6.3.2.2. Experiment 1: Establishing the equilibrium time

Cassette condition: A three millimetres slide-A-Lyzer dialysis cassette with a 10 K (MWCO) pore size was used. Distilled water was loaded on the cassette and immersed in buffer to rule out any leakages**. Figure 6.1** is an illustration of a dialysis experimental procedure with steps 1 to 4.

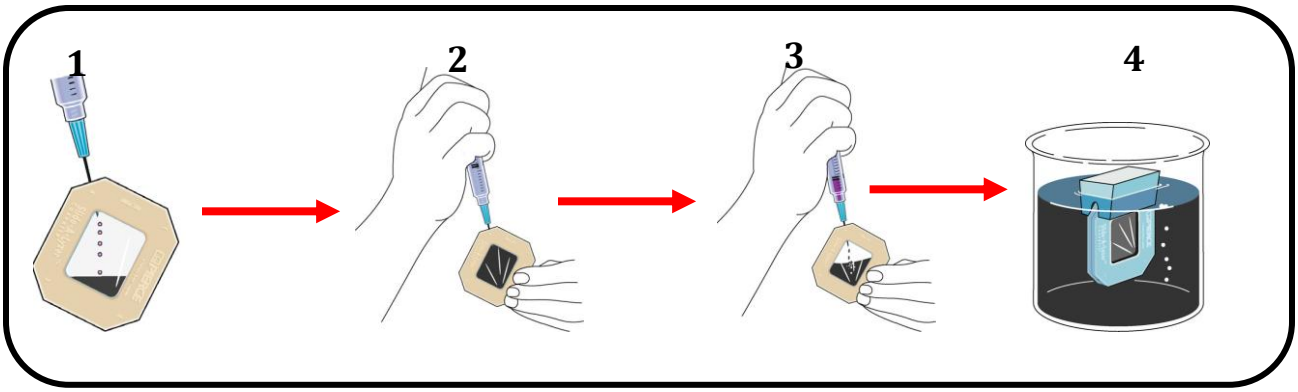


Figure 6.1: An illustration of dialysis experimental procedure (www.piercenet.com) (Accessed 20 February 2014)

Step 1*:* A cassette was cautiously filled to capacity with plasma spiked with either CsA, CP or Dex (3 ml) using a syringe and needle to avoid tearing the membrane.

Step 2*:* Air bubbles were removed from the cassette.

Step 3**:** Aliquots (200 µl) were sampled from plasma and buffer chambers every 2 hours for the duration of the study.

***Step 4*:** Spiked plasma as dialyzed against 20 ml of 0.1 M phosphate buffer (pH 7.4), incubated in a water bath at 37 °C and shaken for 12 - 24 hours. CsA samples were analysed by a chemiluminescent immuno assay (CMIA) kits. CP and Dex samples were analysed by a HPLC method validated in **chapter 5**.

6.3.2.3. Experiment 2: Screening for potential interaction of *Phela* with immune suppressants

At equilibrium-time, *Phela* was added to spiked plasma and sampling was done for 6 more hours. Analysis was done as described above in **Section 6.3.2.4 and 6.3.2.5**.

6.3.2.4. High performance liquid chromatography method

Aliquots from plasma and buffer chambers were spiked with surprofen (internal-standard), extracted with ethyl acetate and centrifuged for 10 min at 6 705g. The supernatant was evaporated under nitrogen, the residue reconstituted with mobile phase and 50 µl injected on the HPLC. Sample separation was on a C_{18} (250 mm x 4.6 mm x 5 µm) column, eluted with 45 % of acetonitrile in 0.05 M sodium phosphate-buffer (pH 3.7; containing 0.5 ml triethylamine) at a flow rate of 1 ml/min and 10 minutes runtime. The UV wavelength was set at 190 nm for cyclophosphamide and switched to 240 nm for dexamethasone and internal-standard detection. A calibration curve was used to extrapolate CP and Dex concentration respectively.

6.3.2.5. Immuno assay analysis

Whole blood was withdrawn from healthy volunteers. CsA samples were diluted equally with whole blood (1:1) and further analysed using CMIA kits in the toxicology laboratory, department of Pharmacology University of the Free State. The concentration was directly read.

6.3.3. STATISTICAL ANALYSIS

The free (unbound) fraction was determined as the concentration ratio of analyte in buffer to that in plasma. The effect of *Phela* on each drug was established by comparison of drug-only groups and drug+*Phela* groups. Data was analysed by Instat graph pad and reported as mean and standard deviation.

6.4. RESULTS

6.4.1. CYCLOPHOSPHAMIDE

6.4.1.1. Cyclophosphamide chromatographic performance

Figure 6.2 shows chromatograms of blank (A), spiked plasma (B) with CP at 4.1 minutes, Dex at 5.5 minutes and IS at 8.1 minutes. CP eluted at 4.4 minutes in plasma from dialysis (C) at 6 hours with a concentration of 26.68 µg/ml in the cassette chamber.

6.4.1.2. Effect of *Phela* on cyclophosphamide

Figure 6.3 is a diagram of CP concentration in plasma and buffer vs time over 10 hours of CP-only group. Mean plasma concentration was higher than the mean buffer concentration. CP equilibrated at 4 hours. CP plasma concentration remained unaltered throughout the study. Furthermore, CP concentration in buffer declined after 8 and 10 hours though not statistically significant.

Figure 6.4 is a diagram of CP concentration in plasma and buffer vs time over 10 hours of cyclophosphamide+*Phela* group. CP concentration in plasma increased and peaked (151.85 µg/ml) after 8 hours and followed by a decline. The concentration of CP in buffer increased slightly to 55.03 µg/ml after 6 hours and declined for the duration of the study. The mean concentration in plasma was higher than the mean buffer concentration, though not statistically significant.

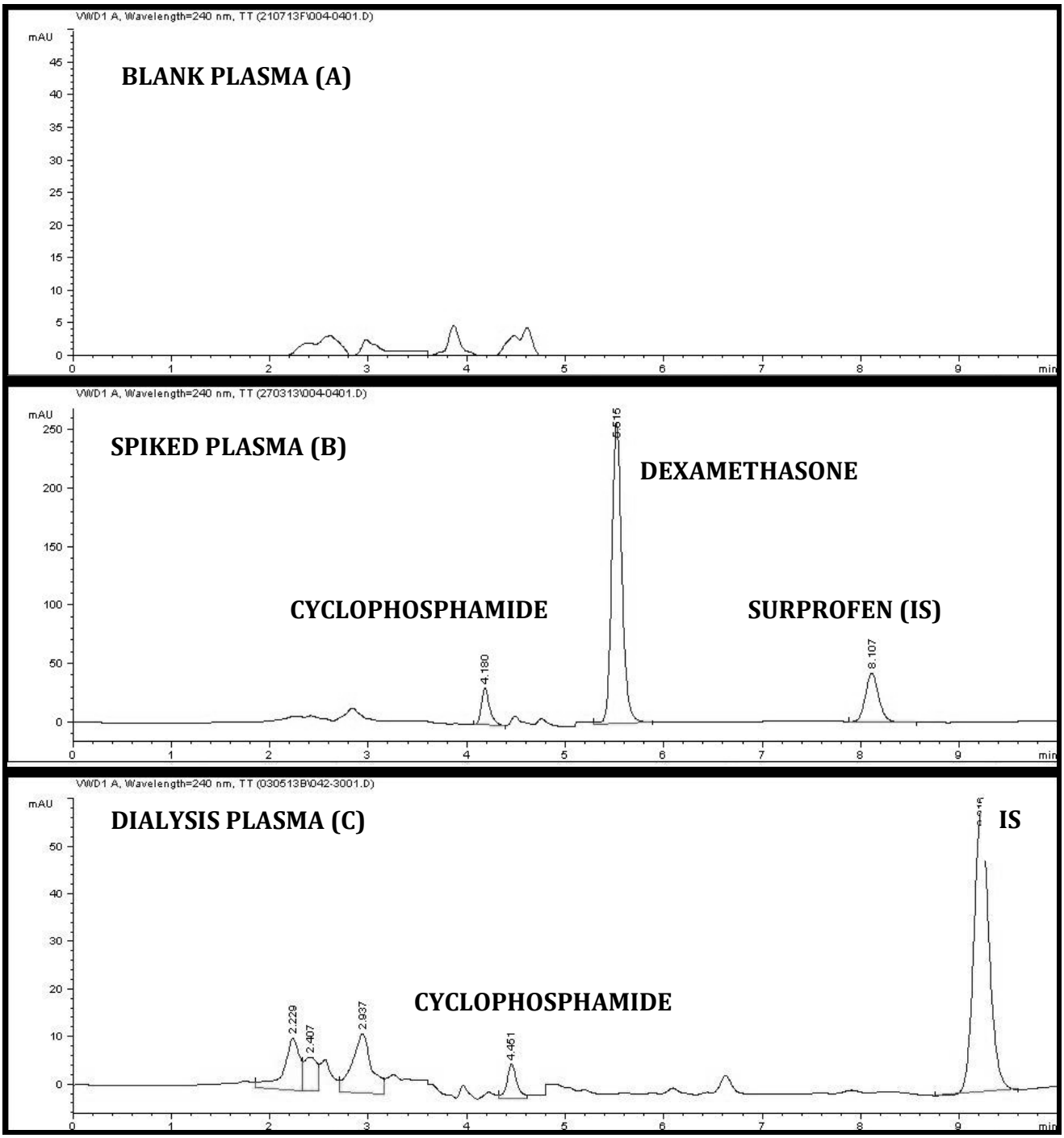


Figure 6.2: Chromatogram of blank plasma (A), spiked plasma (B) with CP at 4.1 minutes, Dex at 5.5 minutes and IS at 8.1 minutes. Cyclophosphamide eluted at 4.4 minutes in plasma from dialysis (C) at 6 hours.

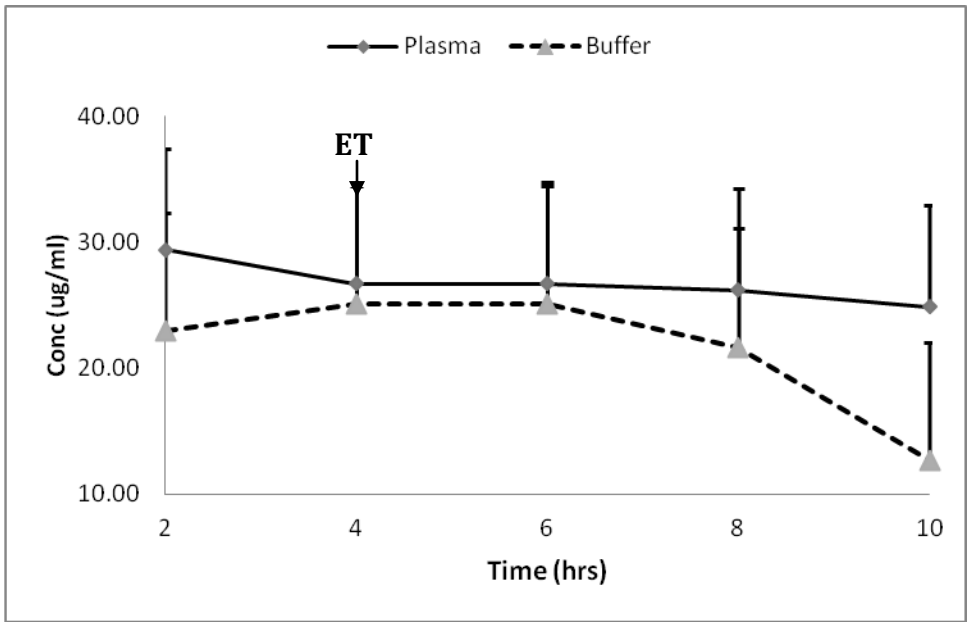


Figure 6.3: A diagram of concentration (µg/ml) in plasma and buffer chambers versus time (hours) over 10 hours of **cyclophosphamide-only** group.
***Abbreviations; ET**: Equlibrium time at 4 hours*

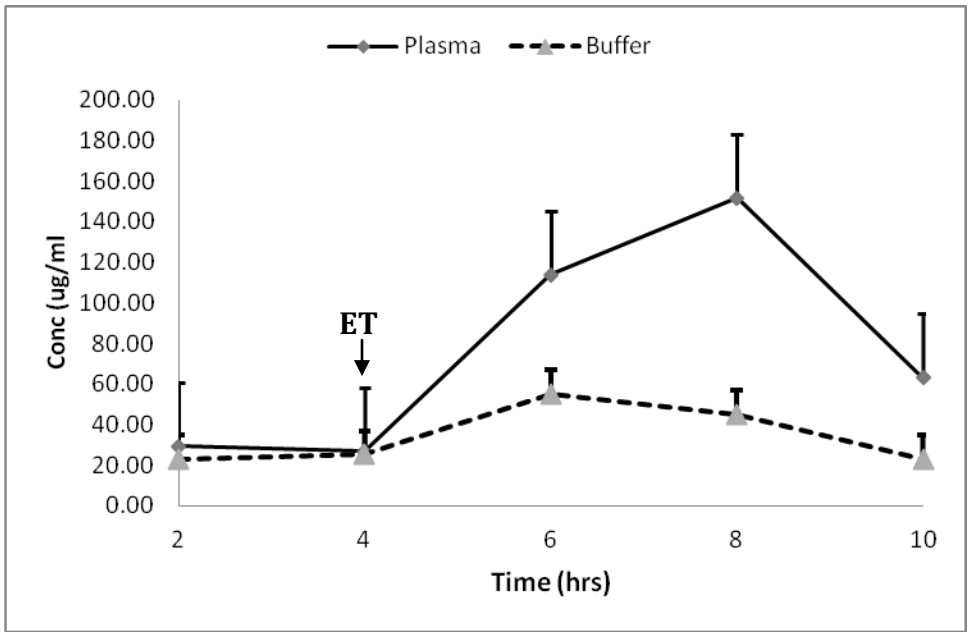


Figure 6.4: A diagram of concentration (µg/ml) in plasma and buffer chambers versus time (hours) over 10 hours of **cyclophosphamide+*Phela*** group
***Abbreviations; ET**: Equlibrium time at 4 hours*

Table 6.1 is a summary of the effect of *Phela* on CP concentration and its free fraction percentage. CP free fraction was 93.85 % after 4 hours in the CP-only treatment and decreased to 50.94% after 8 hours. Whereas, in the *Phela* treated group (CP+*Phela*) CP free fraction was 48.30 % after 4 hours and slightly decreased to 35.94% after 8 hours. CP mean free fraction percentage was 75.83±22.26 % in CP-only group and 37.90±9.57 % in CP+*Phela* group. *Phela* did not alter (P = 0.054) CP free fraction.

Table 6.1: Summarised data of the effect of ***Phela* on cyclophosphamide**, with free fraction concentration (µg/ml) and percentage (%).

Time (hours)	**Cassette (µg/ml)**	**Buffer (µg/ml)**	**Free (µg/ml)**	**Free (%)**
		CYCLOPHOSPHAMIDE – ONLY		
4	26.68	25.04	0.94	93.85
6	26.19	21.66	0.83	82.70
8	24.87	12.67	0.51	50.94
MEAN	**25.91**	**19.79**	**0.76**	**75.83**
SD	0.94	6.39	0.22	22.26
		***PHELA* + CYCLOPHOSPHAMIDE**		
4	113.94	55.03	0.48	48.30
6	151.85	44.73	0.29	29.46
8	63.02	22.65	0.36	35.94
MEAN	**109.60**	**40.80**	**0.38**	**37.90***
SD	44.57	16.54	0.10	9.57

P = 0.054(P < 0.05 vs control)*

6.4.2. DEXAMETHASONE

6.4.2.1. Dexamethasone chromatographic performance

Figure 6.5 shows chromatograms of plasma from the Dex dialysis at 6 hours. Dex eluted at 5.6 min and the internal standard (IS) at 9.9 min. The peaks were sharp and symmetrical. A calibration curve was used to derive the concentration in buffer. Dex concentration in the cassette chamber was of 5.68 µg/ml after 6 hours.

6.4.2.2. Effect of *Phela* on Dexamethasone

Figure 6.6 is a diagram of Dex concentration in plasma and buffer vs time over 10 hours of Dex-only group. Dex equilibrated at 4 hours. Dex concentration in buffer remained unchanged for the duration of the study. Dex concentration in plasma decreased to 2.45 µg/ml after 6 hours, and remained unchanged for the remainder of the study.

Figure 6.7 is a diagram of Dex concentration in plasma and buffer vs time over 10 hours of Dex+*Phela* group. Dex concentration decreased in both groups after 6 hours and remained the same for the duration of the study. There was no difference of mean concentration in buffer and plasma.

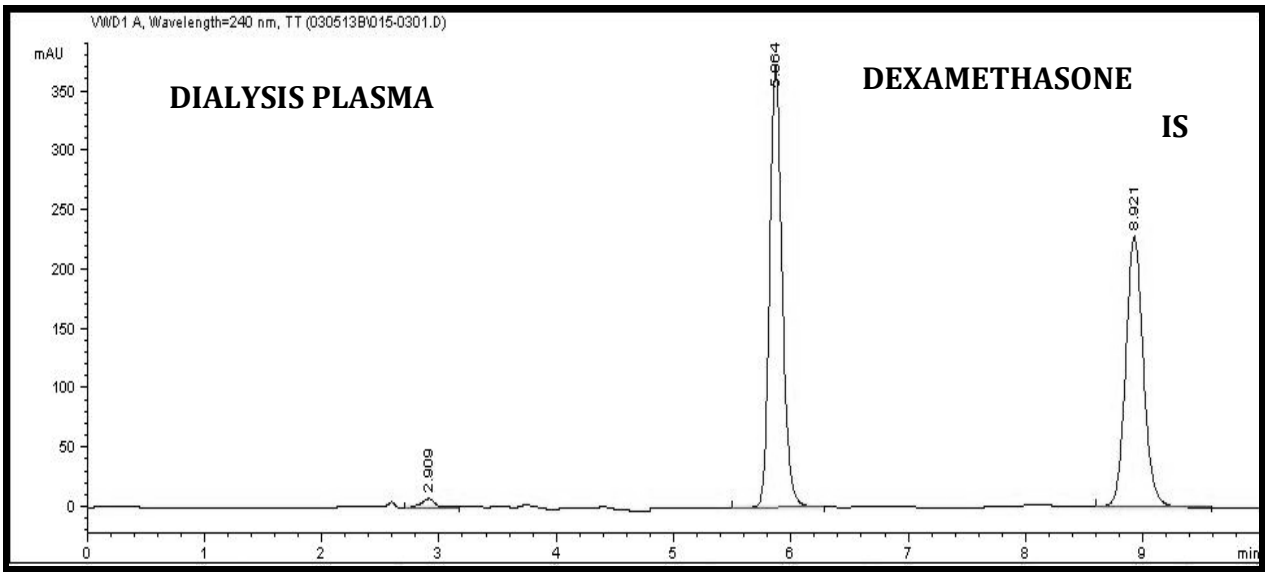


Figure 6.5: Chromatogram of plasma from dexamethasone dialysis at 6 hours. The blank and spiked plasma chromatograms are included in **Figure 6.2**.

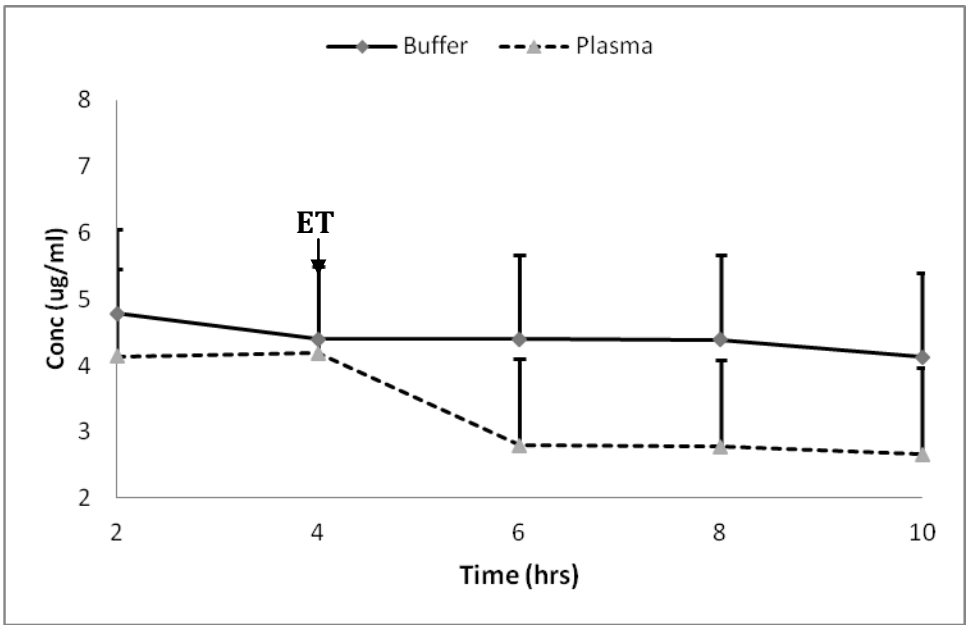


Figure 6.6: A diagram of concentration (µg/ml) in plasma and buffer chambers versus time (hours) over 10 hours of **dexamethasone-only** group.
***Abbreviations; ET**: Equlibrium time at 4 hours*

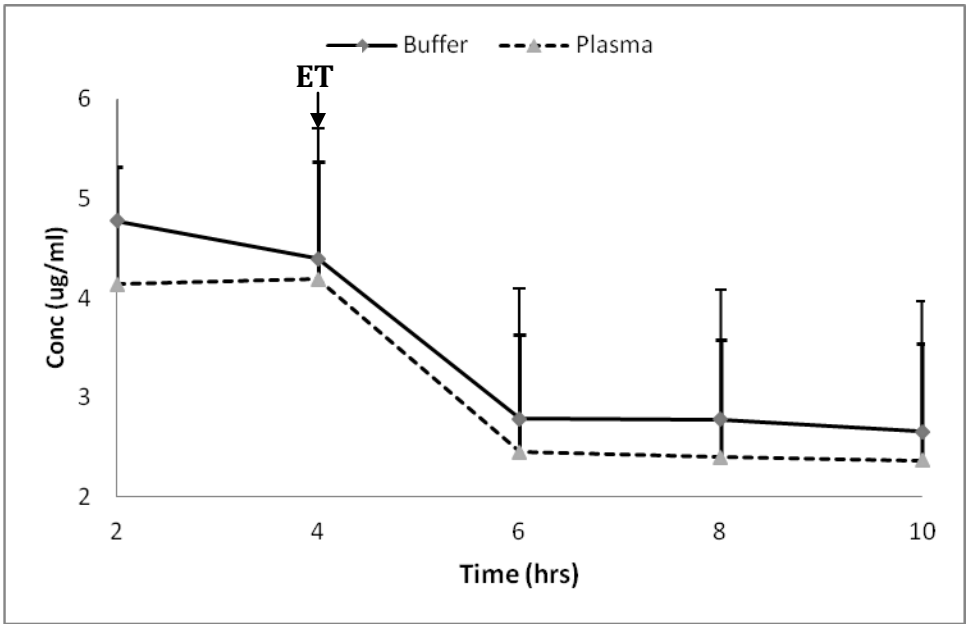


Figure 6.7: A diagram of concentration (µg/ml) in plasma and buffer chambers versus time (hours) over 10 hours of **dexamethasone+*Phela*** group.
***Abbreviations; ET**: Equlibrium time at 4 hours*

Table 6.2 is a summary of the effect of *Phela* on Dex concentration and its free fraction percentage. Dex free fraction was 91.21±5.14 % in the Dex-only group and 87.75±1.38 % in the Dex+*Phela* group. *Phela* had no effect on (P = 0.323) dexamethasone free fraction.

Table 6.2: Summarized data of the effect of ***Phela* on dexamethasone**, with free fraction concentration (µg/ml) and percentage (%).

Time (hours)	Cassette (µg/ml)	Buffer (µg/ml)	Free (µg/ml)	Free (%)
DEXAMETHASONE – ONLY				
4	4.40	4.19	0.95	95.23
6	4.39	3.75	0.85	85.42
8	4.13	3.84	0.93	92.98
MEAN	**4.31**	**3.93**	**0.91**	**91.21**
SD	0.15	0.23	0.05	5.14
***PHELA* + DEXAMETHASONE**				
4	2.79	2.45	0.87	86.61
6	2.78	2.40	0.88	87.81
8	2.66	2.37	0.89	89.10
MEAN	**2.74**	**2.41**	**0.88**	**87.75**
SD	0.07	0.04	0.01	1.38

P = 0.323(P < 0.05 vs control)*

6.4.3. CYCLOSPORINE

CsA equilibrium time was established in previous studies in our laboratory [(Modise *et al*., 2007) results not included].

6.4.3.1. Effect of *Phela* on cyclosporine

Figure 6.8 is a diagram of CsA concentration in plasma and buffer vs time over 28 hours of CsA-only group. CsA equilibrated at 22 hours. CsA concentration in plasma decreased after 24 hours and remained unchanged thereafter. Moreover, CsA concentration in buffer fluctuated from 24 to 28 hours. There was no difference of mean CsA concentration in buffer and plasma (P = 0.167).

Figure 6.9 is a diagram of CsA concentration in plasma and buffer vs time over 28 hours of CsA+*Phela* group. *Phela* did not alter the concentration of CsA in both plasma and buffer. There was no difference of mean concentration in buffer and plasma (P = 0.100).

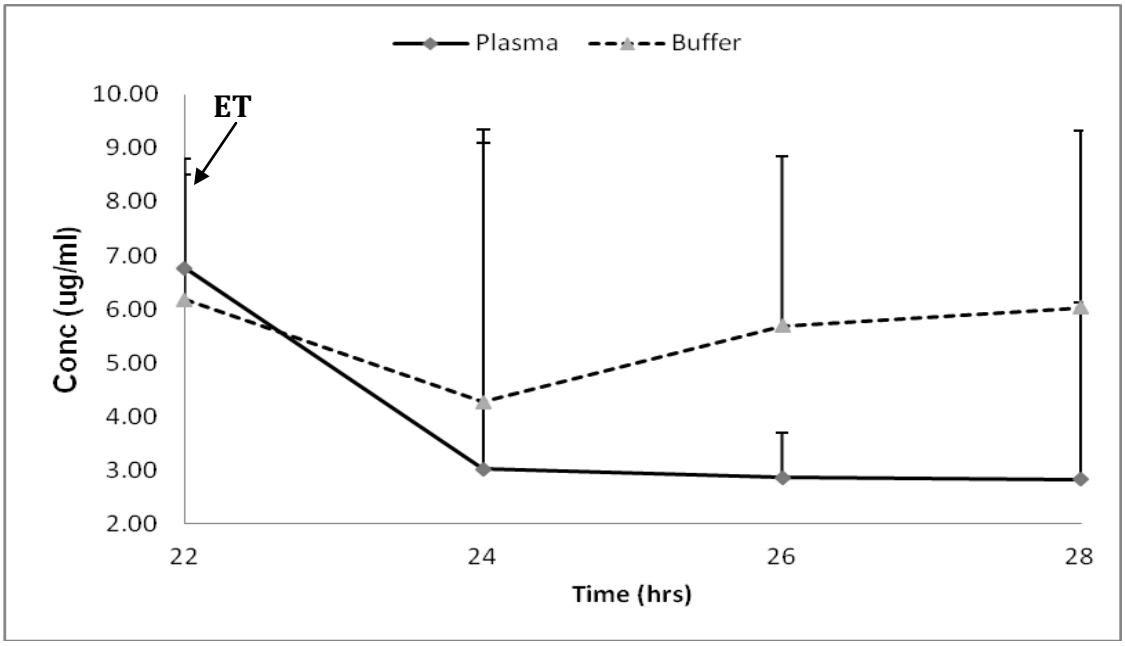


Figure 6.8: A diagram of concentration (µg/ml) in plasma and buffer chambers versus time (hours) of **cyclosporine-only** group.

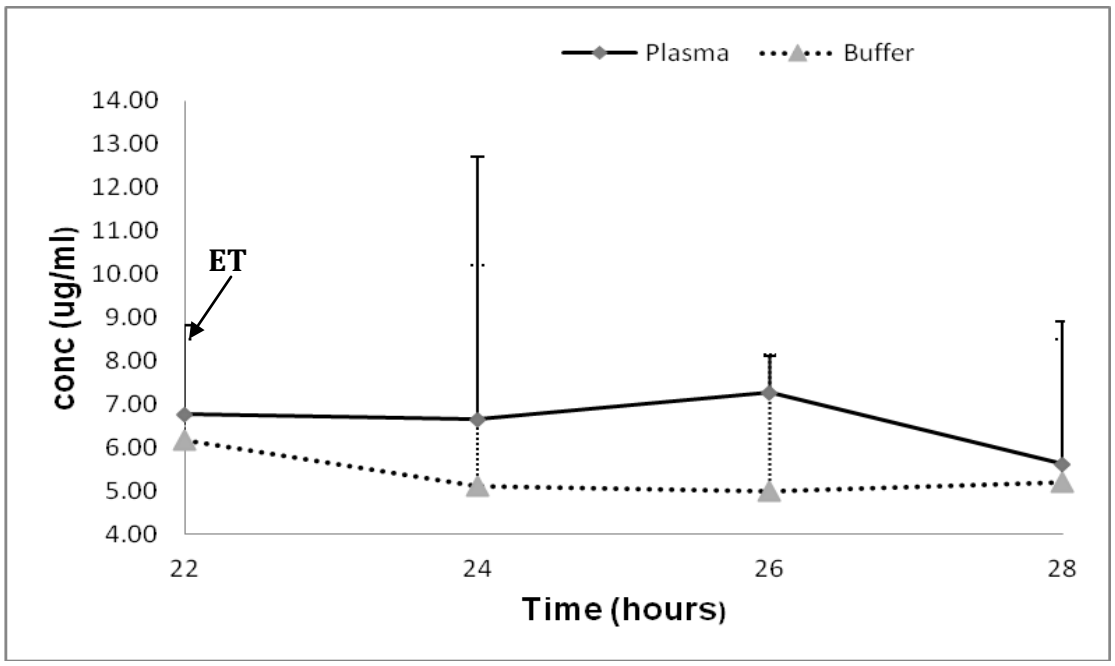


Figure 6.9: A diagram of concentration (µg/ml) in plasma and buffer chambers versus time of **cyclosporine+*Phela*** group.
***Abbreviations; ET**: Equlibrium time at 4 hours*

Table 6.3 is a summary of the effect of *Phel*a on CsA concentration and its free fraction percentage. CsA free fraction was 55.95±12.92 % in CsA-only group and 79.34±12.18 % in CsA+ *Phela* group. *Phela* did not alter (P = 0.085) CsA free fraction. CsA free fraction was 70.73 % after 4 hours and 46.77 % after 8 hours. Whereas, in the *Phela* treated group CsA free fraction was 76.99 % after 4 hours and 92.51 % after 8 hours. The results indicate that *Phela* had varied effect on CsA free fraction though not significant statistically

Table 6.3 Summarised data of the effect of ***Phela* on cyclosporine**, with free fraction concentration (µg/ml) and percentage (%) over 6 hours.

Time (Hours)	Cassette (µg/ml)	Buffer (µg/ml)	Free (µg/ml)	Free (%)
CYCLOSPORINE – ONLY				
24	4.27	3.02	0.71	70.73
26	5.68	2.86	0.50	50.35
28	6.03	2.82	0.47	46.77
MEAN	**5.33**	**2.90**	**0.56**	**55.95**
SD	0.93	0.11	0.13	12.92
***PHELA* + CYCLOSPORINE**				
24	6.65	5.12	0.77	76.99
26	7.27	4.98	0.69	68.50
28	5.61	5.19	0.93	92.51
MEAN	**6.51**	**5.10**	**0.79**	**79.34***
SD	0.84	0.11	0.12	12.18

*P = 0.085 *(P < 0.05 vs control)*

6.5. DISCUSSION

Absorption, distribution, metabolism and elimination (ADME) impact costs during drug development and it is for this reason that understanding the affinity of drugs to plasma proteins at an early stage is essential (Ghuman *et al*., 2005; Gan *et al*., 2013). The fraction of drug that is unbound is only available for pharmacological interaction; therefore it's necessary to determine the free fraction of the total drug concentration (Banker *et al*., 2003).

Equilibrium dialysis is the widely accepted method for assessing plasma protein binding. Its non-specific binding effects are minimized compared with other methods, such as ultra filtration; however equilibrium dialysis is a relatively slow process with an incubation time spanning from 4 to 24 hours at 37 °C (www.piercenet.com, accessed 30 March 2014). Equilibrium dialysis is considered a gold standard due to the reliability of results and robustness of the procedure (Gunn *et al*., 2012; Waters *et al*., 2008).

The results indicate wide variation of mean concentration of drug-only groups in comparison to *drug+Phela* groups. This observation is more pronounced in the CP+Phela test group, which could be due to the plurality nature of *Phela*. However the variations were not statistically significant.

During equilibrium dialysis, movement across the membrane is regulated by drug concentration gradient from a high to a low concentration. Overtime, osmotic pressure in the plasma compartment increases; as thus movement of buffer into the plasma can dilute the drug concentration (Gonzalez *et al.,* 2013). According to Banker (2003) equilibrium dialysis assay is associated with volume shifts due to the dialysate (in this study; phosphate buffer) moving to the plasma side due to osmotic pressure. Therefore, the observed variations in free fraction of some drug+*Phela* (i.e. CsA+*Phela* and CP+*Phela*) groups could be associated to a volume shifts.

6.6. CONCLUSION

In conclusion, the *in vitro* studies indicate that *Phela* had no interactions with CsA, CP and Dex. Moreover, this observation needs to be confirmed in an *in vivo* system. This implies that the drugs can be co-administered with *Phela* without interference.

7.

DETERMINATION OF THE DOSE OF *PHELA* FOR IMMUNE STIMULATION IN RATS

Presented at the American college of clinical pharmacology annual meeting (2013)

7.1. SUMMARY

Introduction: *Phela* is an herbal extract derived from four South Africa plants and under development for use as an immune booster in immune compromised individuals. Unfortunately, the current dose for *Phela* was not evaluated scientifically in rats. Therefore, the aim of this study was to determine the appropriate dose of *Phela* for immunomodulation in healthy rats.

Methodology: Five groups of 15 Sprague-Dawley rats were each treated daily with normal-saline, levamisole, and *Phela* 5, 15.4 or 75 mg/kg. Furthermore, 5 rats were sacrificed after 7, 14 and 21 days of treatment per group. Blood was analyzed for liver, renal and haematology functions. The CD_4 and CD_8 cell counts were determined by flow cytometry. Kidneys, liver, spleen, thymus, were weighed and examined for any pathology. Rat IgG, IgM, IL-2 and IL-10 concentration analysis was done by ELISA.

Results: Physiological function tests and CD_8 count were normal. All the three doses of *Phela* led to increased white blood cell count after 14 days of treatment, and this was significant for doses 5 mg/kg ($P = 0.02$) and 15.4 mg/kg ($P = 0.03$). This observation was associated with increased lymphocyte count which correlated with increased CD_4 count from day 7 to 14 of treatment. At 5 mg/kg, the CD_4 count was 1.10±0.15 at 7 days, and 2.48±0.29 at 14 days ($P = 0.005$), while for 15.4 mg/kg, it increased from 1.30±0.14 to 2.90±0.19 ($P = 0.001$). The CD_4 count in the control dropped progressively but remained in the normal range. IL-2 count (pg/ml) increased on day 7 [Control, 108.34±10.24; 5mg/kg, 156.19±5.62; 15.4mg/kg, 150.88±44.11; 75 mg/kg, 114.89±6.76 and levamisole, 161.51±56.62] and remained high for the duration of the study.

This observation correlates with our previous report where *Phela* led to increased IL-2 (Lekhooa *et al*., 2012).

Conclusion: *Phela* led to ample stimulation of the immune system as indicated by increased CD_4 count and IL-2 at doses of 5 and 15.4 mg/kg. This selective effect implies that *Phela* can be indicated in diseases that interfere with CD_4 and IL-2 count, but these needs to be confirmed in a diseased model.

7.2. INTRODUCTION

The aim of this study was to determine the dose of *Phela* for immune stimulation in healthy rats.

7.3. MATERIALS

7.3.1. 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. Weighing of milligrams and grams quantities of standards and chemicals was done on a precision balances, SBC 31 and SPB 52 (Scaltec Instruments; Germany) respectively. A micro plate reader with an Ascent software program was used for ELISA analysis. A Tecan micro-plate washer (Tecan Austria GmbH; Austria) was used for the washing of the wells during ELISA. The oral gavage was a 16 G-3 curved with a 3.00 mm ball and a metallic catherer purchased from Popper and Sons inc. (U.S.A), and used for the oral administration of drugs. Ugo Basile gas anesthesia system (Ugo Basile Biological research) apparatus, from Italy was used for induction and maintenance of anesthesia.

7.3.2. CHEMICALS AND REAGENTS

Phela was manufactured and supplied by the Indigenous Knowledge Systems Lead Programme of the South African Medical Research Council. Formalin, phosphoric acid, mono sodium, disodium, tween, potassium chloride and sodium chloride were purchased from Sigma Aldrich© (Germany). Deionised and distilled water was prepared in our laboratory by millipore water system (Milli –Q^{Tm}). Isoflor inhalation anaesthetic

(Isoflurane) was supplied by Safeline pharmaceuticals (PTY) Ltd (S.A) and Afrox (S.A) provided medical air (oxygen). EDTA, lithium-heparin and serum activator blood tubes were purchased from Lasec (S.A). Rat IL-2 and IL-10 ELISA kits were purchased from Invitrogen (USA) while rat IgG and IgM ELISA kits were from Affymetrix eBioscience (USA).

7.4. PROCEDURES

7.4.1. ANIMAL CARE

Experimental animals were bred and obtained from the University of the Free State (UFS) animal house. The animal ethics committee of the UFS approved the study (ETOVS NR: 11/2012). The UFS animal care unit provided male Sprague-Dawley (SD) rats and cared for them through-out the study. The animals were housed and acclimatized in ambient temperature (22 °C) and light controlled (light/dark cycle of 12/12hr) animal care facility. The rats had free access to standard rat chow and water, *ad libitum.* The husbandry conditions were kept constant throughout the whole study.

7.4.2. DRUG PREPARATION AND ROUTE OF ADMINISTRATION

All doses were adjusted accordingly based on the most recent body weight per group.

Drug preparation: The recommended dose of *Phela* is 15.4 mg/kg (Lekhooa *et al*., 2012a). The sub-therapeutic and 5x recommended doses were calculated to 5 and 75 mg/kg respectively.

Phela was dissolved in normal saline to achieve the following doses: 5, 15.4 and 75 mg/kg. LEV, a known immune stimulant, was prepared in normal saline to a dose of 2 mg/kg (Bani *et al.,* 2006).

Oral Route: *Phela*, LEV and normal saline were administered by oral gavage in a volume of 4 ml/kg, using an oral gavage attached to a 2 ml syringe.

7.4.3. EXPERIMENTAL DESIGN

Seventy-five rats were divided into five groups of 15 rats each as illustrated in **Figure 7.1**. The groups were then randomly assigned to the different treatment options, i.e. normal saline (control group; SAL), levamisole 2 mg/kg (positive control; LEV), *Phela*

5mg/kg (Low dose; PHL1), *Phela* 15.4 mg/kg (medium dose; PHL2) and *Phela* 75 mg/kg (high dose; PHL3). The animals were treated with the respective therapeutic options daily for 21 days, during which 5 animals from each group were sacrificed after 7, 14 and 21 days of treatment. Of note, on day 0 (the day before the experiment started), five rats that were not treated with anything were sacrificed to obtain baseline parameters.

7.4.4. OBSERVATION AND WEIGHING OF RATS

Rats were observed daily for any physical signs of infection and/or disease progression. *Rats weighing*: Rats were weighed on day 0 and weekly for the duration of the study.

7.4.5. SACRIFICING PROCEDURE AND BLOOD COLLECTION

Sacrificing was done at the department of pharmacology. Rats were placed in a chamber with Isoflurane & oxygen carrier gas for anesthesia induction. Thereafter, the rats were placed on a surgical table in supine position while maintained under isoflorane anesthesia. The rats were sacrificed by exsanguination whilst still under anesthesia. Blood (8 – 10 ml) was withdrawn through cardiac puncture (**Figure 7.2**) and aliquoted into the appropriate test tubes (EDTA, lithium and yellow-toped tubes) and send for analysis of the relevant parameters (described in the next section).

7.4.6. PHYSIOLOGICAL TESTS

Whole blood (3 – 4 ml) was collected in EDTA tubes; and send to the National Health Laboratory Services (NHLS) veterinary section for full blood count analysis. Whilst, CD_4, and CD_8 cells count flow cytometry analysis was done by the department of haematology at the UFS.

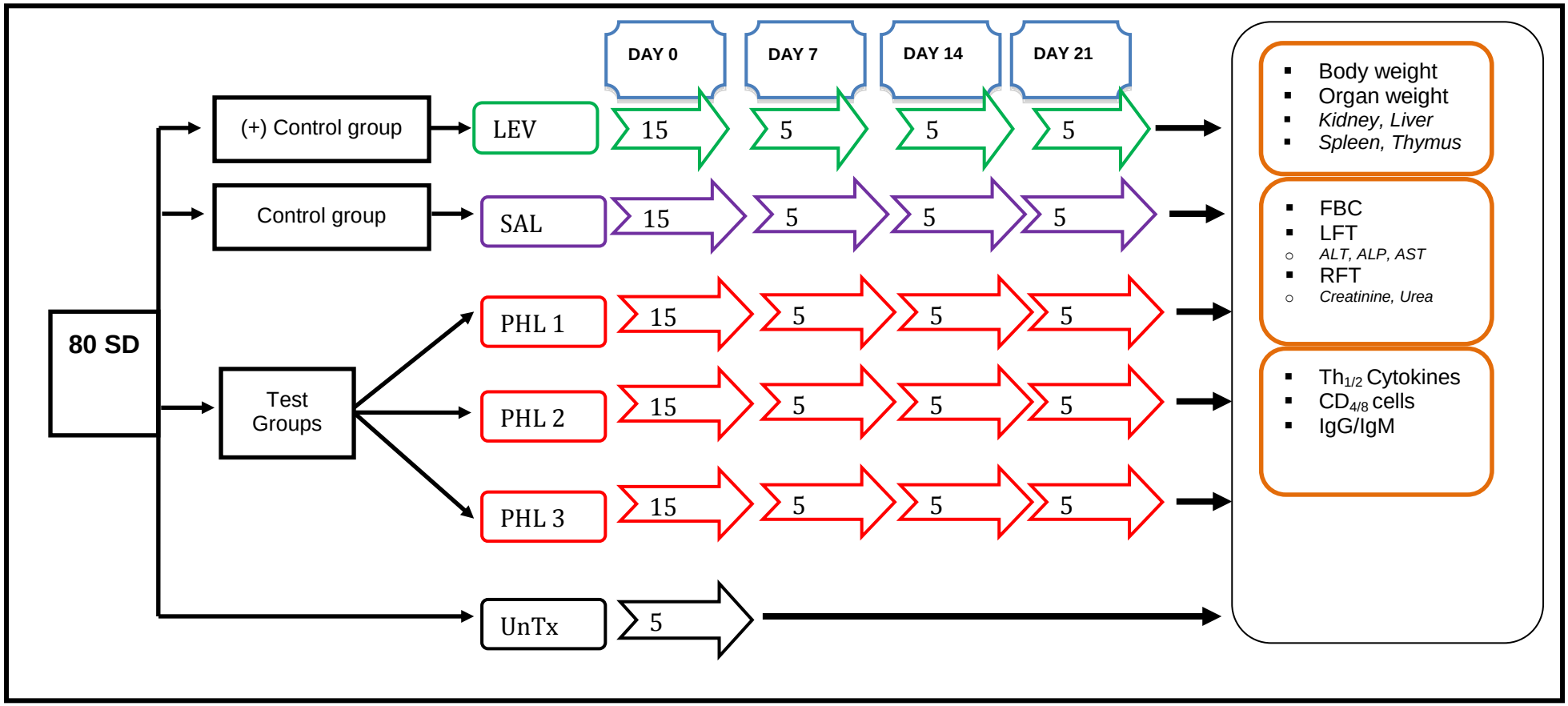


Figure 7.1: Experimental design to determine the dose of *Phela* in healthy rats

Abbreviations: untx: untreated, **Sal**: saline, **Lev**: Levamisole, **FBC**: full blood count, **LFT:** Liver function tests, **ALP**: alkaline phosphatase, **ALT**: Alanine transaminase, **AST**: Aspartate transaminase, **RFT**: Renal function tests, **Urea**: Blood urea nitrogen.

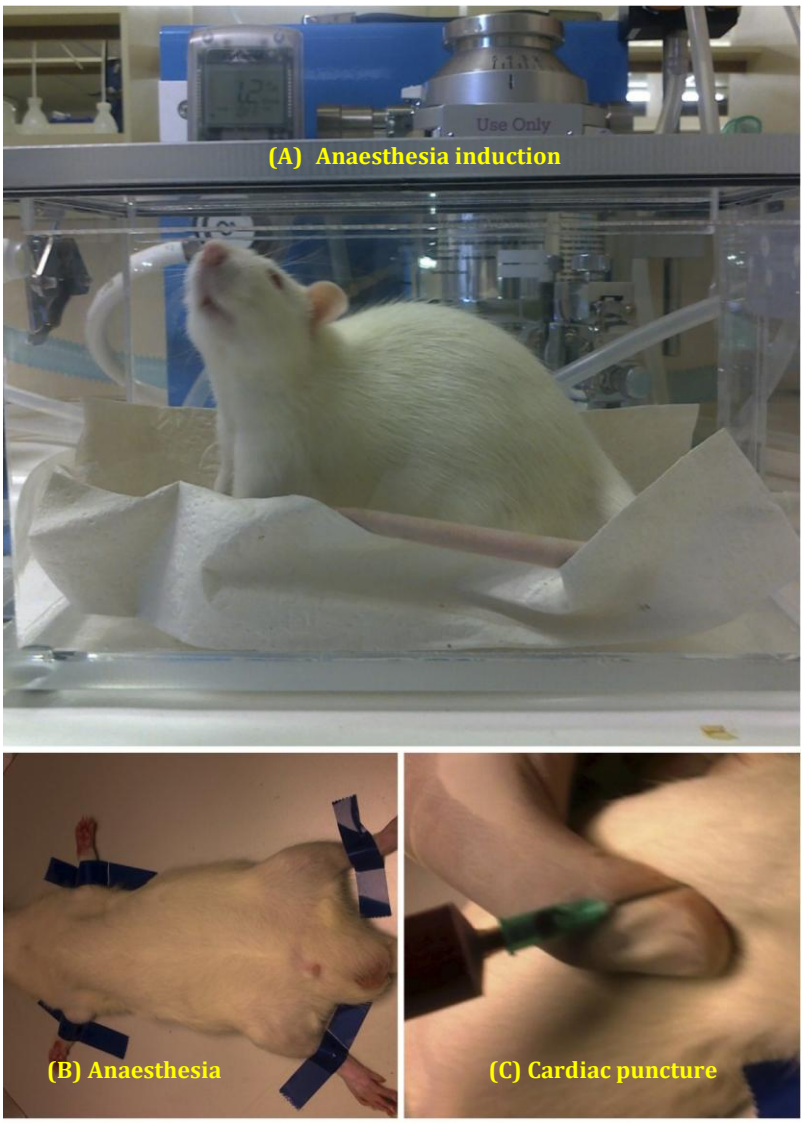


Figure 7.2: Picture illustrating the sacrificing procedure. *(A)The gas chamber for anesthesia induction of rats. (B) Rat in supine position during anesthesia maintenance. (C) Cardiac puncture for blood collection*.

The yellow top tubes were used for serum (3 – 4 ml) collection and stored at –80 °C until time of analysis. Serum liver and renal function tests were analysed by the department of pharmacology's toxicology laboratory. The liver function tests evaluated were alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP). Creatinine and blood urea nitrogen (BUN) were markers for renal function tests.

7.4.7. ORGAN HARVESTING PROCEDURE

Figure 7.3 is a picture illustrating the organ harvesting procedure in rats. The rats' abdominal cavity was dissected to remove the liver, kidney and spleen. Thereafter, the thoracic cavity was opened to access the thymus. All the organs were rinsed with water to remove excess blood and placed in potassium chloride. Furthermore, the organs were weighed and a small piece cut off and preserved in 10 % neutral formalin, to be used later for histopathology analysis for confirmation if necessary.

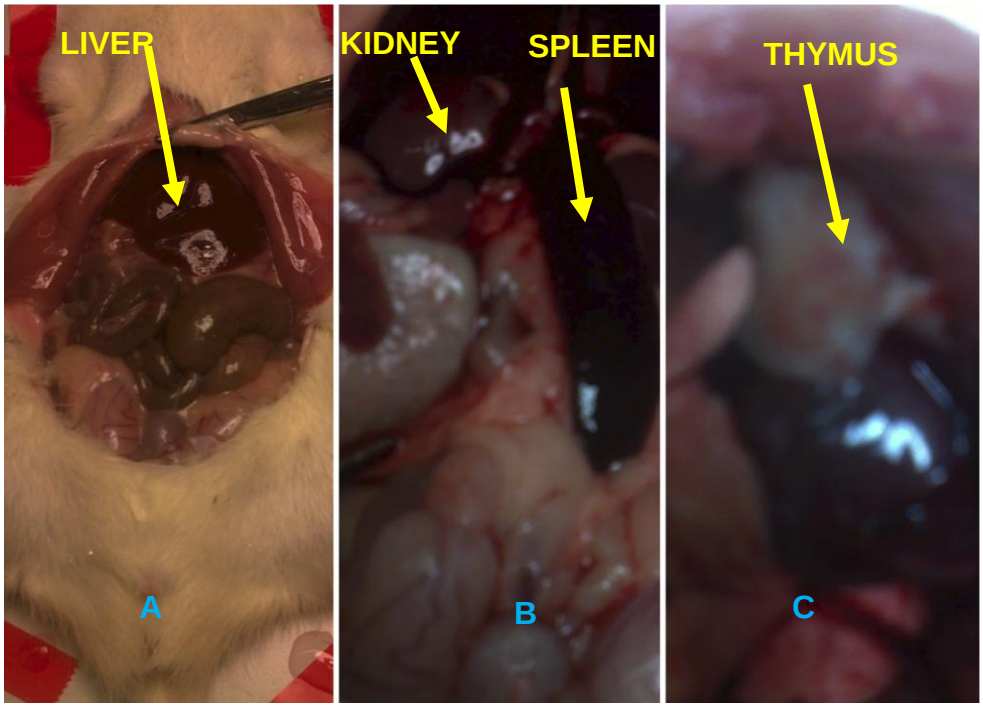


Figure 7.3: Picture of the harvested organs during the sacrificing procedure: (A) is the liver, (B) is the kidneys and spleen and (C) is the thymus.

7.5. ELISA PROCEDURE

IgG, IgM, Interleukin 2 (IL-2), and interleukin 10 (IL-10), concentrations were determined using ELISA kits. Rat specific ELISA kits were applied according to each manufacturer's instructions to determine cytokines and immunoglobins concentration. IL-2 and IL-10 were measured in pg/ml, IgG in mg/ml and IgM in µg/ml. IL-2 and IL-10 had pre-coated micro well plates while IgG and IgM plates required overnight blocking with the specific anti-MAb. From each group three samples were randomly selected and analyzed while

the standards were prepared in duplicate. **Figure 7.4** is an illustration of the ELISA experiment explained with steps from 1 to 5.

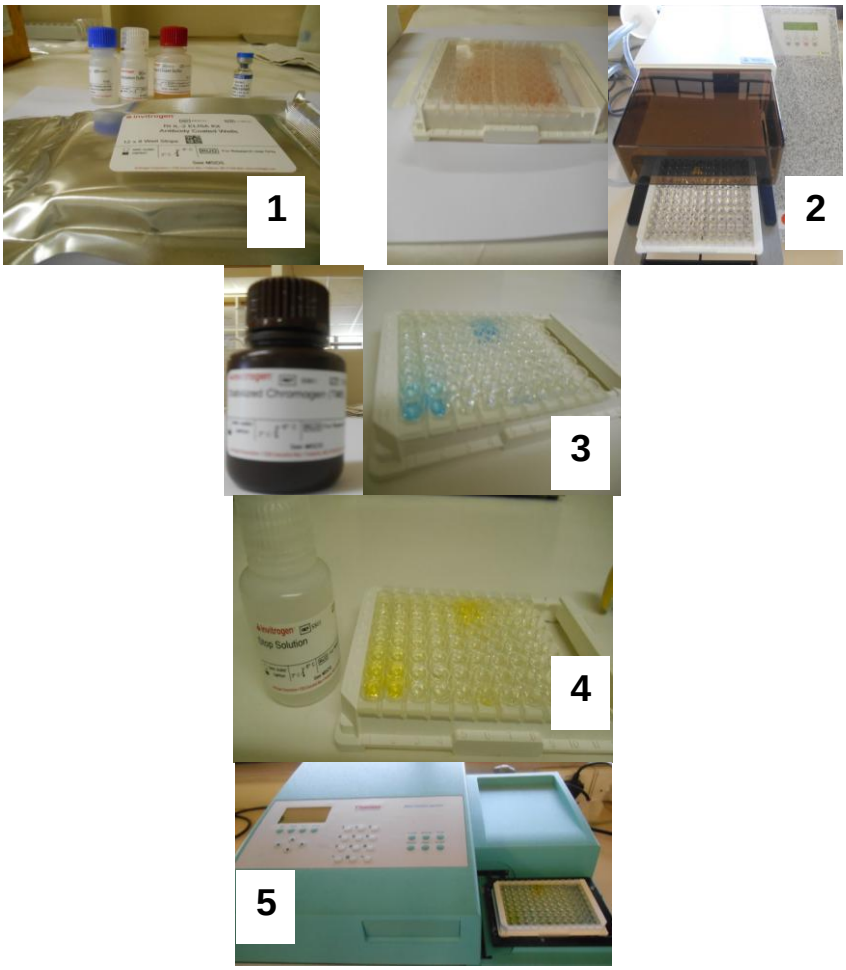


Figure 7.4: Schematic representation of ELISA experiment.

STEP 1: Pre-coated plates with rat specific anti-MAb.

STEP 2: Calibration standards and diluted rat serum samples were added in wells, incubated and shaken for 2hrs; thereafter the plate washed. After which biotinylated detection antibody is added, incubated for 1 hr, followed by a wash step. Thereafter, stabilized streptavidin-HRP was added and incubated for 30 minutes and allow developing in the dark.

STEP 3: Addition of chromogen and incubation for 30 minutes followed. A blue color formation was proportional to the amount of protein present in the sample.

STEP 4: Either the stop solution or phosphoric acid was added to stop the reaction and a yellowish color formed.

STEP 5: Absorbance was read on the micro plate reader at 405 nm. A standard curve with calibration standards against absorbance was plotted to determine cytokine or immunoglobins concentration.

7.6. STATISTICALLY ANALYSIS

All statistical analysis were done using the Graph Pad© Instat program and graphs drawn on an excel program. Data was analyzed by non-parametric methods using the Graph Pad© Instat statistical program. Accordingly, summary data were reported as mean and SD, and the student *t-test* was used for data comparison with the level of significance set at $p < 0.05$.

7.7. RESULTS

7.7.1. PHYSIOLOGICAL PARAMETERS RESPONSE TO TREATMENT WITH ESCALATING DOSES OF *PHELA*

In general *Phela* had no effect on all the physiological parameters in healthy rats. The means of *Phela* treated groups were similar to those of the control and were within the acceptable limits.

7.7.1.1. Liver functions tests

Table 7.1 is a summary of alkaline phosphatase (ALP) levels after treatment with either saline, LEV or the three doses of *Phela* (5, 15.4 and 75 mg/kg) over 21 days. *Phela* (5 and 15.4 mg/kg) treated groups had minimal changes through-out the study; when compared to control group though not statistical significant.

Table 7.2 indicates alanine transaminase (ALT) levels after treatment with either saline, LEV or the three doses of *Phela* (5, 15.4 and 75 mg/kg) over 21 days. Seven days treatment with *Phela* 15.4 mg/kg decreased ALT and recovered after fourteen days of treatment. Treatment of animals with saline and *Phela* over 21 days did not have an effect on ALT.

Table 7.3 is a summary of aspartate transaminase (AST) levels after treatment with either saline, LEV or the three doses of *Phela* (5, 15.4 and 75 mg/kg) over 21 days. AST levels were slightly increased after 14 days treatment with *Phela* 5mg/Kg ($P < 0.071$) though not quite significant.

Table 7.1: Summary of **alkaline phosphatase (U/L)** levels recorded as mean ± SD after 7, 14 and 21 days of *Phela* treatment.

	Saline	PHL 5 mg/kg	PHL 15.4 mg/kg	PHL 75 mg/kg	Levamisole
DAY 0	352.3±75.6	352.3±75.6	352.3±75.6	352.3±75.6	352.3±75.6
DAY 7	399.7±6.5	265.7±16.9	220.5±10.61	324.2±54.4	219.0±90.7
DAY 14	303.7±12.7	354.0±33.6	308.2±72.6	260.0±18.7	237.7±46.4
DAY 21	282.4±29.9	264.0±12.4	264.6±13.9	237.3±11.0	204.0±34.5

P < 0.05 vs control **P < 0.001 vs control* ***Abbreviations*: **PHL**: *Phela,* alkaline phosphatase (289 – 436 U/L)

Table 7.2: Summary of **alanine transaminase (U/L)** levels recorded as mean ± SD after 7, 14 and 21 days of *Phela* treatment.

	Saline	PHL 5 mg/kg	PHL 15.4 mg/kg	PHL 75 mg/kg	Levamisole
DAY 0	49.7±4.7	49.7±4.7	49.7±4.7	49.7±4.7	49.7±4.7
DAY 7	46.0±2.0	47.0±2.0	29.5±3.4	45.2±2.0	43.8±1.7
DAY 14	49.0±10.4	51.4±4.8	45.4±5.0	50.8±3.9	41.7±5.5
DAY 21	47.2±3.6	52.0±6.4	48.4±2.6	48.0±4.6	39.3±4.5

P < 0.05 vs control **P < 0.001 vs control* ***Abbreviations*: **PHL**: *Phela,* Alanine transaminase (46 – 55 U/L)

Table 7.3: Summary of **aspartate transaminase (U/L)** levels recorded as mean ± SD after 7, 14 and 21 days of *Phela* treatment.

	Saline	PHL 5 mg/kg	PHL 15.4 mg/kg	PHL 75 mg/kg	Levamisole
DAY 0	88.0±13.9	88.0±13.9	88.0±13.9	88.0±13.9	88.0±13.9
DAY 7	90.0±6.6	116.7±23.4	86.3±33.7	79.8±9.0	76.0±27.2
DAY 14	103.0±25.2	131.8±8.6	92.8±12.1	99.5±24.4	124.7±19.4
DAY 21	110.0±24.9	134.4±12.6	109.0±16.7	96.0±6.1	64.0±16.8

P < 0.05 vs control **P < 0.001 vs control* ***Abbreviations*: **PHL**: *Phela***,** Aspartate transaminase (81 – 104 U/L).

7.7.1.2. Renal function tests

Table 7.4 is a summary of creatinine levels after treatment with either saline, LEV or the three doses of *Phela* (5, 15.4 and 75 mg/kg) over 21 days. The control group increased after 7 days of treatment and returned to baseline after three weeks. Creatinine levels of *Phela* (5 and 15.4 mg/kg) treated groups decreased after seven days and recovered in 21 days. There was no significant difference between the test groups and the control groups after 21 days of treatment.

Table 7.5 indicates blood urea nitrogen (BUN) levels after treatment with either saline, LEV or the three doses of *Phela* (5, 15.4 and 75 mg/kg) over 21 days. Animals treated with *Phela* 15.4 mg/kg had a higher BUN level after 7 days treatment and returned to baseline by day 14. Seven days treatment with *Phela* 5 and 75 mg/kg resulted in lower readings compared with the control group. All groups returned to baseline after two weeks treatment.

7.7.1.3. Haematological parameters

Table 7.6 describes red blood cell count after treatment with either saline, LEV or the three doses of *Phela* (5, 15.4 and 75 mg/kg) over 21 days. Red blood cell count increased weekly and peaked after three weeks treatment in both saline and *Phela* groups. Moreover, after seven days treatment with *Phela* 5 and 15.4 mg/kg, platelets increased, thereafter returning to baseline by day fourteen of treatment.

Table 7.7 summarizes white blood cell readings after treatment with either saline, LEV or the three doses of *Phela* (5, 15.4 and 75 mg/kg) over 21 days. White cells count decreased after one week treatment in both the control and test groups. *Phela* treatment in all test groups' led to increased WCC after 14 days and returned to baseline by 21 days. Eosinophilia set in after 14 days with saline, *Phela* 5 and 75 mg/kg treatment. Wide variations in the means of the test groups were observed, but were not statistically significant. Monocytes were more pronounced in all *Phela* treated groups when compared to the saline group.

Table 7.4: Summary of **Creatinine (µmol/L**) levels recorded as mean ± SD after 7, 14 and 21 days of *Phela* treatment.

	Saline	PHL 5 mg/kg	PHL 15.4 mg/kg	PHL 75 mg/kg	Levamisole
DAY 0	36.7±8.1	36.7±8.1	36.7±8.1	36.7±8.1	36.7±8.1
DAY 7	45.7±6.7	36.0±11.5	30.0±5.2	34.8±10.2	56.3±4.2
DAY 14	39.0±2.7	32.6±14.3	30.8±4.1	41.0±1.4	57.7±11.6
DAY 21	32.6±9.4	35.4±4.8	38.0±6.4	33.0±6.1	54.3±3.2

P < 0.05 vs control **P < 0.001 vs control* ***Abbreviations +reference values*: **PHL**: *Phela,* Creatinine (31 – 46 µmol/L)

Table 7.5: Summary of **Blood urea nitrogen (mmol/L)** levels recorded as mean ± SD after 7, 14 and 21 days of *Phela* treatment.

	Saline	PHL 5 mg/kg	PHL 15.4 mg/kg	PHL 75 mg/kg	Levamisole
DAY 0	7.20±0.26	7.20±0.26	7.20±0.26	7.20±0.26	7.20±0.26
DAY 7	8.10±0.26	6.17±0.41	11.40±0.72	6.28±0.37	6.00±0.62
DAY 14	7.50±0.69	7.72±0.68	7.24±0.58	6.93±0.82	6.17±0.95
DAY 21	7.48±1.02	7.24±0.72	7.50±0.77	6.93±0.38	5.73±0.81

P < 0.05 vs control **P < 0.001 vs control* ***Abbreviation + reference values*: **PHL**: *Phela, Blood* urea nitrogen (6.5 – 8.2 mmol/L)

Table 7.6: Summary of full blood tests (**red blood cells & Platelets**) levels recorded as mean ± SD after 7, 14 and 21 days of *Phela* treatment.

GROUPS	RCC (x10^12/l)	Haemoglobin (g/dl)	Haematocrit (l/l)	MCV (fl)	MCH (pg)	MCHC (g/dl)	Platelet count (x10^9/l)
Untreated	6.14±0.48	12.60±0.78	0.38±0.03	62.17±0.03	20.50±0.36	33.03±0.70	961.33±63.57
7 DAYS TREATMENT							
Saline (n = 3)	7.53±0.92	15.30±1.73	0.45±0.04	60.13±2.48	20.33±0.21	33.87±1.19	1033.33±79.76
***Phela* 5 mg/kg**	6.37±0.50	12.97±0.58	0.40±0.01	63.03±3.29	20.37±0.92	32.33±0.38	814.33±227.81
***Phela* 15.4 mg/kg**	7.43±0.51	14.37±0.81	0.45±0.02	60.20±2.40	19.40±0.70	32.17±0.38	1033.33±201.08
***Phela* 75 mg/kg**	6.97±0.07	13.53±0.15	0.40±0.01	58.00±0.26	19.43±0.06	33.47±0.21	1188.33±209.19
Levamisole	7.02±0.34	14.17±0.51	0.41±0.01	58.27±1.59	20.23±0.67	34.70±0.62	952.00±77.95
14 DAYS TREATMENT							
Saline	7.04±0.13	14.70±0.28	0.44±0.01	62.60±2.83	20.90±0.78	33.30±0.35	929.00±166.00
***Phela* 5 mg/kg**	7.63±0.24	15.30±0.46	0.46±0.02	59.60±0.53	20.07±0.15	33.67±0.49	955.00±62.95
***Phela* 15.4 mg/kg**	7.60±0.28	15.40±0.50	0.46±0.01	60.30±2.82	20.27±0.57	33.67±0.91	707.67±150.32
***Phela* 75 mg/kg**	8.23±0.48	16.20±0.28	0.49±0.00	59.40±3.82	19.70±0.85	33.20±0.71	745.00±142.84
Levamisole	6.96±0.45	13.50±1.04	0.39±0.03	56.13±1.01	19.40±0.36	34.57±0.06	934.00±112.41
21 DAYS TREATMENT							
Saline	8.14±0.24	16.03±0.70	0.55±0.04	67.57±2.67	19.70±0.35	29.13±0.68	524.33±130.21
***Phela* 5 mg/kg**	8.25±0.16	16.00±0.14	0.54±0.00	65.35±1.06	19.40±0.57	29.70±0.42	563.00±29.70
***Phela* 15.4 mg/kg**	8.16±0.28	15.70±0.35	0.46±0.02	56.67±0.50	19.23±0.42	33.97±0.38	774.33±86.89
***Phela* 75 mg/kg**	8.20±0.83	15.27±0.99	0.46±0.01	55.90±4.89	18.63±0.67	33.47±1.76	815.67±118.75
Levamisole	7.18±0.40	14.50±0.85	0.44±0.04	61.13±2.95	20.20±0.53	33.07±0.81	544.33±22.14

Abbreviations + Reference values: **RCC**: Red cell count (5.99 -6.42), Haemoglobin (12.5 – 13.1), haematocrit (0.393 – 0.406) **MCV**: Mean corpuscular volume (61.2 – 66.1), **MCH**: Mean corpuscular hemoglobin (20.2 – 20.9), **MCHC**: Mean corpuscular hemoglobin concentration (31.1 – 32.3), Platelets (611 – 1034)

Table 7.7: Summary of Full blood test (**white blood cells**) levels recorded as mean ± SD after 7, 14 and 21 days of *Phela* treatment.

GROUPS	WCC (x10^9/l)	Neutrophils (x10^9/l)	Lymphocytes (x10^9/l)	Monocytes (x10^9/l)	Eosinophils (x10^9/l)	Basophils (x10^9/l)
Untreated	6.02±1.63	0.77±0.17	5.03±1.44	0.19±0.11	0.02±0.00	0.01±0.01
			7 DAYS TREATMENT			
Saline	5.44±2.40	1.03±0.81	4.07±1.95	0.30±0.28	0.04±0.02	0.01±0.01
***Phela* 5mg/kg**	3.81±1.02	0.44±0.17	3.14±0.93	0.19±0.04	0.03±0.02	0.01±0.01
***Phela* 15.4 mg/kg**	4.42±1.66	0.77±0.51	3.51±1.14	0.09±0.08	0.05±0.01	0.00±0.01
***Phela* 75 mg/kg**	5.40±1.04	0.63±0.21	4.56±0.77	0.14±0.06	0.06±0.02	0.01±0.01
Levamisole	6.69±1.23	1.06±0.31	5.38±0.84	0.21±0.09	0.03±0.01	0.01±0.01
			14 DAYS TREATMENT			
Saline	5.45±0.13	0.34±0.04	3.79±0.03	0.09±0.03	0.27±0.01	0.05±0.03
***Phela* 5mg/kg**	7.09±0.78	0.88±0.34	5.82±0.48*	0.33±0.15	0.06±0.03	0.01±0.00
***Phela* 15.4 mg/kg**	8.91±1.03	1.13±0.33	6.63±0.94*	0.36±0.09	0.78±0.62	0.01±0.00
***Phela* 75 mg/kg**	6.34±1.25	0.87±0.43	7.31±0.81	0.25±0.12	0.92±0.11	0.01±0.00
Levamisole	7.15±0.44	1.16±0.33	5.55±0.38*	0.38±0.19	0.05±0.02	0.01±0.00
			21 DAYS TREATMENT			
Saline	5.90±0.60	0.64±0.20	4.81±0.39	0.17±0.16	0.26±0.36	0.01±0.00
***Phela* 5mg/kg**	5.36±1.48	2.10±0.32	3.20±1.12	0.05±0.03	0.03±0.03	0.01±0.00
***Phela* 15.4 mg/kg**	5.51±1.62	0.62±0.17	3.78±1.40	0.19±0.16	0.92±0.61	0.01±0.00
***Phela* 75 mg/kg**	6.82±2.30	1.58±1.36	4.38±0.54	0.21±0.23	0.64±0.58	0.01±0.00
Levamisole						

Abbreviations+ Reference values: **WCC**: white cell count (4.16 – 9.55), Neutrophils (0.65 - 0.89), Lymphocytes (3.37 – 5.99), Monocytes (0.11 – 0.27), Eosinophils (0.00 – 0.02), Basophils (0.01 – 9.55)

7.7.2. GENERAL IMMUNE MARKERS RESPONSE TO TREATMENT WITH ESCALATING DOSES OF *PHELA*

Table 7.8 is a summary of body weight (g) and changes in body weight (%) of all groups treated over 21 days with either saline, LEV or three doses of *Phela* (5, 15.4 and 75 mg/kg). The weight change was ±17 % after 7 days in all groups with the exception of *Phela* 15.4 mg/kg with only ±4 % increase. After two and three week's treatment, weight gain doubled in all groups. The changes were within the expected ranges of normal growth.

Table 7.9 – 7.12 is a synopsis of the effect of *Phela* on four organs; kidney, liver, spleen and thymus weight. *Phela* 15.4 mg/kg and levamisole had no effect on the organs' weight while *Phela* 5 and 75 mg/kg had variable effects as described below.

Table 7.9 indicates the effect of *Phela* on kidneys' weight. The kidneys' weight increased rapidly after a week treatment with 5 mg/kg of *Phela* (P = 0.016), however after three weeks treatment the weight remained unchanged (P = 0.056). Furthermore, in other test groups (*Phela* 5 and 75 mg/kg) the weight increased according to normal growth.

Table 7.10 is the effect of *Phela* on liver weight. The liver weight increased rapidly after one week treatment with *Phela* 5 mg/kg (P = 0.016). After three weeks treatment, the weight dropped significantly in groups treated with *Phela* 5 mg/kg (P = 0.05) and 75 mg/kg (P = 0.032).

Table 7.11 summarizes the effect of *Phela* on spleen weight. Spleen weight decreased after three weeks treatment with *Phela* 5 mg/kg. The highest dose of *Phela (Phela* 75 mg/kg*)* significantly prevented weight gain after 14 (P = 0.05) and 21 (P = 0.008) days.

Table 7.12 briefly outlines the effect of *Phela* on thymus weight. Thymus weight in *Phela* treated groups was higher than in the control groups for the duration of the study, though not statistically significant. The 75 mg/kg dose of *Phela* was the only exception with a slight decrease in thymus weight (P = 0.086) after 21 days of treatment.

Table 7.8: Summary of **body weight (g)** before and after treatment and **change** (%) in body weight recorded as mean ± SD after 7, 14 and 21 days of *Phela* treatment.

GROUPS	BEFORE (g)	AFTER (g)	Change (%)
Untreated	224±16	224±16	0
	7 DAYS TREATMENT		
Saline	214±6	250±5	+17±4
***Phela* 5mg/kg**	219±12	258±18	+19±14
***Phela* 15.4 mg/kg**	213±8	221±3	+4±4
***Phela* 75 mg/kg**	208±6	232±12	+12±6
Levamisole	215±14	252±26	+17±8
	14 DAYS TREATMENT		
Saline	222±10	306±12	+38±2
***Phela* 5mg/kg**	213±9	269±10	+27±8
***Phela* 15.4 mg/kg**	215±7	275±6	+28±7
***Phela* 75 mg/kg**	222±8	264±18	+18±13
Levamisole	207±4	258±12	+25±8
	21 DAYS TREATMENT		
Saline	218±12	299±11	+38±10
***Phela* 5mg/kg**	215±9	274±19	+28±7
***Phela* 15.4 mg/kg**	222±11	308±20	+39±15
***Phela* 75 mg/kg**	221±12	285±20	+23±11
Levamisole	213±10	275±16	+30±9

Table 7.9: Summary of **kidney weight (g**) recorded as mean ± SD after 7, 14 and 21 days of *Phela* treatment.

	Saline	PHL 5 mg/kg	PHL 15.4mg/kg	PHL 75 mg/kg	Levamisole
DAY 0	1.73±0.11	1.73±0.11	1.73±0.11	1.73±0.11	1.73±0.11
DAY 7	1.88±0.02	2.08±0.12	1.87±0.08	1.83±0.08	1.99±0.28
DAY 14	2.15±0.15	2.10±0.11	2.32±0.13	1.98±0.17	2.07±0.06
DAY 21	2.34±0.25	2.07±0.10	2.57±0.20	2.24±0.29	2.15±0.23

Abbreviations: **PHL**: *Phela*

Table 7.10: Summary of **liver weight (g)** recorded as mean ± SD after 7, 14 and 21 days of *Phela* treatment.

	Saline	PHL 5 mg/kg	PHL 15.4 mg/kg	PHL 75 mg/kg	Levamisole
DAY 0	10.04±0.85	10.04±0.85	10.04±0.85	10.04±0.85	10.04±0.85
DAY 7	10.08±0.32	12.16±1.19	9.34±0.92	11.23±1.15	11.18±1.12
DAY 14	11.66±0.68	11.38±0.76	12.71±1.11	10.61±0.98	11.17±0.94
DAY 21	12.88±1.46	10.84±0.72	12.54±0.68	10.13±1.60	11.22±0.94

Abbreviations: **PHL**: *Phela*

Table 7.11: Summary of **spleen weight (g)** recorded as mean ± SD after 7, 14 and 21 days of *Phela* treatment.

	Saline	PHL 5 mg/kg	PHL 15.4 mg/kg	PHL 75 mg/kg	Levamisole
DAY 0	0.52±0.06	0.52±0.06	0.52±0.06	0.52±0.06	0.52±0.06
DAY 7	0.55±0.02	0.62±0.12	0.56±0.04	0.54±0.08	0.63±0.03
DAY 14	0.68±0.05	0.69±0.09	0.67±0.07	0.60±0.06	0.57±0.05
DAY 21	0.73±0.03	0.61±0.07	0.68±0.09	0.60±0.06	0.59±0.09

Abbreviations: **PHL**: *Phela*

Table 7.12: Summary of **thymus weight (g)** recorded as mean ± SD after 7, 14 and 21 days of *Phela* treatment.

	Saline	PHL 5 mg/kg	PHL 15.4 mg/kg	PHL 75 mg/kg	Levamisole
DAY 0	0.47±0.04	0.47±0.04	0.47±0.04	0.47±0.04	0.47±0.04
DAY 7	0.51±0.05	0.54±0.07	0.58±0.12	0.54±0.07	0.57±0.06
DAY 14	0.48±0.07	0.59±0.04	0.57±0.09	0.53±0.10	0.55±0.08
DAY 21	0.53±0.04	0.55±0.11	0.52±0.03	0.44±0.04	0.49±0.08

Abbreviations: **PHL**: *Phela*

7.7.3. IMMUNE CELLS RESPONSE TO TREATMENT WITH ESCALATING DOSES OF *PHELA*

Figure 7.5 – 7.7 are plots illustrating the effect of *Phela* on various immune cells after 21 days of treatment. Treatment with *Phela* for fourteen days had maximum effect on the immune system confirmed by an increase in white cell, neutrophils and lymphocytes count.

Figure 7.5 is an illustration of white cell count response to treatment with either saline, LEV or three doses of *Phela* (5, 15.4 and 75 mg/kg) over 21 days. Two weeks treatment with *Phela* 15.4 mg/kg led to the highest response of WCC. Furthermore *Phela* led to increased WCC after 7 and 14 days treatment, returning to baseline after 21 days.

Figure 7.6 is a diagram of neutrophils count response to treatment with either saline, LEV or three doses of *Phela* (5, 15.4 and 75 mg/kg) over 21 days. *Phela* 5 and 75 mg/kg inhibited neutrophils after 7 days treatment but slightly increased by day 21. LEV treatment did not alter neutrophils response through-out the study.

Figure 7.7 exemplifies lymphocyte cell count response to treatment with either saline, LEV or three doses of *Phela* (5, 15.4 and 75 mg/kg) over 21 days. Lymphocytes proliferation peaked after 14 days in LEV (5.55±0.38) and *Phela* (PHL 5 mg/kg, 5.82±0.48; PHL 5 mg/kg, 6.63±0.94; PHL 75 mg/kg, 4.31±0.81) treated groups.

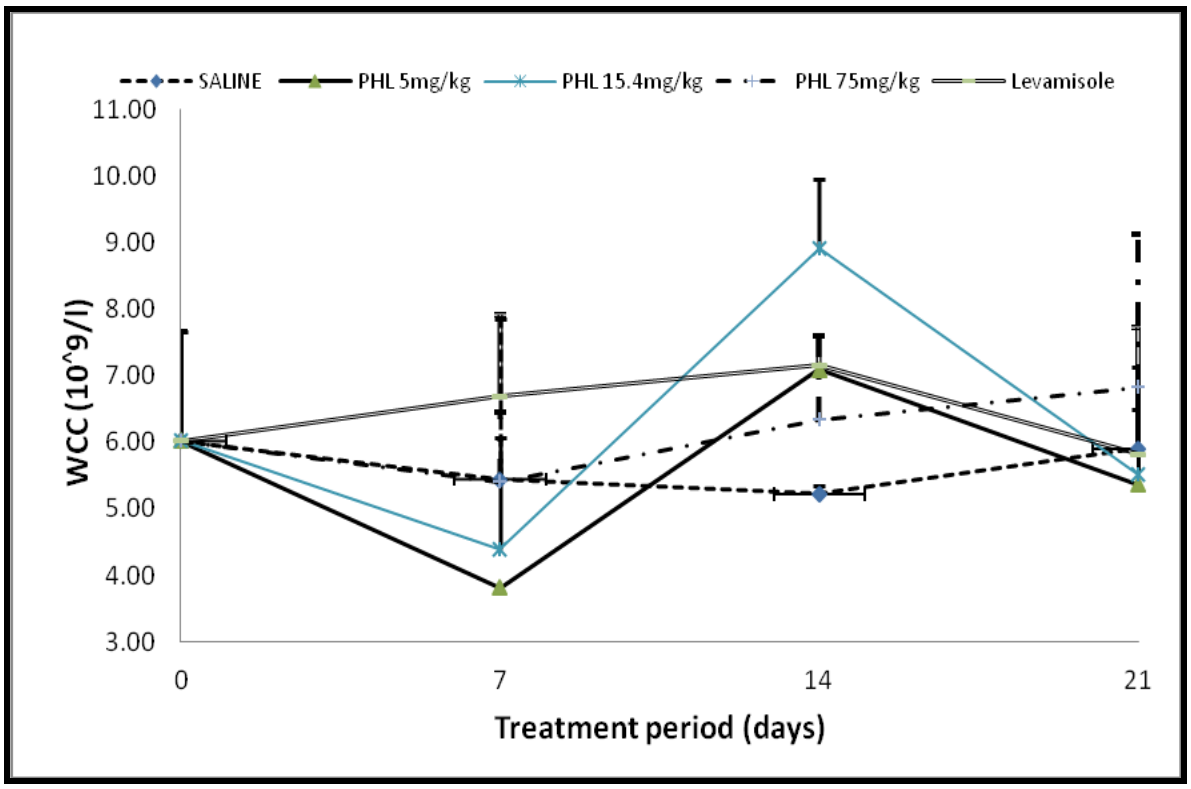


Figure 7.5: Effect of *Phela* on WCC count after 7, 14 and 21 days treatment.

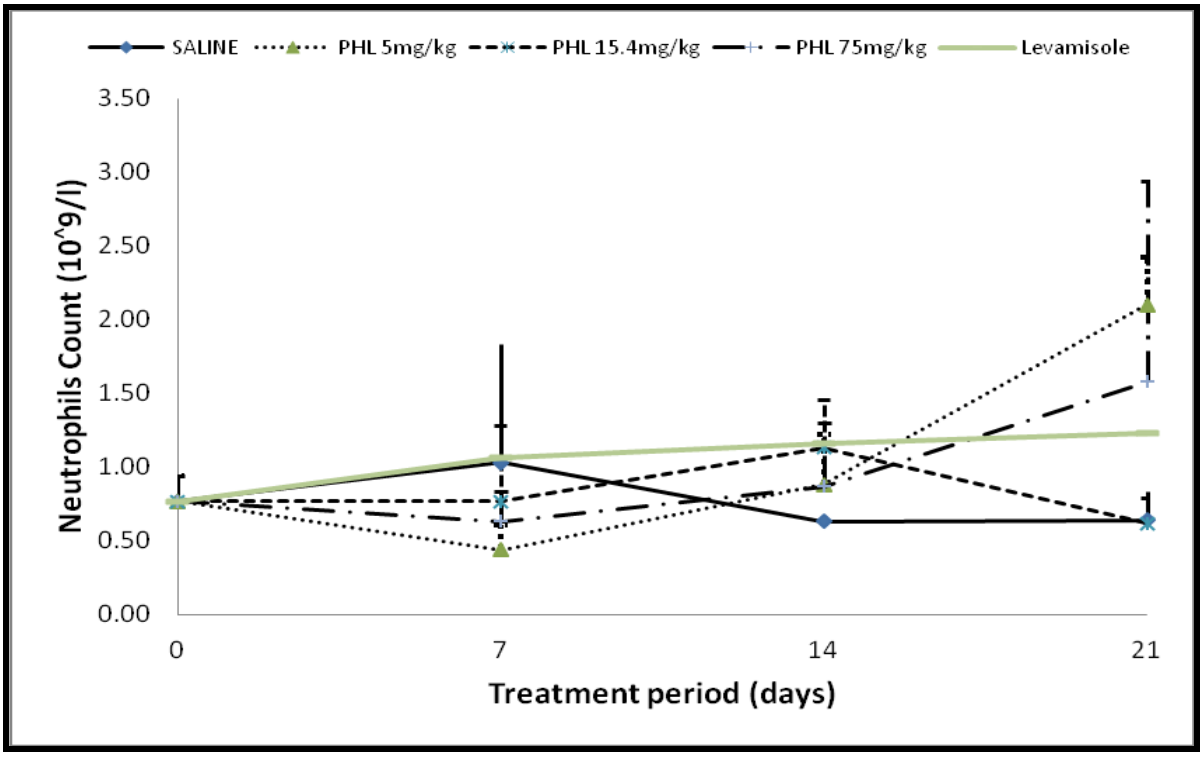


Figure 7.6: Effect of *Phela* on neutrophils count after 7, 14 and 21 days treatment.

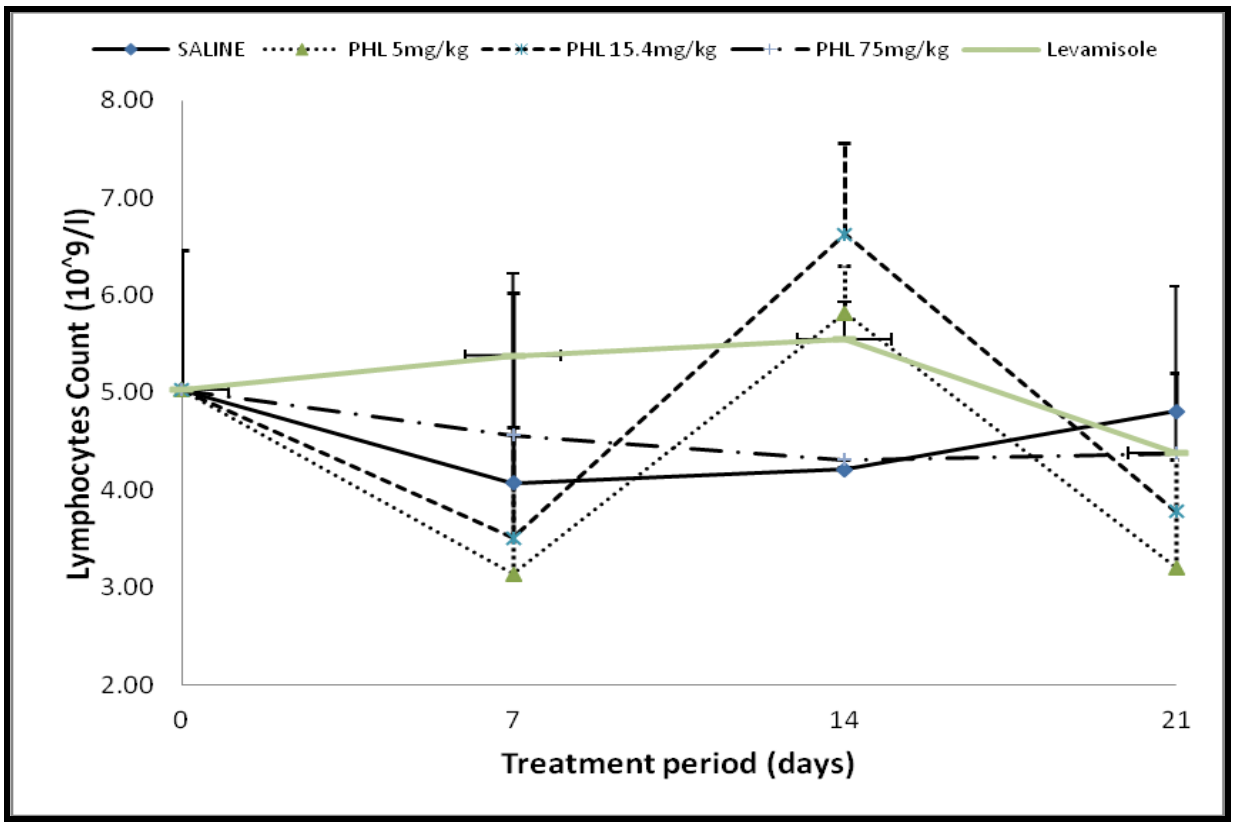


Figure 7.7: Effect of *Phela* on Lymphocytes count after 7, 14 and 21 days treatment.

Figure 7.8 demonstrates CD_4 cell count response to treatment with either saline, LEV or three doses of *Phela* (5, 15.4 and 75 mg/kg) over 21 days. Seven days treatment with *Phela* 5 and 15.4 mg/kg caused a slight decline in CD_4 cell count. *Phela* treated groups steadily increased and CD_4 cell count response peaked in rats treated with *Phela* 5, 15.4 and 75 mg/kg after fourteen days. *Phela* 75 mg/kg and LEV groups returned to baseline on day 21, while *Phela* (5 and 15.4 mg/kg) groups were slightly below baseline.

Figure 7.9 is an illustration of CD_8 cell count response to treatment with either saline, LEV or three doses of *Phela* (5, 15.4 and 75 mg/kg) over 21 days. Seven days treatment caused a decline in CD_8 cell count in both control and *Phela* 5 mg/kg group. Furthermore, the control group slowly recovered from the decrease on day 7. Rats treated with *Phela* recovered and peaked after 14 days treatment. Rats treated with LEV also had the highest CD_8 count response on day 14. In general, *Phela* had a similar effect on CD_4 and CD_8 cell through-out the study.

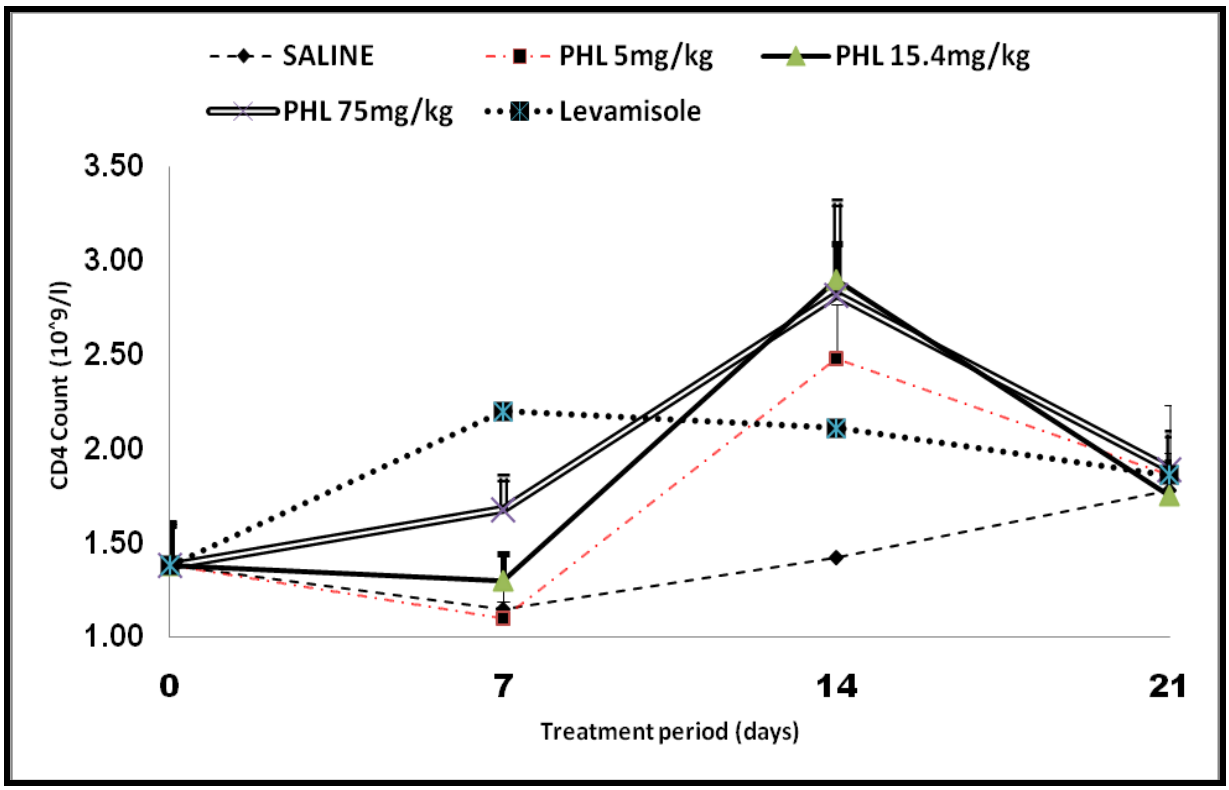


Figure 7.8: Effect of *Phela* on CD_4 count after 7, 14 and 21 days treatment.

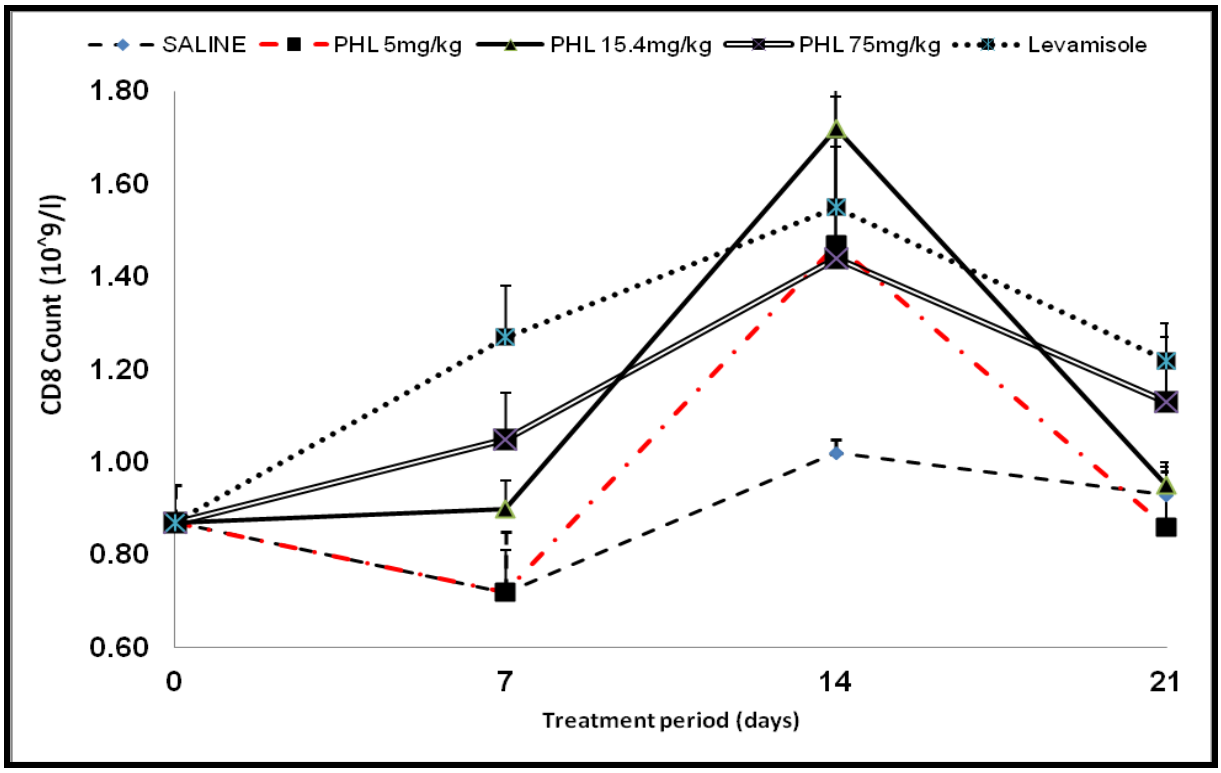


Figure 7.9: Effect of *Phela* on CD_8 count after 7, 14 and 21 days treatment.

7.7.4. IMMUNOGLOBULINS RESPONSE TO TREATMENT WITH ESCALATING DOSES OF *PHELA*

Figure 7.10 is an illustration of IgG count response to treatment with either saline, LEV or three doses of *Phela* (5, 15.4 and 75 mg/kg) over 21 days. IgG response was inhibited in rats treated with *Phela* 5 mg/kg and saline after 7 days. Thereafter, Saline, *Phela* 15.4 mg/kg groups slightly recovered from the decline. On the same note, *Phela* 75 mg/kg and LEV significantly increased IgG count after 7 days of treatment. Other *Phela* treated-groups were slightly increased on day 14 though not significant. All groups returned to baseline on day 21 whilst *Phela* 75 mg/kg group was above baseline.

Figure 7.11 is a schematic presentation of IgM count response to treatment with either saline, LEV or three doses of *Phela* (5, 15.4 and 75 mg/kg) over 21 days. IgM proliferation was inhibited after seven days treatment in all groups and remained low for the duration of the study. *Phela* and LEV had no effect on IgM count.

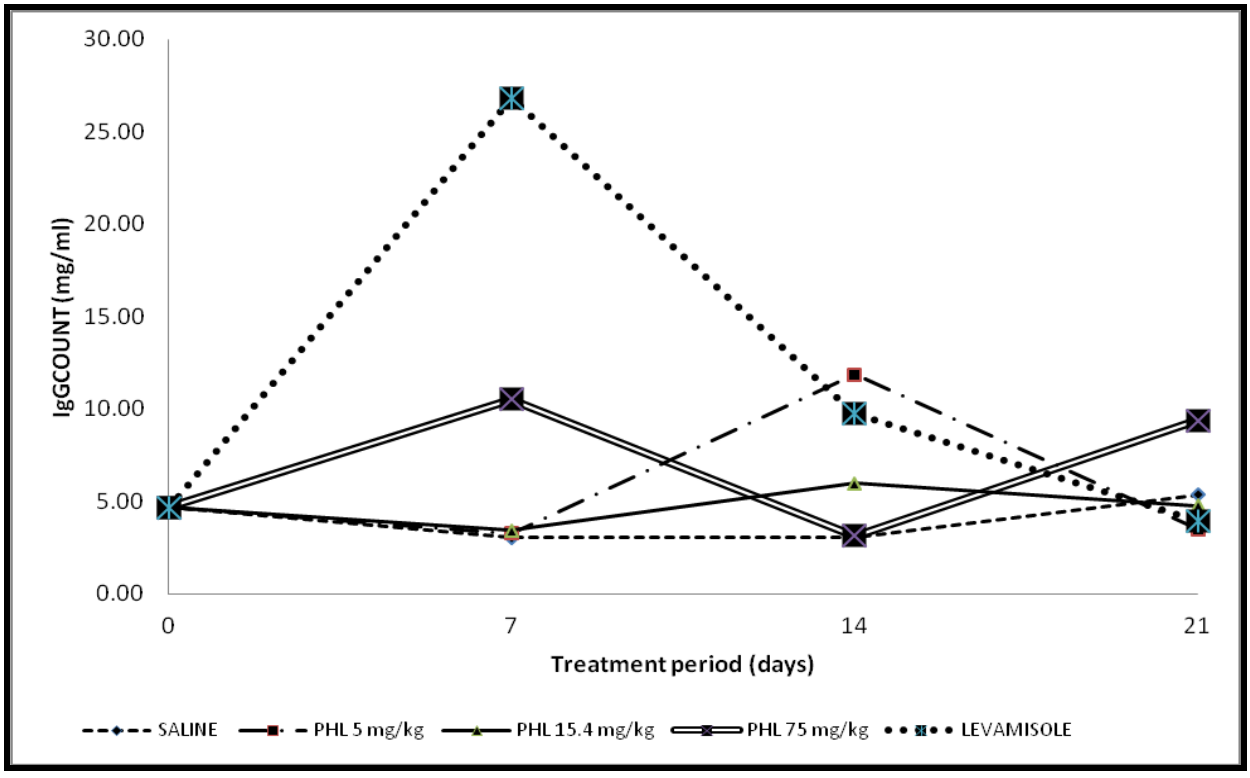


Figure 7.10: Effect of *Phela* on IgG count (mg/ml) after 7, 14 and 21 days treatment**.**

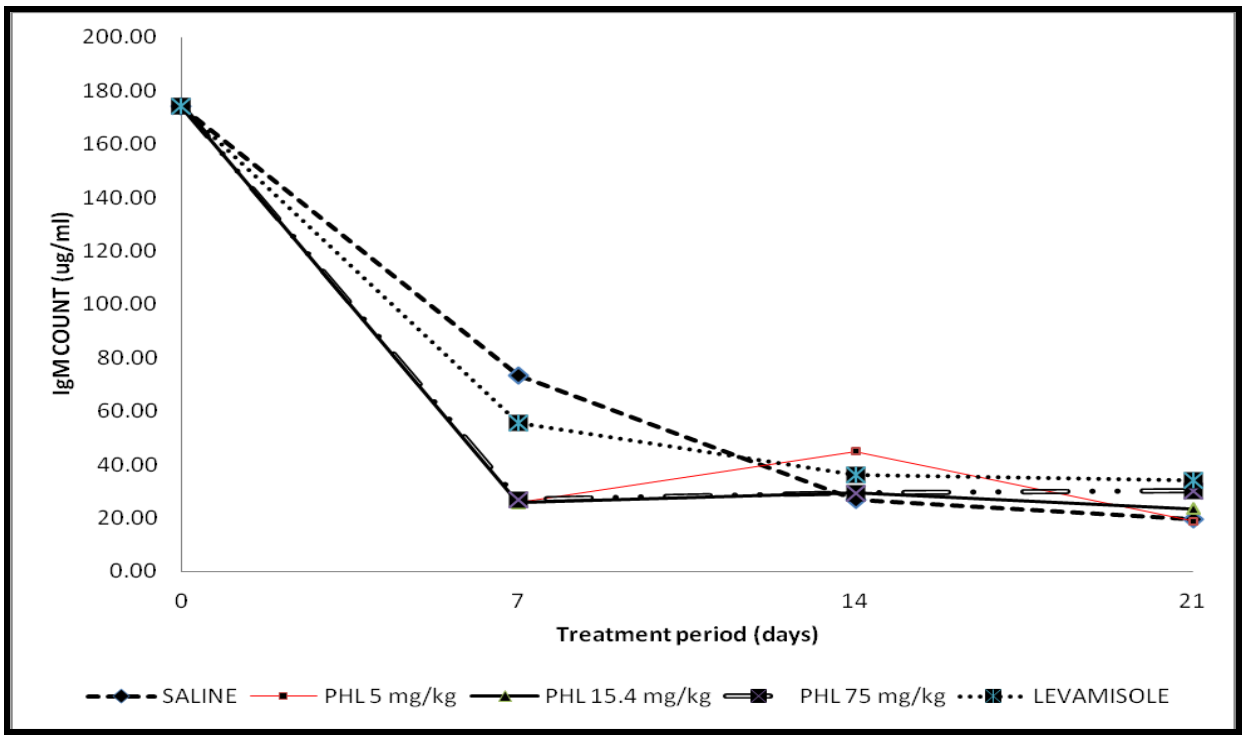


Figure 7.11: Effect of *Phela* on IgM count (µg/ml) after 7, 14 and 21 days treatment.

7.7.5. CYTOKINES RESPONSE TO TREATMENT WITH ESCALATING DOSES OF *PHELA*

Figure 7.12 is a plot of IL-2 count response to treatment with either saline, LEV or three doses of *Phela* (5, 15.4 and 75 mg/kg) over 21 days. IL-2 increased after seven days treatment with LEV and *Phela* 5 and 15.4 mg/kg, thereafter only LEV group remained high, and *Phela* treated groups declined. After 21 days of treatment IL-2 count returned to baseline in all control and test-groups.

Figure 7.13 is an illustration of IL-10 count response to treatment with either saline, LEV or three doses of *Phela* (5, 15.4 and 75 mg/kg) over 21 days. Rats IL-10 increased after seven days treatment with LEV and *Phela* 5 and 15.4 mg/kg respectively, thereafter only LEV group remained high. All groups returned to slightly above baseline by day 21 of treatment.

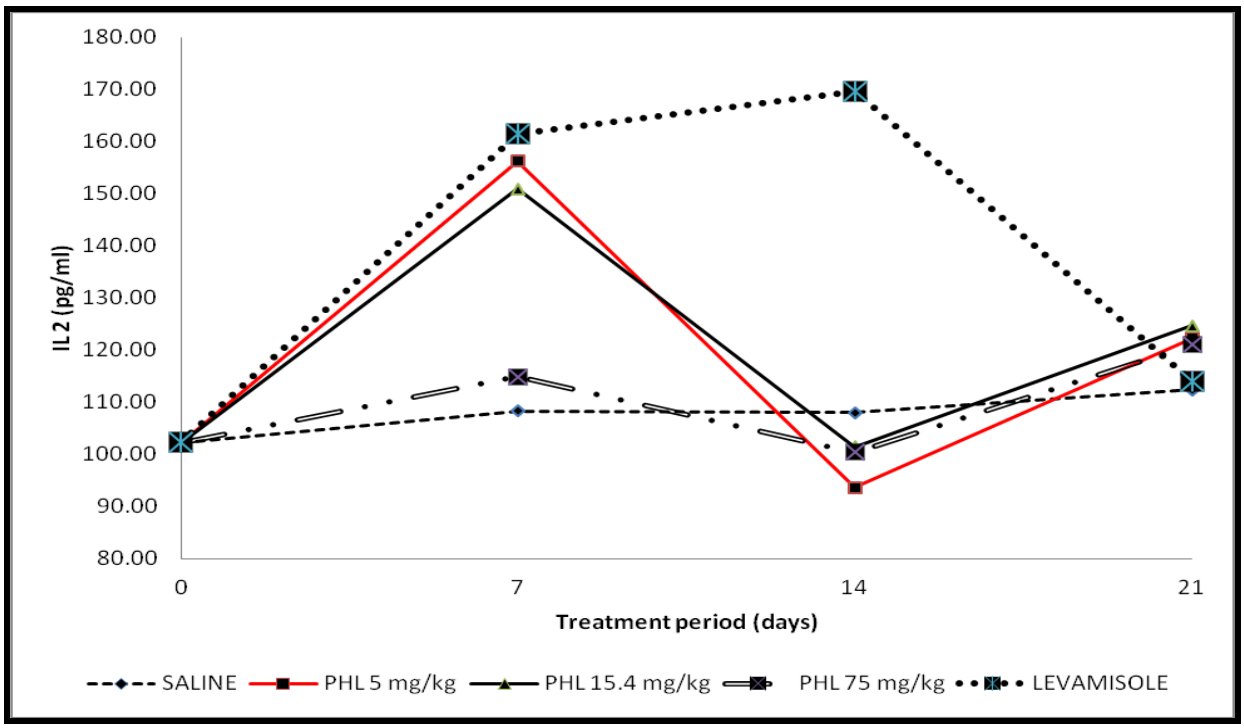


Figure 7.12: Effect of *Phela* on IL-2 count (pg/ml) after 7, 14 and 21 days treatment.

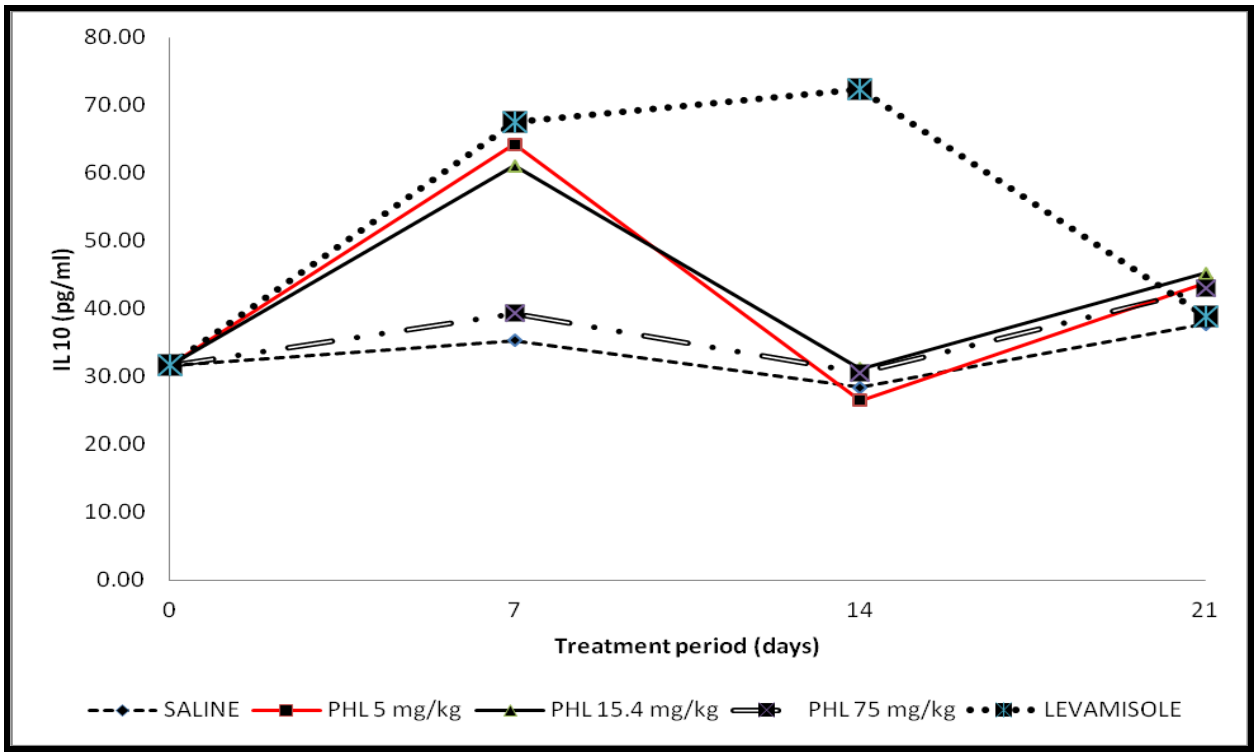


Figure 7.13: Effect of *Phela* on IL-10 count (pg/ml) after 7, 14 and 21 days treatment.

7.8. DISCUSSION

For many generations, *Phela* has been used in South Africa to treat wasting conditions in bed-ridden patients to increase the energy in these patients. In this study, we investigated the immunomodulatory effects of three doses of *Phela* in healthy rats to establish the dose that gives optimum results in rats. It is relevant and important to evaluate the escalating doses of herbal medicine as they are known to behave differently at different doses. Low doses are often sub therapeutic whereas high doses can cause interference resulting in false (-/+) and possible toxicity. Moreover, preliminary experiments are necessary to establish therapeutic doses in healthy animal and/or humans.

The results show that the haematology, liver and renal functions test were normal. The absence of growth retardation in the study of animals; lack of effect on liver and kidney weight suggest safety even at a high dose further proving that *Phela* is safe in rats. Also, results obtained after weight measurements of the body and lymphoid organs' weight (spleen and thymus) indicated slight increase that correlated with immune stimulation. LEV was an appropriate positive control because it exhibits non-specific immuno stimulation on both cellular and humoral immunity (Krakowski *et al.*, 1999).

T-lymphocytes play a pivotal role in regulating the immune response and the first step in proliferation of acquired immune response following stimulation. Furthermore they produce various cytokines which activate other immune cells to proliferate and differentiate into effector cells (Auttachoat *et al.*, 2004). Moreover, *Phela* produced non-specific immune response effects through modulation of leukocytes subsets. The significant rise in the white cell, lymphocytes, CD_4 and CD_8 cell may potentiate usefulness of *Phela* as an immune stimulant a factor that was reported in the anecdotal reports of healers and previous studies in our department (Matsabisa *et al.*, 2006; Lekhooa *et al.*, 2012a). It was observed therefore that the effect of *Phela* on the immune system for all doses was more pronounced after 14 days of treatment and returned to baseline after 21 days.

Comparison of the rats' immune response after treatment with *Phela* 5 and 75 mg/kg illustrated a dose-dependent pattern. *Phela* (5 mg/kg) was sub-therapeutic and the

higher dose (75 mg/kg) gave inconsistent levels which could be associated with possible interference common in herbal medicines high doses treatment. The intermediate dose (*Phela* 15.4 mg/kg) effect on test groups resulted in up-regulation of immune response and proliferation of leukocytes *in vivo*. Traditionally, *Phela* is prepared by combining the four plants in specific ratio, boiled over an hour, allowed to cool, sifted and taken as 3 cups daily. Based on the traditional preparation the recommended dose for humans is 15.4 mg/kg per day, however this to be scientifically validated *in vivo (MRC report; 2011).* The intermediate dose is similar to the recommended dose in humans.

A large number of plant species and their components have been shown to be potential immunomodulators acting as anti-inflammatory, antistress and anti cancer agents (Bin-Hafeez *et al*., 2003). Furthermore, the classification of a drug can either be immune stimulant, immune suppressant and/or both depending on the receptors being activated at a certain time. In phytotherapy, immunomodulators may be defined as botanicals that alter the activities of the immune system via cytokines, hormones, neurotransmitters and other peptides (Spelman *et al.,* 2006; Leckeweg *et al.,* 2007). Since targeting cytokines is now considered to be one of the logical approaches for the prevention and treatment of infectious disease ELISA serum quantification of cytokines and immunoglobulins was necessary.

Phela had no effect on IgG, IgM, and IL-10. IL-2 was up-regulated after 7 days of treatment with all doses of *Phela*. This correlates with our previous study that *Phela* led to increase in IL-2 (Lekhooa *et al.*, 2012a). IL-2 is not just the potent pro-inflammatory cytokine, along with IL-1 and IL-17 it has been reported to have shown potential augmentation of immune response in various infectious conditions and malignancies. TH_1 cells induce cell mediated immunity that fights intracellular organism, and eliminates cancerous whereas TH_2 cells up regulate antibody proliferation to fight extracellular organisms. The results have shown that *Phela* augmented the cell mediated response by stimulating leukocytes subsets and IL-2 and reached peak level in 14 days. Furthermore, the use of the effector functions of the TH cells is considered to be the more innovative therapeutic strategy to fight infections (Patwardhan *et al.*, 2000; Szeto *et al*., 2000).

Though, the changes in immune parameters of *Phela* treated groups peaked at 14 days and declined to baseline, it was expected because most herbal preparation and/or medicines do not modulate a healthy immune system. Botanicals and traditional herbal medicines that modulate the immune system have a potential to provide supportive therapy to conventional chemotherapy and other disease of immunopathology.

The results have proved that escalating doses of *Phela* modulated the immune system of healthy rats via proliferation of lymphocytes and increase in IL-2 count, potentiating it as an ideal candidate to evaluate the animal model of immune suppression.

7.9. CONCLUSION

Phela 15.4 mg/kg is a therapeutic dose for immune stimulation in rats. This dose of *Phela* needs to be further investigated in diseased rats to understand the mechanism of immunomodulation of *Phela in vivo.*

8.

ESTABLISHING THE RAT MODEL FOR IMMUNE SUPPRESSION USING CYCLOSPORINE, CYCLOPHOSPHAMIDE AND DEXAMETHASONE

8.1. SUMMARY

Introduction: Owing to the general lack of disease models, by which to evaluate drugs for immune modulation, there is a need to develop alternative models that can accurately assess for the mechanism and resultant effects of such drugs on the immune system. Consequently, the development of a rat model of drug-induced immunosuppression by which to test new drugs for immune modulation was embarked on. Here, the aim of the study was to determine the optimum dose and time of optimum immunesuppression by known immune suppressants; cyclosporine (CsA), cyclophosphamide (CP) and dexamethasone (Dex).

Methodology: Different groups of twelve rats each were treated with either CsA or CP or Dex only, and along with a respective control group in each case. Thereafter, three rats were sacrificed at regular intervals. Physiological function tests and selected immune markers (CD_4, CD_8, IgG, IgM, IL-2 and IL-10) were analysed.

Results: As expected, the animals exhibited abnormal physiological function tests in association with progressive immunesuppression. By day 10, cyclosporine had suppressed the CD_4 and CD_8 cell counts, and inhibited IL-2 proliferation, while cyclophosphamide suppression of CD_4 and CD_8 cell counts, IL-2 and IgM was observed on day 7. Dexamethasone immunesuppression was similar to cyclophosphamide effect on day 5. In all cases, the immunesuppression continued for the duration of the study. These results imply that cyclosporine inhibited cell mediated immunity, while cyclophosphamide and dexamethasone suppressed both the cell mediated and humoral immunity.

Conclusion: This also implies that a rat model of drug induced immune suppression was successfully developed. This rat model will be validated when the immune

suppressed rats are co-administered with a test drug, in this case *Phela,* to understand its mechanism of immune modulation.

8.2. INTRODUCTION

The aim of this study was to develop a rat model of cyclosporine, cyclophosphamide and dexamethasone induced immune-suppression, by optimizing the dose and time for optimal immune suppression.

8.3. MATERIALS

The apparatuses and reagents used in this part of the study were the same as those used in **Sections 7.3.1 and 7.3.2**. The syringes (2.5 and 10 ml) and 21G needles were used during animal treatment and 25 G for blood withdrawal were purchased from Lasec (S.A). Dexamethasone phosphate (Pharma–Q) injections (4 mg/ml) and Endoxan (Sanofi Aventis) 50 mg tablets were purchased from the Bloemfontein Medi-Clinic pharmacy. Cyclosporine was purchased from Sigma Aldrich© (Germany). Normal-saline (Adcock Ingram, S.A). Olive oil was purchased from checkers© hyper market.

8.4. PROCEDURES

8.4.1. ANIMAL CARE, DOSE SELECTION AND DRUG PREPARATION

The animal care remained the same as described in **chapter 7**, **Section 7.4.1**. In order to develop a rat model of immune induced suppression, three doses per drug under investigation were considered. The doses were as follows: Cyclophosphamide once weekly doses (Low, 100 mg/kg; medium, 150 mg/kg and high 200 mg/kg) [Murganandan, 2005; Abraham, 2007; Radojcic, 2009], cyclosporine daily doses (low, 2 mg/kg; medium 5 mg/kg and high 10 mg/kg) [Zhang, 1993; Li, 2003] and dexamethasone daily doses (low, 1.5 mg/kg; medium, 3 mg/kg and high, 6 mg/kg) [Barlett, 1998; Minet-Quinard *et al.*, 2000 and Fan, 2009].

However, due to cost constraints and limitation of rats only one dose per drug was selected. The drug preparation was as follows: Cyclosporine (CsA) was dissolved in olive oil to achieve 2 mg/kg (Rafferty *et al*., 2012).

Cyclophosphamide (CP) tablets were ground with a mortar and pestle, dissolved in normal saline and homogenized with a blender to 100 mg/kg (Muruganandan *et al*., 2005). Dexamethasone solution (4 mg/ml) was aliquoted (100 µl) into dosing syringes. As thus the dose was 1.5 mg/kg (Minet-Quinard *et al*., 2000).

8.4.2. ROUTES OF ADMINISTRATION

Subcutaneous Route: CsA and olive oil were administered subcutaneously in a volume of 0.4 µl/kg using a 25G needle attached to 1 ml syringe. The rat was held in a towel in the left hand such that it lay on its back and the drug was administered subcutaneously by the right hand.

Intraperitoneal Route: CP (4 ml/kg) and dexamethasone (0.4 µl/kg) were administered intraperitoneally (IP). The rat was held in a towel in the left hand such that it lay on its back and the drug was administered intraperitoneally by the right hand.

8.4.3. EXPERIMENTAL DESIGN FOR THE DEVELOPMENT OF A RAT MODEL

Figure 8.1 is an illustration of the experimental design for the development of rat model. Sprague dawley male rats (n = 54) were used for this experiment. Rats were divided into three test groups of twelve rats each and the three control groups of six rats each. Vehicle treated groups were control groups.

Cyclosporine

Six rats were treated daily subcutaneously with 100 µl of vehicle (n = 6) and with 2 mg/kg CsA (n = 12) similarly for 31 days. The vehicle was olive oil. Three rats were sacrificed after 10, 17, 24 and 31 days from each group.

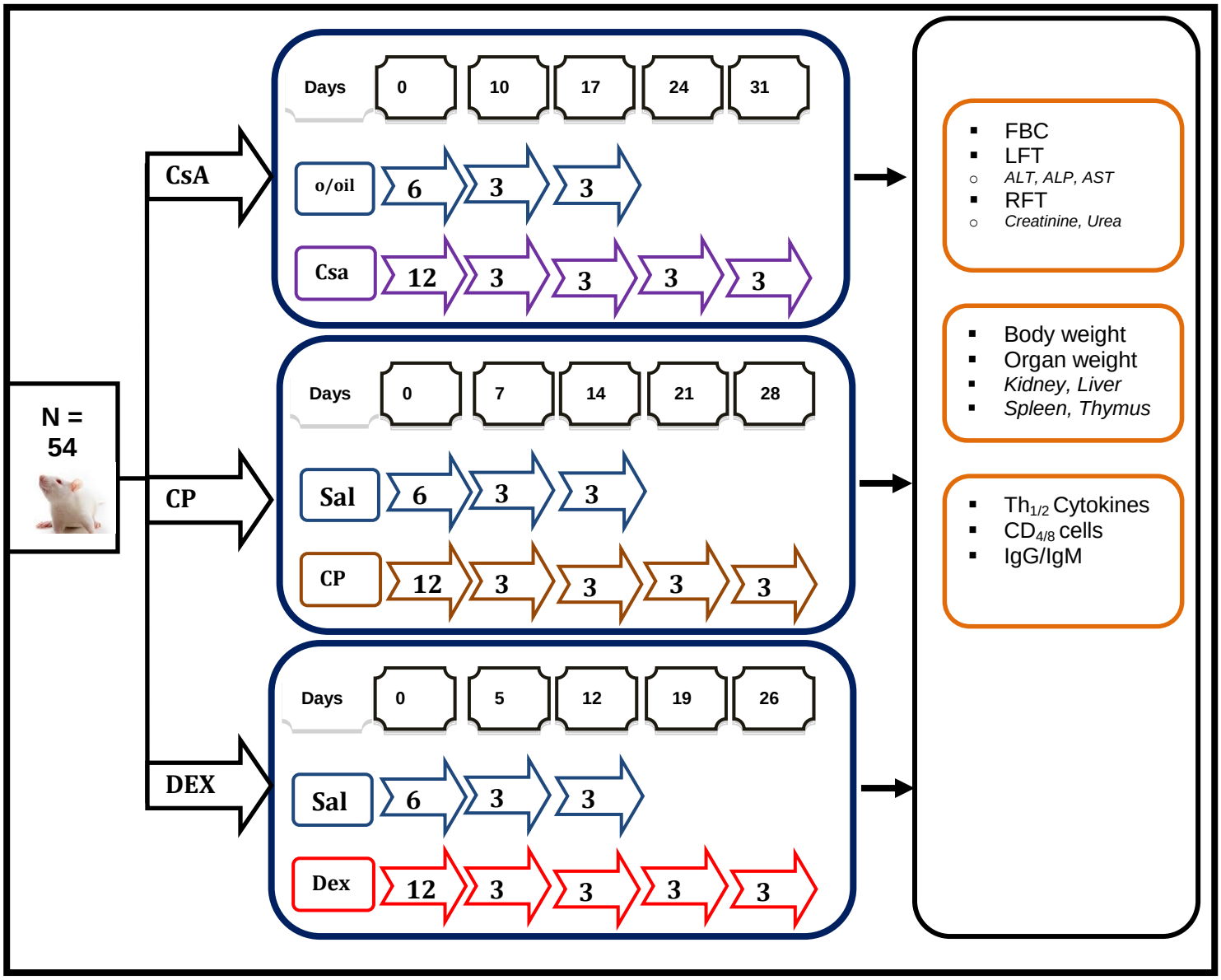


Figure 8.1: The experimental design a rat model of cyclosporine, cyclophosphamide and dexamethasone induced immune-suppression.

Abbreviations: untx: untreated, **Sal**: saline, **Lev**: Levamisole, **FBC**: full blood count, **LFT:** Liver function tests, **ALP**: alkaline phosphatase, **ALT**: Alanine transaminase, **AST**: Aspartate transaminase, **RFT**: Renal function tests, **Urea**: Blood urea nitrogen

Cyclosphosphamide

Rats were treated IP with either 100 mg/kg of cyclophosphamide (n = 12) or 1 ml of saline, the vehicle (n = 6) once weekly over 4 weeks. Three rats were sacrificed after 7, 14, 21 and 28 days of treatment in each group.

Dexamethasone

The fifth group (n = 12) was treated daily with 1.5 mg/kg (IP) of dexamethasone for twenty six days. The last group (n = 6) was similarly treated with 100 µl of normal-saline. Three rats were sacrificed after 5, 7, 12, 19 and 26 days from each group.

8.4.4. OBSERVATIONS AND WEIGHING OF RATS

Rats were observed daily for any physical signs of infection and/or disease progression and weighed weekly as described in **Chapter 7, Section 7.4.4**.

8.4.5. SACRIFICING PROCEDURE

Sacrificing was done at the department of pharmacology according to the timeframe of each drug. The sacrificing steps were as described in **Chapter 7, Section 7.4.5.**

8.4.6. MEASUREMENT OF PHYSIOLOGICAL PARAMETERS AND IMMUNE MARKERS' CONCENTRATION

EDTA tubes were used to collect whole blood for full blood count, CD_4 and CD_8 count analysis. Plasma (2 ml) was collected in lithium-heparin tubes for each drug concentration determination. CP and Dex were analysed by an HPLC-UV method validated in **Chapter 5** and CsA was by chemiluminescent immuno assay. Similarly, Serum was collected for liver and renal function tests as elaborated in **Chapter 7, Section 7.4.6**.

8.4.7. ORGAN HARVESTING PROCEDURE

The liver, kidney, spleen and thymus were harvested as described **Chapter 7, Section 7.4.7**.

8.4.8. MEASUREMENT OF CYTOKINES AND IMMUNOGLOBULINS RESPONSE BY ELISA

Rat specific ELISA kits were applied according to each manufacturer's instructions to determine IgG, IgM, IL-2 and IL-10, concentration in serum as described in **chapter 7, section 7.5.**

8.5. STATISTICAL ANALYSIS

All statistical analysis was done using the Graph Pad© Instat program and graphs drawn on an excel program as elaborated on in **chapter 7, section 7.6**.

8.6. RESULTS

8.6.1. EFFECT OF CYCLOSPORINE ON RATS

8.6.1.1. Clinical observations

All rats survived the treatment until the end of the study.

8.6.1.2. Physiological parameters

8.6.1.2.1. Liver functions tests

Table 8.1 is a summary of the effect of CsA on the liver function tests. There is a wide variation of liver function tests in the test groups when compared to the respective control groups; however the readings are within the normal ranges. CsA treatment had no effect on the liver function tests.

8.6.1.2.2. Renal function tests

Table 8.2 is a summary of the effect of CsA treatment on the renal function tests. CsA treatment had no effect on the renal function tests.

8.6.1.2.3. Haematological parameters

Table 8.3 is a summary of the effect of CsA treatment on full blood count. On day 10 elevations of red cell count, haemoglobin and haematocrit was observed though not statistically significant. Furthermore, thrombocytosis was observed on day 17. No changes were observed in the white cells and lymphocyte count when compared to the control group.

Table 8.1: Summary of **liver function test** recorded as mean ± SD after treatment with **cyclosporine** in rats.

	OLIVE OIL (CONTROL)			CYCLOSPORINE (TEST)		
DAYS	**ALP (U/L)**	**ALT (U/L)**	**AST (U/L)**	**ALP (U/L)**	**ALT (U/L)**	**AST (U/L)**
0	47.0±4.7	310.5±75.6	80.0±13.9	47.0±4.7	310.5±75.6	80.0±13.9
10	52.8±4.1	270.8±18.1	122.0±15.2	60.0±0.0	38.0±0.0	107.5±2.1
17	52.0±10.6	12.5±13.4	112.0±38.3	33.5±4.2	164.5±21.0	100.8±37.6
24				62.3±12.9	180.5±36.7	150.3±39.2
31				59.0±8.6	179.0±38.8	184.8±24.4

Abbreviations + Reference values: **ALP:** alkaline phosphatase (289 – 436 U/L), **ALT:** Alanine transaminase (46 – 55 U/L), **AST:** Aspartate transaminase (81 – 104 U/L).

Table 8.2: Summary of **renal function tests** recorded as mean ± SD after treatment with **cyclosporine** in rats.

	OLIVE OIL (CONTROL)		CYCLOSPORINE (TEST)	
DAYS	**Creatinine (µmol/L)**	**BUN (mmol/L)**	**Creatinine (µmol/L)**	**BUN (mmol/L)**
0	7.20±0.89	36.67±8.14	7.20±0.89	36.67±8.14
10	6.80±0.22	60.25±6.34	7.70±0.14	50.50±3.54
17	6.73±0.55	14.67±4.93	11.60±1.20	55.40±4.34
24			6.86±1.32	10.60±6.27
31			6.59±0.89	48.0±4.95

Abbreviations + Reference values: **BUN**: Blood urea nitrogen (6.5 – 8.2 mmol/L), Creatinine (31 – 46 µmol/L)

Table 8.3: Summary of **Full blood count test** levels recorded as mean ± SD after treatment with **cyclosporine** in rats.

PARAMETERS	DAYS				
	0	10	17	24	31
OLIVE OIL (CONTROL)					
RCC (x10^12/l)	6.21±0.30	6.25±0.70	6.78±0.13		
Haemoglobin (g/dl)	12.75±0.35	12.73±1.01	13.45±0.21		
Haematocrit (l/l)	0.39±0.00	0.38±0.02	0.38±0.01		
MCV (fl)	63.65±3.46	61.60±2.78	56.35±2.05		
MCH (pg)	20.55±0.49	20.43±0.64	19.95±0.49		
MCHC (g/dl)	32.35±1.06	33.20±0.50	35.45±0.49		
Platelets (x10^9/l)	822±299	600±36	749±165		
WCC (x10^9/l)	6.02±1.63	4.82±0.59	5.36±1.31		
Neutrophils (x10^9/l)	0.77±0.17	0.98±0.59	0.60±0.18		
Lymphocytes (x10^9/l)	5.03±1.44	3.54±1.09	3.86±0.42		
Monocytes (x10^9/l)	0.19±0.11	0.20±0.09	0.27±0.03		
Eosinophils (x10^9/l)	0.02±0.00	0.09±0.06	0.10±0.01		
Basophils (x10^9/l)	0.01±0.01	0.01±0.01	0.04±0.01		
CYCLOSPORINE (TEST)					
RCC (x10^12/l)	6.21±0.30	7.09±0.74	8.20±0.05	7.73±0.40	7.10±0.80
Haemoglobin (g/dl)	12.75±0.35	14.05±1.05	15.77±0.64	16.13±0.29	13.27±1.27
Haematocrit (l/l)	0.39±0.00	0.42±0.03	0.45±0.02	0.45±0.02	0.37±0.04
MCV (fl)	63.65±3.46	58.70±2.06	55.20±1.57	58.80±1.97	52.10±1.14
MCH (pg)	20.55±0.49	19.80±0.87	19.23±0.67	20.93±0.81	18.73±0.35
MCHC (g/dl)	32.35±1.06	33.75±0.52	34.80±0.26	35.53±0.67	35.93±0.38
Platelets (x10^9/l)	822±299	671±254	1107±30	219±210	656±61
WCC (x10^9/l)	6.02±1.63	3.95±0.49	5.46±0.65	4.48±0.92	3.56±0.83
Neutrophils (x10^9/l)	0.77±0.17	0.62±0.04	0.91±0.23	0.85±0.35	0.63±0.23
Lymphocytes (x10^9/l)	5.03±1.44	4.27±1.14	4.01±0.48	3.30±0.71	2.41±0.51
Monocytes (x10^9/l)	0.19±0.11	0.20±0.16	0.39±0.24	0.14±0.11	0.17±0.19
Eosinophils (x10^9/l)	0.02±0.00	0.09±0.04	0.09±0.10	0.23±0.20	0.35±0.17
Basophils (x10^9/l)	0.01±0.01	0.03±0.03	0.05±0.03	0.01±0.01	0.00±0.00

Abbreviations + Reference values: **RCC**: Red cell count (5.99 -6.42), Haemoglobin (12.5 – 13.1), haematocrit (0.393 – 0.406) **MCV**: Mean corpuscular volume (61.2 – 66.1), **MCH**: Mean corpuscular hemoglobin (20.2 – 20.9), **MCHC**: Mean corpuscular hemoglobin concentration (31.1 – 32.3), Platelets (611 – 1034) **WCC**: white cell count (4.16 – 9.55), Neutrophils (0.65 - 0.89), Lymphocytes (3.37 – 5.99), Monocytes (0.11 – 0.27), Eosinophils (0.00 – 0.02), Basophils (0.01 – 9.55)

8.6.1.3. General Immune markers response

Table 8.4 is a summary of the changes in body weight before and after treatment with CsA. Rats body weight increased by 17 and 34 % after 10 and 17 days treatment with olive oil. Furthermore, the CsA treated rats also had a similar rate of growth. CsA treatment did not alter rats' body weight as indicated by the percentage weight change.

Table 8.5 shows changes in kidney, liver, spleen and thymus weight after treatment with cyclosporine. Kidneys and spleen weight were not affected by CsA treatment. CsA treatment decreased thymus weight and significantly decreased liver weight (P = 0.0001) on day 17.

Table 8.4: Summary of **body weight** recorded as mean ± SD treatment with **cyclosporine** in rats.

DAYS	**OLIVE OIL (CONTROL)**			**CYCLOSPORINE (TEST)**		
	BEFORE (g)	***AFTER (g)***	***Change (%)***	***BEFORE (g)***	***AFTER (g)***	***Change (%)***
0	224±16	224±16	0	224±16	224±16	0
10	229±10	269±10	+17±5	225±11	257±19	+14±6
17	205±5	275±4	+34±0	212±4	278±5	+32±4
24				238±7	295±12	+24±8
31				229±4	312±9	+36±6

Table 8.5: Summary of **organs weight (g)** recorded as mean ± SD after treatment with **cyclosporine** in rats.

DAYS	OLIVE OIL (CONTROL)	CYCLOSPORINE (TEST)
	KIDNEYS	
0	1.73±0.11	1.73±0.11
10	2.11±0.08	2.05±0.22 (P = 0.6751)
17	2.21±0.20	2.00±0.08 (P = 0.0732)
24		2.38±0.10
31		2.38±0.08
	LIVER	
0	10.04±0.85	10.04±0.85
10	12.32±0.77	11.19±1.01 (P = 0.1965)
17	12.54±0.53	9.31±0.38 (P = 0.0001)**
24		11.81±0.97
31		11.44±0.27
	SPLEEN	
0	0.52±0.06	0.52±0.06
10	0.64±0.06	0.67±0.09 (P = 0.6868)
17	0.56±0.04	0.58±0.03 (P = 0.6085)
24		0.60±0.03
31		0.60±0.04
	THYMUS	
0	0.47±0.04	0.47±0.04
10	0.59±0.09	0.62±0.12 (P = 0.5587)
17	0.69±0.02	0.48±0.12 (P = 0.2782)
24		0.52±0.09
31		0.34±0.04

**P < 0.05 vs control **P < 0.001 vs control*

8.6.1.4. Immune cells response

Figure 8.2 shows plots of the effect of CsA chronic treatment on WCC (A), neutrophils (B) and lymphocyte (C) count versus time. CsA had no effect on WCC as indicated by similar concentrations in both groups. CsA inhibited neutrophils count on day 10 (Control, 0.98±0.59 vs CsA, 0.62±0.58), after which they declined on day 24. CsA decreased lymphocyte count over time [(CsA): Day 0, 5.03±1.44; Day 31, 2.40±0.31).

Figure 8.3 shows plots of the effect of CsA chronic treatment on CD_4 (A) and CD_8 (B) count versus time. CsA treatment significantly decreased CD_4 and CD_8 count [($CD_{4:}$ control, 2.45±0.33 vs CsA, 1.62±0.06) ($CD_{8:}$ control, 1.53±0.24 vs CsA, 0.78±0.01)] on day 10 and continued for the duration of the study.

8.6.1.5. Immunoglobulins response

Figure 8.4 shows plots of the effect of CsA chronic treatment on IgG (A) and IgM (B) count versus time. CsA treatment increased IgG count after 10 days, peaked at day 17, thereafter returned to baseline. IgM count decreased on day 10 in both the control and test group, furthermore, remaining low for the duration of the study.

8.6.1.6. Cytokines response

Figure 8.5 shows plots of the effect of CsA chronic treatment on IL-2 (A) and IL-10 (B) count versus time. CsA inhibited IL-2 count proliferation from 10 days (102.82±4.34) and the suppression continued till the end of the study. IL-10 was inhibited by CsA treatment thought not statistically significant.

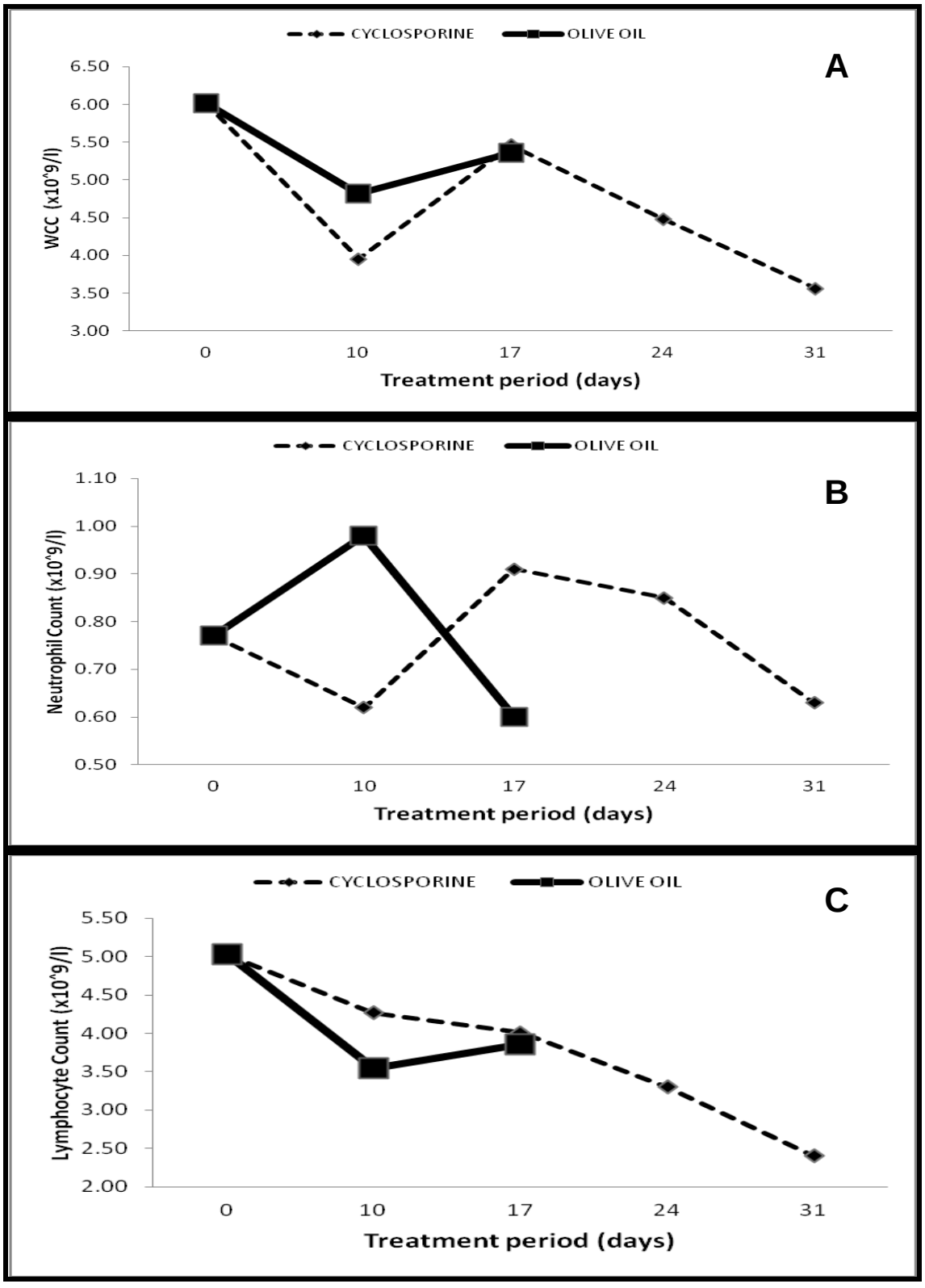


Figure 8.2: The effect of cyclosporine chronic treatment on **white cell count (A), neutrophils (B) and lymphocyte (C)** count in rats.

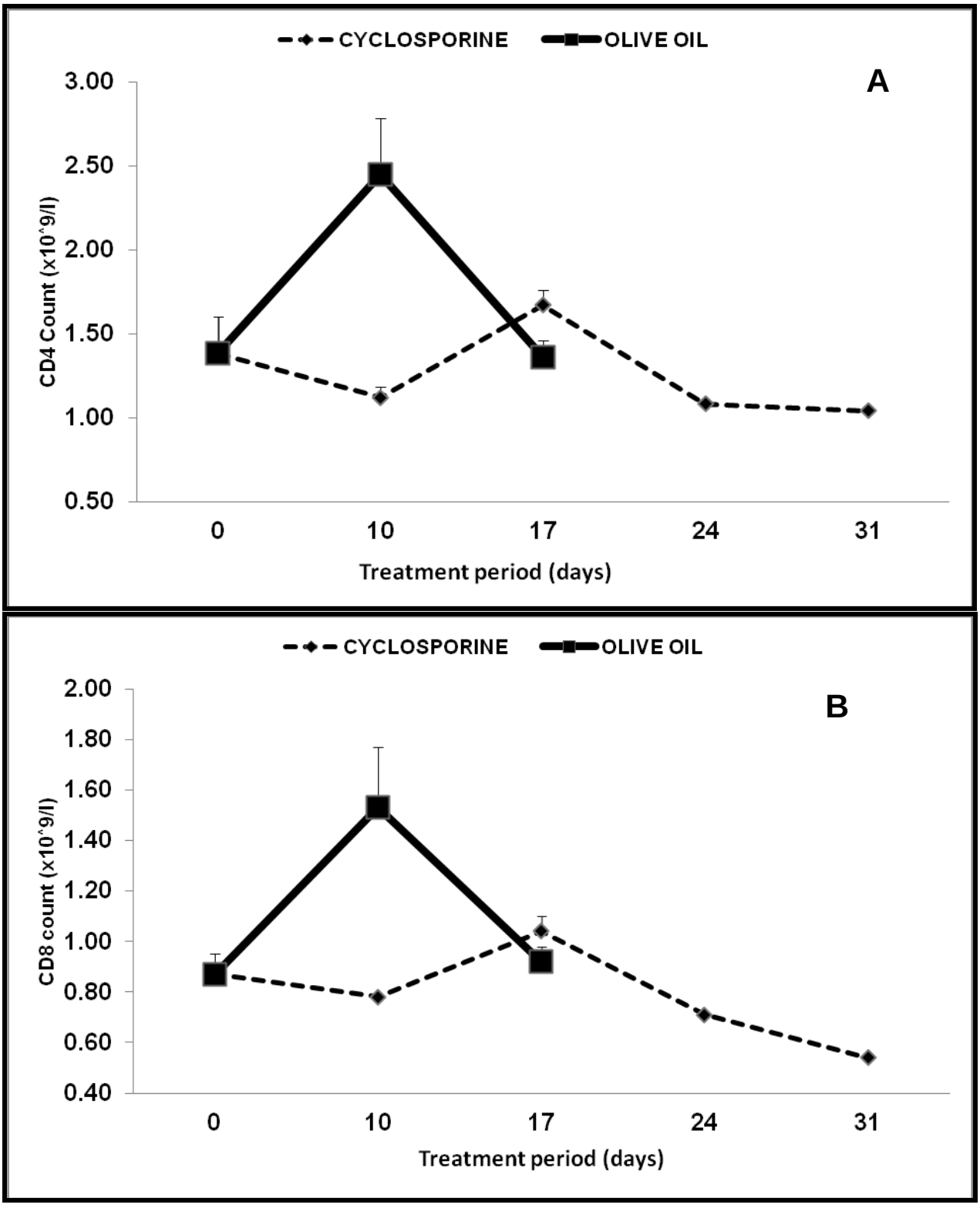


Figure 8.3: The effect of cyclosporine chronic treatment on **CD_4 (A) and CD_8 (B)** cells count in rats.

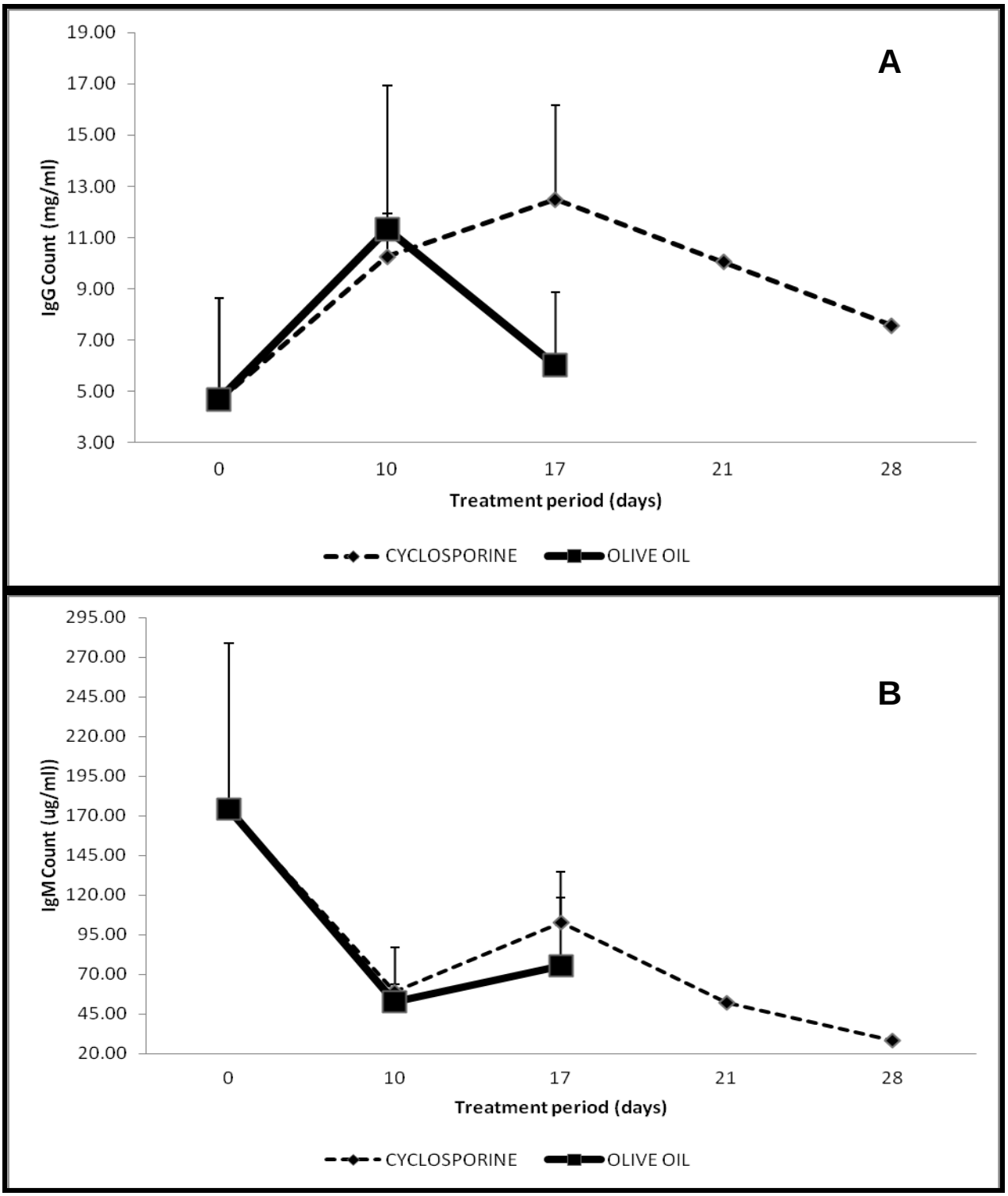


Figure 8.4: The effect of cyclosporine chronic treatment on **IgG (A) and IgM (B)** response in rats.

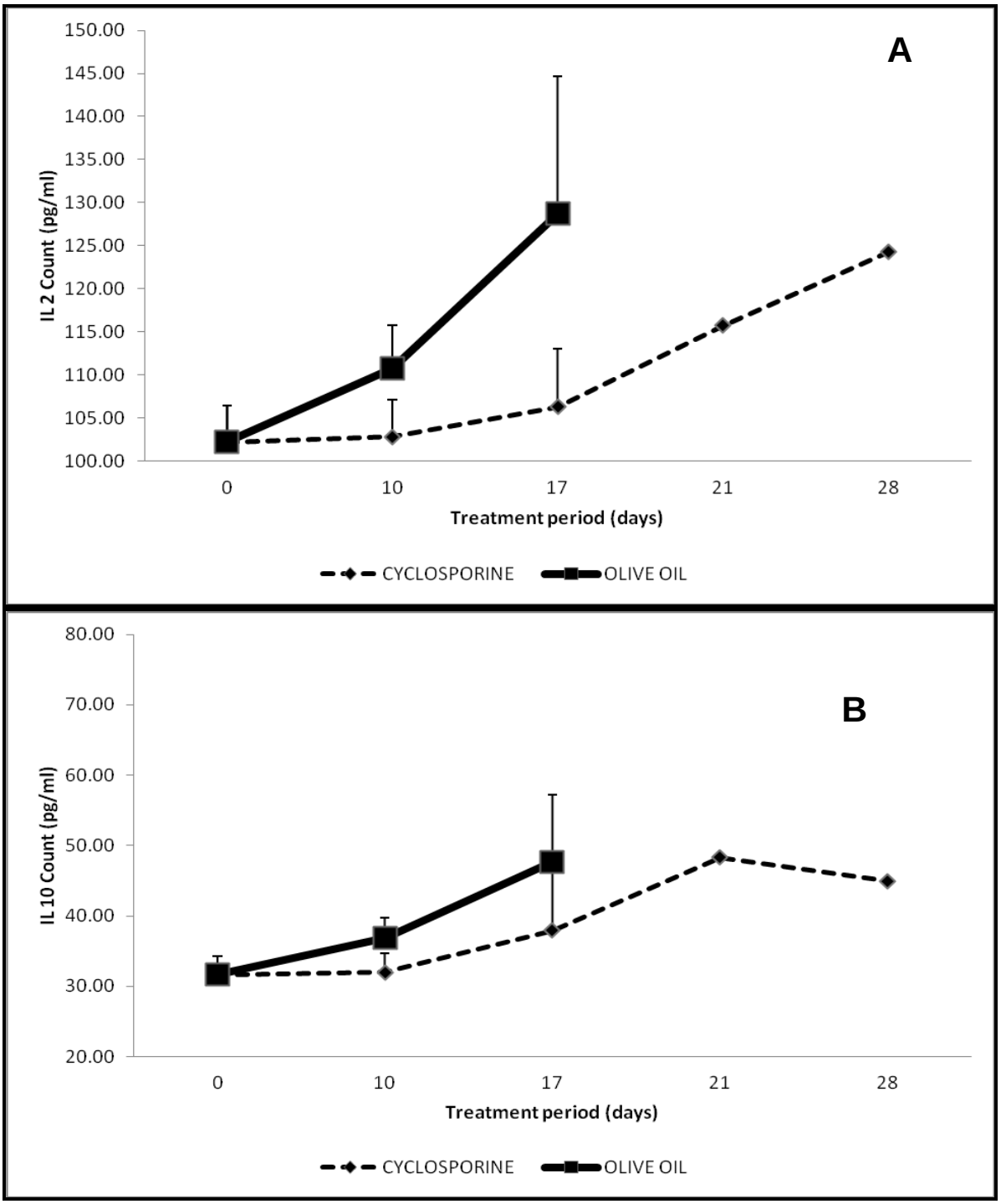


Figure 8.5: The effect of cyclosporine chronic treatment on **IL-2 (A) and IL-10 (B)** response in rats.

8.6.2. EFFECT OF CYCLOPHOSPHAMIDE ON RATS

8.6.2.1. Clinical observation

All rats survived treatment until the end of the study. CP treated rats looked frail and ill by the end of the study.

8.6.2.2. Physiological parameters

8.6.2.2.1. Liver functions tests

Table 8.6 is a summary of the effect of CP on the liver function tests. CP treatment did not alter liver function test levels.

8.6.2.2.2. Renal function tests

Table 8.7 is a summary of the effect of CP on the renal function tests. CP had no effect on the renal function marker response.

8.6.2.2.3. Haematological parameters

Table 8.8 shows changes in full blood count after treatment with CP. On day 7, red cells and haemoglobin count declined as expected. Similarly, platelets decreased by CP treatment. Results show that CP inhibited WCC and lymphocytes in a time dependent manner in rats. Neutrophils count doubled after 28 days of CP treatment in rats.

Table 8.6: Summary of **liver function tests** recorded as mean ± SD after treatment with **cyclophosphamide** in rats.

	SALINE (CONTROL)			CYCLOPHOSPHAMIDE (TEST)		
DAYS	**ALP (U/L)**	**ALT (U/L)**	**AST (U/L)**	**ALP (U/L)**	**ALT (U/L)**	**AST (U/L)**
0	47.0±4.7	310.5±75.6	80.0±13.9	47.0±4.7	310.5±75.6	80.0±13.9
10	48.0±1.7	302.7±5.5	78.0±7.0	48.0±1.7	224.3±7.2	95.0±6.2
17	39.7±4.9	248.5±3.5	76.3±7.6	39.7±4.9	130.3±32.3	149.0±63
24				38.5±20.5	146.0±126	113.5±68.6
31				37.8±2.5	145.3±11.7	99.8±18.1

Abbreviations + Reference values: **ALP:** alkaline phosphatase (289 – 436 U/L), **ALT:** Alanine transaminase (46 – 55 U/L), **AST:** Aspartate transaminase (81 – 104 U/L).

Table 8.7: Summary of **renal function tests** recorded as mean ± SD after treatment with **cyclophosphamide** in rats.

	SALINE (CONTROL)		CYCLOPHOSPHAMIDE (TEST)	
DAYS	**Creatinine (µmol/L)**	**BUN (mmol/L)**	**Creatinine (µmol/L)**	**BUN (mmol/L)**
0	7.20±0.89	36.67±8.14	7.20±0.89	36.67±8.14
10	5.93±0.40	24.0±5.56	5.53±0.40	30.0±4.58
17	6.05±1.06	58.0±14.14	6.67±0.90	69.67±5.86
24			6.60±1.13	26.00±14.14
31			5.88±0.91	32.00±8.16

Abbreviations +Reference values: Blood urea nitrogen (6.5 – 8.2 mmol/L), Creatinine (31 – 46 µmol/L)

Table 8.8: Summary of **Full blood count** levels recorded as mean ± SD after treatment with **cyclophosphamide** in rats.

	DAYS				
PARAMETERS	**0**	**10**	**17**	**24**	**31**
SALINE (CONTROL)					
RCC (x10^12/l)	6.21±0.30	6.71±0.11	6.25±0.89		
Haemoglobin (g/dl)	12.75±0.35	13.50±0.26	12.53±1.86		
Haematocrit (l/l)	0.39±0.00	0.41±0.01	0.37±0.06		
MCV (fl)	63.65±3.46	60.63±2.10	58.85±0.75		
MCH (pg)	20.55±0.49	20.13±0.29	20.03±0.15		
MCHC (g/dl)	32.35±1.06	33.20±0.79	34.03±0.15		
Platelets (x10^9/l)	822±299	1132±18	746±147		
WCC (x10^9/l)	6.02±1.63	5.69±1.61	5.65±2.07		
Neutrophils (x10^9/l)	0.77±0.17	0.71±0.20	0.70±0.27		
Lymphocytes (x10^9/l)	5.03±1.44	4.70±1.36	4.59±1.79		
Monocytes (x10^9/l)	0.19±0.11	0.21±0.07	0.30±0.05		
Eosinophils (x10^9/l)	0.02±0.00	0.05±0.02	0.06±0.02		
Basophils (x10^9/l)	0.01±0.01	0.01±0.01	0.01±0.00		
CYCLOPHOSPHAMIDE (TEST)					
RCC (x10^12/l)	6.21±0.30	5.98±0.20	5.96±0.31	5.98±0.13	5.06±0.63
Haemoglobin (g/dl)	12.75±0.35	12.40±0.36	11.70±0.99	11.45±0.49	10.10±1.45
Haematocrit (l/l)	0.39±0.00	0.37±0.01	0.35±0.03	0.35±0.02	0.31±0.04
MCV (fl)	63.65±3.46	61.37±1.46	57.95±1.63	57.80±1.70	61.53±2.74
MCH (pg)	20.55±0.49	20.77±0.32	19.65±0.64	19.15±0.35	19.97±0.81
MCHC (g/dl)	32.35±1.06	33.80±0.44	33.85±0.21	33.15±0.21	32.43±0.92
Platelets (x10^9/l)	822±299	965±117	743±159	954±44	476±104
WCC (x10^9/l)	6.02±1.63	2.68±0.47	2.22±0.51	3.88±0.98	3.13±1.71
Neutrophils (x10^9/l)	0.77±0.17	0.62±0.22	0.94±0.13	2.26±0.11	1.47±1.05
Lymphocytes (x10^9/l)	5.03±1.44	1.82±0.31	0.89±0.23	1.25±0.83	0.99±0.75
Monocytes (x10^9/l)	0.19±0.11	0.22±0.03	0.39±0.13	0.26±0.08	0.64±0.20
Eosinophils (x10^9/l)	0.02±0.00	0.01±0.00	0.01±0.01	0.10±0.12	0.02±0.03
Basophils (x10^9/l)	0.01±0.01	0.01±0.00	0.01±0.01	0.02±0.01	0.01±0.00

Abbreviations + Reference values: **RCC**: Red cell count (5.99 -6.42), Haemoglobin (12.5 – 13.1), haematocrit (0.393 – 0.406) **MCV**: Mean corpuscular volume (61.2 – 66.1), **MCH**: Mean corpuscular hemoglobin (20.2 – 20.9), **MCHC**: Mean corpuscular hemoglobin concentration (31.1 – 32.3), Platelets (611 – 1034) **WCC**: white cell count (4.16 – 9.55), Neutrophils (0.65 - 0.89), Lymphocytes (3.37 – 5.99), Monocytes (0.11 – 0.27), Eosinophils (0.00 – 0.02), Basophils (0.01 – 9.55)

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8.6.2.3. General Immune markers response

Table 8.9 is a summary of the changes in body weight before, after and change percentage after treatment with CP. Rats treated with CP had stunted growth when compared with the control group from day 7 until the end of the study.

Table 8.10 shows changes in kidney, liver, spleen and thymus weight after treatment with CP. The kidney and liver growth was stunted after 7 and 14 days though not statistically significant. Spleen weight was lost after 7 days of CP treatment (P = 0.05) though not statistically different. Thymus weight was significantly decreased after 7 (P = 0.0004) and 14 (P = 0.0001) days and continued to decline for the duration of the study.

Table 8.9: Summary of **body weight** recorded as mean ± SD treatment with **cyclophosphamide** in rats.

DAYS	**SALINE (CONTROL)**			**CYCLOPHOSPHAMIDE (TEST)**		
	BEFORE (g)	***AFTER (g)***	***Change (%)***	***BEFORE (g)***	***AFTER (g)***	***Change (%)***
0	224±16	224±16	0	224±16	224±16	0
7	214±5	250±10	+17±7	218±4	234±6	+7±1
14	226±12	277±1	+23±6	212±4	239±11	+13±5
21				230±5	254±18	+10±8
28				225±11	238±13	+6±3

Table 8.10: Summary of **organs' weight (g)** recorded as mean ± SD after treatment with **cyclophosphamide** in rats.

DAYS	SALINE (CONTROL)	CYCLOPHOSPHAMIDE (TEST)
	KIDNEYS	
0	1.73±0.11	1.73±0.11
7	2.01±0.08	1.81±0.13 (P = 0.0810)
14	2.19±0.13	1.97±0.13 (P = 0.0838)
21		2.00±0.20
28		1.85±0.13
	LIVER	
0	10.04±0.85	10.04±0.85
7	12.36±0.26	11.72±0.38 (P = 0.0745)
14	12.45±0.68	11.32±0.85 (P = 0.1083)
21		11.01±1.66
28		10.63±0.43
	SPLEEN	
0	0.52±0.06	0.52±0.06
7	0.71±0.13	0.49±0.06 (P = 0.051)*
14	0.70±0.13	0.62±0.09 (P = 0.4068)
21		0.71±0.17
28		0.89±0.06
	THYMUS	
0	0.47±0.04	0.47±0.04
7	0.55±0.01	0.26±0.05 (P = 0.0004)*
14	0.60±0.05	0.19±0.02 (P = 0.0001)**
21		0.17±0.08
28		0.16±0.02

**P < 0.05 vs control **P < 0.001 vs control*

8.6.2.4. Immune cells response

Figure 8.6 shows plots of the effect of CP chronic treatment on WCC (A), neutrophils (B) and lymphocyte (C) count versus time. WCC declined after 7 days and the inhibition was evident for the duration of the study. Neutrophils remained unchanged for the first 14 days, followed by a tremendous increase on day 21 (2.26±0.11) and the latter of the study. CP inhibited lymphocytes proliferation throughout the study.

Figure 8.7 shows plots of the effect of CP chronic treatment on CD_4 (A) and CD_8 (B) count versus time. CD_4 and CD_8 suppression confirmed myelosuppression observed with lymphocyte count due to CP treatment. On day 0, CD_4 and CD_8 counts were (CD_4, 1.38±0.22; CD_8, 0.87±0.08) and were almost depleted by day 28 (CD_4, 0.13±0.07; CD_8, 0.12±0.10) of treatment.

8.6.2.5. Immunoglobulins response

Figure 8.8 shows plots of the effect of CP chronic treatment on IgG (A) and IgM (B) count versus time. CP treatment had no effect on IgG count. CP treatment significantly decreased IgM count by day 7 ($P = 0.02$) and more so on day 28 (Day 0, 174.24±104.42; day 28, 46.96±26.17).

8.6.2.6. Cytokines response

Figure 8.9 shows plots of the effect of CP chronic treatment on IL-2 (A) and IL-10 (B) count versus time. CP inhibited IL-2 proliferation on day 7 (100.57±7.89) and day 28 (85.10±32.25) though not statistically significant. CP treatment did not alter IL-10 count, since the concentration was similar in both groups.

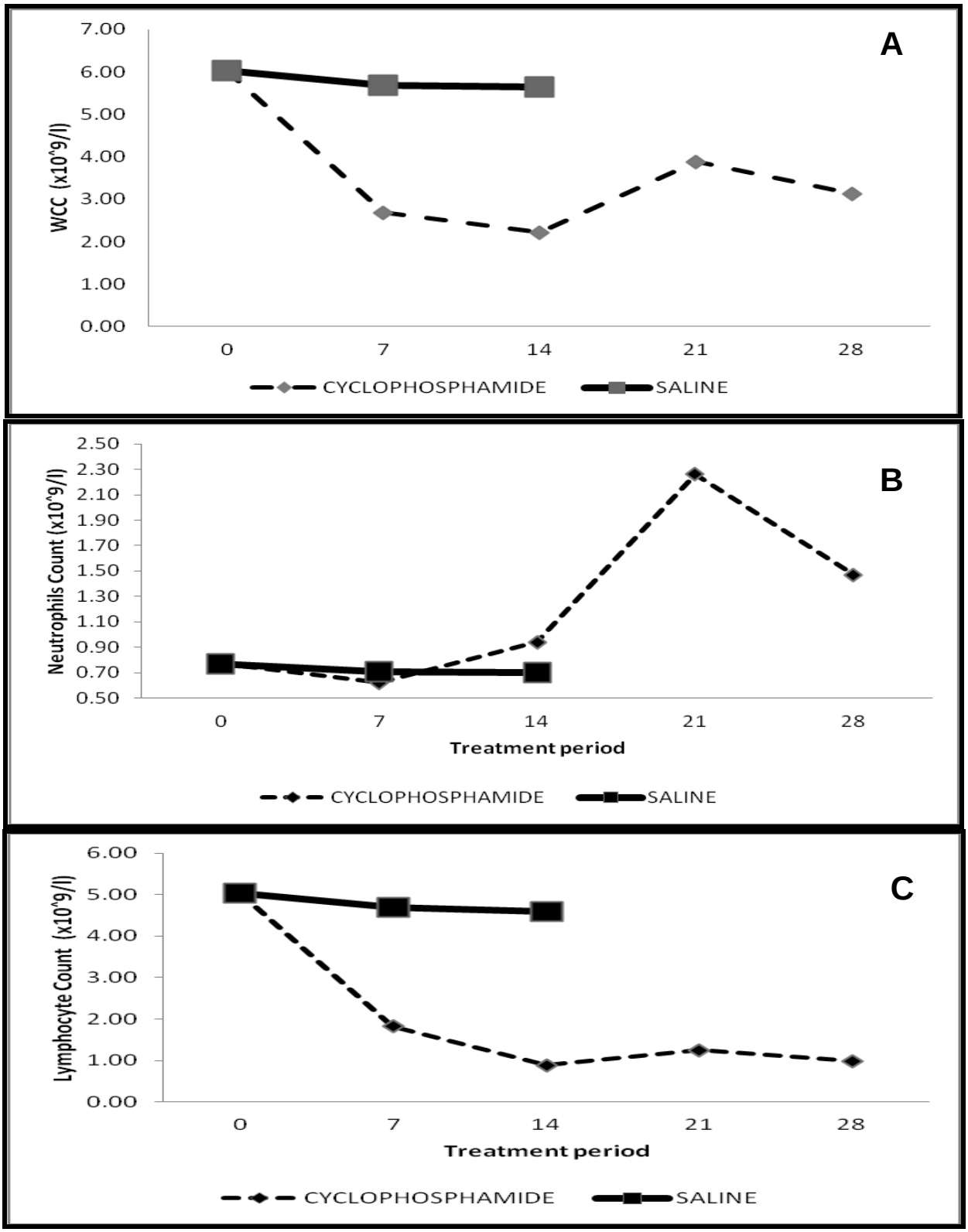

Figure 8.6: The effect of CP treatment on the **white cell count (A), neutrophils (B) and lymphocytes (C)** in rats.

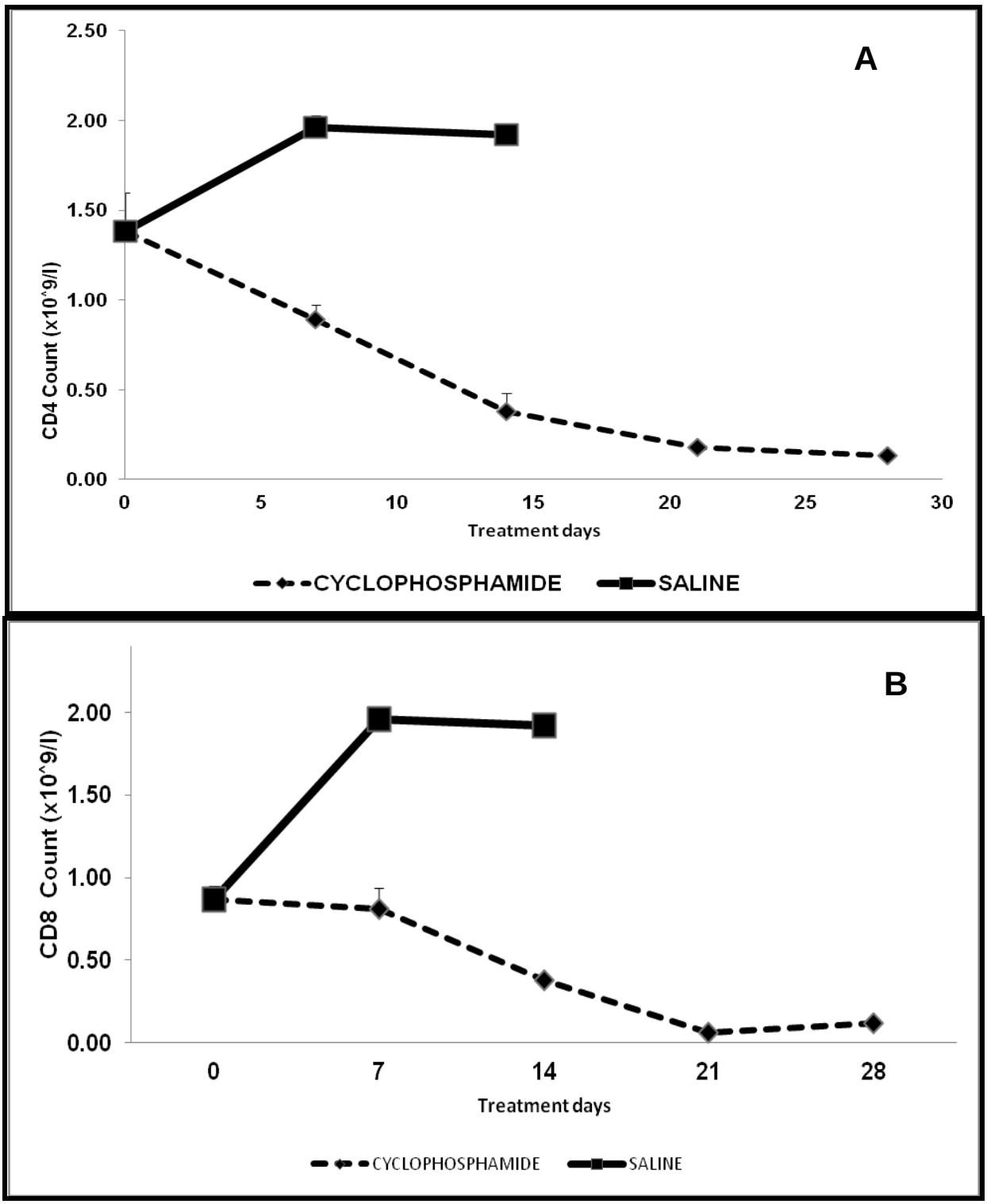

Figure 8.7: The effect of CP treatment on **CD_4 (A) and CD_8 (B) cells** in rats.

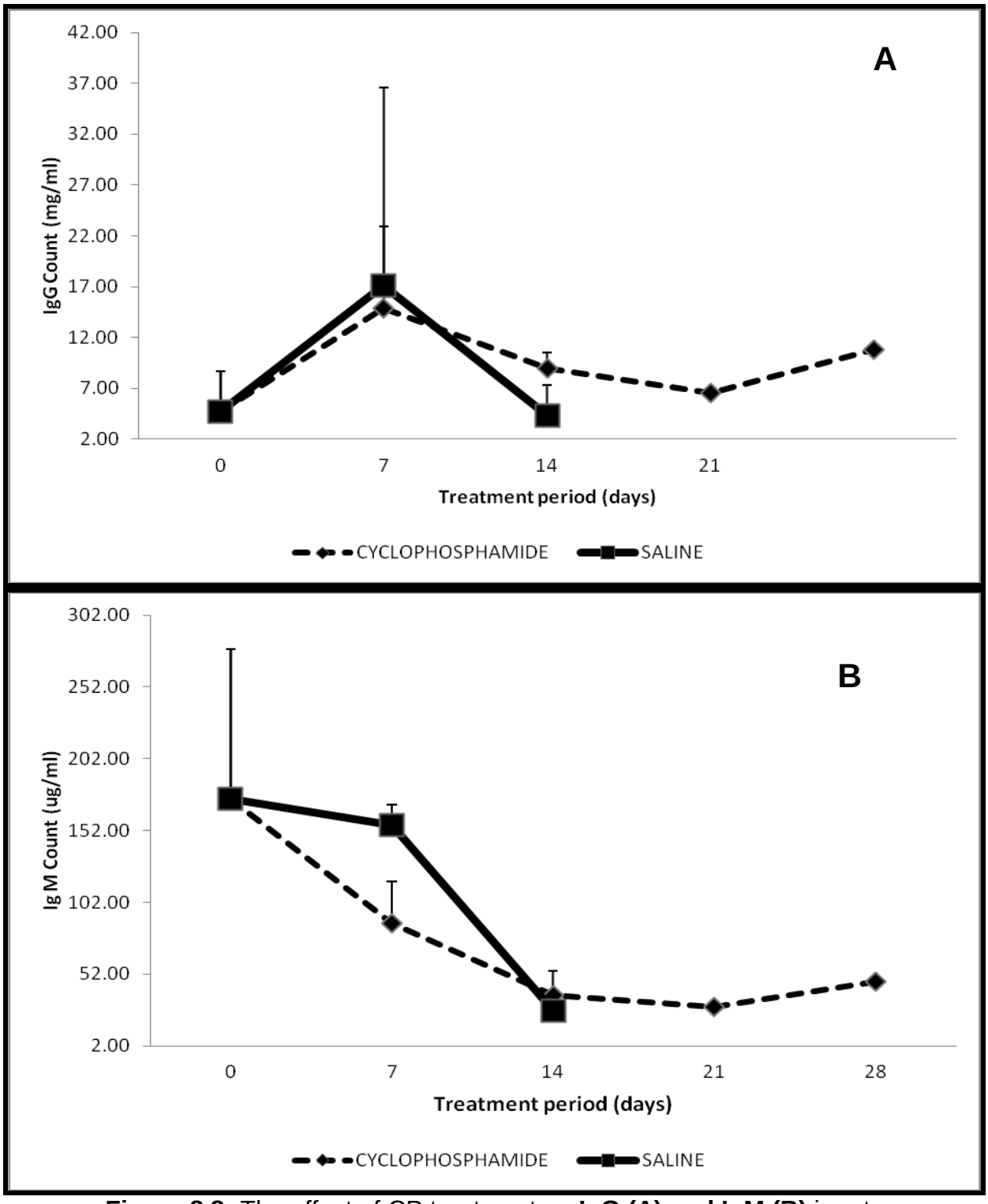

Figure 8.8: The effect of CP treatment on **IgG (A) and IgM (B)** in rats

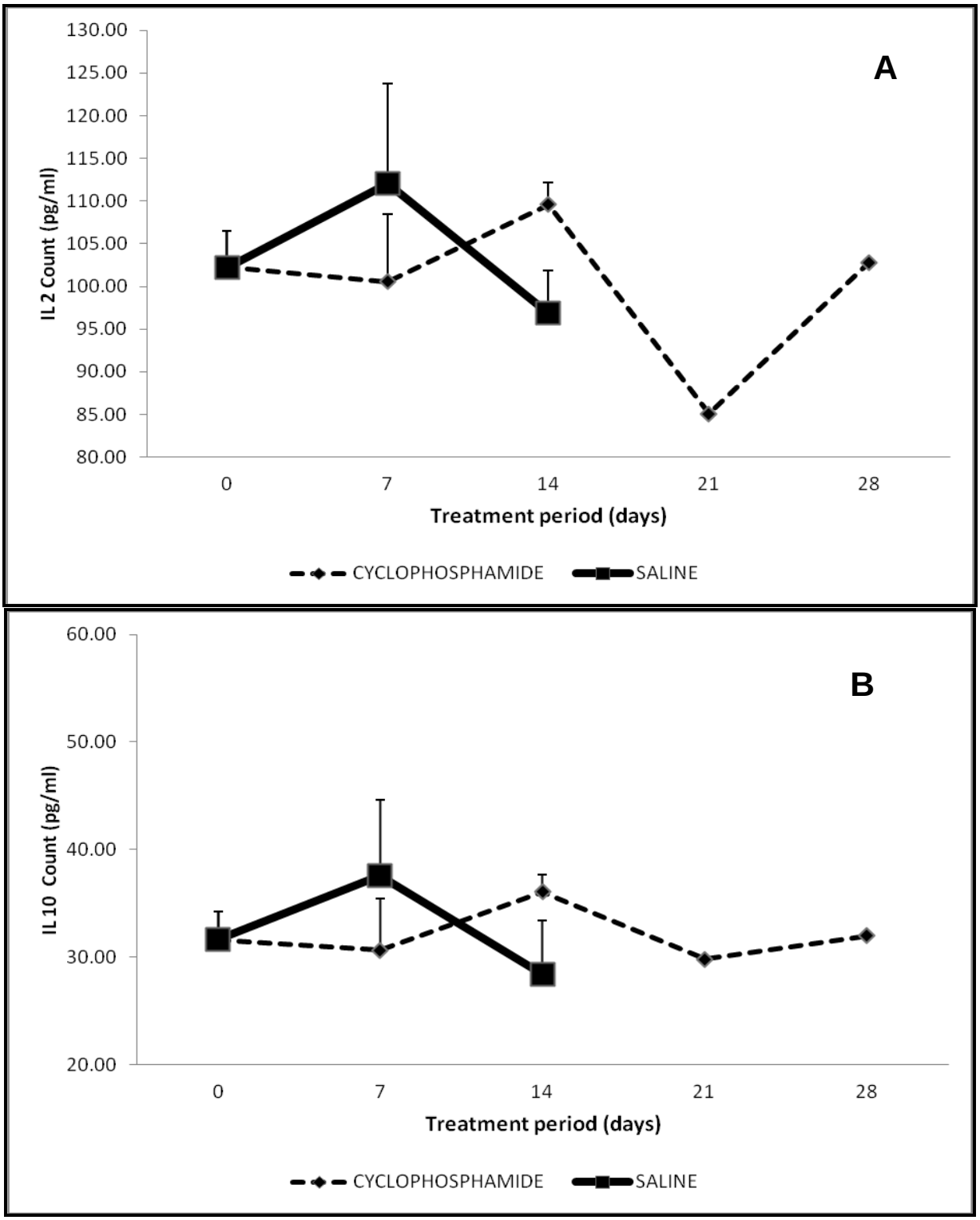

Figure 8.9: The effect of CP treatment on **IL-2 (A) and IL-10 (B)** in rats.

8.6.3. EFFECT OF DEXAMETHASONE ON RATS

8.6.3.1. Clinical observation

All rats survived treatment until the end of the study. Rats treated with Dex were frail and ill and this observation was worsened with prolonged treatment.

8.6.3.2. Physiological parameters

8.6.3.2.1. Liver functions tests

Table 8.11 is a summary of the effect of Dex on the liver function tests. ALT decreased after 5 (P = 0.05) and 7 (P = 0.03) days of Dex treatment.

8.6.3.2.2. Renal function tests

Table 8.12 is a summary of the effect of Dex on the renal function tests. Creatinine was increased significantly after 7 days (P = 0.03) and returned to baseline at the end of the study. BUN was not altered by dexamethasone treatment.

8.6.3.2.3. Haematological parameters

Table 8.13 shows changes full blood count after treatment with dexamethasone. After 12 days of dexamethasone treatment haemoglobin (20.87±8.95) and haematocrit (0.44±0.60) count increased from 0.41±0.01 respectively. Thrombocytopenia was observed on days 12 (260.33±164.49) and 19 (341.00±104.10) of treatment in the test group. Dexamethasone increased neutrophils from 0.77±0.14 on day 0 to 4.89±3.00 on day 26. Lymphocytes were inhibited by dexamethasone through-out the study (day 0, 5.03±1.44; day 26, 0.40±0.25).

Table 8.11*:* Summary of **liver function tests** recorded as mean ± SD after treatment with **dexamethasone** in rats.

	SALINE (CONTROL)			DEXAMETHASONE (TEST)		
DAYS	**ALP (U/L)**	**ALT (U/L)**	**AST (U/L)**	**ALP (U/L)**	**ALT (U/L)**	**AST (U/L)**
0	47.0±4.7	310.5±75.6	80.0±13.9	47.0±4.7	310.5±75.6	80.0±13.9
5	58.7±4.6	314.0±59.0	125.0±23.0	72.5±7.8	215.5±53.0	99 ±4.2
7	52.0±3.6	326.3±20.6	149.7±30.1	52.5±4.9	175.5±110	65.0±19.8
12				74.3±19.3	145.5±3.50	131.0±61.6
19				83.0±26.1	57.0±0.00	165.8±14.9
26				71.3±15.9	49.70±8.50	87.7±19.1

Abbreviations + Reference values: **ALP:** alkaline phosphatase (289 – 436 U/L), **ALT:** Alanine transaminase (46 – 55 U/L), **AST:** Aspartate transaminase (81 – 104 U/L).

Table 8.12: Summary of **renal function tests** recorded as mean ± SD after treatment with **dexamethasone** in rats.

	SALINE (CONTROL)		DEXAMETHASONE (TEST)	
DAYS	**Creatinine (µmol/L)**	**BUN (mmol/L)**	**Creatinine (µmol/L)**	**BUN (mmol/L)**
0	7.20±0.89	36.67±8.14	7.20±0.89	36.67±8.14
5	6.40±0.52	54.33±8.50	8.25±0.78	58.50±0.71
7	6.00±0.70	55.33±3.21	8.00±1.13	57.50±6.36
12			9.07±1.75	57.67±4.51
19			8.18±0.39	57.25±9.00
26			6.40±0.66	54.33±12.3

Abbreviations + Reference values: Blood urea nitrogen (6.5 – 8.2 mmol/L), Creatinine (31 – 46 µmol/L)

Table 8.13: Summary of **Full blood count** levels recorded as mean ± SD after treatment with **dexamethasone** in rats.

	DAYS					
PARAMETERS	**0**	**5**	**7**	**12**	**19**	**26**
SALINE (CONTROL)						
RCC (x10^12/l)	6.21±0.30	5.73±0.58	5.93±0.28			
Haemoglobin (g/dl)	12.75±0.35	11.97±0.93	12.03±0.95			
Haematocrit (l/l)	0.39±0.00	0.35±0.03	0.35±0.02			
MCV (fl)	63.65±3.46	61.93±1.53	59.30±0.53			
MCH (pg)	20.55±0.49	20.93±0.55	20.30±0.79			
MCHC (g/dl)	32.35±1.06	33.80±0.17	34.23±1.31			
Platelets (x10^9/l)	822±299	815±150	563±210			
WCC (x10^9/l)	6.02±1.63	3.83±0.23	5.17±1.55			
Neutrophils (x10^9/l)	0.77±0.17	0.57±0.17	0.74±0.26			
Lymphocytes (x10^9/l)	5.03±1.44	2.89±0.26	3.86±1.31			
Monocytes (x10^9/l)	0.19±0.11	00.09±0.04	0.44±0.20			
Eosinophils (x10^9/l)	0.02±0.00	0.26±0.20	0.12±0.06			
Basophils (x10^9/l)	0.01±0.01	0.02±0.01	0.01±0.00			
DEXAMETHASONE (TEST)						
RCC (x10^12/l)	6.21±0.30	6.68±0.69	6.87±1.07	7.48±0.92	7.02±1.17	6.87±0.93
Haemoglobin (g/dl)	12.75±0.35	13.63±1.66	13.73±2.16	20.87±8.95	13.90±1.57	13.83±1.57
Haematocrit (l/l)	0.39±0.00	0.36±0.04	0.40±0.06	0.44±0.60	0.38±0.05	0.37±0.03
MCV (fl)	63.65±3.46	57.80±1.35	57.97±1.33	57.20±1.47	54.70±2.91	54.00±3.44
MCH (pg)	20.55±0.49	20.37±0.46	20.00±0.20	19.87±0.95	19.93±1.21	20.20±1.15
MCHC (g/dl)	32.35±1.06	35.23±0.57	34.50±0.56	34.70±0.75	36.43±0.32	37.43±0.38
Platelets (x10^9/l)	822±299	781±228	511±289	260±164	341±104	879±326
WCC (x10^9/l)	6.02±1.63	2.78±1.59	3.96±2.22	5.84±1.47	3.28±0.69	6.09±3.74
Neutrophils (x10^9/l)	0.77±0.17	1.87±1.11	1.33±0.45	4.51±1.14	2.70±0.62	4.89±3.00
Lymphocytes (x10^9/l)	5.03±1.44	0.72±0.40	0.93±0.54	0.90±0.23	0.29±0.04	0.40±0.25
Monocytes (x10^9/l)	0.19±0.11	0.16±0.16	0.13±0.04	0.21±0.18	0.08±0.05	0.04±0.03
Eosinophils (x10^9/l)	0.02±0.00	0.02±0.01	0.06±0.09	0.02±0.02	0.16±0.11	0.42±0.46
Basophils (x10^9/l)	0.01±0.01	0.00±0.01	0.01±0.01	0.00±0.00	0.01±0.00	0.00±0.0

Abbreviations + Reference values: **RCC**: Red cell count (5.99 -6.42), Haemoglobin (12.5 – 13.1), haematocrit (0.393 – 0.406) **MCV**: Mean corpuscular volume (61.2 – 66.1), **MCH**: Mean corpuscular hemoglobin (20.2 – 20.9), **MCHC**: Mean corpuscular hemoglobin concentration (31.1 – 32.3), Platelets (611 – 1034) **WCC**: white cell count (4.16 – 9.55), Neutrophils (0.65 - 0.89), Lymphocytes (3.37 – 5.99), Monocytes (0.11 – 0.27), Eosinophils (0.00 – 0.02), Basophils (0.01 – 9.55)

8.6.3.3. General Immune markers response

Table 8.14 is a summary of the changes in body weight before and after Dex treatment. Dex treated rats lost weight from day 5 and continued for the duration of the study. The percentage change in the Dex group was -21±2 % by day 26 of treatment.

Table 8.15 shows changes in kidney, liver, spleen and thymus weight after treatment with Dex. The kidney and liver weight decreased significantly after 7 days (kidney, P = 0.009; liver, P = 0.006). Dex treatment caused significant spleen weight loss after 5 days (P = 0.007) and 7 days (P = 0.0001). The thymus shrunk in weight after 5 days (P = 0.007) and 7 days (P = 0.003) treatment and continued to decline for the duration of the study.

Table 8.14: Summary of **body weight** recorded as mean ± SD treatment with **dexamethasone** in rats.

DAYS	***SALINE (CONTROL)***			***DEXAMETHASONE (TEST)***		
	BEFORE (g)	***AFTER (g)***	***Change (%)***	***BEFORE (g)***	***AFTER (g)***	***Change (%)***
0	224±16	224±16	0	224±16	224±16	0
5	204±5	228±3	+11±1	218±	196±7	-10±3
7	228±5	265±8	+16±1	221±5	197±10	-11±3
12				214±4	177±3	-17±2
19				219±9	175±6	-20±5
26				220±7	174±8	-21±2

Table 8.15: Summary of **organs' weight (g)** recorded as mean ± SD after treatment **dexamethasone** in rats.

DAYS	SALINE (CONTROL)	DEXAMETHASONE (TEST)
	KIDNEYS	
0	1.73±0.11	1.73±0.11
5	1.98±0.19	1.88±0.20 (P = 0.5544)
7	2.10±0.07	1.75±0.11 (P = 0.009)*
12		1.61±0.07
19		1.79±0.04
26		1.80±0.11
	LIVER	
0	10.04±0.85	10.04±0.85
5	11.88±0.61	11.07±0.43 (P = 0.1335)
7	12.47±0.44	10.61±0.40 (P = 0.0056)*
12		9.53±0.17
19		8.68±0.42
26		9.00±0.74
	SPLEEN	
0	0.52±0.06	0.52±0.06
5	0.68±0.10	0.34±0.06 (P = 0.006)*
7	0.70±0.03	0.27±0.01 (P =0.0001)*
12		0.25±0.01
19		0.26±0.04
26		0.32±0.04
	THYMUS	
0	0.47±0.04	0.47±0.04
5	0.59±0.09	0.11±0.01 (P = 0.007)*
7	0.69±0.02	0.02±0.07 (P = 0.003)*
12		0.13±0.01
19		0.22±0.06
26		0.28±0.04

**P < 0.05 vs control **P < 0.001 vs control*

8.6.3.4. Immune cells response

Figure 8.10 shows plots of the effect of Dex chronic treatment on white cell (A), neutrophils (B) and lymphocyte (C) count versus time. White cell count was inhibited by Dex, though not significant. Dex treatment led to increased neutrophils count over time and was more pronounced on day 19. Lymphocytes were significantly suppressed by dexamethasone on day 5 ($P = 0.001$) reaching a low of 0.40±0.25 on day 26.

Figure 8.11 shows plots of the effect of Dex chronic treatment on CD_4 (A) and CD_8 (B) count versus time. Dex treatment inhibited CD_4 and CD_8 count and led to depletion (CD_4, 0.02±0.01; CD_8, 0.01±0.01) on day 26.

8.6.3.5. Immunoglobulins response

Figure 8.12 shows plots of the effect of Dex chronic treatment on IgG (A) and IgM (B) count versus time. IgG was inhibited by Dex for the first 12 days and increased to 16.51±8.48 on day 19 and returned to baseline thereafter. IgM was inhibited throughout the study by Dex treatment.

8.6.3.6. Cytokines response

Figure 8.13 shows plots of the effect of Dex chronic treatment on IL-2 (A) and IL-10 (B) count versus time. Treatment with Dex led to variable effect on IL-2 count through-out the study. Dex treatments had no effect on IL-10 count.

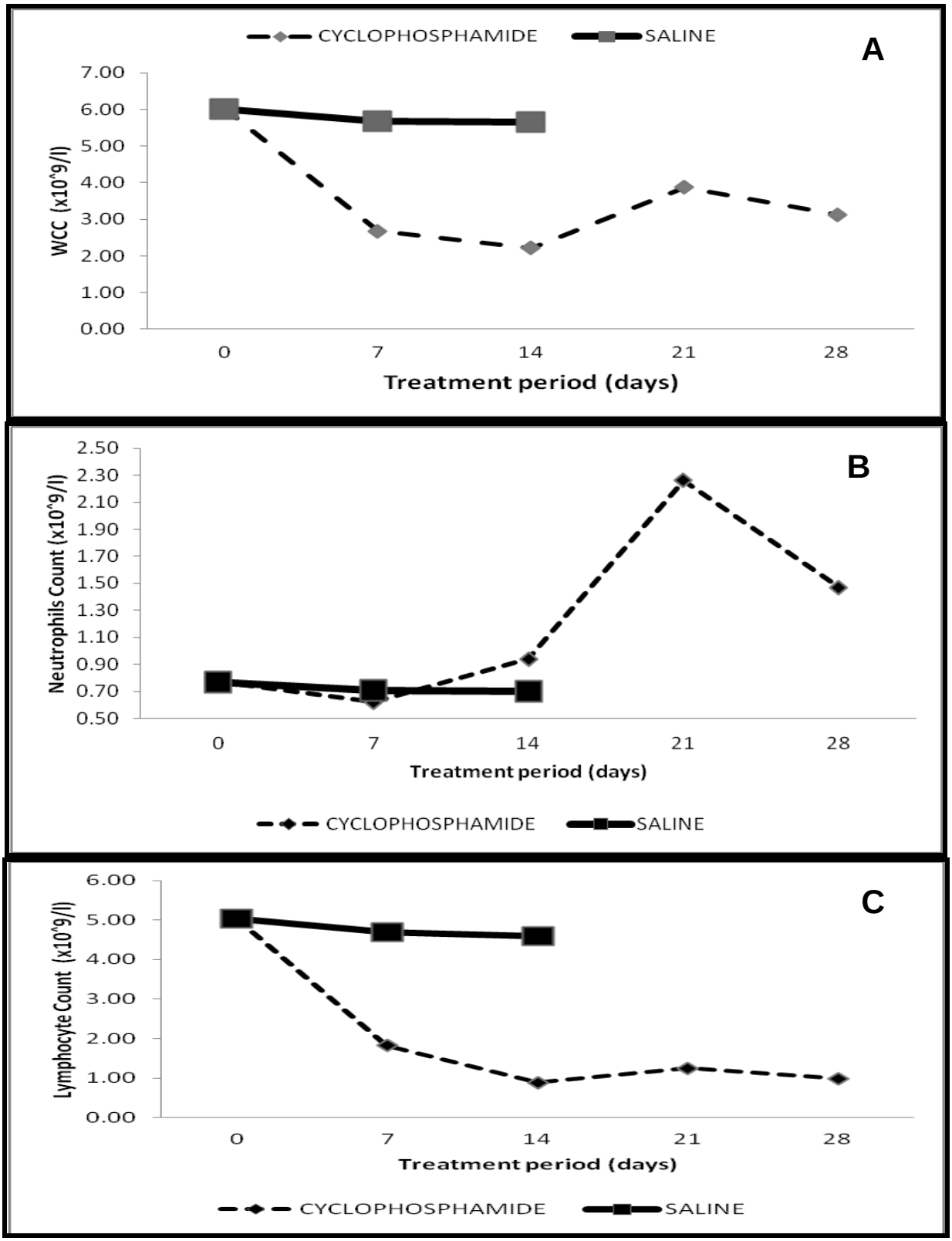

Figure 8.10: The effect of Dex chronic treatment on the **white cell count (A), neutrophils (B) and lymphocytes (C)** in rats.

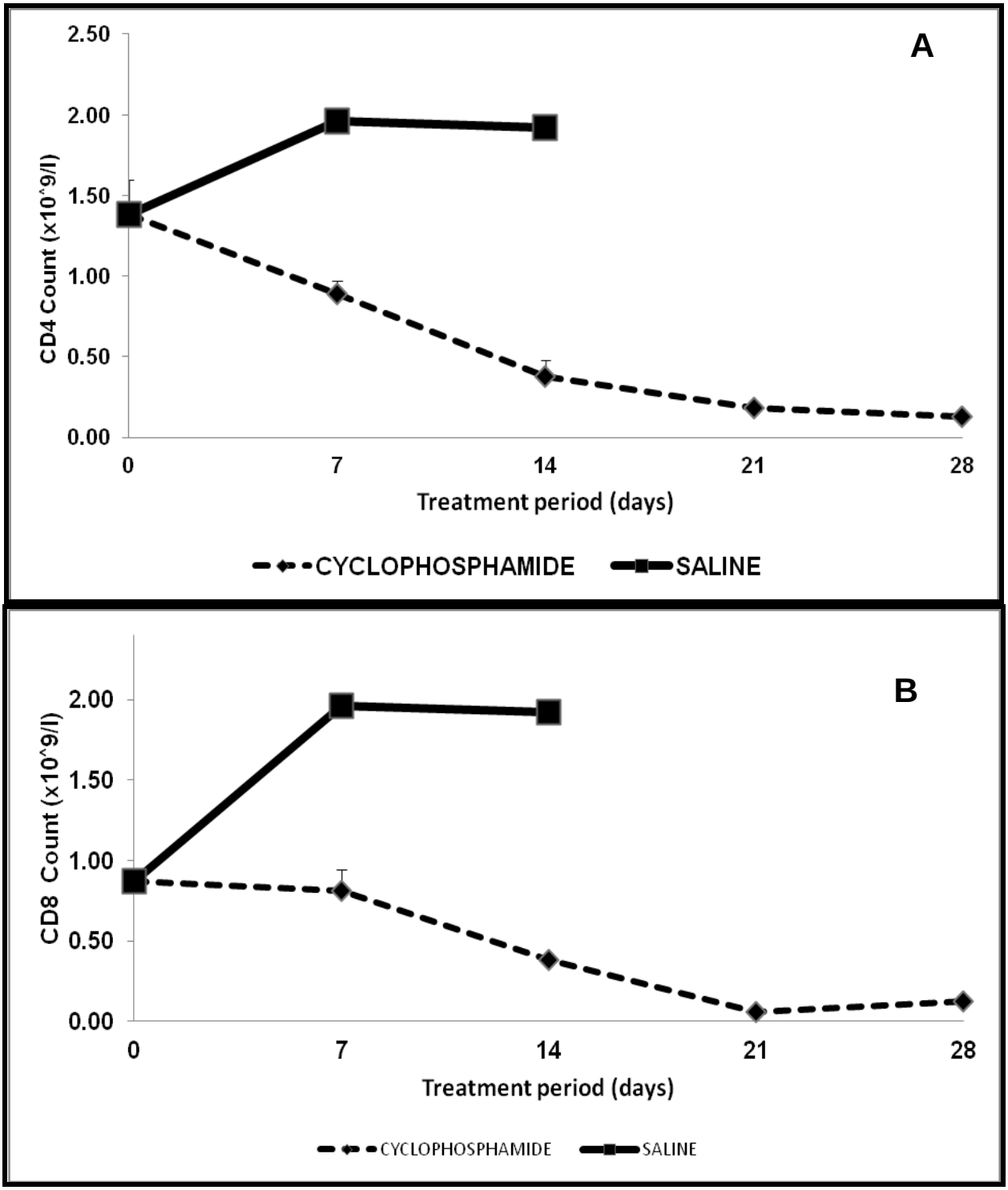

Figure 8.11: The effect of Dex chronic treatment on **CD_4 (A) and CD_8 (B) cells** in rats.

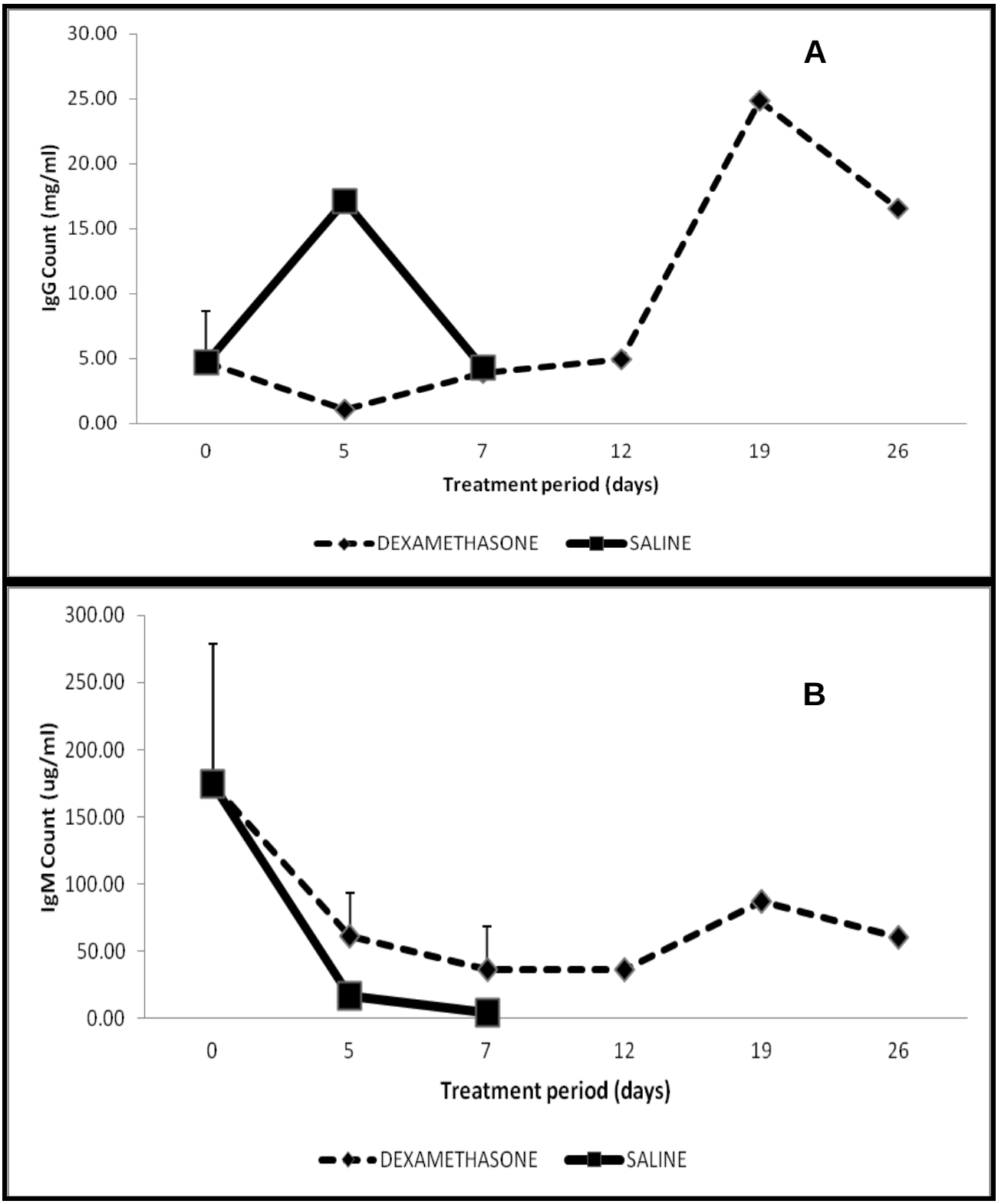

Figure 8.12: The effect Dex chronic treatment on **IgG (A) and IgM (B)** in rats.

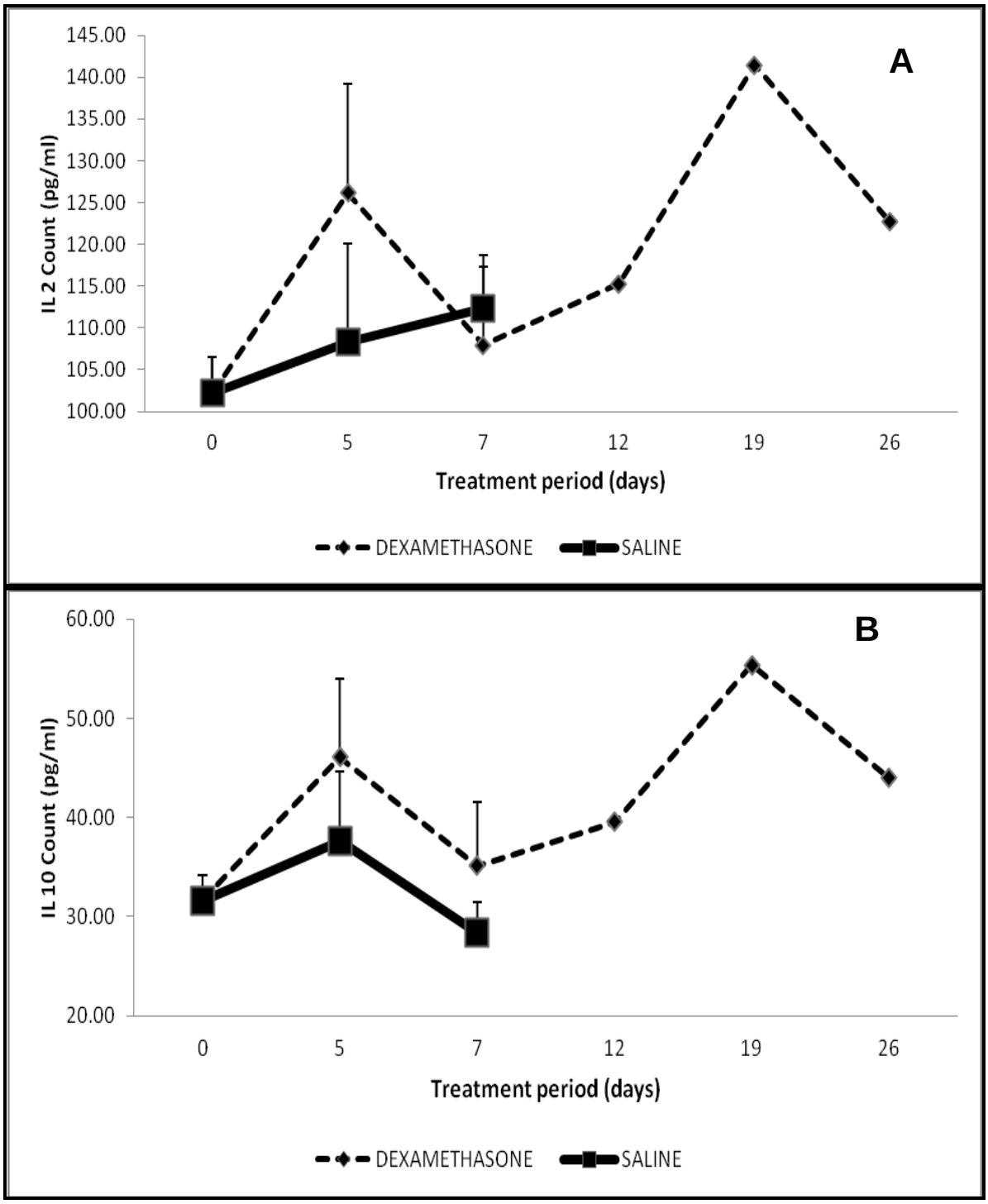

Figure 8.13: The effect of Dex chronic treatment on **IL-2 (A) and IL-10 (B)** in rats.

8.6.3.7. Summary of the effect of cyclosporine, cyclophosphamide and dexamethasone on physiological and immune markers in rats

Table 8.16 is the summary of effect on cyclosporine, cyclophosphamide and dexamethasone on physiological and immune markers in rats. Cyclosporine suppressed white cell count and lymphocytes [Untreated, 6.02±1.63 & 5.03±1.44; cyclosporine, 3.56±0.83 & 2.41±0.51] by the end of the study. On day 10, CD_4 and CD_8 counts were significantly suppressed ($P < 0.0001$), furthermore, IL-2 proliferation was inhibited [control, 128.79±15.82; cyclosporine, 106.30±6.70]. Cyclophosphamide treatment decreased white cell count and lymphocyte after 7 days [control, 5.69±1.61 & 4.70±1.36; cyclophosphamide, 2.68±0.47 & 1.82±0.31]. The immune suppression was significant for IgM ($P = 0.02$) CD_4 ($P = 0.0001$) and CD_8 ($P = 0.009$) count. After 5 days treatment with dexamethasone CD_4 and CD_8 counts [control, 1.27±0.16 & 0.82±0.02; dexamethasone, 0.43±0.05 & 0.23±0.03] declined and were depleted by the end of the study. Moreover, dexamethasone significantly suppressed lymphocytes ($P = 0.001$) and IgM ($P = 0.001$) on day 5. Cyclosporine inhibited cell mediated immunity, cyclophosphamide and dexamethasone suppressed both cell mediated and humoral and dexamethasone effect was more systemic.

Table 8.16: Summary of the effect of cyclosporine, cyclophosphamide and dexamethasone on physiological and immune markers in rats.

Parameters	Cyclosporine	Cyclophosphamide	Dexamethasone
PHYSIOLOGICAL MARKERS			
Liver function tests	↓ ALP	↓ ALP	↓ ALP
Renal function tests	↑ creatinine	no effect	↑ creatinine
Red cell count	↑	↑	↑
Haemoglobin	↑	↑	↑
Haematocrit	↑	↑	↑
Platelets	thrombocytosis	thrombocytopenia	thrombocytopenia
Eosionophils	eosinophilia		
IMMUNE MARKERS			
Body weight	no effect	stunted	↓
Kidney weight	no effect	stunted	↓
Liver weight	↓	stunted	↓
Spleen weight	no effect	↓	↓
Thymus weight	↓	↓	↓
White cell count	↓	↓	↓
Neutrophils	no effect	↑	↑
Lymphocytes	↓	↓	↓
CD_4	↓	↓	↓ & depleted
CD_8	↓	↓	↓& depleted
IgG	↑	no effect	↓
IgM	no effect	↓	↓
IL-2	↓	no effect	variable
IL-10	↓	no effect	no effect

↓= loss/inhibition/decrease, ↑= increase/proliferation

8.6.4. DISCUSSION

Cyclosporine, cyclophosphamide and dexamethasone are all potent immune suppressive agents with diverse functional mechanism and were used to induce leukopenia, myelosuppression and immune suppression. The present study was performed to develop an immunesuppression rat model. This was achieved by evaluating changes in selected immune markers after chronic treatment with cyclosporine, cyclophosphamide and dexamethasone to better understand immune disease progression in rats. Physiological parameters were evaluated to test normality, whilst immune markers gave insight on the mechanism of the immune response, the level of suppression and/or the extend of the immune system impairment. Also, monitoring of cytokines elaborates on the mechanism of immune response at a specific time. Measurement of antigen-specific IgM, IgG and total IgG allows investigator to monitor the total accumulated humoral immunity in circulating blood, while blood leukocytes and their respective subsets elaborate on the cell mediated immune response (Lilly *et al*., 2005).

Test-drug related effect on erythrocytes was illustrated with changes in the red blood cell subsets. All the immune suppressants altered kidney and liver function. Evidence of liver disease was confirmed by liver weight loss and low alkaline phosphate count in immune suppressed rats. Similarly cyclophosphamide and dexamethasone stunted the liver growth time dependently. Moreover, creatinine was elevated in cyclosporine and dexamethasone treated rats. Anaemic disease in cyclophosphamide treated rats was confirmed by reduced red blood cells, haemoglobin and haematocrit. A mild case of polycythaemia was observed in groups treated with cyclosporine and dexamethasone. Furthermore, the results indicated thrombocytopenia after immune suppression with cyclophosphamide and dexamethasone; increasing the risk of bleeding in rats. Overall, the immune suppressants led to atrophy of immune organs and liver and renal injury that is time-dependent.

The used dose of each drug had been previously used for acute immune suppression; however, none was used individually for chronic treatment prior to this study. Observations from the results confirmed that cyclosporine treated rats were immune suppressed via the inhibition of IL-2 proliferation. Also, cyclosporine

inhibited leukocytes proliferation by inhibiting their subsets such as white cell count, lymphocytes, CD_4 and CD_8 cells. According to von Horsten and his colleagues (1998) cyclosporine is known to induce alteration of blood leukocyte number and function. Cyclosporine is a cyclic polypeptide that can bind to cyclophilin and inhibits calcineurin phosphate activity causing decrease in some cytokines especially IL-2. Cyclosporine induced immune suppression in rats was mostly cell mediated and more pronounced over time. Cell-mediated immunity involves activation of immune cells to combat infection and it's regulated by cytokines. These cytokines initiate cell mediated immunity by activating macrophages, natural killer cells and cytotoxic T-cells, which kill bacteria and virus infected cells respectively. It is most effective in removing virus-infected cells and participating in defending against fungi, protozoans, cancers and intracellular bacteria.

Rats treated with cyclophosphamide were stunted in body and organ growth. Moreover, T-lymphocytes suppression and inhibited immunoglobulin proliferation was evident with a low white cell count, lymphocytes, CD_4, CD_8, IgG and IgM count during the study. Cyclophosphamide is a nitrogen mustard alkylating agent that causes the formation of cross links within DNA, reducing the number of circulating lymphocytes and impairing function of humoral immunity and cell mediated immunity (Smith *et a*l., 2003). Humoral immune response activates plasma cells to mature into immunoglobulin secreting cells. These immunoglobulins counteract the invading pathogens to eliminate infection.

Dexamethasone effect in rats was systemic and more pronounced as the immune disease set in. The white cell count and lymphocytes were suppressed, while CD_4 and CD_8 cells were almost depleted at the end of the study after dexamethasone treatment. IgG and IgM count was suppressed throughout the study. This could be related to dexamethasone's mechanism of action that occurs at gene transcription level, and achieved by altering regulation of gene expressions. Dexamethasone is a glucocortocoid with anti-inflammatory and immunosuppressive effects. Changes in immune markers have confirmed immune suppression in all test groups was drug and time dependent.

A drug's mechanism can either be cell mediated, humoral response or both. Decrease of TH_1 pathway leads to a loss of cell mediated immunity and thus ineffectiveness to fight viral-infections. While decline of TH_2 pathway inhibits humoral immune response hence failure to overcame bacteria-infections. However the $TH_{1/2}$ pathways co-exist because the regulate each other regarding their actions whereby a shift towards TH_1 promotes cell mediated response and decrease TH_2 pathway and vice versa. This observation can be used to investigate an immune booster that is being developed for viral infections, whereby restoration of cell mediated immunity is vital.

Immune suppression led to loss of the white cell count significantly in a time dependent manner. This was supported by neutropenia and declining counts of lymphocytes, CD_4 cells and CD_8 cells. Moreover decrease in granulocytes and lymphocytes population in the entire tested subsets showed comparable decline. Furthermore, Loss of immune cells observed in rats, correlates with a loss of cell mediated immunity, occurring during viral infections. Moreover, the results show that cyclosporine and cyclophosphamide inhibited IL-2 production and slightly increased IgG, further supporting a declining and/or shifting of TH_1 pathway towards TH_2 pathway.

Disorders of the immune system can result in autoimmune diseases, inflammatory disease, various forms of cancer and immunodeficiency and it's caused by loss of either cell-mediated or humoral immune response or both. During immunodeficiency, the immune system is less active than normal increasing occurrences of infections such as during HIV/AIDS infection. For instance during HIV progression, deterioration of the cellular immune system leads to severe immuno depression and renders the individual more susceptible to opportunistic disease and/or certain types or neoplasm (Tasca, 2012; Macallan, 2013; Li and Ling, 2012; Fabbiane, 2013).

Knowledge of the changes in immune parameters gives an inside into the immune system's mechanism of pathology caused by pro-longed immune suppression that can occur during immune deficiency. Hence it of utmost importance to monitor

immune function over time with immune markers. Moreover, defines the specific time to start intervention with an immune booster being evaluated.

In summary, treatment of rats with cyclosporine, cyclophosphamide and dexamethasone altered physiological function as confirmed by nephrotoxicity, thrombocytopenia, anaemic and liver disease. The findings of this study confirmed immune suppression mechanism that is drug specific and time dependent. Cyclosporine inhibited cell mediated immunity, cyclophosphamide and dexamethasone suppressed both cell mediated and humoral and dexamethasone effect was more systemic. As thus a rat model of induced immune suppression using cyclosporine, cyclophosphamide and dexamethasone has been developed. This model can be used to scientifically evaluate any purported immune boosters. For the purpose of this study co-treatment with *Phela* will be started at the time frame [CsA, 10 days; CP, 7 days; Dex, 5 days] of initial immune suppression.

8.7. CONCLUSION

An immunosuppression rat model has been established. It will be applied in the proceeding chapters to study mechanism of modulation of *Phela* an herbal medicine. However for further studies it could be necessary to intervene at all time frames defined in this study per drug, in order to gather the ideal time for intervention and also to gather advantages and limitations of the immune booster being studied.

9.

EFFECT OF PHELA ON A RAT MODEL OF CYCLOSPORINE, CYCLOPHOSPHAMIDE AND DEXAMETHASONE INDUCED IMMUNE SUPPRESSION

Presented at the 17th world conference of basic and clinical pharmacology (2014)

9.1. SUMMARY

Introduction: Traditional medicines have gained interest as a source of therapy for ailments; however, a gap remains from traditional usage to potential clinical use, hence a need for scientific validation *in vivo* to understand their efficacy and scope of application. As several medicinal plants are employed throughout the world to improve the immunological disorders, it has been established that the evaluation of plants products that either promote or inhibit immunocyte proliferation is crucial to the study of immunomodulation and drug discovery (Nudo *et al.*, 2011). Lekhooa (2012) reported that *Phela* is an immune booster and does not interact with the major CYP450 isoforms. *Phela* is an herbal medicine that is currently being developed for patients with a compromised immune system (i.e. HIV positive patients). Its efficacy was first acclaimed in anecdotal reports of traditional healers. However, there is no supporting scientific information on its mechanism of action on the purported abilities hence; *Phela* is an ideal immune booster to evaluate the developed rat model of induced immunesuppression. Having established a rat model of drug-induced immune suppression in **Chapter 8**, here the model is applied to establish the mechanism of action of a purported immune booster *Phela* on the immune system. As thus, the aim of this chapter is to establish the mechanism of immunomodulation of *Phela* in a rat model of induced immune suppression of cyclosporine, cyclophosphamide and dexamethasone.

Methodology: Different groups of 15 rats each were pre-treated with CsA, CP or Dex only to induce immunesuppression. Thereafter, the control-groups continued on the immunesuppresant only and the test groups were co-treated with immunesuppresants (CsA, CP or Dex) and *Phela* for 21 days. Thereafter, 5 rats were sacrificed once weekly. Physiological function tests and immune markers (CD_4, CD_8, IgG, IgM, IL-2 and IL-10) were analysed.

Results: *Phela's* immunesuppression reversal was drug-specific and time dependent. *Phela* stopped the progression of immunesuppression in rats treated with cyclosporine as indicated by the reversal or resistance to CsA induced changes in the WCC, neutrophils, lymphocytes, CD_4, CD_8, and IL-2. Also, *Phela* increased IL-2 count after 7 and 14 days [*CsA-group: (106.30±6.70); (115.71±19.61) vs Phela*+CsA-*group: (138.31±36.02), (178.28±48.75)*] of treatment. Furthermore, *Phela* prevented progression of CP induced body and thymus weight loss, suppression of IgG and IgM, and minimal effect on CD_4 and CD_8 cell count. IgG concentration was significantly increased in the *Phela*-treated group over 7 days (P = 0.01) and 14 days (P = 0.008). IgM levels remained high through-out the study though not statistically significant. *Phela* prevented Dex induced suppression on WCC, neutrophils, CD_8 cells, IgG and IgM on day 7. As such, *Phela* stimulated the cell mediate immunity in the CsA rat model via IL-2 mechanism, while minimally resisting CP and Dex induced changes in rats.

Conclusion: *Phela's* mechanism of immunomodulation is mainly cell mediated immunity. Therefore, *Phela* can be a perfect candidate for testing against diseases or disorders associated with suppressed the cell mediate immunity, such as HIV and TB.

9.2. INTRODUCTION

The aim of this study was to establish the mechanism of immunomodulation of *Phela* using a rat model of cyclosporine, cyclophosphamide and dexamethasone -induced immune suppression.

9.3. MATERIALS

The apparatuses and reagents used in this part of the study were the same as those used in **Chapter 7**, **Sections 7.3**.

9.4. PROCEDURES

9.4.1. ANIMAL CARE AND DRUG PREPARATION

The animal care remained the same as described in **Chapter 7, Section 7.4.1**. *Phela* was dissolved in normal saline to 15.4 mg/kg. CsA, CP and Dex were prepared as described in **Chapter 8, Sections 8.4.1 and 8.4.2**.

9.4.2. EXPERIMENTAL DESIGN FOR THE EFFECT OF *PHELA* ON IMMUNE SUPPRESSED RATS.

Figure 9.1 is an illustration of the experimental design of the effect of *Phela* on CsA, CP and Dex induced immune suppression in a rat model.

Cyclosporine

Thirty male rats were immune suppressed with CsA (2 mg/kg) for 10 days prior. Thereafter, on the 11th day, rats were divided into 2 groups (n = 15). *Phela* (15.4 mg/kg) was given daily by gastric lavage as intervention to the test-group with continued cyclosporine treatment over 21 days. The control group was continued on the cyclosporine-only treatment for the duration of the study. Rats (n = 5) were sacrificed weekly from each group.

Cyclophosphamide

Thirty male rats were intraperitoneally inoculated with CP (100 mg/kg) once weekly to induce immune suppression. On the 8th day the rats were divided into 2 groups (n = 15). *Phela* (15.4 mg/kg) was given daily with a gastric lavage as intervention to the test-group with continued cyclophosphamide treatment over 21 days. The control group was continued on the cyclophosphamide-only treatment for the duration of the study. Rats (n = 5) were sacrificed weekly from each group.

Dexamethasone

Thirty male rats were intraperitoneally inoculated with Dex (1.5 mg/kg) daily over 5 days to induce immune suppression. On the sixth day the rats were divided into 2 groups (n = 15). *Phela* (15.4 mg/kg) was given daily with a gastric lavage as intervention to the test group with continued dexamethasone treatment over 21 days. The control group was continued on the dexamethasone-only treatment for the duration of the study. Rats (n = 5) were sacrificed weekly from each group.

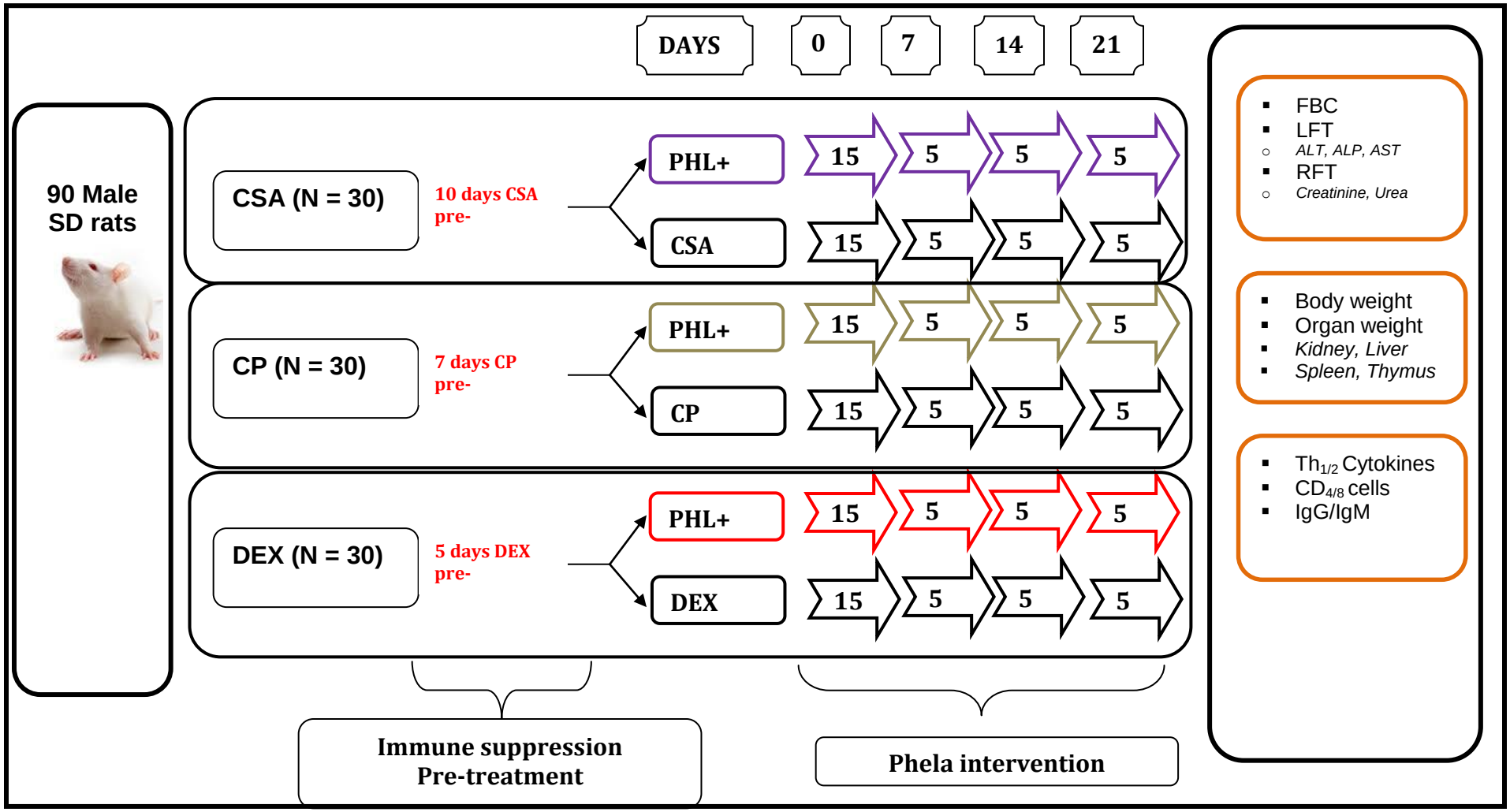

Figure 9.1: Experimental design to establish the mechanism of immunomodulation of *Phela* using a rat model of drug-induced immune suppression.

Abbreviations: CsA: cyclosporine, **CP**: cyclophosphamide, **Dex:** dexamethasone, **PHL**: *Phela*, **SD**: Sprague dawley, **FBC**: full blood count, **LFT**: Liver function tests, **ALP:** alkaline phosphatase, **ALT:** Alanine transaminase, **AST**: Aspartate transaminase, **RFT:** Renal function tests

9.4.3. OBSERVATIONS AND WEIGHING RATS

Rats were observed daily for any physical signs of infection and/or disease progression and weighed weekly as described in **Chapter 7**, **Section 7.4.4**.

9.4.4. SACRIFICING PROCEDURE

Sacrificing was done at the department of Pharmacology according to the timeframe of each drug. The sacrificing steps were as described in **chapter 7**, **section 7.4.5.**

9.4.5. MEASUREMENT OF PHYSIOLOGICAL PARAMETERS AND IMMUNE MARKERS' CONCENTRATION

EDTA tubes were used to collect whole blood (2 ml) for full blood, CD_4 and CD_8 count analysis. Plasma (2 ml) was collected in lithium-heparin tubes for each drug (i.e. cyclosporine, cyclophosphamide and dexamethasone) concentration determination. Cyclophosphamide and dexamethasone were analysed by an HPLC-UV method validated in **Chapter 5**, while cyclosporine was analyzed by chemiluminescent immuno assay (CMIA). Similarly, serum was collected for liver and renal function tests as elaborated in **Chapter 7**, **Section 7.4.6**.

9.4.6. ORGAN HARVESTING

The liver, kidney, spleen and thymus were harvested as described **Chapter 7**, **Section 7.4.7**.

9.4.7. MEASUREMENT OF CYTOKINES AND IMMUNOGLOBULINS BY ELISA

Rat specific ELISA kits were applied according to each manufacturer's instructions to determine IL-2, IL-10, IgG and IgM concentration in serum as described in **Chapter 7**, **Section 7.5.**

9.5. STATISTICAL ANALYSIS

All statistical analysis was done using the Graph Pad© Instat program and graphs drawn on an excel program as elaborated on in **Chapter 7**, **Section 7.6**.

9.6. RESULTS

9.6.1. EFFECT OF *PHELA* ON A RAT MODEL OF CsA, INDUCED IMMUNE SUPPRESSION

9.6.1.1. Physiological Parameters

9.6.1.1.1. Liver functions tests

Table 9.1 is a summary of liver function test after treatment with *Phela* on cyclosporine induced suppression. *Phela* had no effect on liver function tests.

9.6.1.1.2. Renal function tests

Table 9.2 is a summary of the renal function tests after treatment with *Phela* on cyclosporine induced suppression. The reading on day 7 in the Phela treated group was undetectable. *Phela* had no effect on renal function tests.

9.6.1.1.3. Haematological parameters

Table 9.3 is a summary of full blood count after treatment with *Phela* on cyclosporine induced suppression. *Phela* increased white cell count and neutrophils after 7 days of treatment and had no effect on lymphocytes.

Table 9.1: Summary of **liver function test** recorded as mean ± SD after 7,14 and 21 days of treatment *Phela* in CsA suppressed rats.

	CYCLOSPORINE (Only)			**CYCLOSPORINE (+*PHELA*)**		
DAYS	**ALP**	**ALT**	**AST**	**ALP**	**ALT**	**AST**
	(U/L)	**(U/L)**	**(U/L)**	**(U/L)**	**(U/L)**	**(U/L)**
0	38.0±0.0	60.0±0.0	107.5±2.1	38.0±0.0	60.0±0.0	107.5±2.1
7	164.5±21.0	33.5±4.2	100.8±37.6	38.0±0.0	52.3±7.5	107.5±2.1
14	180.5±36.7	62.3±12.9	150.3±39.2	185.8±25.5	72.0±22.0	212.5±119.1
21	179.0±38.8	59.0±8.6	184.8±24.4	140.0±42.3	50.5±8.3	135.0±18.0

Abbreviations + Reference values: **ALP:** alkaline phosphatase (289 – 436 U/L), **ALT:** Alanine transaminase (46 – 55 U/L), **AST:** Aspartate transaminase (81 – 104 U/L).

Table 9.2: Summary of **renal function tests** recorded as mean ± SD after 7,14 and 21 days of treatment with *Phela* in CsA suppressed rats.

	CYCLOSPORINE (Only)		**CYCLOSPORINE (+*PHELA*)**	
DAYS	**Creatinine**	**BUN**	**Creatinine**	**BUN**
	(µmol/L)	**(mmol/L)**	**(µmol/L)**	**(mmol/L)**
0	50.50±3.54	7.70±0.14	50.50±3.54	7.70±0.14
7	55.40±4.34	11.60±1.20	_____	5.80±0.77
14	10.60±6.27	6.86±1.32	13.33±3.79	6.47±0.06
21	48.00±4.95	65.90±0.87	44.80±7.26	5.48±0.64

Abbreviations + Reference values: Blood urea nitrogen (6.5 – 8.2 mmol/L), Creatinine (31 – 46 µmol/L)

Table 9.3: Summary of **Full blood count test** levels recorded as mean ± SD after 7,14 and 21 days of treatment with *Phela* in CsA suppressed rats.

	DAYS			
PARAMETERS	**0**	**7**	**14**	**21**
		CYCLOSPORINE (Only)		
RCC (10^12/l)	7.09±0.74	8.02±0.05	7.73±0.40	7.10±0.80
Haemoglobin (g/dl)	14.05±1.05	15.77±0.64	16.13±0.29	13.27±1.27
Haematocrit (l/l)	0.42±0.02	0.45±0.02	0.45±0.02	0.37±0.04
MCV (fl)	58.70±2.06	55.20±1.57	58.80±1.97	52.10±1.14
MCH (pg)	19.80±0.87	19.23±0.67	20.93±0.81	18.73±0.35
MCHC (g/dl)	33.75±0.52	34.80±0.26	35.53±0.67	35.93±0.38
Platelets (10^9/l)	671±254	1107±30	219±210	656±61
WCC (10^9/l)	3.95±0.49	5.46±0.65	4.48±0.92	3.56±0.83
Neutrophils (10^9/l)	0.62±0.04	0.91±0.23	0.85±0.35	0.63±0.23
Lymphocytes (10^9/l)	4.27±1.14	4.01±0.48	3.30±0.71	2.41±0.51
Monocytes (10^9/l)	0.20±0.16	0.39±0.24	0.14±0.11	0.17±0.19
Eosinophils (10^9/l)	0.09±0.04	0.09±0.10	0.23±0.20	0.35±0.17
Basophils (10^9/l)	0.03±0.03	0.05±0.03	0.01±0.01	0.00±0.00
		CYCLOSPORINE (+*PHELA*)		
RCC (10^12/l)	7.09±0.74	7.08±0.88	8.18±0.21	7.84±0.22
Haemoglobin (g/dl)	14.05±1.05	14.40±1.40	17.88±0.44	14.80±0.26
Haematocrit (l/l)	0.42±0.02	0.40±1.40	0.48±0.01	0.42±0.00
MCV (fl)	58.70±2.06	56.83±1.34	58.90±0.45	53.73±1.25
MCH (pg)	19.80±0.87	20.400.56	21.85±0.93	18.90±0.17
MCHC (g/dl)	33.75±0.52	35.90±0.40	37.13±1.34	35.10±0.53
Platelets (10^9/l)	671±254	770±366	479±267	841±156
WCC (10^9/l)	3.95±0.49	7.07±2.60	5.15±0.98	4.99±1.64
Neutrophils (10^9/l)	0.62±0.04	3.24±2.37	1.56±1.27	1.05±0.27
Lymphocytes (10^9/l)	4.27±1.14	3.72±0.86	2.73±0.44	3.66±1.32
Monocytes (10^9/l)	0.20±0.16	0.22±0.17	0.19±0.05	0.22±0.10
Eosinophils (10^9/l)	0.09±0.04	0.50±0.09	0.32±0.06	0.06±0.03
Basophils (10^9/l)	0.03±0.03	0.01±0.02	0.01±0.00	0.00±0.01

Abbreviations + Reference values: **RCC**: Red cell count (5.99 -6.42), Haemoglobin (12.5 – 13.1), haematocrit (0.393 – 0.406) **MCV**: Mean corpuscular volume (61.2 – 66.1), **MCH**: Mean corpuscular hemoglobin (20.2 – 20.9), **MCHC**: Mean corpuscular hemoglobin concentration (31.1 – 32.3), Platelets (611 – 1034) **WCC**: white cell count (4.16 – 9.55), Neutrophils (0.65 - 0.89), Lymphocytes (3.37 – 5.99), Monocytes (0.11 – 0.27), Eosinophils (0.00 – 0.02), Basophils (0.01 – 9.55)

9.6.1.2. Cyclosporine concentration in plasma

Table 9.4 is a summary of cyclosporine concentration in plasma after the rats were treated with *Phela* for three weeks. There was no difference in the test group's (CsA+*Phela*) concentration when compared with the control group (CsA-only).

Table 9.4: Summary of **cyclosporine concentration** (ng/ml) in plasma recorded as mean ± SD after 7, 14 and 21 days of treatment with *Phela* in CsA suppressed rats.

DAYS	Cyclosporine (Only)	Cyclosporine (+*PHELA*)
0	32 ±0	32 ±0
7	70±18	45±17
14	34±8	44±3
21	59±28	45±5

Abbreviations: CsA= cyclosporine

9.6.1.3. General immune markers response

Table 9.5 is a summary of the effect of *Phela* on rats' body weight and weight change percentage in CsA rat model. There was no effect on body weight change levels after *Phela* treatment. The rats grew as expected and the change percentage was similar in both groups

Table 9.5: Summary of **body weight (g**) recorded as mean ± SD after 7, 14 and 21 days after treatment with *Phela* in CsA suppressed rats.

DAYS	CYCLOSPORINE (only)			CYCLOSPORINE (+ PHELA)		
	BEFORE (g)	***AFTER (g)***	***Change (%)***	***BEFORE (g)***	***AFTER (g)***	***Change (%)***
0	225±11	257±19	+14±6	225±11	257±19	+14±6
7	212±4	278±5	+32±4	208±10	254±9	+31±5
14	238±7	295±12	+24±8	238±14	290±17	+22±4
21	229±4	312±9	+36±6	219±5	315±14	+44±8

Table 9.6 is a summary of the effect of *Phela* on organ weight in CsA rat model. *Phela* prevented kidney swelling on day 7 (P = 0.05) and 14 (P = 0.008). *Phela* had no effect on liver and spleen weight loss throughout the study, while thymus weight loss was reversed after 21 days (P = 0.0001) of treatment.

Table 9.6: Summary **organs weight (g)** recorded as mean ± SD after 7, 14 and 21 days of treatment with *Phela* in CsA suppressed rats.

DAYS	CYCLOSPORINE (only)	CYCLOSPORINE (+ *PHELA*)
	KIDNEYS	
Day 0	2.05±0.22	2.05±0.22
Day 7	2.00±0.08	2.16±0.14
Day 14	2.38±0.10	2.18±0.07 (P = 0.0082)*
Day 21	2.38±0.08	2.32±0.19
	LIVER	
Day 0	11.19±1.01	11.19±1.01
Day 7	9.31±0.38	10.60±1.16 (P = 0.0463)*
Day 14	11.81±0.97	11.10±0.64
Day 21	11.44±0.27	12.22±1.09
	SPLEEN	
Day 0	0.67±0.09	0.67±0.09
Day 7	0.58±0.03	0.59±0.06
Day 14	0.60±0.03	0.54±0.09
Day 21	0.60±0.04	0.61±0.02
	THYMUS	
Day 0	0.62±0.12	0.62±0.12
Day 7	0.48±.12	0.59±0.10
Day 14	0.52±0.09	0.47±0.10
Day 21	0.34±0.04	0.53±0.05 (P = 0.0001)**

**P < 0.05 vs control **P < 0.001 vs control*

9.6.1.4. Immune cells response

Figure 9.2 shows plots of white cell count (A), neutrophils (B) and lymphocytes (C) count versus time in a cyclosporine-induced rat model after *Phela* treatment. White cell count levels remained higher in the *Phela* treated group through-out the study though not statistically significant. Moreover, *Phela* reversed neutrophils inhibition after 7 and 14 returning to baseline on day 21. *Phela* overcame cyclosporine induced suppression effect on lymphocytes after 21 days of treatment.

Figure 9.3 shows plots of CD_4 (A) and CD_8 (B) cell count versus time in a cyclosporine-induced rat model after *Phela* treatment. *Phela* reversed cyclosporine effect on CD_4 count suppression on day 14 (CsA, 1.08±0.30; CsA+*Phela*: 1.43±0.14) and 21 (P = 0.05). Similarly, *Phela* prevented CD_8 count suppression on day 14 (CsA, 0.71±0.25; CsA+*Phela*: 0.78±0.04) and was significant after 21 days (P = 0.002).

9.6.1.5. Immunoglobulins response

Figure 9.4 shows plots of IgG (A) and IgM (B) count versus time in a cyclosporine-induced rat model after *Phela* treatment. *Phela* had no effect on IgG count. *Phela* reversed IgM count suppression on day 14 (CsA, 51.91±14.85; CsA+*Phela*: 132.86±103.33) and 21 (CsA, 28.06±14.18; CsA+*Phela*: 42.82±28.32) though not statistically significant.

9.6.1.6. Cytokines response

Figure 9.5 shows plots of IL-2 (A) and IL-10 (B) count versus time in a cyclosporine-induced rat model after *Phela* treatment. *Phela* stopped IL-2 count suppression on day 7 (CsA, 106.30±6.70; CsA+*Phela*, 138.31±36.02) and 14 (CsA, 115.71±19.61; CsA+*Phela*, 178.28±48.75) though not statistically significant. IL-10 was increased after 7 days (CsA, 37.93±8.36; CsA+*Phela*, 48.32±20.67) and 14 days (CsA, 48.32±19.62; CsA+*Phela*, 69.64±28.87) in the *Phela* treated group.

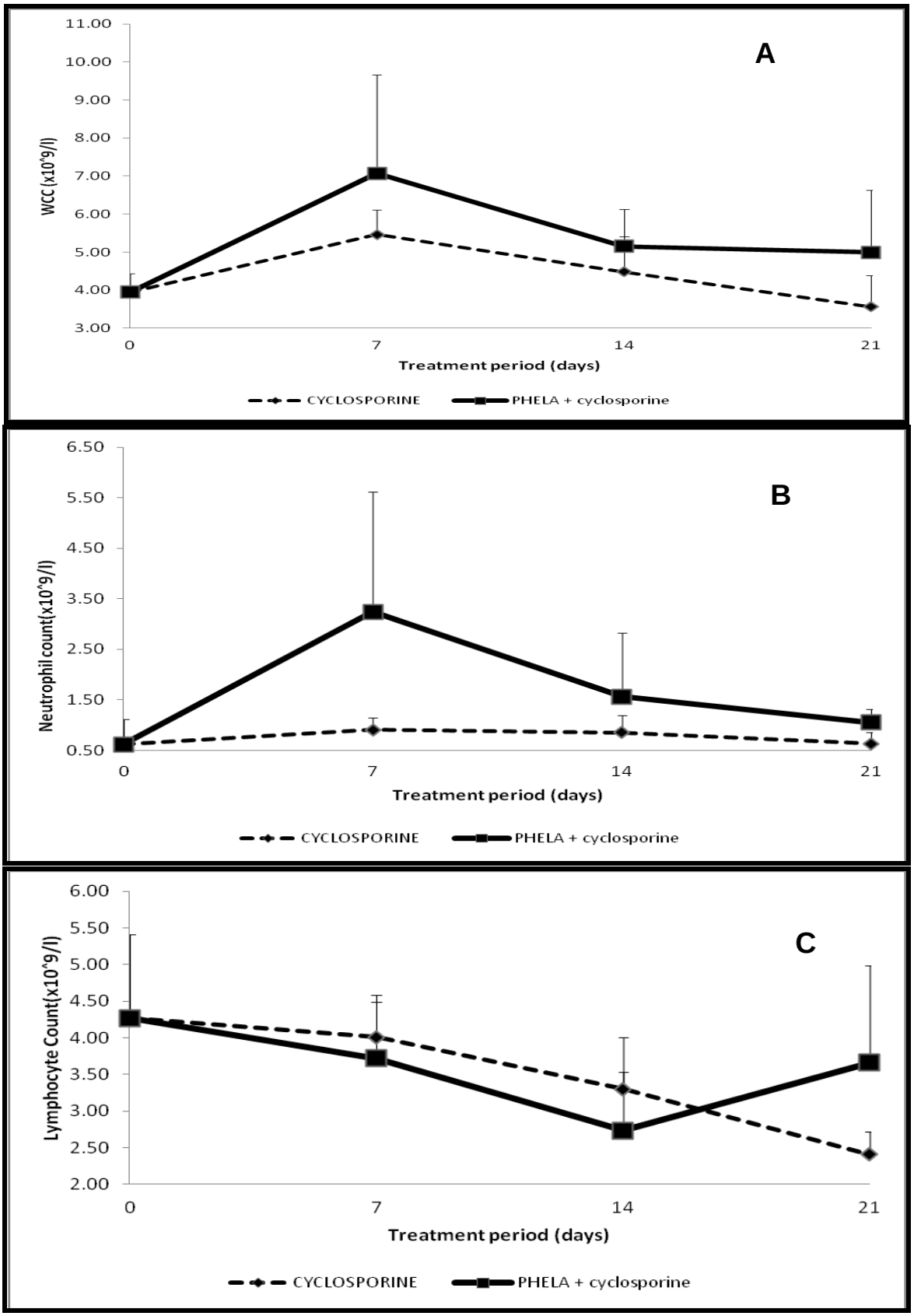

Figure 9.2: Effect of *Phela* on **WCC(A) neutrophils (B) and lymphocytes (C)** count after 7, 14 and 21 days treatment in CsA suppressed rats.

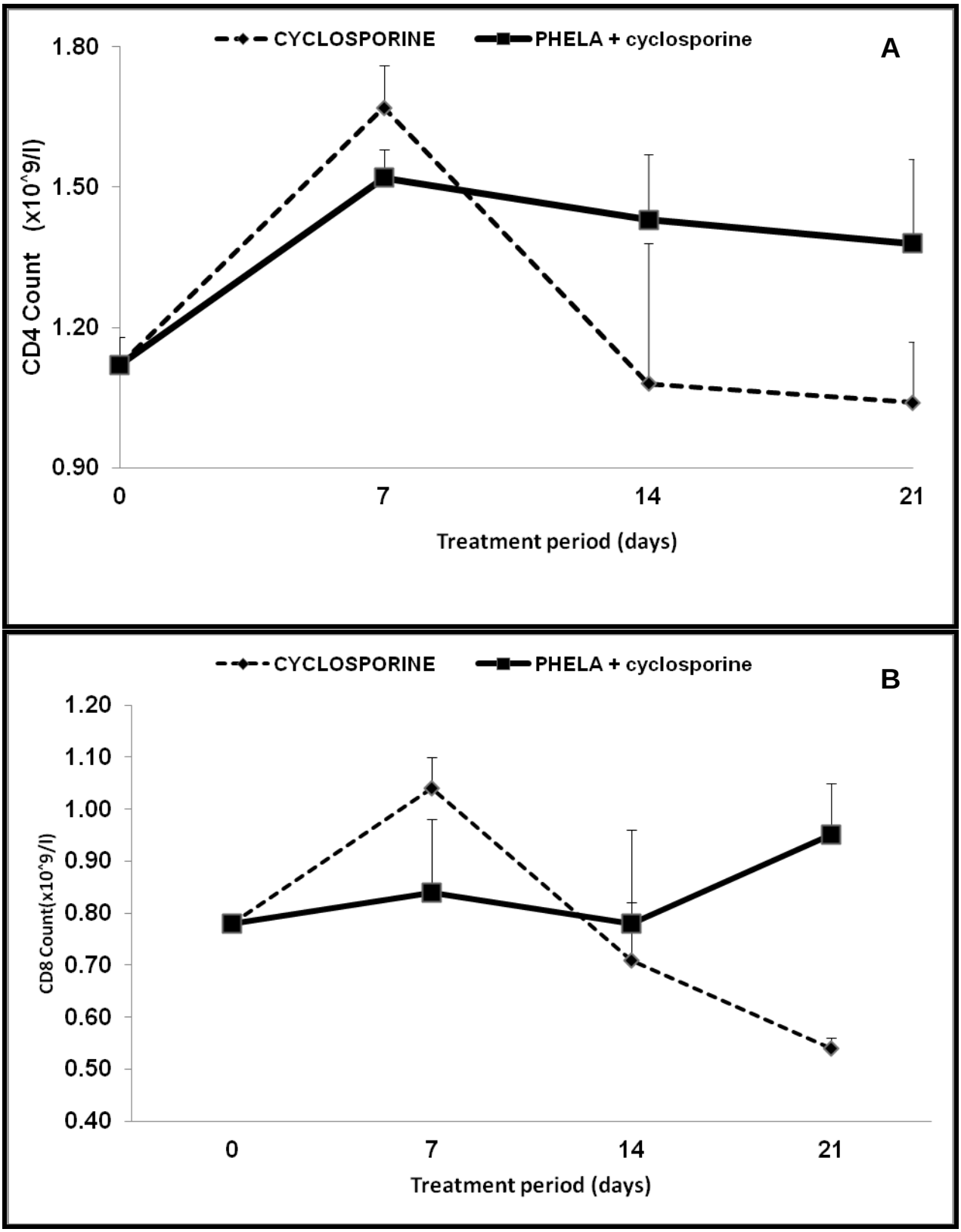

Figure 9.3: Effect of *Phela* on **CD_4 (A) and CD_4 (B)** cell count after 7, 14 and 21 days treatment in CsA suppressed rats.

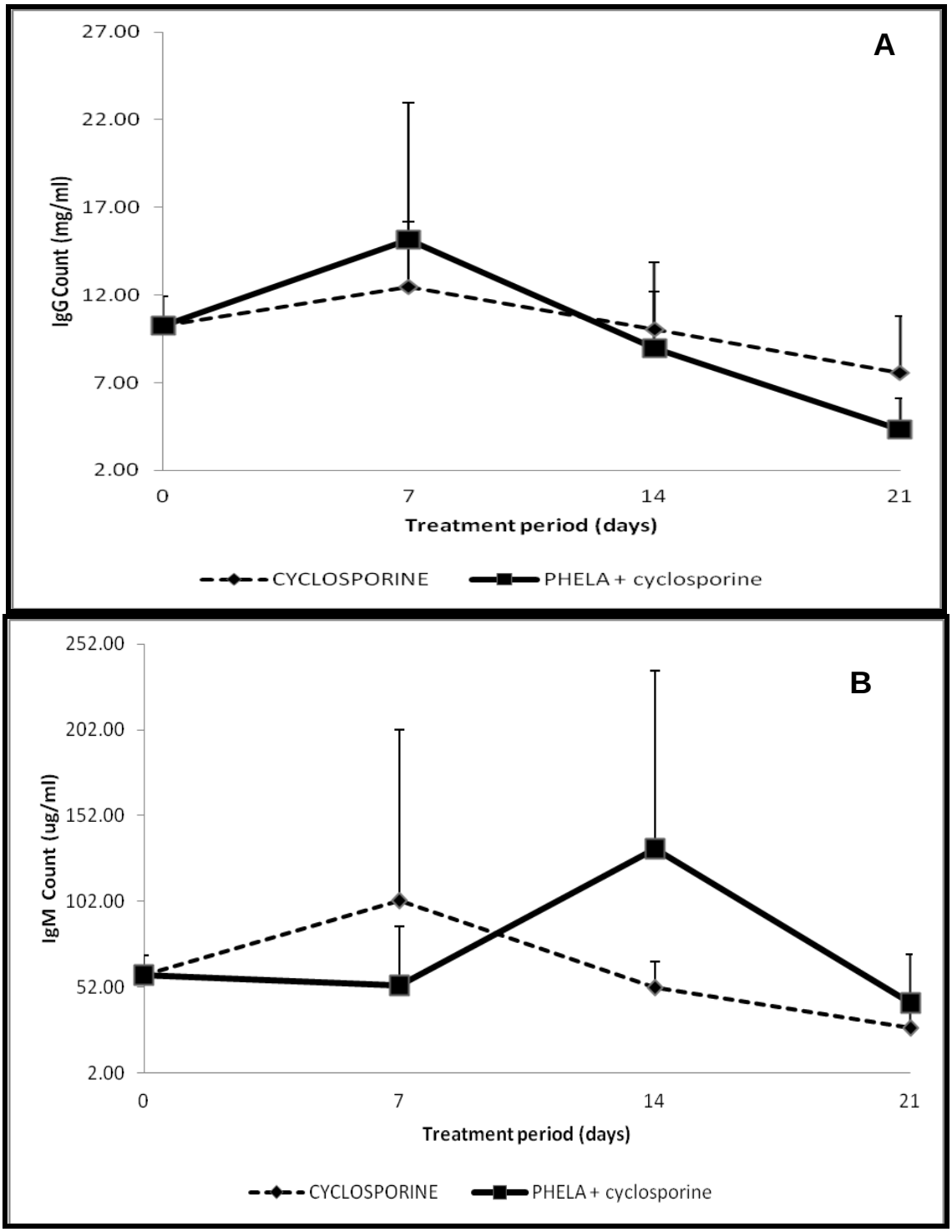

Figure 9.4: Effect of *Phela* on **IgG (A) and IgM (B)** count after 7, 14 and 21 days treatment in CsA suppressed rats.

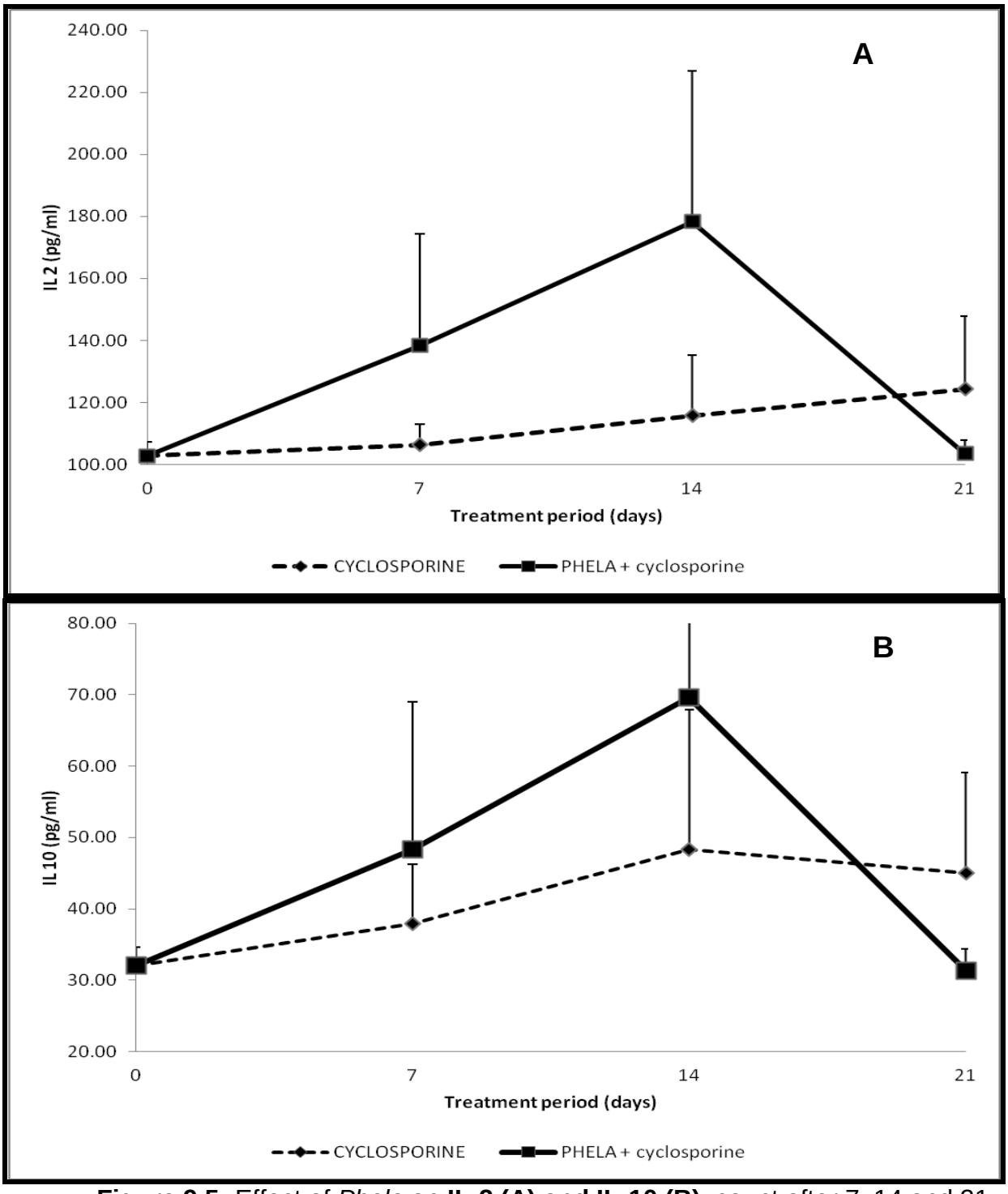

Figure 9.5: Effect of *Phela* on **IL-2 (A) and IL-10 (B)** count after 7, 14 and 21 days treatment in CsA suppressed rats.

9.6.2. EFFECT OF *PHELA* ON A RAT MODEL OF CYCLOPHOSPHAMIDE INDUCED IMMUNE SUPPRESSION

9.6.2.1. Physiological Parameters

9.6.2.1.1. Liver functions tests

Table 9.7 is a summary of liver function test after treatment with *Phela* on CP induced suppression. *Phela* had no effect on liver function tests over 21 days of treatment.

9.6.2.1.2. Renal function tests

Table 9.8 is a summary of renal function test after treatment with *Phela* on CP induced suppression. Creatinine levels significantly decreased after 7 days of treatment with *Phela*. *Phela* had no effect on BUN levels over 21 days of treatment.

9.6.2.1.3. Haematological parameters

Table 9.9 is a summary of full blood count after treatment with *Phela* on CP induced suppression. *Phela* decreased platelets throughout the study, while white cell count increased after 21 days in the *Phela* treated group.

Table 9.7: Summary of **liver function test** recorded as mean ± SD after 7, 14 and 21 days of treatment with *Phela* in CP suppressed rats.

	CYCLOPHOSPHAMIDE (ONLY)			**CYCLOPHOSPHAMIDE (+*PHELA*)**		
DAYS	**ALT** (U/L)	**ALP** (U/L)	**AST** (U/L)	**ALP** (U/L)	**ALT** (U/L)	**AST** (U/L)
0	48.0±1.7	224.3±7.2	78.0±7.0	48.0±1.7	224.3±7.2	78.0±7.0
7	39.7±4.9	130.3±32.3	76.3±7.6	53.3±2.9	157.3±24.8	96.0±9.5
14	38.5±20.5	146.0±126	113.5±69	56.3±7.1	159.7±42	129.3±32
21	37.8±2.5	145.3±12	99.8±18	44.5±4.2	165.3±38.1	97.3±8.4

Abbreviations + Reference values: **ALP:** alkaline phosphatase (289 – 436 U/L), **ALT:** Alanine transaminase (46 – 55 U/L), **AST:** Aspartate transaminase (81 – 104 U/L).

Table 9.8: Summary of **renal function tests** recorded as mean ± SD after 7, 14 and 21 days of treatment with *Phela* in CP suppressed rats.

	CYCLOPHOSPHAMIDE (ONLY)		**CYCLOPHOSPHAMIDE (+*PHELA*)**	
DAYS	**Creatinine** (µmol/L)	**BUN** (mmol/L)	**Creatinine** (µmol/L)	**BUN** (mmol/L)
0	30.00±4.58	5.53±0.40	30.00±4.58	5.53±0.40
7	69.67±5.86	6.67±0.90	9.00±4.00	5.20±0.50
14	26.00±14.14	6.60±1.13	59.00±1.41	5.55±1.06
21	32.00±8.16	5.88±0.91	198±67	4.53±1.90

Abbreviations + Reference values: Blood urea nitrogen (6.5 – 8.2 mmol/L), Creatinine (31 – 46 µmol/L)

Table 9.9: Summary of **Full blood count test** levels recorded as mean ± SD after 7, 14 and 21 days of treatment with Phela in CP suppressed rats.

	DAYS			
PARAMETERS	**0**	**7**	**14**	**21**
	CYCLOPHOSPHAMIDE (ONLY)			
RCC (10^12/l)	5.98±0.20	5.96±0.31	5.98±0.13	5.06±0.63
Haemoglobin (g/dl)	12.40±0.36	11.70±0.99	11.45±0.49	10.10±1.45
Haematocrit (l/l)	0.37±0.01	0.35±0.03	0.35±0.02	0.31±0.04
MCV (fl)	61.37±1.46	57.95±1.63	57.80±1.70	61.53±2.74
MCH (pg)	20.77±0.32	19.65±0.64	19.15±0.35	19.97±0.81
MCHC (g/dl)	33.80±0.44	33.85±0.21	33.15±0.21	32.43±0.92
Platelets (10^9/l)	965±117	743±159	954±44	476±104
WCC (10^9/l)	2.68±0.47	2.22±0.51	3.88±0.98	3.13±1.71
Neutrophils (10^9/l)	0.62±0.22	0.94±0.13	2.26±0.11	1.47±1.05
Lymphocytes (10^9/l)	1.82±0.31	0.89±0.23	1.25±0.83	0.99±0.75
Monocytes (10^9/l)	0.22±0.03	0.39±0.13	0.26±0.08	0.64±0.20
Eosinophils (10^9/l)	0.01±0.00	0.01±0.01	0.10±0.02	0.02±0.03
Basophils (10^9/l)	0.01±0.00	0.01±0.00	0.02±0.01	0.01±0.00
	CYCLOPHOSPHAMIDE (+*PHELA*)			
RCC (10^12/l)	5.98±0.20	5.99±0.32	5.51±0.22	4.76±0.22
Haemoglobin (g/dl)	12.40±0.36	12.43±0.64	11.27±0.38	9.75±0.21
Haematocrit (l/l)	0.37±0.01	0.37±0.01	0.34±0.02	0.29±0.01
MCV (fl)	61.37±1.46	62.00±0.35	60.90±0.50	60.75±4.45
MCH (pg)	20.77±0.32	20.77±0.06	20.47±0.21	20.55±1.34
MCHC (g/dl)	33.80±0.44	33.47±0.23	33.57±0.42	33.80±0.14
Platelets (10^9/l)	965±117	463±119	536±181	344±59
WCC (10^9/l)	2.68±0.47	1.70±0.28	1.78±0.30	3.93±2.02
Neutrophils (10^9/l)	0.62±0.22	0.70±0.12	0.79±0.32	2.43±1.58
Lymphocytes (10^9/l)	1.82±0.31	0.73±0.30	0.75±0.19	0.97±0.06
Monocytes (10^9/l)	0.22±0.03	0.09±0.04	0.20±0.09	0.50V0.40
Eosinophils (10^9/l)	0.01±0.00	0.05±0.05	0.03±0.03	0.03±0.02
Basophils (10^9/l)	0.01±0.00	0.01±0.00	0.01±0.01	0.01±0.01

Abbreviations + Reference values: **RCC**: Red cell count (5.99 - 6.42), Haemoglobin (12.5 – 13.1), haematocrit (0.393 – 0.406) **MCV**: Mean corpuscular volume (61.2 – 66.1), **MCH**: Mean corpuscular hemoglobin (20.2 – 20.9), **MCHC**: Mean corpuscular hemoglobin concentration (31.1 – 32.3), Platelets (611 – 1034) **WCC**: white cell count (4.16 – 9.55), Neutrophils (0.65 - 0.89), Lymphocytes (3.37 – 5.99), Monocytes (0.11 – 0.27), Eosinophils (0.00 – 0.02), Basophils (0.01 – 9.55)

9.6.2.2. Cyclophosphamide concentration in plasma

Table 9.10 is a summary of CP concentration in plasma after the rats were treated with *Phela* for three weeks. There was no difference in the test group's (CP+*Phela*) concentration when compared with the control group (CP-only).

Table 9.10: Summary of **Cyclophosphamide concentration** (µg/ml) in plasma recorded as mean ± SD after 7, 14 and 21 days of treatment with Phela in CP-suppressed rats.

DAYS	Cyclophosphamide (Only)	Cyclophosphamide (+*PHELA*)
0	10.00±0.71	10.00±0.71
7	8.95±1.11	8.41±0.70
14	8.25±0.88	7.79±1.13
21	9.38±1.03	11.13±1.44

Abbreviations: CP= cyclophosphamide

9.6.2.3. General immune markers response

Table 9.11 indicates rat body weight before and after treatment, also weight change percentage after *Phela* treatment. *Phela* overcame the stunted growth on day 14 (CP, +10±8 %; *Phela*, +21±5 %) and 21 days (CP, +6±3 %; *Phela*, +15±8 %).

Table 9.11: Summary of **body weight (g)** recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in CP suppressed rats.

DAYS	CYCLOPHOSPHAMIDE (only)			CYCLOPHOSPHAMIDE (+ *PHELA*)		
	BEFORE (g)	***AFTER (g)***	***Change (%)***	***BEFORE (g)***	***AFTER (g)***	***Change (%)***
0	218±4	234±6	+7±1	218±4	234±6	+7±1
7	212±4	239±11	+13±5	209±8	235±12	+12±7
14	230±5	254±18	+10±8	216±5	262±11	+21±5
21	225±11	238±13	+6±3	211±7	237±18	+15±8

Table 9.12 indicates organ weight after *Phela* treatment and summarized as mean and standard deviation. *Phela* had no effect on the liver and kidneys' weight. *Phela* prevented spleen swelling on day 7 (P = 0.02) and day 14 (CP, 0.71±0.17; CP+*Phela*, 0.59±0.10) caused by CP chronic treatment. *Phela* antagonized thymus weight loss after 7 (P = 0.05) and 14 (CP, 0.17±0.08; CP+*Phela*, 0.24±0.05) days treatment.

Table 9.12: Summary of **organs' weight (g)** recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in CP suppressed rats.

DAYS	CYCLOPHOSPHAMIDE (only)	CYCLOPHOSPHAMIDE (+ *PHELA*)
KIDNEYS		
Day 0	1.81±0.13	1.81±0.13
Day 7	1.97±0.13	1.88±0.11
Day 14	2.00±0.20	2.00±0.10
Day 21	1.85±0.13	1.92±0.19
LIVER		
Day 0	11.72±0.38	11.72±0.38
Day 7	11.32±0.85	9.78±1.06
Day 14	11.01±1.66	11.29±0.59
Day 21	10.63±0.43	10.36±0.62
SPLEEN		
Day 0	0.49±0.06	0.49±0.06
Day 7	0.62±0.09	0.45±0.05 (P = 0.0177)*
Day 14	0.71±0.17	0.59±0.10
Day 21	0.89±0.06	0.81±0.40
THYMUS		
Day 0	0.26±0.05	0.26±0.05
Day 7	0.19±0.02	0.28±0.06 (P = 0.0458)*
Day 14	0.17±0.08	0.24±0.05
Day 21	0.16±0.02	0.18±0.04

**P < 0.05 vs control*

9.6.2.4. Immune cells response

Figure 9.6 shows plots of the effect of *Phela* on white cell count (A), neutrophils (B) and lymphocytes (C) count versus time in a CP-induced rat model. *Phela* did not reverse the immune suppression on WCC, neutrophils and lymphocytes count.

Figure 9.7 shows plots of effect of *Phela* on CD_4 (A) and CD_8 (B) count versus time in a cyclophosphamide-induced rat model. *Phela* reversed immune suppression on CD_4 count from 14 days (CP, 0.18±0.12; CP+*Phela*, 0.24±0.11) and significantly after 21 days ($P = 0.04$). *Phela* overcame CP induced immune suppression on CD_8 count from 14 days ($P = 0.08$) and after 21 days (CP, 0.12±0.10; CP+*Phela*, 0.23±0.16).

9.6.2.5. Immunoglobulins response

Figure 9.8 shows plots of the effect of *Phela* on IgG (A) and IgM (B) count versus time in a CP-induced rat model. IgG levels were stimulated in *Phela* treated group after 7 ($P = 0.01$) and 14 days ($P = 0.008$) and returned to baseline after 21 days. IgM levels were stimulated in *Phela* treated group after 7 (CP, 37.40±16.83; *Phela*, 72.80±56.27) and 14 (CP, 29.32±9.87; *Phela*, 85.94±63.35) days and decreased after 21 days.

9.6.2.6. Cytokines response

Figure 9.9 shows plots of the effect of *Phela* on IL-2 (A) and IL-10 (B) count versus time in a CP-induced rat model. *Phela* antagonized CP suppression by slightly increasing IL-2 count after 14 (CP, 85.10±32.26; CP+*Phela*, 106.81±6.13,) and 21 days (CP, 102.82±5.4; CP+*Phela*, 118.77±36.47) though not significant. *Phela* overcame IL-10 inhibition after seven days ($P = 0.05$).

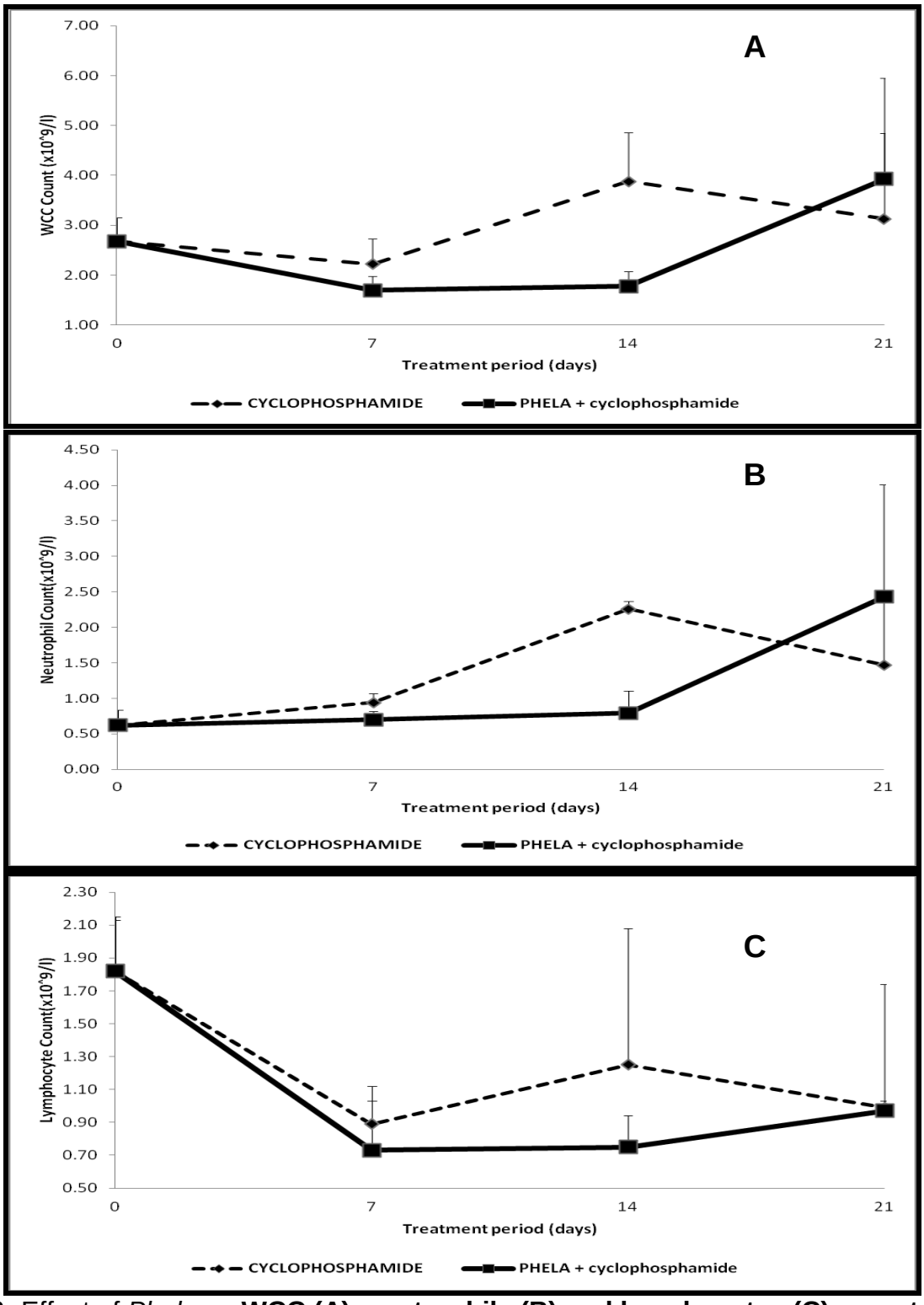

Figure 9.6: Effect of *Phela* on **WCC (A), neutrophils (B) and lymphocytes (C) count** after 7, 14 and 21 days treatment in CP suppressed rats.

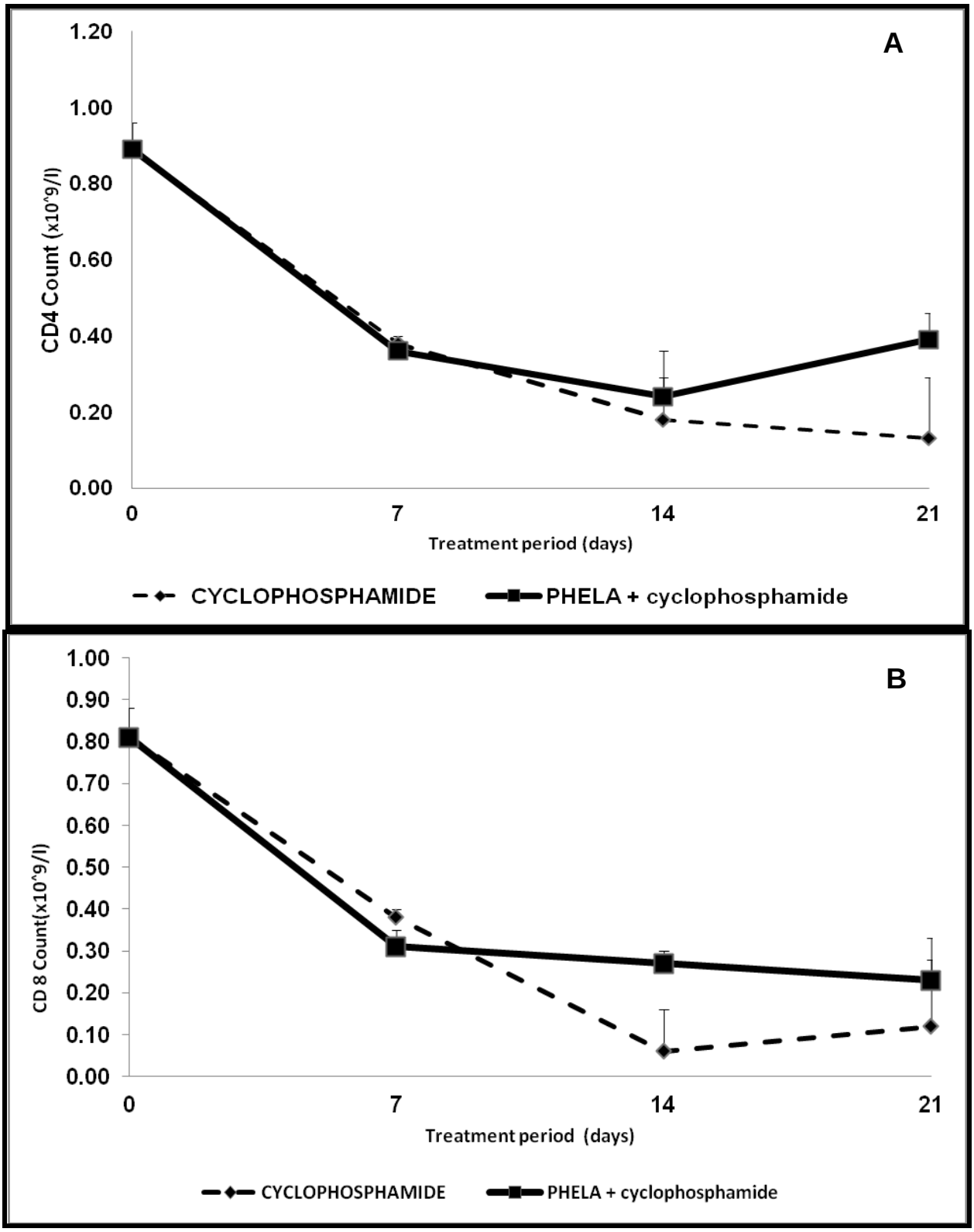

Figure 9.7: Effect of *Phela* on **CD_4 (A) and CD_8 (B) cell count** after 7, 14 and 21 days treatment in CP suppressed rats.

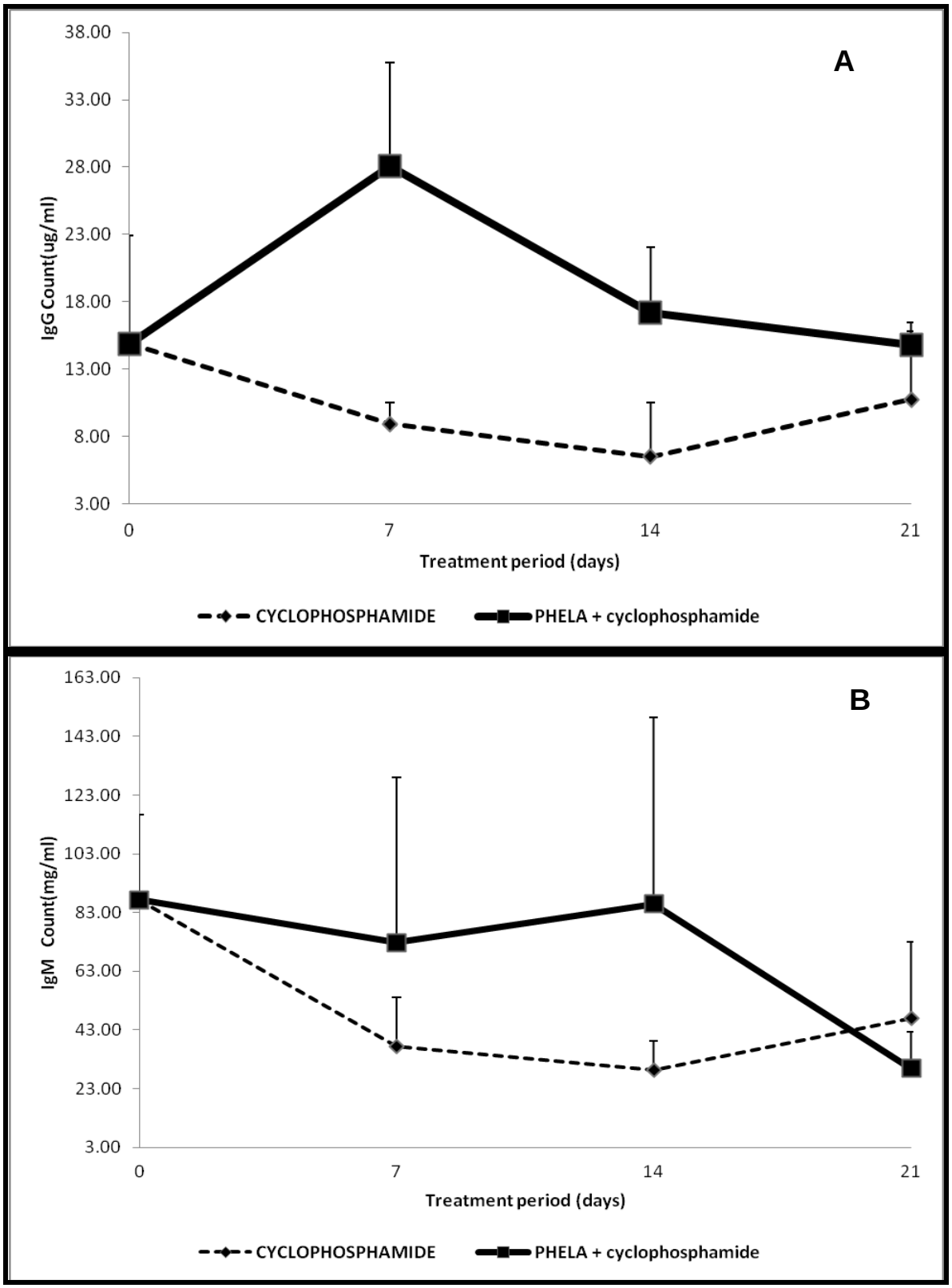

Figure 9.8: Effect of *Phela* on **IgG (A) ang IgM (B) count** after 7, 14 and 21 days treatment in CP suppressed rats.

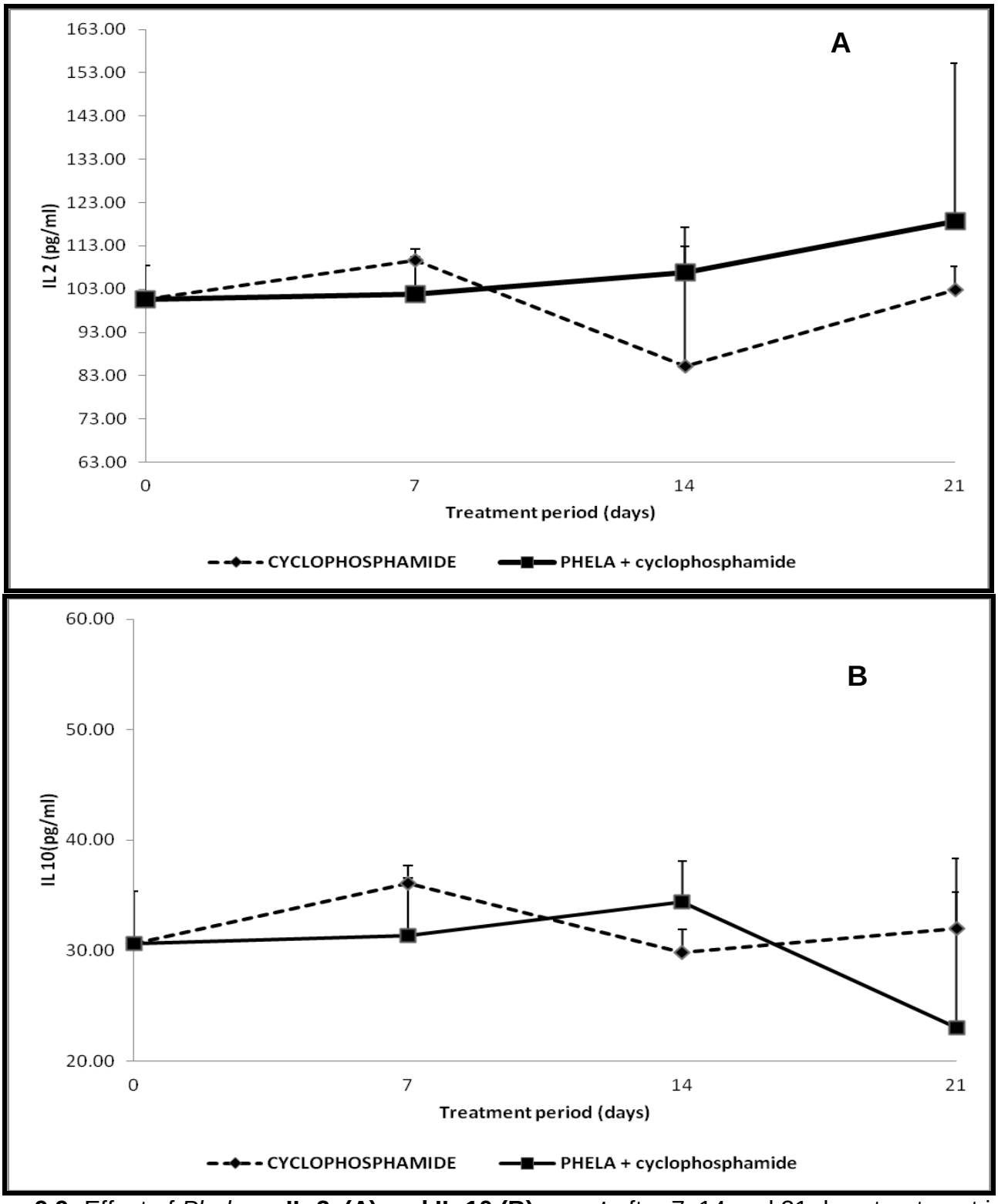

Figure 9.9: Effect of *Phela* on **IL-2 (A) and IL-10 (B) count** after 7, 14 and 21 days treatment in CP suppressed rats

9.6.3. EFFECT OF *PHELA* ON A RAT MODEL OF DEXAMETHASONE INDUCED IMMUNE SUPPRESSION

9.6.3.1. Physiological Parameters

9.6.3.1.1. Liver functions tests

Table 9.13 a summary of liver function test after treatment with *Phela* in Dex treated rats*. Phela* had no effect on liver function tests over 21 days of treatment. Though a wide variation was observed, the response had not reached toxic levels.

9.6.3.1.2. Renal function tests

Table 9.14 is a summary of renal function test after treatment with *Phela* on Dex induced suppression. *Phela* had no effect on renal function tests over 21 days of treatment.

9.6.3.1.3. Haematological parameters

Table 9.15 is a summary of full blood count tests after treatment with *Phela* in Dex suppressed rats. *Phela* prevented red blood count elevation on day and reversed WCC suppression on day 7 (P = 0.01) and 14 (Dex, 5.84±1.47; Dex+*Phela*, 10.54±1.31).

Table 9.13*:* Summary of **liver function test** recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in Dex suppressed rats.

	DEXAMETHASONE (ONLY)			**DEXAMETHASONE (+*PHELA*)**		
DAYS	**ALP**	**ALT**	**AST**	**ALP**	**ALT**	**AST**
	(U/L)	**(U/L)**	**(U/L)**	**(U/L)**	**(U/L)**	**(U/L)**
0	72.5±7.8	215.5±53.0	99.0±4.2	72.5±7.8	215.5±53.0	99.0±4.2
7	74.3±19.3	145.5±3.50	131.0±61.6	71.3±16.3	215.5±53.0	202.3±80.5
14	83.0±26.1	57.0±0.00	165.8±14.9	53.3±2.1	110.0±58.8	132.3±25.3
21	71.3±15.9	49.70±8.50	87.7±19.1	103.00±37	83.0±0.00	156.8±105.4

Abbreviations + Reference values: **ALP:** alkaline phosphatase (289 – 436 U/L), **ALT:** Alanine transaminase (46 – 55 U/L), **AST:** Aspartate transaminase (81 – 104 U/L).

Table 9.14*:* Summary of **renal function tests** recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in Dex suppressed rats.

	DEXAMETHASONE (ONLY)		**DEXAMETHASONE (+*PHELA*)**	
DAYS	**Creatinine**	**BUN**	**Creatinine**	**BUN**
	(µmol/L)	**(mmol/L)**	**(µmol/L)**	**(mmol/L)**
0	58.50±0.71	8.25±0.78	58.50±0.71	8.25±0.78
7	57.67±4.51	9.07±1.75	49.25±10.81	8.25±0.78
14	57.25±9.00	8.18±0.39	55.00±10.36	6.40±1.21
21	54.33±12.34	6.40±0.66	49.50±11.82	5.43±0.88

Abbreviations +Reference values: Blood urea nitrogen (6.5 – 8.2 mmol/L), Creatinine (31 – 46 µmol/L)

Table 9.15: Summary of **Full blood count test** levels recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in Dex suppressed rats.

	DAYS			
PARAMETERS	**0**	**7**	**14**	**21**
	DEXAMETHASONE (ONLY)			
RCC (10^12/l)	6.68±0.69	7.48±0.92	7.02±1.17	6.87±0.93
Haemoglobin (g/dl)	13.63±1.66	20.87±8.95	13.90±1.57	13.83±1.34
Haematocrit (l/l)	0.36±0.04	0.44±0.60	0.38±0.05	0.37±0.03
MCV (fl)	57.80±1.35	57.20±1.47	54.70±2.91	54.00±3.44
MCH (pg)	20.37±0.46	19.87±0.95	19.93±1.21	20.20±1.15
MCHC (g/dl)	35.23±0.57	34.70±0.75	36.43±0.32	37.43±0.38
Platelets (10^9/l)	781±228	260±164	341±104	879±326
WCC (10^9/l)	2.78±1.59	5.84±1.47	3.28±0.69	6.09±3.74
Neutrophils (10^9/l)	1.87±1.11	4.51±1.14	2.70±0.62	4.89±3.00
Lymphocytes (10^9/l)	072±0.40	0.90±0.23	0.29±0.04	0.40±0.25
Monocytes (10^9/l)	0.16±0.16	0.20±0.18	0.08±0.05	0.04±0.03
Eosinophils (10^9/l)	0.02±0.01	0.02±0.02	0.16±0.11	0.42±0.46
Basophils (10^9/l)	0.00±0.01	0.00±0.00	0.01±0.00	0.00±0.00
	DEXAMETHASONE (+PHELA)			
RCC (10^12/l)	6.68±0.69	7.48±0.59	6.62±1.22	7.28v0.96
Haemoglobin (g/dl)	13.63±1.66	15.30±0.75	13.37±1.91	14.20±1.59
Haematocrit (l/l)	0.36±0.04	0.44±0.02	0.36±0.05	0.38±0.05
MCV (fl)	57.80±1.35	58.83±2.34	53.97±2.54	52.27±0.72
MCH (pg)	20.37±0.46	20.50±0.62	20.33v1.38	19.53±0.45
MCHC (g/dl)	35.23±0.57	34.83±0.67	37.67±0.81	37.40±0.44
Platelets (10^9/l)	781±228	321±135	512±123	757±52
WCC (10^9/l)	2.78±1.59	10.54±1.31	3.29±0.98	7.88±1.79
Neutrophils	1.87±1.11	9.18±1.24	2.67±0.86	6.32±1.43
Lymphocytes (10^9/l)	072±0.40	1.09±0.10	0.28±0.18	0.52±0.12
Monocytes (10^9/l)	0.16±0.16	0.28±0.11	0.08±0.04	0.26±0.23
Eosinophils (10^9/l)	0.02±0.01	0.06±0.08	0.26±0.23	0.39±0.59
Basophils (10^9/l)	0.00±0.01	0.00±0.00	0.00±0.00	0.00±0.00

Abbreviations + Reference values: **RCC**: Red cell count (5.99 -6.42), Haemoglobin (12.5 – 13.1), haematocrit (0.393 – 0.406) **MCV**: Mean corpuscular volume (61.2 – 66.1), **MCH**: Mean corpuscular hemoglobin (20.2 – 20.9), **MCHC**: Mean corpuscular hemoglobin concentration (31.1 – 32.3), Platelets (611 – 1034) **WCC**: white cell count (4.16 – 9.55), Neutrophils (0.65 - 0.89), Lymphocytes (3.37 – 5.99), Monocytes (0.11 – 0.27), Eosinophils (0.00 – 0.02), Basophils (0.01 – 9.55)

9.6.3.2. Dexamethasone concentration in plasma

Table 9.16 is summary of Dex concentration in plasma after the rats were treated with *Phela* for three weeks. There was no difference in the test group's (Dex+*Phela*) concentration when compared with the control group (Dex-only).

Table 9.16: Summary of **dexamethasone concentration** (µg/ml) in plasma recorded as mean ± SD after treatment with *Phela* in Dex suppressed rats.

DAYS	Dexamethasone (Only)	Dexamethasone (+*PHELA*)
0	12.98±3.50	12.98±3.50
7	11.28±0.94	11.28±0.32
14	17.47±2.58	15.19±6.78
21	13.01±1.56	11.61±2.30

Abbreviations: Dex = dexamethasone

9.6.3.3. General immune markers response

Table 9.17 indicates rat body weight before and after treatment, also weight change percentage recorded as mean and standard deviation. Phela had no effect on rats' body weight levels. The rats lost weight at the same rate in both control and test groups.

Table 9.17: Summary of **body weight** recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in Dex suppressed rats.

DAYS	DEXAMETHASONE (only)			DEXAMETHASONE (+ *PHELA*)		
	BEFORE (g)	***AFTER (g)***	***Change (%)***	***BEFORE (g)***	***AFTER (g)***	***Change (%)***
0	218±3	196±7	-10±3	218±3	196±7	-10±3 %
7	214±4	177±3	-17±2	210±4	172±6	-18±2 %
14	219±9	175±6	-20±5	214±15	175±2	- 18±6 %
21	220±7	174±8	-21±2	212±9	163±8	-23±7%

Table 9.18 indicates organ weight after 7, 14 and 21 days treatment with *Phela.* All organs were not affected b*y Phela* treatment over 21 days with the exception of the kidneys. Here, *Phela* reversed the swelling after 21 days treatment (P = 0.0114).

Table 9.18*:* Summary of **organs' weight (g)** recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in Dex suppressed rats.

DAYS	DEXAMETHASONE (only)	DEXAMETHASONE (+ *PHELA*)
	KIDNEYS	
Day 0	1.88±0.20	1.88±0.20
Day 7	1.61±0.07	1.70±0.10
Day 14	1.79±0.04	1.80±0.11
Day 21	1.80±0.11	1.60±0.08 (P = 0.0114)*
	LIVERS	
Day 0	11.07±0.43	11.07±0.43
Day 7	9.53±0.17	9.52±0.56
Day 14	8.68±0.42	8.50±0.32
Day 21	9.00±0.74	8.42±0.94
	SPLEEN	
Day 0	0.34±0.06	0.34±0.06
Day 7	0.25±0.01	0.27±0.05
Day 14	0.26±0.04	0.25±0.01
Day 21	0.32±0.04	0.31±0.04
	THYMUS	
Day 0	0.20±0.07	0.20±0.07
Day 7	0.13±0.01	0.13±0.04
Day 14	0.22±0.06	0.23±0.06
Day 21	0.28±0.04	0.28±0.03

**P < 0.05 vs control*

9.6.3.4. Immune cells response

Figure 9.10 shows plots of WCC (A), neutrophils (B) and lymphocytes (C) count versus time in a Dex-induced rat model after *Phela* treatment. *Phela* reversed WCC suppression on day 7 (P = 0.01) and 14 (Dex, 5.84±1.47; Dex+*Phela*, 10.54±1.31). Similarly, *Phela* overcame neutrophils suppression after 7 days (Dex, 4.51±1.14; Dex+*Phela*, 9.18±1.24) of treatment but failed to reverse lymphocyte suppression.

Figure 9.11 shows plots of CD_4 (A) and CD_8 (B) cell count versus time in a Dex-induced rat model after *Phela* treatment. *Phela* overcame CD_4 count suppression on day 21 (P = 0.001). The CD_8 count remained high in the *Phela* treated group after 7 days (Dex, 0.03±0.00; Dex+*Phela*, 0.12±0.01) of treatment.

9.6.3.5. Immunoglobulins response

Figure 9.12 shows plots of IgG (A) and IgM (B) count versus time in a Dex-induced rat model after *Phela* treatment. IgG count response was higher in the *Phela* treated group after 7 days (P = 0.007) treatment. *Phela* increased IgM after 7 days (Dex, 36.18±46.40; Dex+*Phela*, 196.54±257.47) though not statistically significant.

9.6.3.6. Cytokines response

Figure 9.13 shows plots of IL-2 (A) and IL-10 (B) count versus time in a Dex-induced rat model after *Phela* treatment. *Phela* had no effect on IL-2 and IL-10 count in Dex treated rats.

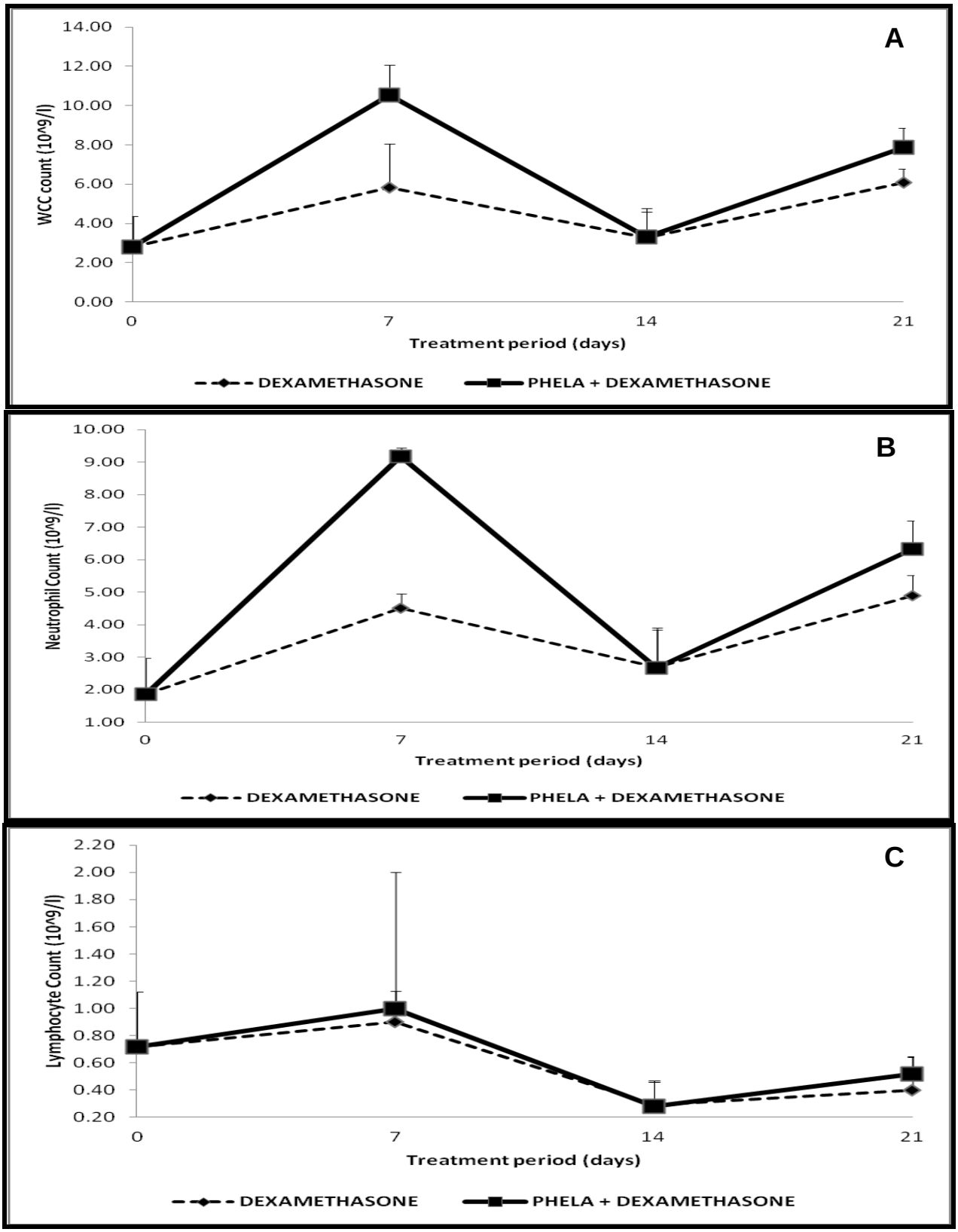

Figure 9.10: Effect of *Phela* on **WCC(A), neutrophils (B) and lymphocytes** count after 7, 14 and 21 days treatment in Dex suppressed rats.

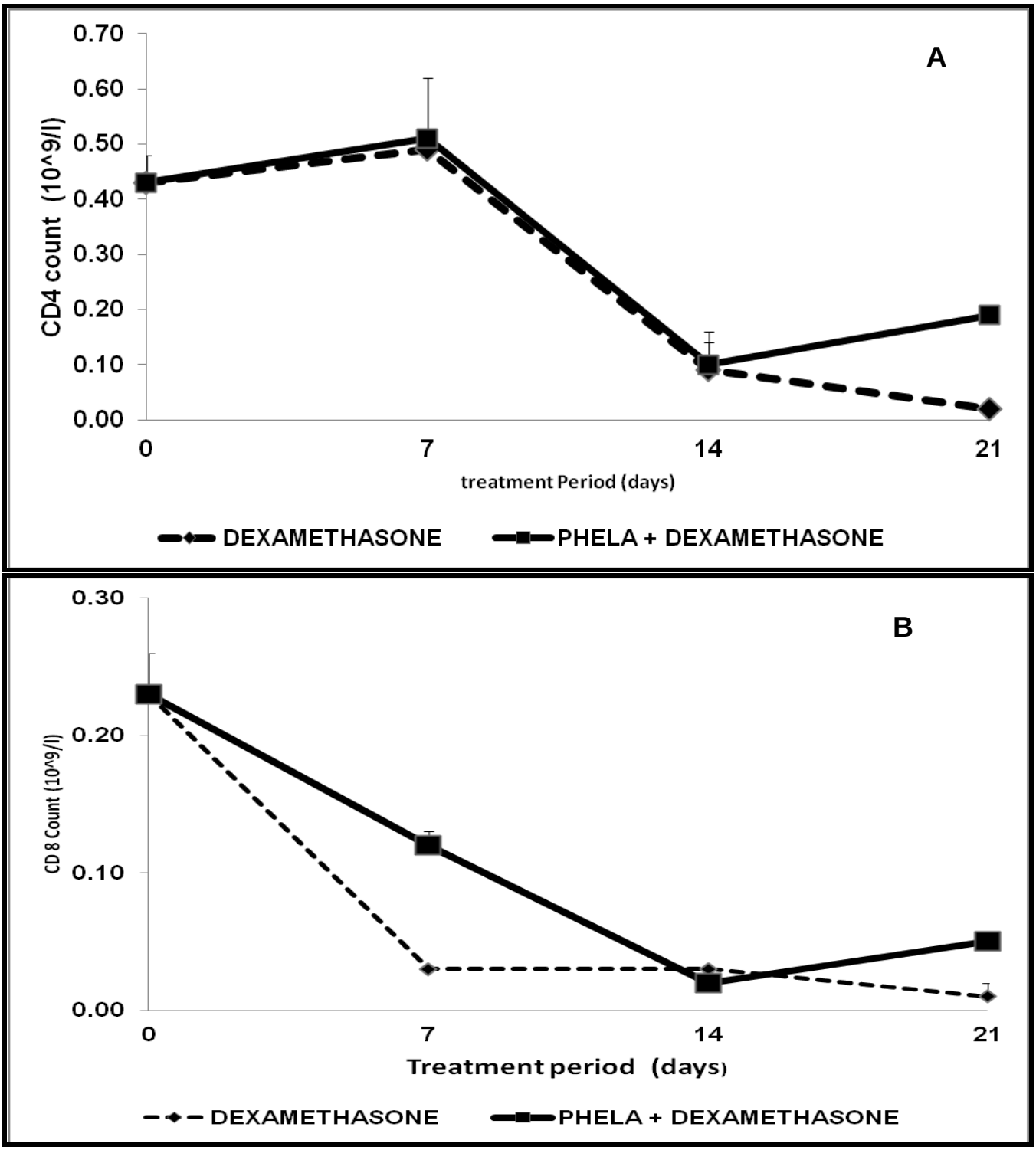

Figure 9.11: Effect of *Phela* on **CD_4 (A) and CD_8 (B)** cell count after 7, 14 and 21 days treatment in Dex suppressed rats

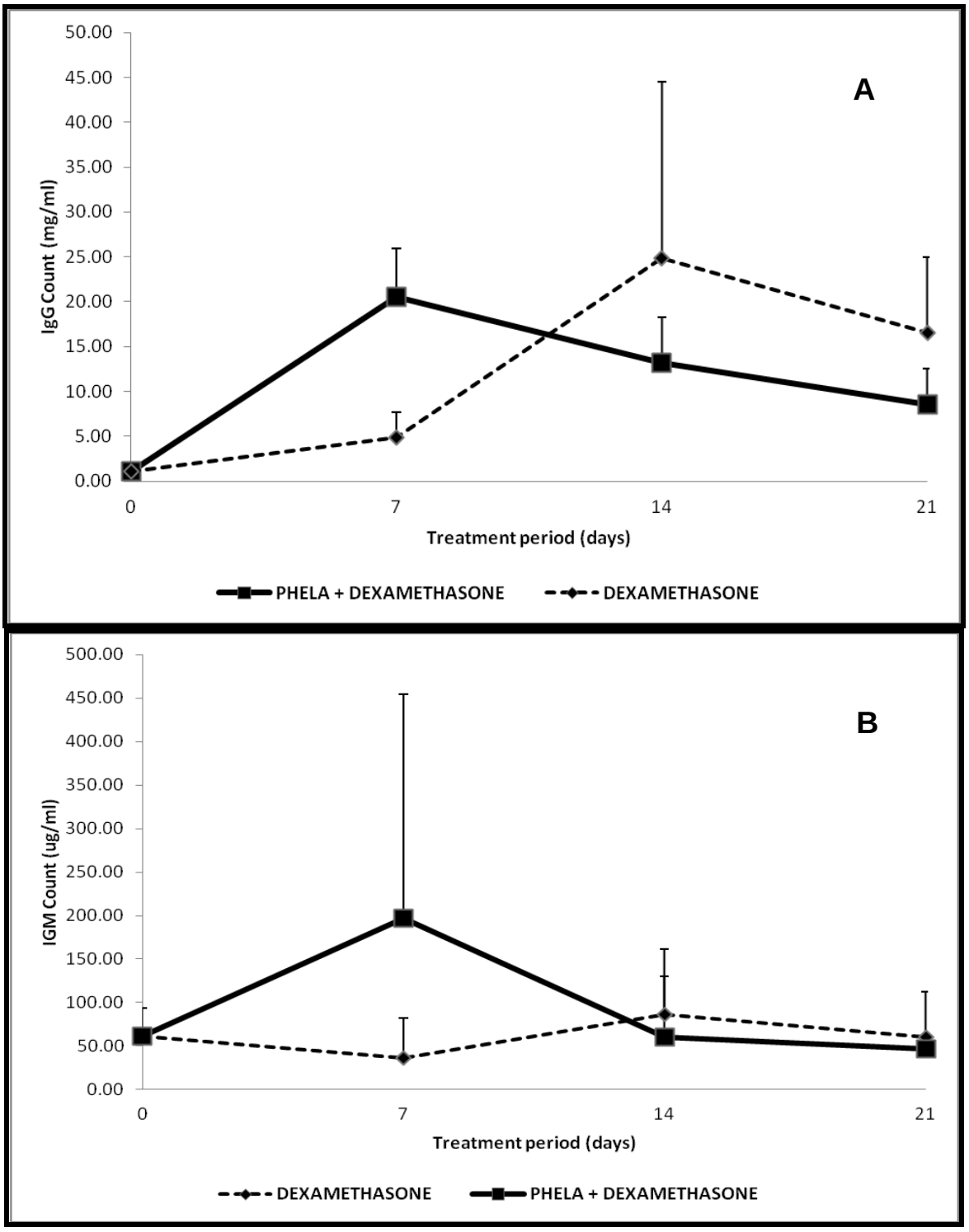

Figure 9.12: Effect of *Phela* on **IgG (A) and IgM (B)** count after 7, 14 and 21 days treatment in Dex suppressed rats

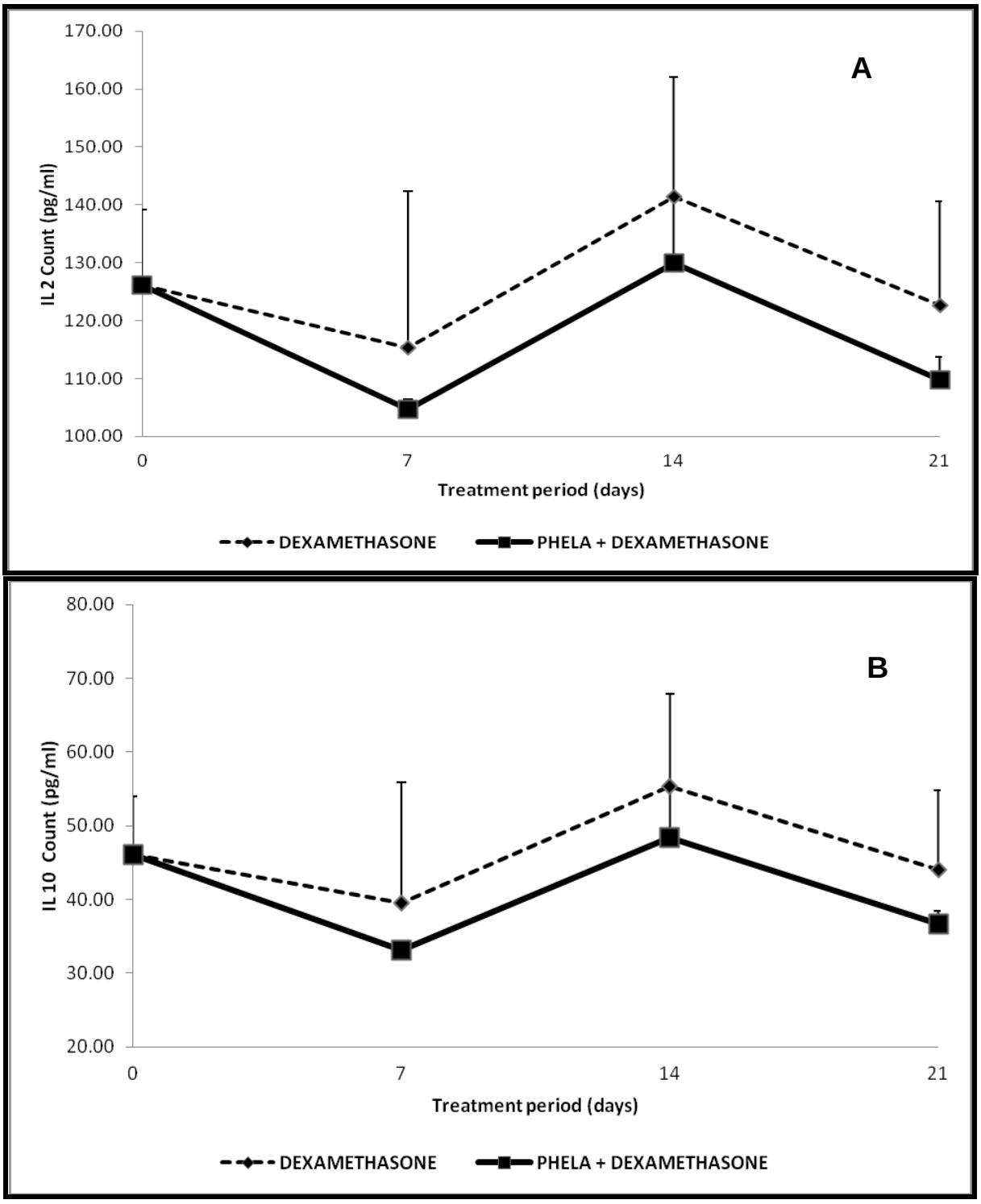

Figure 9.13: Effect of *Phela* on **IL-2 (A) and IL-10 (B)** count after 7, 14 and 21 days treatment in Dex suppressed rats

9.7. DISCUSSION

Table 9.19 is the summary of the observations during treatment with CsA, CP and Dex only and *Phela*, respectively. Although various changes in the parameters studied were observed at different times between the test and respective controls groups, the main findings were under the CsA disease model. Here, *Phela* intervention led to increased WCC mainly due to increased neutrophils and CD_4 count, and increased IL-2 along with IL-10 (see coloured cells in **Table 9.19**). On the other hand, *Phela* intervention in the CP model led to increased IL-2 and IgM, while other parameters, including those of the Dex model, were not consistent. This implies that *Phela* modulates mainly TH_1 response which associates with CMI.

Phela effect on cyclophosphamide induced immunosuppression

Phela prevented progression of CP-induced body and thymus weight loss, and suppression of IgG and IgM, with minimal effect on CD_4 and CD_8 cells. Mild myelosuppression and leukopenia observed in the control group correlated with continuous treatment with cyclophosphamide. This was expected owing to the fact that cyclophosphamide treatment reduces the number of circulating lymphocytes and impaired function of both humoral and cell mediated immune response (Smith *et al.*, 2003). Also cyclophosphamide induced immune suppression mechanism entails selectively damaging the B-lymphocytes of birds and rats and reduction in weight of spleen and thymus (Ahmad *et al*., 2012; Lee *et al.*, 2011).

The production of antigen-specific antibodies presents a major defense mechanism of humoral immune response and protects against infectious agents by neutralizing viruses, destroying agents, antibody-dependant cellular cytotoxicity (Xiao *et al*., 2008). However, *Phela* moderately overcame the CP-induced myelosuppression by reversing weight reduction in treated rats. Moreover, *Phela* restored TH_2 immunity by restoring IgG and IgM concentration in serum. It is well known that TH_2 cells mediate the activation and maintenance of the humoral or antibody-mediated immune response against extracellular parasites, bacteria, allergens and toxins.

Table 9.19. Summary of the observations during treatment with CsA, CP and Dex-only and *Phela* respectively over 21 days

Drugs	CsA-only	CsA+PHL	CP-only	CP+PHL	Dex-only	Dex+PHL
Body ΔW	———	———	Stunted growth from d7	Prevented loss by d14 - 21	Steep ↓ by d7-21	———
Spleen ΔW	———	———	Swollen from d7 - 12	Stopped the swelling from d	Steep ↓ by d7-21	———
Thymus ΔW	Reduced by d7 -21	Restored by d 21 (P =0.0001)	Reduced from d7 - 21	Steep ↓ by d7-21	Steep ↓ by d7-21	———
WCC	Suppressed by d7 -21	Relatively higher than CsA-only gp from d7 - 21	Suppressed by d7 -21	Relatively higher than CP-only gp by d 21	Suppressed by d7 -21	Moderate↑ by d7 thereafter fell to Dex-only gp
Neutrophils	Suppressed by d7 -21	Peak at d7, was higher than CsA-only gp from d14 -21	Suppressed by d7 -21	Relatively higher than CP-only gp by d 21	Suppressed by d7 -21	Moderate↑ by d7 (P= 0.01) thereafter fell to Dex-only gp
Lymphocytes	Suppressed by d7 -21	Relatively higher than CsA-only gp from d7 - 21	Suppressed by d7 -21	———	Suppressed by d7 -21	———
CD_4 count	Slightly increased on d7 but inhibited thereafter	Antagonized by d14-21 (P = 0.05)	Suppressed by d7 -21	Restored by d21 (P= 0.04)	Suppressed by d7 -21	Reversed suppression by d21 (P= 0.001)
CD_8 count	Slightly increased by d7 but inhibited thereafter	Antagonized by d14-21 (P = 0.002)	Suppressed by d7 -21	Restored by d14 -21(P = 0.05)	Suppressed by d7 -21	Moderate↑ by d7 thereafter fell to Dex-only gp
IgG	Inhibited through out	Slight ↑ by d7 but ↓ thereafter	Peak at d14 & ↓ thereafter	Peak at d7 & remained ↑ by d14 (P< 0.01) thereafter fell to CP-only gp	Peak at d14 & thereafter fell back to Dex-only gp	Peak at d7 & thereafter fell below Dex-only gp
IgM	Slightly increased by d7 but inhibited thereafter	Peak at d14 & thereafter fell back to CsA-only gp	Suppressed by d7 -21	Antagonized by d7-14 & thereafter fell below CP-only gp	Remained lower through out	Peak at d7 (P= 0.007) & thereafter fell below Dex-only gp
IL-2	Slightly ↑ by d7 but inhibited thereafter	Peak at d14 & thereafter fell below CsA-only gp	Suppressed by d7 -21	Moderate ↑ by d14-21	Remained lower through out	Failed to overcome Suppression
IL-10	Slightly increased by d7 but inhibited thereafter	Peak at d14 & thereafter fell below CsA-only gp	Suppressed by d7 -21	Slight ↑ by d7 & thereafter fell below CP-only gp	Remained lower through out	Failed to overcome Suppression

Abbreviations: _____ = no effect, ↑ = increase, ↓ = decreased, **CsA** = cyclosporine, **gp** = group **CP** = cyclophosphamide, **Dex** = dexamethasone, **IL-2** = interleukin 2, **IL-10** = interleukin 10, **WCC** = white cell count, **d** = day.

Furthermore, TH_2 cells produce cytokines such as IL-4, IL-5, IL-6 and IL-10 and these cytokines counteract TH_1 response. These results demonstrate that *Phela* stimulates the humoral immunity.

Phela effect on cyclosporine induced immunosuppression

Phela stopped the progression of immunesuppression in rats treated with cyclosporine as indicated by the reversal or resistance to CsA induced changes in the WCC, neutrophils, lymphocytes, CD_4, CD_8, and confirmed with elevated IL-2 concentration. Rats' body weight reduction was prevented in the test group by *Phela* intervention. Cyclosporine induced immune suppression observed in the control group was due to the inhibition of calcineurin phosphatase activity, causing a decreased synthesis of several cytokines such as IL-2, IL-3 and IL-4.

These results imply that *Phela* is an immune stimulant whereby, one such mechanism is via stimulation of IL-2 which was responsible for stimulation of the lymphocytes and neutrophils. IL-2 plays an important role to promote T-cells proliferation, cytokines production and functional properties of B cells, macrophages and natural killer cells. Whereas IL-10 inhibits the production of IL-2 and IFN-γ by TH_1, decreased pro-inflammatory cytokines and down regulated eosinophils function activity. *Phela* restored TH_1 immune response in a CsA induced rat model.

Phela effect on dexamethasone induced immunosuppression

Phela minimally reversed dexamethasone effect after 7 days treatment, on the WCC, neutrophils, CD_8 cell count, IgG and IgM concentration after which the immunesuppression overcame the effect of *Phela*. This observation could be related to dexamethasone's mechanism of action that occurs at gene transcription level, and achieved by altering regulation of gene expressions. Dexamethasone is a steroid indicated for the treatment of several pathologies sues to anti-inflammatory and immunosuppressor effects (Urban *et al.*, 2009). Myelosuppression in both test and control group correlates with changes in red blood cells, platelets and haemoglobin. Thrombocytopenia and leukopenia were due to loss of stem cells and inability of the bone marrow to generate new blood cells.

Immunomodulation

$TH_{1/2}$ immune response is self-regulatory and it's very crucial in determining the immunomodulation effect of a drug. In most cases disease pathology affects $TH_{1/2}$ balance, any extreme change effects makes the immune system incompetent, thus more susceptible to disease (Yamada *et al*., 2011; Ziauddin *et al*., 1996). Furthermore, the essence of immunomodulation is that a pharmacological agent acting under various doses and time regimens display an immunomodulation effect and immunostimulatory effects of a drug and/or nutritional supplements are difficult to evaluate in healthy people or animals (Huang *et al.*, 2007; Mukherjee *et al.*, 2010; Muruganandan *et al*., 2005; Tan *et al*., 2004). Hence, it was of utmost importance to evaluate *Phela* in a diseased model.

The results of the present study showed that *Phela* prevented spleen and thymus weight reduction in test groups in comparison with the respective controls. Spleen and thymus are the outlets of lymphocytes growth and proliferation and alterations of the immune organs weights correlate with the immune system loss of functionality and susceptibility to immune suppression and inability to resist infection. This indicates that *Phela* overcame the effect of CP and CsA induced immunesuppression on the development of these immune organs in rats (**Table 9.19**).

Modulation of $TH_{1/2}$ response is emerging as one of the biological targets for immune stimulation of traditional medicines to assess therapeutic efficacy (Borchers *et al*., 1997; Romagnani *et al.*, 1997; Gautam *et al.*, 2009; Sharififar *et al*., 2009). The results are compelling evidence demonstrating that *Phela* modulates the functions of various levels of the immune function. Observation from the results indicated that treatment with *Phela* promoted recovery of lymphocytes and its subsets CD_4 and CD_8 cells in immune suppressed rats in a time-dependent manner. Furthermore, *Phela* reversed drug-specific immune suppression by modulating the $TH_{1/2}$ immune response which indicative of the mechanism of immunomodulation. Similarly, *Phela* induced interleukin-2 mechanism in cyclosporine treated rats, activated humoral immunity in cyclophosphamide treated rats and the effect on the dexamethasone model was inconsistent.

Helper T-cells differentiate into either TH_1 activators that promote cell mediated immunity while TH_2 are humoral immunity and can be monitored through cytokines (e.g. IL-2, IFN-γ, IL-4 and IL-10) secretion. Based on the affinity towards the afore-mentioned subsets immunomodulators can be classified as either TH_1 or TH_2 or mixed. Observation form the results indicate that *Phela* has a strong TH_1 immuno modulatory properties, though there is evidence of a minimal effect on TH_2 and is believed that its due to the plural effect common for traditional medicines. Herbal preparations have synergistic interactions of many constituents, thus activity from the presence of multiple active principle that must be taken together to create the desired response (Pezzuto, 1997; Kumar *et al*., 2012).

It appears that, by day 21, the efficacy of *Phela* was overcome by the continued immunosuppression. This is akin to an ongoing/advancing disease process that can overwhelm the drug efficacy. The findings suggest that *Phela* can intervene in diseases where there is a shift in $TH_{1/2}$ balance. Up-regulated IL-2 and lymphocytes has been reported to have protective cellular and humoral immunity in patients with immune compromised conditions such as cancer, AIDS and TB (Lucy and Bird 2004). As such, *Phela* stimulates the cell mediate immunity (CMI) which would render it a candidate for testing against diseases or disorders associated with suppressed the CMI, such as HIV and TB.

9.8. CONCLUSION

Here, a rat model of drug-induced immunosuppression was successfully used to establish that the mechanism of immunomodulation of *Phela* is cell mediated.

10.

EVALUATION OF THESIS STUDY AND FUTURE WORKS

10.1 EVALUATION OF THE THESIS STUDY

This chapter evaluates the achievements of this research in relation with the objectives set at the beginning of this study. **Chapter 4 to 9** successfully answered the research questions that were outlined in the objectives in **Chapter 3.**

- All batches of *Phela* were fingerprinted using an HPLC-DAD and HPLC-FLD methods. Analysis of *Phela* by HPLC-DAD indicated two peaks with a retention time of 10.4±2.2 and 23.4±1.0 minutes. Whereas, HPLC-FLD analysis had four marker peaks with retention time of 13.0±0.8 for A, 23.0±2.4 for B, 31.2±0.7 for C, 39.2±0.6 for D (**Chapter 4**).
- An HPLC-UV method was validated and applied for the simultaneous detection of cyclophosphamide and dexamethasone and their respective quantification in rat plasma (**Chapter 5**).
- There was no interaction between *Phela* and cyclosporine, cyclophosphamide and dexamethasone *in vitro* after equilibrium dialysis analysis (**Chapter 6**).
- All the evaluated doses of *Phela* stimulated the immune system in healthy rats. *Phela* 15.4 mg/kg was the dose to be further tested on the rat model (**Chapter 7**).
- An immune suppression rat model of cyclosporine, cyclophosphamide and dexamethasone for testing of purported immune boosters' mechanism of action was successfully developed (**Chapter 8**).
- The mechanism of immunomodulation of *Phela* in rats is cell mediated. (**Chapter 9**).

The rat model was successfully applied to establish *Phela*'s mechanism of immunomodulation. *Phela'*s mechanism is mostly cell mediated. Therefore, *Phela* would be an ideal candidate for testing against diseases or disorders associated with suppressed the CMI, such as HIV/AIDS and TB.

10.2 FUTURE WORK

- In South Africa a lot of traditional medicines that are available on the various markets have no scientific evidence of their recommended use. As thus the rat model must be applied to evaluate and characterize the commonly used immune boosters that have no evidence of their purported use.
- Further studies are necessary to intervene with *Phela* at different stages of the disease progression and over a longer time period (i.e. 6 months) in order to establish the best time to start *Phela* treatment in immune suppressed patients. Moreover this will give an indication of the long-term effect of *Phela* on the immune system.
- To evaluate *Phela* in animal models of diseases (i.e. HIV/AIDS & TB) associated with loss of cell mediated immunity.
- To test for potential interaction of *Phela* with drugs such as anti-TB and/or ARV's which might be used in co-administration if *Phela* is approved for use in diseases with loss of cell immunity.
- To test the effect of *Phela* in humans during clinical trials. This will outline how *Phela* can be incorporated for clinical use for immune suppressed patients.

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APPENDICES

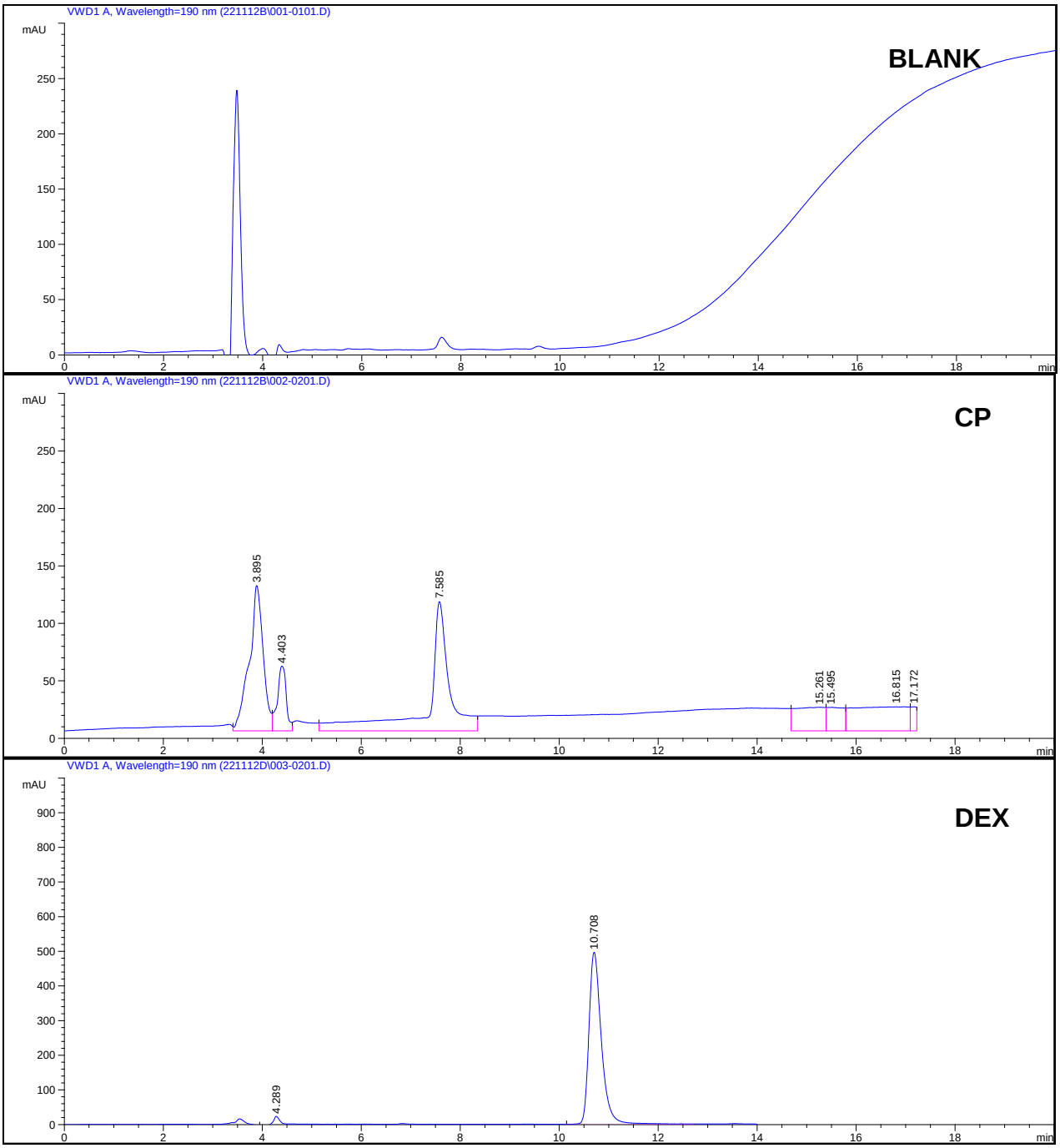

Figure A1: A blank chromatogram and chromatogram of two drugs CP (no peak), Dex (10.8 min) under adapted method conditions.

***Abrreviations**: CP = cyclophosphamide, Dex = dexamethasone*

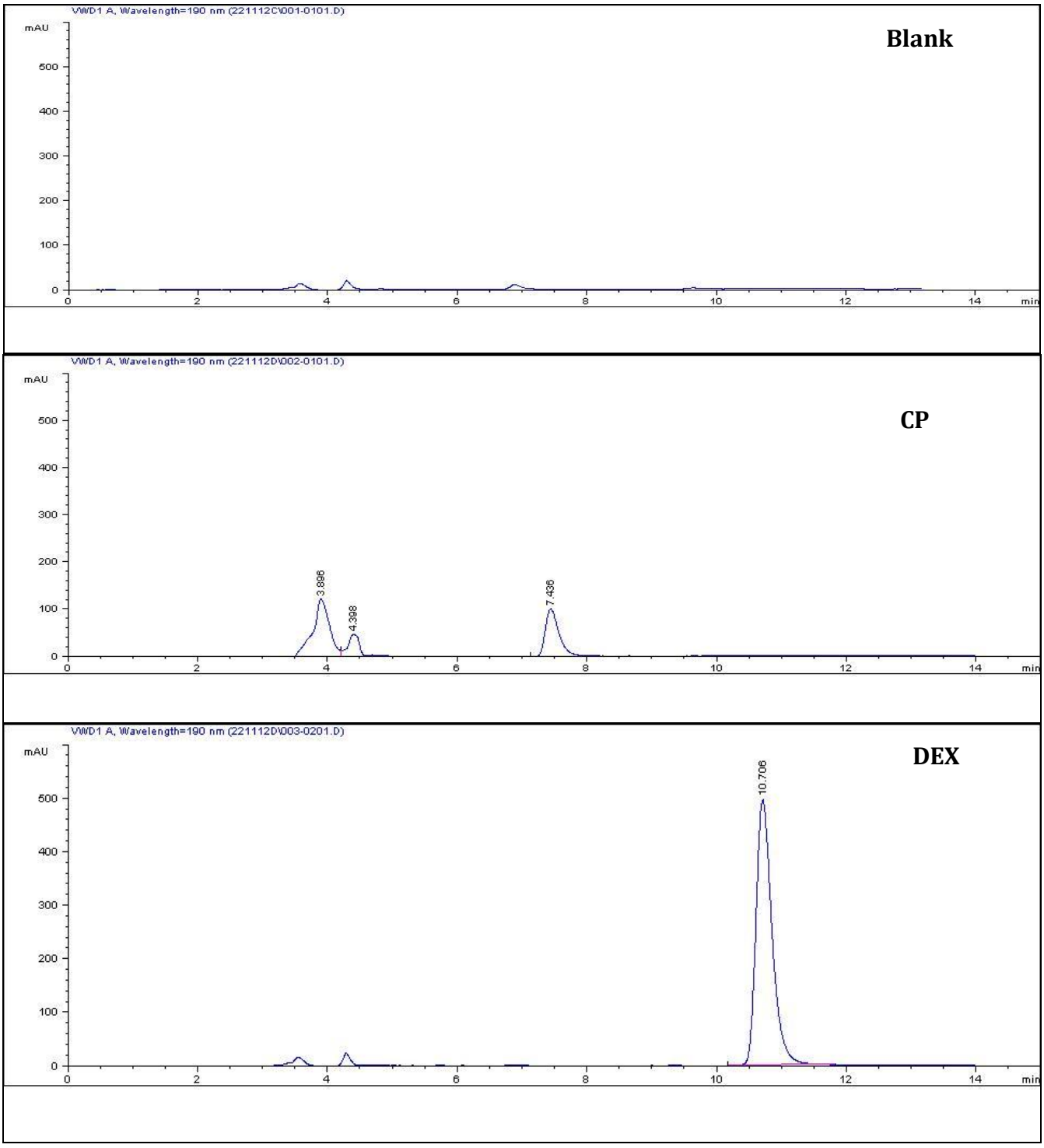

Figure A2: A blank chromatogram and chromatogram of two drugs CP (7.4 min), Dex (10.8 min) at a wavelength of 190 nm.

***Abrreviations**: CP = cyclophosphamide, Dex = dexamethasone*

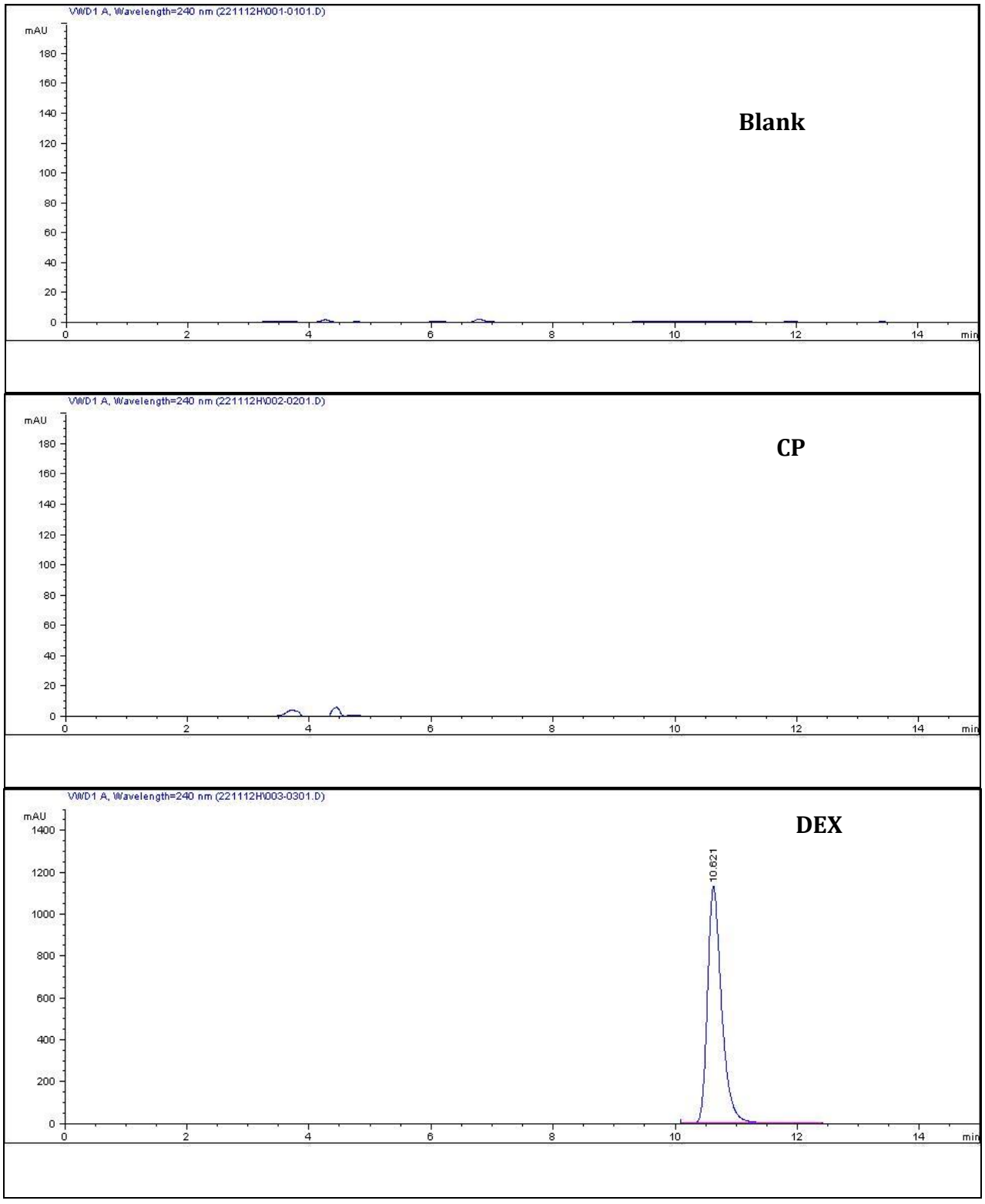

Figure A3: A blank chromatogram and chromatogram of two drugs CP (no peak), Dex (10.5 min) at a wavelength of 240 nm.

Abrreviations*: CP = cyclophosphamide, Dex = dexamethasone*

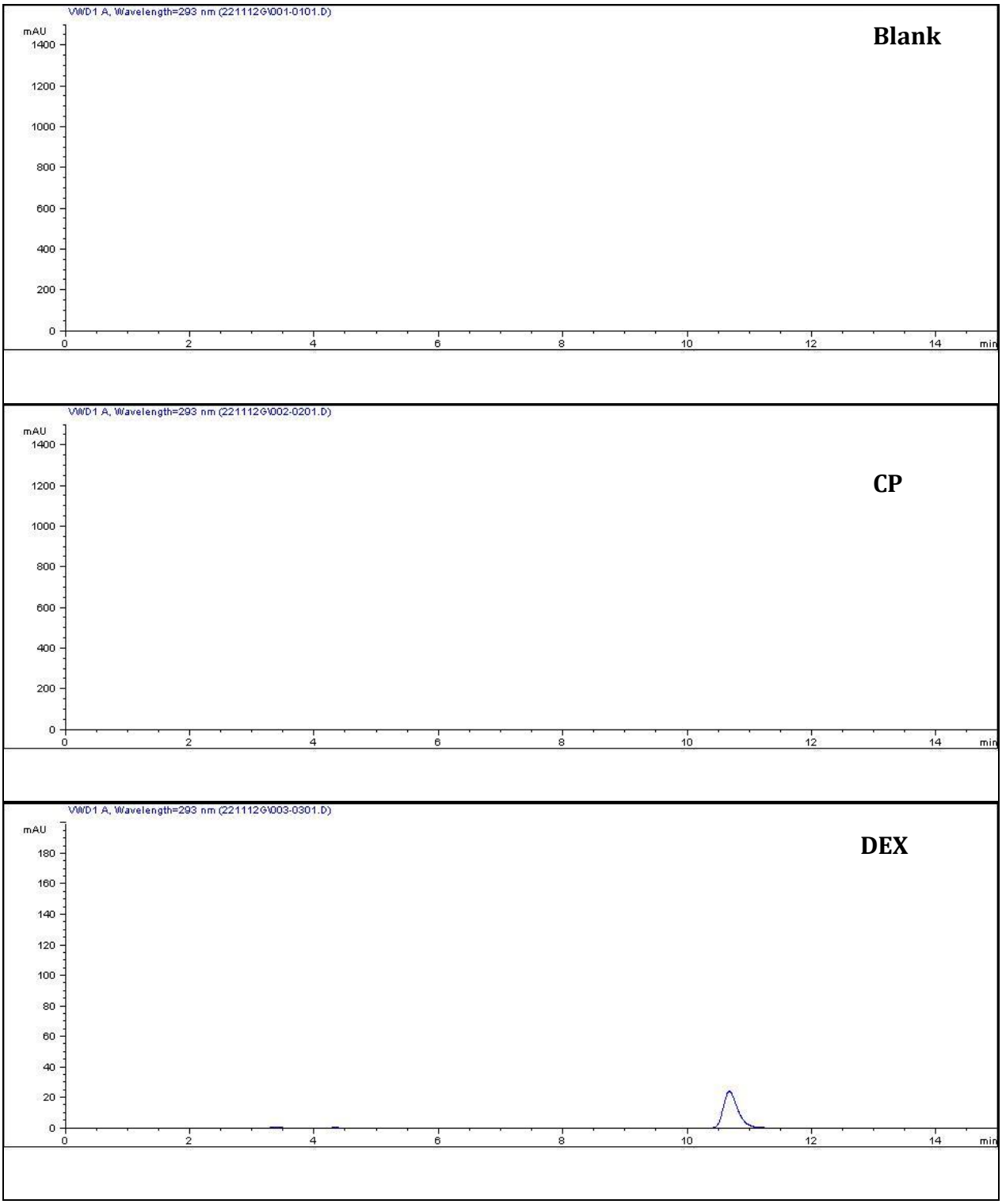

Figure A4: A blank chromatogram and chromatogram of two drugs CP (no peak), Dex (10.8 min) at a wavelength of 293 nm.

Abrreviations*: CP = cyclophosphamide, Dex = dexamethasone*

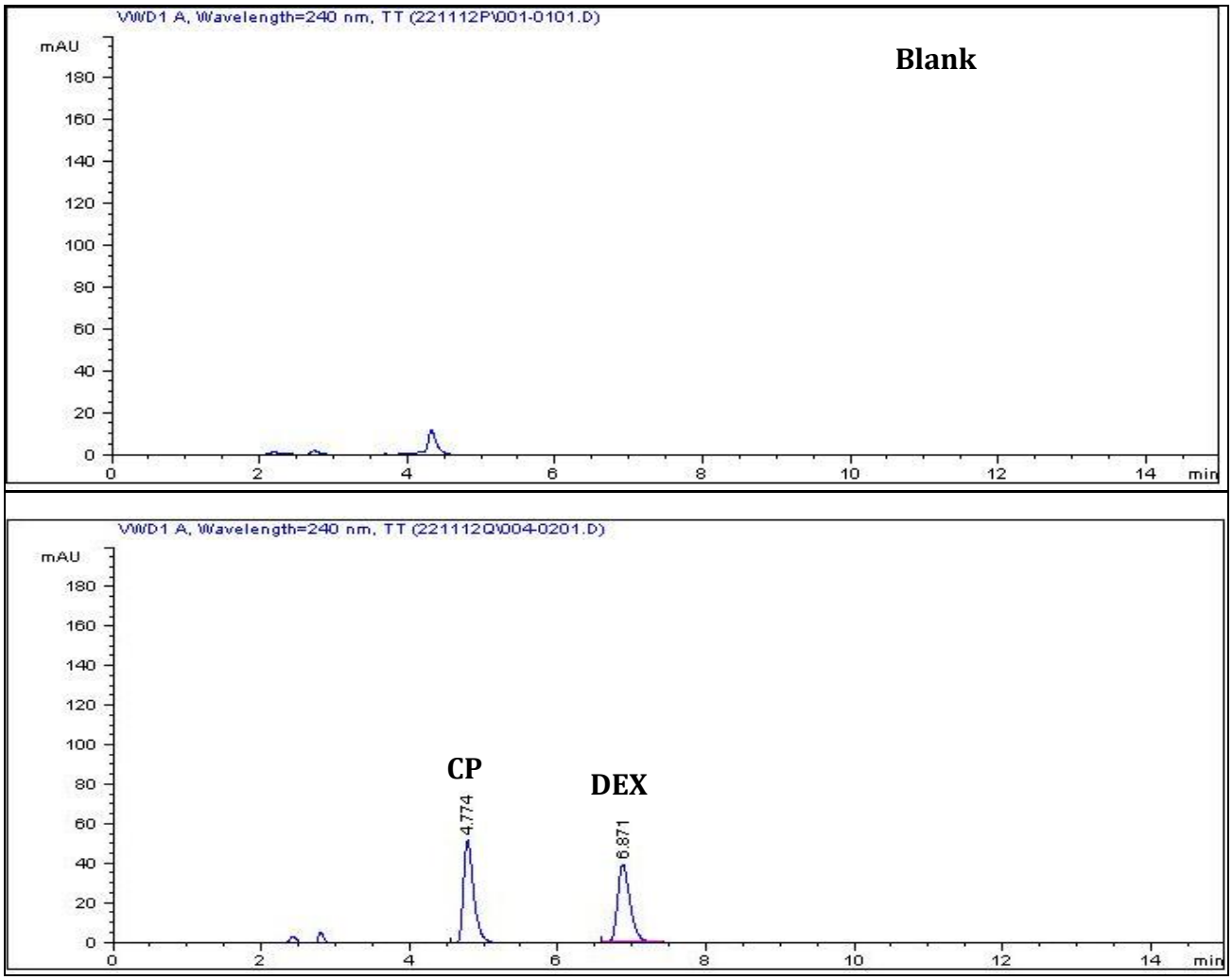

Figure A5: A blank chromatogram and chromatogram of two drugs CP (4.7 min), Dex (6.8 min) at a flow rate of 0.65 ml/min.

Abrreviations*: CP = cyclophosphamide, Dex = dexamethasone*

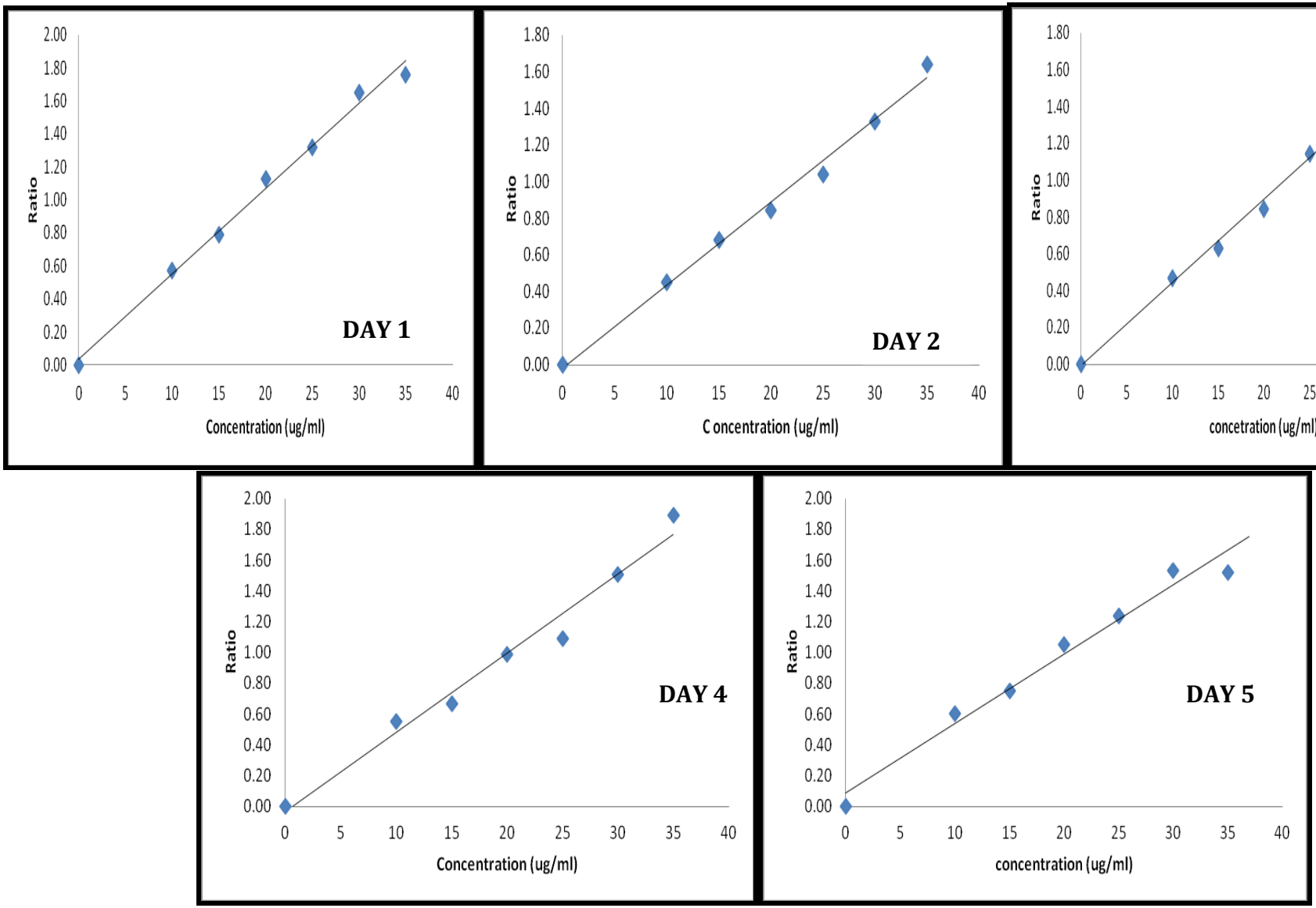

Figure A6: Daily Calibration curve of **cyclophosphamide**

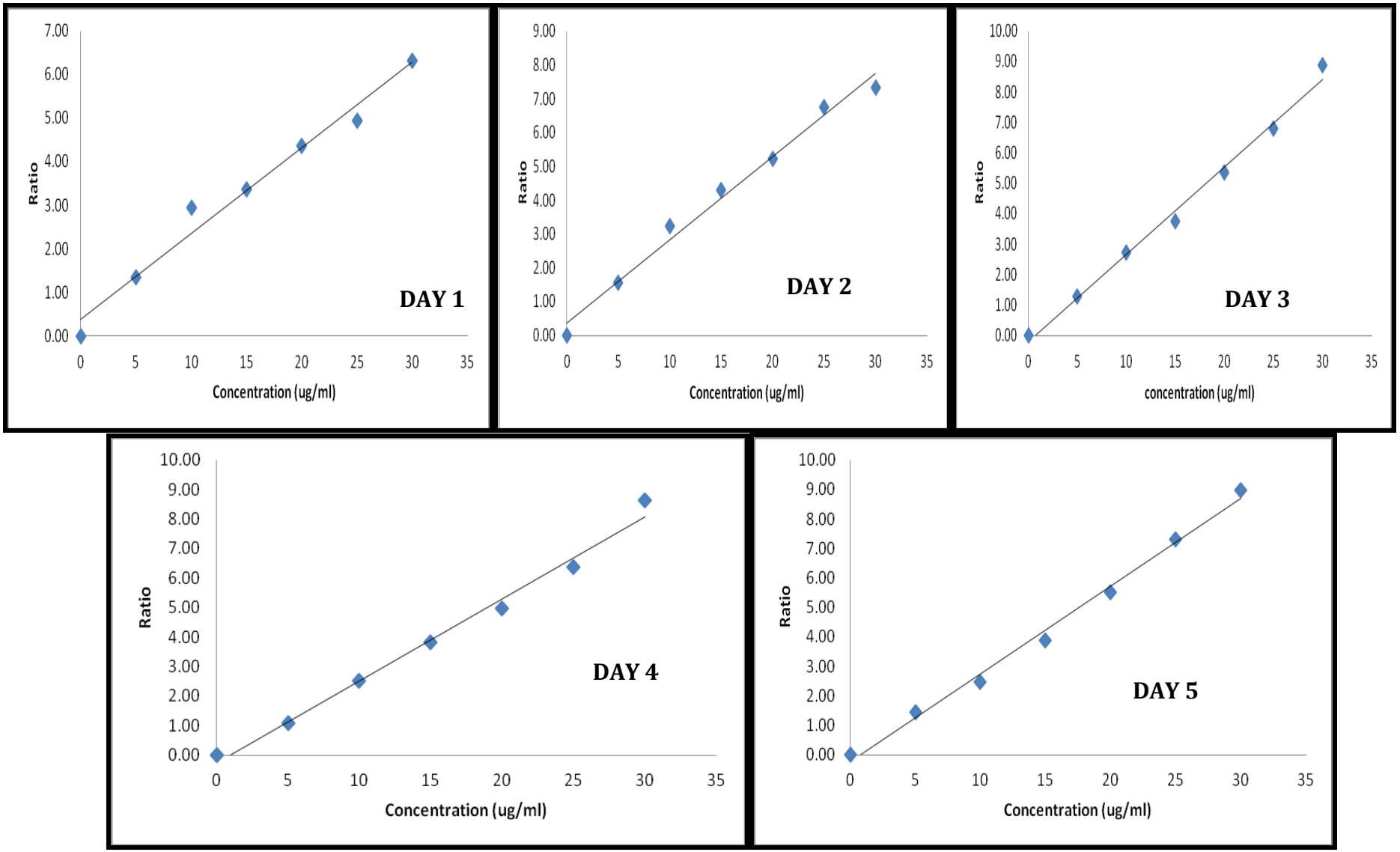

Figure A6: Daily Calibration curve of **Dexamethasone**

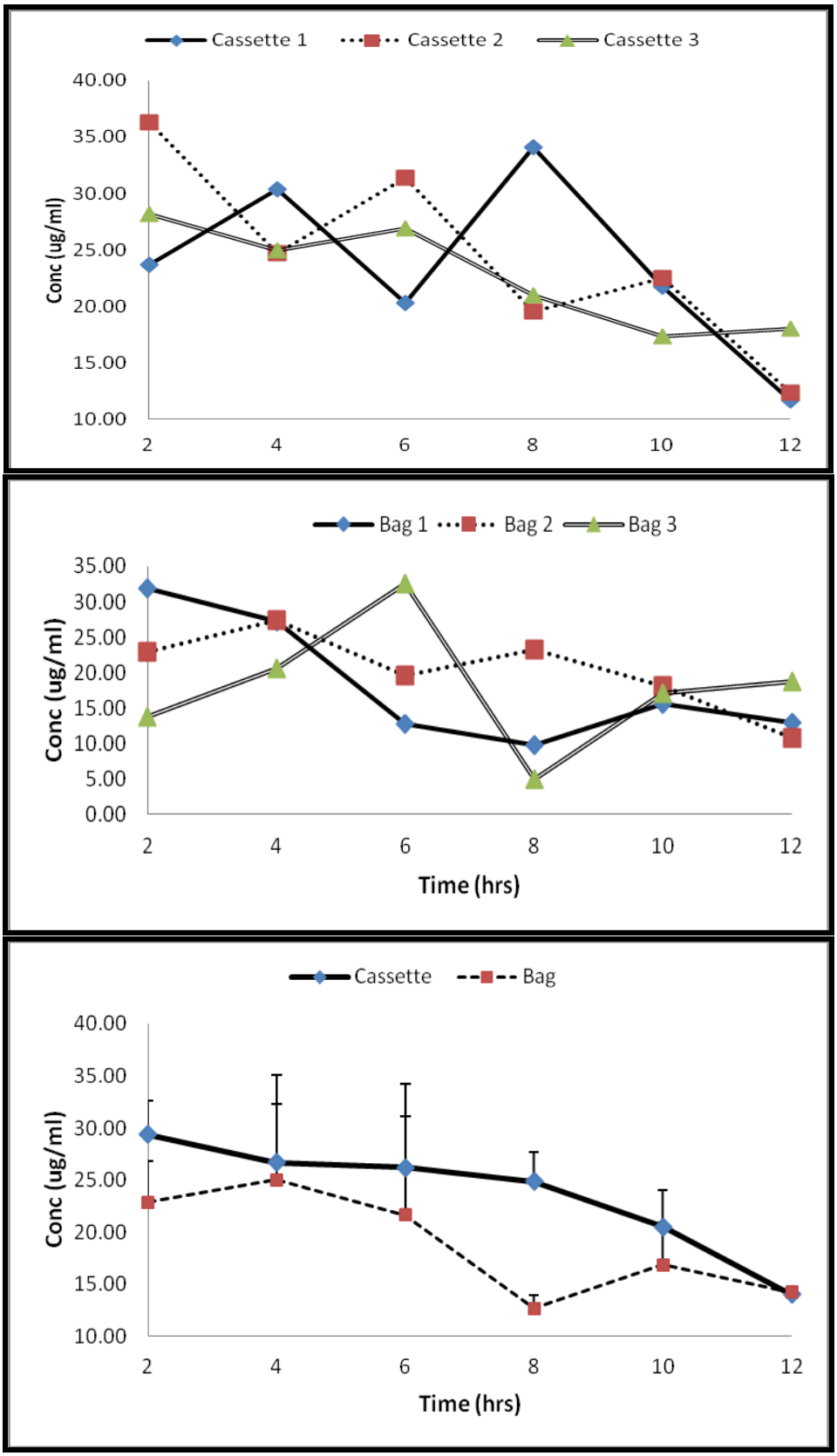

Figure B1: A diagram of **cyclophosphamide** concentration (µg/ml) in plasma and buffer versus time (hours) during over 12 hours, with equilibrium time at 4 hours

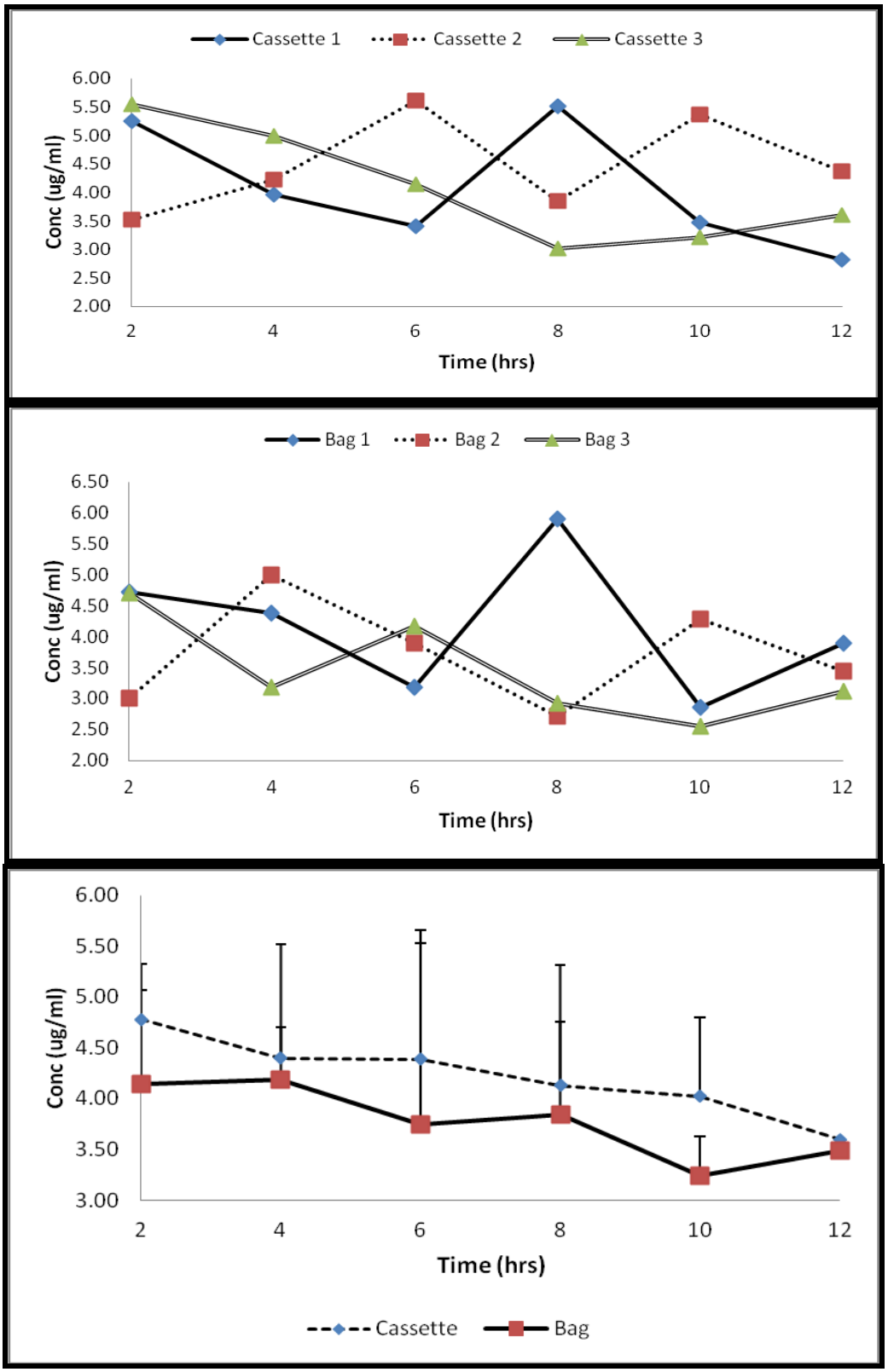

Figure B2: A diagram of **dexamethasone** concentration (µg/ml) in plasma and buffer versus (time) over 12 hours, with equilibrium time at 4 hours

Table *C1*: Summary of WCC count (x10^9/l) recorded as mean ± SD after 7, 14 and 21 days of *Phela* treatment.

	Saline	PHL 5mg/kg	PHL 15.4mg/kg	PHL 75 mg/kg	Levamisole
DAY 0	6.02±1.63	6.02±1.63	6.02±1.63	6.02±1.63	6.02±1.63
DAY 7	3.81±1.02	3.81±1.02	4.42±1.66	5.40±1.04	6.69±1.23
DAY 14	5.45±0.13	7.09±0.78	8.91±1.03	6.34±1.25	7.15±0.44
DAY 21	5.90±0.60	5.36±1.48	5.51±1.62	6.82±2.30	5.82±1.90

Table *C2*: Summary of neutrophils count (x10^9/l) recorded as mean ± SD after 7, 14 and 21 days of *Phela* treatment.

	Saline	PHL 5mg/kg	PHL 15.4mg/kg	PHL 75 mg/kg	Levamisole
DAY 0	0.77±0.17	0.77±0.17	0.77±0.17	0.77±0.17	0.77±0.17
DAY 7	1.03±0.81	0.44±0.17	0.77±0.51	0.63±0.21	1.06±0.31
DAY 14	0.34±0.04	0.88±0.34	1.13±0.33	0.87±0.43	1.16±0.33
DAY 21	0.64±0.20	2.10±0.32	0.62±0.17	1.58±1.36	1.23±0.29

Table *C3*: Summary of lymphocytes count (x10^9/l) recorded as mean ± SD after 7, 14 and 21 days of *Phela* treatment.

	Saline	PHL 5mg/kg	PHL 15.4mg/kg	PHL 75 mg/kg	Levamisole
DAY 0	5.03±1.44	5.03±1.44	5.03±1.44	5.03±1.44	5.03±1.44
DAY 7	4.07±1.95	3.14±0.93	3.51±1.14	4.56±0.77	5.38±0.84
DAY 14	3.79±0.03	5.82±0.48	6.63±0.94	7.31±0.81	5.55±0.38
DAY 21	4.81±0.39	3.20±1.12	3.78±1.40	4.38±0.54	4.38±1.71

Table *C4*: Summary of CD_8 count (x10^9/l) recorded as mean ± SD after 7, 14 and 21 days of *Phela* treatment.

	Saline	PHL 5mg/kg	PHL 15.4mg/kg	PHL 75 mg/kg	Levamisole
DAY 0	0.87±0.08	0.87±0.08	0.87±0.08	0.87±0.08	0.87±0.08
DAY 7	0.72±0.13	0.72±0.09	0.90±0.06	1.05±0.10	1.27±0.11
DAY 14	1.14±0.03	1.47±0.32	1.72±0.23	1.44±0.24	1.55±0.15
DAY 21	0.93±0.05	0.86±0.13	0.95±0.05	1.13±0.11	1.22±0.08

Table *C5*: Summary of CD_4 count (x10^9/l) recorded as mean ± SD after 7, 14 and 21 days of *Phela* treatment.

	Saline	PHL 5mg/kg	PHL 15.4mg/kg	PHL 75 mg/kg	Levamisole
DAY 0	1.38±0.22	1.38±0.22	1.38±0.22	1.38±0.22	1.38±0.22
DAY 7	1.15±0.04	1.10±0.15	1.30±0.14	1.68±0.17	2.20±0.02
DAY 14	1.58±0.01	2.48±0.29	2.90±0.19	2.82±0.49	2.11±0.33
DAY 21	1.78±0.45	1.86±0.12	1.75±0.18	1.89±0.19	1.86±0.27

Table *C6*: Summary of IgG count (mg/ml) recorded as mean ± SD after 7, 14 and 21 days of *Phela* treatment.

	Saline	PHL 5mg/kg	PHL 15.4mg/kg	PHL 75 mg/kg	Levamisole
DAY 0	4.60±3.95	4.60±3.95	4.60±3.95	4.60±3.95	4.60±3.95
DAY 7	3.05±1.28	3.26±0.04	3.42±2.04	10.52±8.19	26.83±24.25
DAY 14	4.56±1.09	11.86±14.72	5.99±5.49	3.15±1.87	9.74±5.83
DAY 21	5.36±2.61	3.50±2.63	4.77±3.39	9.38±2.50	3.94±3.38

Table *C7*: Summary of IgM count (µg/ml) recorded as mean ± SD after 7, 14 and 21 days of *Phela* treatment.

	Saline	**PHL 5mg/kg**	**PHL 15.4mg/kg**	**PHL 75 mg/kg**	**Levamisole**
DAY 0	174.24±104.42	174.24±104.42	174.24±104.42	174.24±104.42	174.24±104.42
DAY 7	73.39±61.99	25.70±13.17	25.70±20.77	27.03±8.69	55.65±34.81
DAY 14	26.88±10.02	45.04±38.83	29.36±13.80	29.27±33.46	36.29±11.28
DAY 21	19.64±7.42	18.84±15.37	23.49±8.94	30.27±17.01	34.08±13.35

Table *C8*: Summary of IL-2 count (pg/ml) recorded as mean ± SD after 7, 14 and 21 days of *Phela* treatment.

	Saline	**PHL 5mg/kg**	**PHL 15.4mg/kg**	**PHL 75 mg/kg**	**Levamisole**
DAY 0	102.21±4.25	102.21±4.25	102.21±4.25	102.21±4.25	102.21±4.25
DAY 7	108.34±10.48	156.19±5.62	150.88±44.11	114.89±6.76	161.51±56.62
DAY 14	108.34±10.48	93.62±24.29	101.39±3.09	100.37±0.87	169.69±57.06
DAY 21	112.33±9.58	122.25±19.68	124.70±1.42	121.02±11.07	114.07±20.25

Table *C9*: Summary of IL-10 count (pg/ml) recorded as mean ± SD after 7, 14 and 21 days of *Phela* treatment.

	Saline	**PHL 5mg/kg**	**PHL 15.4mg/kg**	**PHL 75 mg/kg**	**Levamisole**
DAY 0	31.63±2.57	31.63±2.57	31.63±2.57	31.63±2.57	31.63±2.57
DAY 7	35.34±6.34	64.26±3.40	61.05±26.66	39.29±4.09	67.48±34.22
DAY 14	35.34±6.34	26.44±41.69	31.13±1.87	30.52±0.52	72.42±34.50
DAY 21	37.75±5.79	43.74±11.90	45.23±0.86	43.00±6.69	38.80±12.24

Table D1: Summary of white cell count (WCC) (x10^9/l) count recorded as mean ± SD after treatment with immune suppressants (cyclosporine, cyclophosphamide and dexamethasone) in rats.

	CYCLOSPORINE	**OLIVE OIL**
Untreated	6.95±2.70	6.95±2.70
10 days	6.73±3.27	4.82±0.65 (P = 0.3745)
17 days	5.46±0.65	5.36±1.31 (P = 0.9118)
	CYCLOPHOSPHAMIDE	**SALINE**
Untreated	6.95±2.70	6.95±2.70
7 days	2.68±0.47	5.69±1.61 (P = 0.0359)
14 days	2.22±0.51	5.65±2.07 (P = 0.1162)
	DEXAMETHASONE	**SALINE**
Untreated	6.95±2.70	6.95±2.70
5 days	2.78±1.59	3.83±0.23 (P = 0.3205)
7 days	3.96±2.22	5.17±1.55 (P = 0.4801)

Table D2: Summary of neutrophils (x10^9/l) count recorded as mean ± SD after treatment with immune suppressants (cyclosporine, cyclophosphamide and dexamethasone) in rats.

	CYCLOSPORINE	**OLIVE OIL**
Untreated	0.51±0.46	0.51±0.46
10 days	0.63±0.55	0.98±0.59 (P = 0.4456)
17 days	0.91±0.23	0.60±0.18 (P = 0.2138)
	CYCLOPHOSPHAMIDE	**SALINE**
Untreated	0.51±0.46	0.51±0.46
7 days	0.62±0.22	0.62±0.22 (P = 0.9999)
14 days	0.94±0.13	0.70±0.27 (P =0.3396)
	DEXAMETHASONE	**SALINE**
Untreated	0.51±0.46	0.51±0.46
5 days	1.87±1.11	0.57±0.17 (P = 0.1140)
7 days	1.33±0.45	0.74±0.26 (P = 0.1218)

Table D3: Summary of lymphocytes (x10^9/l) count recorded as mean ± SD after treatment with immune suppressants (cyclosporine, cyclophosphamide and dexamethasone) in rats.

	CYCLOSPORINE	**OLIVE OIL**
Untreated	3.11±2.99	3.11±2.99
10 days	3.07±2.25	3.54±1.09 (P = 0.7560)
17 days	4.01±0.48	3.86±0.42 (P = 0.7239)
	CYCLOPHOSPHAMIDE	**SALINE**
Untreated	3.11±2.99	3.11±2.99
7 days	1.82±0.31	4.70±1.36 (P = 0.0232)
14 days	0.89±0.23	4.59±1.79 (P = 0.0694)
	DEXAMETHASONE	**SALINE**
Untreated	3.11±2.99	3.11±2.99
5 days	0.72±0.40	2.89±0.26 (P = 0.0014)
7 days	0.93±0.54	3.86±1.31 (P = 0.0231)

Table D4: Summary of CD_4 (x10^9/l) count recorded as mean ± SD after treatment with immune suppressants (cyclosporine, cyclophosphamide and dexamethasone) in rats.

DAYS	CYCLOSPORINE	OLIVE OIL
0	1.38±0.22	1.38±0.22
10	1.12±0.06	2.45±0.33 (P = 0.002)*
17	1.67±0.09	1.36±0.10 (P = 0.007)*
24	1.08±0.30	
31	1.04±0.13	
DAYS	**CYCLOPHOSPHAMIDE**	**SALINE**
0	1.38±0.22	1.38±0.22
7	0.89±0.07	1.96±0.08 (P = 0.0001)**
14	0.38±0.01	1.92±0.10 (P = 0.0001)**
21	0.18±0.12	
28	0.13±0.07	
DAYS	**DEXAMETHASONE**	**SALINE**
0	1.38±0.22	1.38±0.22
5	0.43±0.05	1.27±0.16 (P = 0.0010)**
7	0.51±0.06	2.15±0.39 (P = 0.0019)**
12	0.49±0.01	
19	0.09±0.05	
26	0.02±0.01	

**P < 0.05 vs control **P < 0.001 vs control*

Table D5: Summary of $CD_{8\ (}x10^{\wedge}9/l)$ count recorded as mean ± SD after treatment with immune suppressants (cyclosporine, cyclophosphamide and dexamethasone) in rats.

DAYS	CYCLOSPORINE	OLIVE OIL
0	0.87±0.08	0.87±0.08
10	0.78±0.01	1.53±0.24 (P = 0.0057)*
17	1.04±0.06	0.92±0.06 (P = 0.0801)
24	0.71±0.25	
31	0.54±0.02	
DAYS	**CYCLOPHOSPHAMIDE**	**SALINE**
0	0.87±0.08	0.87±0.08
7	0.81±0.05	1.19±0.13 (P = 0.0091)*
14	0.38±0.04	1.29±0.03 (P = 0.0001)**
21	0.06±0.03	
28	0.12±0.10	
DAYS	**DEXAMETHASONE**	**SALINE**
0	0.87±0.08	0.87±0.08
5	0.23±0.03	0.82±0.02 (P = 0.0001)**
7	0.16±0.02	0.96±0.27 (P = 0.0065)*
12	0.03±0.00	
19	0.03±0.00	
26	0.01±0.01	

**P < 0.05 vs control **P < 0.001 vs control*

Table D6: Summary of IgG (mg/ml) count recorded as mean ± SD after treatment with immune suppressants (cyclosporine, cyclophosphamide and dexamethasone) in rats.

DAYS	CYCLOSPORINE	OLIVE OIL
0	4.68±3.95	4.68±3.95
10	10.27±1.68	11.31±5.61 (P = 0.8241)
17	12.48±3.69	6.04±2.84 (P = 0.0547)*
24	10.06±2.12	
31	7.57±3.21	
DAYS	**CYCLOPHOSPHAMIDE**	**SALINE**
0	4.68±3.95	4.68±3.95
7	14.90±8.03	17.14±19.43 (P = 0.9056)
14	8.93±1.61	4.27±3.09 (P = 0.0817)
21	6.50±4.05	
28	10.77±5.06	
DAYS	**DEXAMETHASONE**	**SALINE**
0	4.68±3.95	4.68±3.95
5	1.06±0.16	17.14±19.43 (P = 0.3624)
7	3.96±2.22	4.27±3.09 (P = 0.4369)
12	4.93±2.75	
19	24.87±19.67	
26	16.51±8.48	

**P < 0.05 vs control **P < 0.001 vs control*

Table D7: Summary of IgM (ug/ml) count recorded as mean ± SD after treatment with immune suppressants (cyclosporine, cyclophosphamide and dexamethasone) in rats.

DAYS	CYCLOSPORINE	OLIVE OIL
0	174.24±104.42	174.24±104.42
10	59.00±11.59	52.57±34.19 (P = 0.8221)
17	102.63±99.29	75.46±59.48 (P =0.6950)
24	51.91±14.85	
31	28.06±14.18	
DAYS	**CYCLOPHOSPHAMIDE**	**SALINE**
0	174.24±104.42	174.24±104.42
7	87.27±29.03	155.56±14.72 (P = 0.0230)*
14	37.40±16.83	26.44±5.00 (P = 0.3336)
21	29.32±9.87	
28	46.96±26.17	
DAYS	**DEXAMETHASONE**	**SALINE**
0	174.24±104.42	174.24±104.42
5	61.43±31.95	155.56±14.72 (P = 0.0175)*
7	36.85±31.63	26.44±5.00 (P = 0.0014)*
12	36.18±46.40	
19	86.79±74.71	
26	60.43±51.62	

**P < 0.05 vs control **P < 0.001 vs control*

Table D8: Summary of IL-2 (ug/ml) count recorded as mean ± SD after treatment with immune suppressants (cyclosporine, cyclophosphamide and dexamethasone) in rats .

DAYS	CYCLOSPORINE	OLIVE OIL
0	102.21±4.25	102.21±4.25
10	102.82±4.34	110.80±4.91 (P = 0.1616)
17	106.30±6.70	128.79±15.82 (P = 0.0863)
24	115.71±19.61	
31	124.29±23.40	

DAYS	CYCLOPHOSPHAMIDE	SALINE
0	102.21±4.25	102.21±4.25
7	100.57±7.89	112.02±11.70 (P = 0.2326)
14	109.57±2.65	96.89±4.96 (P = 0.0068)*
21	85.10±32.26	
28	102.82±5.44	

DAYS	DEXAMETHASONE	SALINE
0	102.21±4.25	102.21±4.25
5	126.13±13.01	112.02±11.70 (P = 0.2932)
7	107.93±10.72	96.89±4.96 (P = 0.1807)
12	115.30±27.00	
19	141.47±20.64	
26	122.66±17.88	

**P < 0.05 vs control **P < 0.001 vs control*

Table D9: Summary of IL-10 (pg/ml) count recorded as mean ± SD after treatment with immune suppressants (cyclosporine, cyclophosphamide and dexamethasone) in rats.

DAYS	CYCLOSPORINE	OLIVE OIL
0	31.63±2.57	31.63±2.57
10	32.00±2.62	36.82±2.97 (P = 0.1616)
17	37.93±8.36	47.70±9.57 (P = 0.2087)
24	48.32±19.62	
31	44.98±14.15	
DAYS	**CYCLOPHOSPHAMIDE**	**SALINE**
0	31.63±2.57	31.63±2.57
7	30.64±4.77	37.56±7.08 (P = 0.2326)
14	36.08±1.60	28.41±3.00 (P = 0.0068)*
21	29.81±2.15	
28	32.00±3.29	
DAYS	**DEXAMETHASONE**	**SALINE**
0	31.63±2.57	31.63±2.57
5	46.09±7.86	37.56±7.08 (P = 0.2932)
7	35.09±6.48	28.41±3.00 (P = 0.1807)
12	39.54±16.34	
19	55.36±12.47	
26	43.99±10.81	

**P < 0.05 vs control **P < 0.001 vs control*

Table E1: Summary of White Cell Count (x10^9/l) as mean ± SD after 7, 14 and 21 days treatment with *Phela* in cyclosporine suppressed rats.

	CYCLOSPORINE (only)	**CYCLOSPORINE (+ *PHELA*)**
Day 0	6.73±3.27	6.73±2.73
Day 7	5.46±0.65	7.07±2.60 (P = 0.3550)
Day 14	4.48±0.92	5.15±0.98 (P = 0.3998)
Day 21	3.56±0.83	4.99±1.64 (P = 0.2496)

Table E2: Summary of neutrophils (x10^9/l) count as mean ± SD after 7, 14 and 21 days treatment with *Phela* in cyclosporine suppressed rats.

	CYCLOSPORINE (only)	**CYCLOSPORINE (+ *PHELA*)**
Day 0	0.63±0.55	0.63±0.55
Day 7	0.91±0.23	3.24±2.37 (P = 0.1652)
Day 14	0.85±0.35	1.56±1.27 (P = 0.3987)
Day 21	0.63±0.23	1.05±0.27 (P = 0.1072)

Table E3: Summary of lymphocytes (x10^9/l) count as mean ± SD after 7, 14 and 21 days treatment with *Phela* in cyclosporine suppressed rats

	CYCLOSPORINE (only)	**CYCLOSPORINE (+ *PHELA*)**
Day 0	3.07±2.25	3.07±2.25
Day 7	4.01±0.48	3.72±0.86 (P = 0.6385)
Day 14	3.30±0.71	3.09±0.80 (P = 0.7378)
Day 21	2.40±0.31	3.66±1.32 (P = 0.1992)

Table E4: Summary of CD_4 count (x10^9/l) recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in cyclosporine suppressed rats

	CYCLOSPORINE (only)	**CYCLOSPORINE (+ *PHELA*)**
Day 0	1.12±0.06	1.12±0.06
Day 7	1.67±0.09	1.52±0.06 (P = 0.0640)
Day 14	1.08±0.30	1.43±0.14 (P = 0.1400)
Day 21	1.04±0.13	1.38±0.18 (P = 0.0525)*

**P < 0.05 vs control **P < 0.001 vs control*

Table E5: Summary of CD_8 count (x10^9/l) recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in cyclosporine suppressed rats

	CYCLOSPORINE (only)	**CYCLOSPORINE (+ *PHELA*)**
Day 0	0.78±0.01	0.78±0.01
Day 7	1.04±0.06	0.84±0.14 (P = 0.0801)
Day 14	0.71±0.25	0.78±0.04 (P = 0.6431)
Day 21	0.54±0.02	0.95±0.10 (P = 0.0023)*

**P < 0.05 vs control **P < 0.001 vs control*

Table E6:: Summary of IgG (mg/ml) count recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in cyclosporine suppressed rats.

	CYCLOSPORINE (only)	**CYCLOSPORINE (+ *PHELA*)**
Day 0	10.27±1.68	10.27±1.68
Day 7	12.48±3.69	15.17±7.80 (P =0.5562)
Day 14	10.06±2.12	8.99±4.88 (P = 0.7008)
Day 21	7.57±3.21	4.34±1.79 (P = 0.1298)

**P < 0.05 vs control **P < 0.001 vs control*

***Table E7**:* Summary of IgM (µg/ml) count recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in cyclosporine suppressed rats.

	CYCLOSPORINE (only)	**CYCLOSPORINE (+ *PHELA*)**
Day 0	58.99±11.59	58.99±11.59
Day 7	102.63±99.29	53.12±34.40 (P = 0.3825)
Day 14	51.91±14.85	132.86±103.33 (P = 0.1718)
Day 21	28.06±14.18	42.82±28.32 (P = 0.4647)

**P < 0.05 vs control **P < 0.001 vs control*

Table E8: Summary of IL-2 (pg/ml) recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in cyclosporine suppressed rats.

	CYCLOSPORINE (only)	**CYCLOSPORINE (+ PHELA)**
Day 0	102.82±4.34	102.82±4.34
Day 7	106.30±6.70	138.31±36.02 (P = 0.2048)
Day 14	115.71±19.61	178.28±48.75 (P = 0.1082)
Day 21	124.29±23.40	103.44±4.42 (P = 0.2040)

**P < 0.05 vs control **P < 0.001 vs control*

Table E9: Summary of IL-10 (pg/ml) recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in cyclosporine suppressed rats.

	CYCLOSPORINE (only)	**CYCLOSPORINE (+ *PHELA*)**
Day 0	32.00±2.62	32.00±2.62
Day 7	37.93±8.36	48.32±20.67 (P = 0.3875)
Day 14	48.32±19.62	69.64±28.87 (P = 0.2676)
Day 21	44.98±14.15	31.26±3.12 (P = 0.1103)

**P < 0.05 vs control **P < 0.001 vs control*

Table F1: Summary of WCC count (x10^9/l) recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in cyclophosphamide suppressed rats .

	CYCLOPHOSPHAMIDE (only)	**CYCLOPHOSPHAMIDE (+ *PHELA*)**
Day 0	2.68±0.47	2.68±0.47
Day 7	2.22±0.51	1.70±0.28 (P = 0.2062)
Day 14	3.88±0.98	1.78±0.30 (P = 0.3638)
Day 21	3.13±1.71	3.93±2.02 (P = 0.0497)

Table F2*:* Summary of Neutrophils count (x10^9/l) recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in cyclophosphamide suppressed rats.

	CYCLOPHOSPHAMIDE (only)	**CYCLOPHOSPHAMIDE *(+ PHELA*)**
Day 0	0.62±0.22	0.62±0.22
Day 7	0.94±0.13	0.70±0.12 (P = 0.0548)
Day 14	2.26±0.11	0.79±0.32 (P = 0.0872)
Day 21	1.47±1.05	2.43±1.58 (P = 0.3529)

Table F3: Summary of Lymphocyte count (x10^9/l) recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in cyclophosphamide suppressed rats (n = 5).

	CYCLOPHOSPHAMIDE (only)	**CYCLOPHOSPHAMIDE (+ *PHELA*)**
Day 0	1.82±0.31	1.82±0.31
Day 7	0.89±0.23	0.73±0.30 (P = 0.2062)
Day 14	1.25±0.83	0.75±0.19 (P = 0.3638)
Day 21	0.99±0.75	0.97±0.06 (P = 0.0497)

Table F4: Summary of CD_4 count (x10^9/l) recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in cyclophosphamide suppressed rats

	CYCLOPHOSPHAMIDE (only)	**CYCLOPHOSPHAMIDE (+ *PHELA*)**
Day 0	0.89±0.07	0.89±0.07
Day 7	0.38±0.01	0.36±0.02 (P = 0.2062)
Day 14	0.18±0.12	0.24±0.11 (P = 0.3638)
Day 21	0.13±0.07	0.39±0.16 (P = 0.0497)*

***Table F5**:* Summary of CD_8 count (x10^9/l) recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in cyclophosphamide suppressed rats

	CYCLOPHOSPHAMIDE (only)	**CYCLOPHOSPHAMIDE (+ *PHELA*)**
Day 0	0.81±0.07	0.81±0.07
Day 7	0.38±0.04	0.31±0.02 (P = 0.0548)*
Day 14	0.06±0.03	0.23±0.10 (P = 0.0872)
Day 21	0.12±0.10	0.23±0.16 (P = 0.3529)

Table F6: Summary of IgG count (mg/ml) recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in cyclophosphamide suppressed rats

	CYCLOPHOSPHAMIDE (only)	**CYCLOPHOSPHAMIDE (+ *PHELA*)**
Day 0	14.90±8.03	14.90±8.03
Day 7	8.93±1.61	28.07±7.72 (P = 0.0137)*
Day 14	6.50±4.05	17.16±4.89 (P = 0.0089)*
Day 21	10.77±5.06	14.81±1.69 (P = 0.2598)

**P < 0.05 vs control **P < 0.001 vs control*

Table F7: Summary of IgM count (µg/ml) recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in cyclophosphamide suppressed rats

	CYCLOPHOSPHAMIDE (only)	**CYCLOPHOSPHAMIDE (+ *PHELA*)**
Day 0	87.27±29.03	87.27±29.03
Day 7	37.40±16.83	72.80±56.27 (P = 0.2758)
Day 14	29.32±9.87	85.94±63.35 (P = 0.1288)
Day 21	46.96±26.17	29.84±12.53 (P = 0.3645)

**P < 0.05 vs control **P < 0.001 vs control*

Table F8: Summary of IL-2 count (mg/ml) recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in cyclophosphamide suppressed rats

	CYCLOPHOSPHAMIDE (only)	**CYCLOPHOSPHAMIDE (+ *PHELA*)**
Day 0	100.57±7.89	100.57±7.89
Day 7	109.57±2.65	101.80±8.61
Day 14	85.10±32.26	106.81±6.13
Day 21	102.82±5.44	118.77±36.47

**P < 0.05 vs control **P < 0.001 vs control*

Table F9: Summary of IL-10 count (µg/ml) recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in cyclophosphamide suppressed rats

	CYCLOPHOSPHAMIDE (only)	**CYCLOPHOSPHAMIDE (+ *PHELA*)**
Day 0	87.27±29.03	87.27±29.03
Day 7	37.40±16.83	72.80±56.27 (P = 0.2758)
Day 14	29.32±9.87	85.94±63.35 (P = 0.1288)
Day 21	46.96±26.17	29.84±12.53 (P = 0.3645)

**P < 0.05 vs control **P < 0.001 vs control*

Table G1: Summary of White Cell Count (x10^9/l) recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in dexamethasone suppressed rats

	DEXAMETHASONE (only)	**DEXAMETHASONE (+ *PHELA*)**
Day 0	2.78±1.59	2.78±1.59
Day 7	3.96±2.22	5.17±1.55 (P = 0.0144)
Day 14	3.84±1.47	10.54±1.31 (P = 0.9928)
Day 21	3.28±0.69	3.29±0.98 (P = 0.4970)

Table G2: Summary of neutrophils (x10^9/l) count recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in dexamethasone suppressed rats

	DEXAMETHASONE (only)	**DEXAMETHASONE (+ *PHELA*)**
Day 0	1.87±1.11	1.87±1.11
Day 7	1.33±0.45	0.74±0.26 (P = 0.086)
Day 14	4.51±1.14	7.18±1.24 (P = 0.4331)
Day 21	2.70±0.62	2.67±0.88 (P = 0.4968)

Table G3: Summary of lymphocytes (x10^9/l) count recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in dexamethasone suppressed rats

	DEXAMETHASONE (only)	**DEXAMETHASONE (+ *PHELA*)**
Day 0	0.72±0.40	0.72±0.40
Day 7	0.90±0.23	1.09±1.00 (P = 0.2628)
Day 14	0.29±0.18	0.28±0.18 (P = 0.8821)
Day 21	0.40±0.25	0.52±0.12 (P = 0.4997)

Table G4: Summary of CD_4 (x10^9/l) count recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in dexamethasone suppressed rats (n = 5)

	DEXAMETHASONE (only)	***DEXAMETHASONE (+ PHELA)***
Day 0	0.43±0.05	0.43±0.05
Day 7	0.49±0.01	0.51±0.11 (P = 0.9290)
Day 14	0.09±0.05	0.10±0.06 (P = 0.9396)
Day 21	0.02±0.01	0.19±0.01 (P = 0.0010)*

**P < 0.05 vs control **P < 0.001 vs control*

Table G5: Summary of CD_8 (x10^9/l) count recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in dexamethasone suppressed rats.

	DEXAMETHASONE (only)	**DEXAMETHASONE (+ *PHELA*)**
Day 0	0.23±0.03	0.23±0.03
Day 7	0.03±0.00	0.12±0.01 (P = 0.0032)*
Day 14	0.03±0.00	0.02±0.01 (P = 0.9999)
Day 21	0.01±0.01	0.05±0.01 (P = 0.1510)

**P < 0.05 vs control **P < 0.001 vs control*

Table G6: Summary of IgG (mg/ml) count recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in dexamethasone suppressed rats

	DEXAMETHASONE (only)	**DEXAMETHASONE (+ *PHELA*)**
Day 0	1.06±0.16	1.06±0.16
Day 7	4.93±2.75	20.57±5.35 (P = 0.0073)*
Day 14	24.87±19.67	13.16±5.11 (P = 0.2932)
Day 21	16.51±8.48	8.54±3.96 (P = 0.1397)

**P < 0.05 vs control **P < 0.001 vs control*

Table G7: Summary of IgM (ug/ml) count recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in dexamethasone suppressed rats (n = 5)

	DEXAMETHASONE (only)	**DEXAMETHASONE (+ *PHELA*)**
Day 0	61.43±31.95	61.43±31.95
Day 7	36.18±46.40	196.54±257.47 (P = 0.2417)
Day 14	86.79±74.71	60.76±69.85 (P = 0.7080)
Day 21	60.43±51.62	47.26±14.27 (P = 0.5984)

**P < 0.05 vs control **P < 0.001 vs control*

Table G8: Summary of IL-2 (pg/ml) count recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in dexamethasone suppressed rats (n = 5)

	DEXAMETHASONE (only)	**DEXAMETHASONE (+ *PHELA*)**
Day 0	126.13±13.01	126.13±13.01
Day 7	115.30±27.00	104.66±1.74
Day 14	141.47±20.64	130.02±11.13
Day 21	122.66±17.88	109.72±3.99

**P < 0.05 vs control **P < 0.001 vs control*

Table G9: Summary of IL-10 (pg/ml) count recorded as mean ± SD after 7, 14 and 21 days treatment with *Phela* in dexamethasone suppressed rats.

	DEXAMETHASONE (only)	**DEXAMETHASONE (+ *PHELA*)**
Day 0	46.09±7.86	46.09±7.86
Day 7	39.54±16.34	33.11±1.05 (P = 0.6345)
Day 14	55.36±12.47	48.44±6.73 (P = 0.4454)
Day 21	43.99±10.81	36.63±1.77 (P = 0.3093)

**P < 0.05 vs control **P < 0.001 vs control*

PUBLICATION LIST

1. PRESENTED ABSTRACTS

- M.R. Lekhooa, A.Walubo, J.B. du Plessis and M.G. Matsabisa., Testing for potential interaction between *Phela* a herbal extract and cyclosporine, cyclophosphamide and dexamethasone**.** *29th Japanese society of the study of xenobiotics conference, San Francisco, 19 -23 October 2014***.**

- M.R. Lekhooa, A.Walubo, J.B. du Plessis and M.G. Matsabisa., *Phela* reversed cyclophosphamide-induced suppression of IgG and IgM in a rat model. *17TH world conference of basic and clinical pharmacology, Cape Town, 13 -18 July 2014.*

- M.R. Lekhooa, A.Walubo, J.B. du Plessis and M.G. Matsabisa., *Phela* reversed cyclosporine-induced suppression of lymphocytes and interleukin-2 in a rat model. *17TH world conference of basic and clinical pharmacology, Cape Town, 13 -18 July 2014.*

- M.R. Lekhooa, A.Walubo, J.B. du Plessis and M.G. Matsabisa., Simultaneous determination of cyclophosphamide and dexamethasone by high performance liquid chromatography with UV wavelength switching. *10TH International society of the study of xenobiotics conference, Toronto, 1 – 5 October 2013.*

- M.R. Lekhooa, A.Walubo, J.B. du Plessis and M.G. Matsabisa., Immunomodulation of CD_4 count by Phela a herbal medicine in healthy rats. *Annual American college of clinical pharmacology meeting, Washington DC, 22 - 25 September 2013*.

2. MANUSCRIPTS IN PREPARATION

- M.R. Lekhooa, A.Walubo, J.B. du Plessis and M.G. Matsabisa., Immunomodulation effects of Phela a herbal medicine on some on selected parameters in healthy rats. *To be submitted February 2015*.

- M.R. Lekhooa, A.Walubo, J.B. du Plessis and M.G. Matsabisa., Induction of TH_1 cytokines and protective effect of Phela a herbal medicine in an immune suppression rat model. *To be submitted February 2015.*

- M.R. Lekhooa, A.Walubo, J.B. du Plessis and M.G. Matsabisa., Potential interaction between *Phela* a herbal extract and cyclosporine, cyclophosphamide and dexamethasone. *To be submitted February 2015.*

TESTING FOR POTENTIAL INTERACTION BETWEEN *PHELA* A HERBAL EXTRACT AND CYCLOSPORINE, CYCLOPHOSPHAMIDE AND DEXAMETHASONE

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Background: *Phela* is a traditional herbal medicine product, derived from 4 S.A medicinal plants. It has been used for decades for wasting conditions. Previous anecdotal reports of patients, healers indicated that *Phela* stimulates the immune system. *Currently, Phela* is under development for use as an immune booster in immune-compromised individuals. However its drug-drug interactions profile is unknown. Cyclosporine, cyclophosphamide and dexamethasone are known immunesuppresants with varying mechanism of action. Since the subsequent animal experiment in our laboratory would involve co-administration of the drugs (cyclosporine, cyclophosphamide and dexamethasone) with *Phela*, there was a need to test for potential interactions with *Phela*. Thus, the aim of the experiment was to screen for direct interactions with any of the three drugs using an equilibrium dialysis by slide-a-lyzer technique.

Methods: Plasma (3ml) spiked with the drugs separately was dialyzed against 0.1M of sodium phosphate buffer (pH 7.4) until equilibrium protein binding for each drug was reached, after which Phela was added and dialysis was continued for six more hours. Aliquots (200µl) were taken every 2 hours from plasma in the chamber and buffer, and were analysed for the respective drug concentrations. Thereafter, the free and bound concentrations were determined.

Results: CP and Dex equilibrated 4 hours and CsA at 22 hours. Free fraction (%) was (mean±SD): [CP-only, 75.83±22.26% vs CP+*Phela,* 37.90±9.57%, p=0.054; Dex-only, 91.21±5.14% vs Dex+*Phela,* 87.75±1.38 %, p=0.085; and CsA-only, 55.95±12.92% vs CsA+Phela, 79.34±12.18%, p=0.323] Despite wide variations, there was no significant difference between the free fractions of drug-only group when compared with drug+*Phela* group.

Conclusion: In conclusion, there was no interaction between *Phela* and cyclosporine, cyclophosphamide and dexamethasone. This implies that the drugs can be co-administered with *Phela* without interference.

PHELA REVERSED CYCLOPHOSPHAMIDE-INDUCED SUPPRESSION OF IGG AND IGM IN A RAT MODEL

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Background: *Phela* is a herbal medicine product under development for use as an immune booster in immune-compromised individuals. Unfortunately, the lack of diseased models by which to evaluate the mechanism and efficacy of products purported to be immune boosters has hampered the development of these products. Here, using *Phela* as test compound, a rat model of cyclophosphamide-induced immune suppression was used to investigate the mechanism of immunomodulation by *Phela.*

Methods: Sprague dawley rats were used and approval from animal ethics committee was obtained. Two groups of 15 rats were pre-treated with cyclophosphamide (CP: 100 mg/kg) once weekly to induce immune suppression. On the eighth day the control group continued on the CP-only treatment. The test group was treated with *Phela* (15.4 mg/kg) daily and once weekly dose of CP. Five rats were sacrificed after 7, 14 and 21 days of treatment in each group. Blood was analyzed for liver, renal and haematology functions and the CD_4 and CD_8 counts were analyzed by flow cytometry. IgG, IgM and IL-2 measurements were done by ELISA. The kidney, liver, spleen, thymus, were weighed and examined for any pathology. Body weight was recorded before and after the study.

Results: *Phela* minimized thymus weight loss and prevented body weight reduction (%: SD±mean) after 14 and 21 days [*Phela-group: (+21±5) and (+15±8) vs. Control-group (+10±8) and (+6±3)*]. Furthermore, *Phela* overcame CD_4 and CD_8 count suppression from 14 days ($P < 0.08$) and significantly after 21 days ($P<0.05$). IgG concentration was significantly increased in *Phela*-treated group over 7 days ($P = 0.01$) and 14 days ($P = 0.008$) and returned to baseline after 21 days. IgM levels remained high through-out the study though not statistically significant. After 21 days *Phela* overcame neutrophils and lymphocytes suppression, ($x10^{\wedge}9/l$: SD±mean) [*Phela-group: (1.05±0.27) and (3.66±1.24) vs. Control-group: (0.63±0.23) and (2.40±0.31*].

Conclusion: *Phela* prevented progression of the immunesuppression in rats treated with cyclophosphamide as indicated by increasing IgG and IgM levels. This implies that *Phela* may stimulate antibody production in patients with a compromised immune system and that *Phela*'s mechanism of immunomodulation is partly humoral.

PHELA REVERSED CYCLOSPORINE-INDUCED SUPPRESSION OF LYMPHOCYTES AND INTERLEUKIN-2 IN A RAT MODEL.

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Background: *Phela* is a herbal medicine product under development for use as an immune booster in immune-compromised individuals. Unfortunately, the lack of diseased models by which to evaluate the mechanism and efficacy of products purported to be immune boosters has hampered the development of these products. Here, using *Phela* as test compound, a rat model of cyclosporine-induced immune suppression was used to investigate the mechanism of immunomodulation by *Phela*.

Methods: Sprague dawley rats were used and approval from animal ethics committee was obtained. Two groups of 15 rats were pre-treated with cyclosporine (CSA: 1.5 mg/kg) daily for ten days. On the eleventh day the control group continued on the CSA-only treatment. The test group was treated with *Phela* (15.4 mg/kg) daily and continues on CSA treatment. Five rats were sacrificed after 7, 14 and 21 days of treatment in each group. Blood was analyzed for liver, renal and haematology functions. The CD_4 and CD_8 counts were analyzed by flow cytometry. Rat IgG, IgM and IL-2 were measured by ELISA. The kidney, liver, spleen, thymus, were weighed (g) and examined for any pathology. Body weight was monitored during the study.

Results: *Phela* prevented thymus weight-loss and this was significant after 21 days [*Phela-group: (0.53±0.05g)* (P = 0.0001)*, Control-group (0.34±0.04g)*] of treatment. On day 21 Phela modulated CSA-induced suppression in white cell count, neutrophils and lymphocytes (x10^9/l: SD±mean) [*Phela-group: (4.99±1.64), (1.05±0.27), (3.66±1.24) vs. Control-group (3.56±0.83), (0.63±0.23) (2.40±0.31)*]. Furthermore, *Phela* reversed the immune-suppression on day 14 in CD_4 and CD_8 count and significantly ($P < 0.05$) on day 21. *Phela* increased IL-2 count after 7 and 14 days [*Phela-group: (138.31±36.02), (178.28±48.75) vs. Control-group (106.30±6.70); (115.71±19.61)*], and returned to baseline after 21 days.

Conclusion: *Phela* stopped the progression of immunesuppression in rats treated with cyclosporine as indicated by increasing lymphocytes and IL-2. This implies that *Phela* may stimulate IL-2 in patients with a compromised immune system and that ***Phela's*** mechanism of immunomodulation is probably cell-mediated.

SIMULTANEOUS DETERMINATION OF CYCLOPHOSPHAMIDE AND DEXAMETHASONE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH UV WAVELENGTH SWITCHING

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Background: Cyclophosphamide and dexamethasone are often used for cancer therapy and during *in vivo* experimental pharmacology. Hence, a need to develop and validate an high performance liquid chromatography (HPLC) method with UV wavelength switching detection for rat plasma analysis.

Methodology: Plasma spiked with cyclophosphamide and dexamethasone and surprofen as the internal standard was extracted with ethyl acetate. Centrifugation was 10 min long at 6 705 g and the supernatant was evaporated under nitrogen at 47°C. The residue was reconstituted with 100 µl of mobile phase and 50 µl was injected on an isocratic HPLC system. The sample was eluted with 45 % of acetonitrile in 0.05 M sodium phosphate buffer (pH 3.7; containing 0.5 ml triethylamine) at a flow rate of 1 ml/min and 10 minutes runtime. Separation was achieved on a RP-Luna C_{18} (250 mm x 4.6 mm x 5 µm) column coupled to a guard column. The UV wavelength was set at 190 nm for cyclophosphamide and switched to 240 nm for dexamethasone and internal standard detection. Linearity was evaluated with a 6-point calibration curve over five days. Accuracy was tested with a repeated sampling of low, medium and high concentrations. Short-term stability was evaluated at different temperature settings over 24 and 48 hours. Plasma from rats treated with the two drugs was analyzed with the validated method.

Results: Cyclophosphamide eluted at 4.2 minutes while dexamethasone and internal standard eluted at 5.7 and 8.1 minutes respectively. Cyclophosphamide linear regression was y = 0.04x + 0.11 and y = 0.32x – 1.52 for dexamethasone. The correlation coefficient was 0.999 and 0.998 for cyclophosphamide and dexamethasone respectively. The coefficient of variance was less than 15% for both drugs. Recovery was 101 – 108 % and 99 – 107 % for cyclophosphamide and dexamethasone respectively after accuracy testing. Both drugs were stable in the fridge and ultra freezer over 24 and 48 hours.

Conclusion: The method was successfully used to measure the two drugs after administration to rats. The validated high performance liquid chromatography method with UV wavelength switching is simple, rapid, specific and accurate.

IMMUNOMODULATION OF CD_4 COUNT BY *PHELA*, A HERBAL EXTRACT

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Background: Phela is an extract from a herbal medicine that is under development for use as an immune booster in immune compromised individuals. Unfortunately, the current dose for Phela was not evaluated scientifically. Therefore, the aim of this study was to determine the appropriate dose of Phela for immunomodulation.

Methodology: Four groups of 15 Sprague-Dawley rats each were treated daily with either normal-saline, Phela 5, 15.4 or 75 mg/kg, and in each group 5 rats were sacrificed after 7, 14 and 21 days of treatment. Blood was analysed for liver, renal and haematology functions, and the CD_4 and CD_8 counts (%) were analysed by flow cytometry. The kidney, liver, spleen and thymus were weighed and examined for any pathology.

Results: All the three doses of Phela led to increased white blood cell count after 14 days of treatment, and this was significant for doses 5 mg/kg ($P = 0.02$) and 15.4 mg/kg ($P = 0.03$). This was associated with increased lymphocyte count which correlated with increased CD4 count from day 7 to 14 of treatment. At 5 mg/kg, the CD_4 count was 1.10±0.15 at 7 days, and 2.48±0.29 at 14 days ($p = 0.005$), while for 15 mg/kg, it increased from 1.30±0.14 to 2.90±0.19 ($p = 0.001$). The CD_4 count in the control dropped progressively but remained in the normal range. Eosinophilia was also noted while CD_8 and physiological function tests were normal. This observation correlates with our previous report where Phela led to increased IL-2.

Conclusion: In conclusion, Phela led to ample stimulation of the immune system as indicated by increased CD_4 count at doses of 5 and 15 mg/kg. This selective effect implies that Phela can be indicated in diseases that interfere with CD_4 count, but this needs to be confirmed in a diseased model.

SUMMARY

***Key words*:** Phela, Rat model, mechanism of immunomodulation, cell-mediated, traditional herbal medicines product, TH_1 cytokines.

Traditional medicines have gained interest as a source of therapy; however, a gap remains from traditional usage to potential clinical use. *Phela* a South African traditional herbal medicines is currently being developed for patients with a compromised immune system. Its efficacy was first acclaimed in anecdotal reports of traditional healers however; there is no supporting scientific information on its mechanism of action on the purported abilities. Unfortunately, there is no appropriate animal model for the testing of immune-boosters, based on the understanding of the model of immune response in particular diseases, an *in vivo* model in which the cell mediated, humoral or non-specific immune response can be studied is more appropriate. Here, it was proposed to undertake a study to develop a rat model by which to characterize the effect of *Phela* on selected immune markers in immune-suppressed rats.

Phela quality-control analysis was done by HPLC-DAD and HPLC-FLD methods prior to all experiments and had 2 and 4 mark peaks respectively which were similar with those obtained previously. Thereafter, a simple and accurate HPLC-UV method for the simultaneous detection of cyclophosphamide and dexamethasone was validated and applied to rat plasma. The method was linear with regression and correlation coefficients of y = 0.04x+0.11 and 0.999 for cyclophosphamide, and y = 0.32x–1.52 and 0.998 for dexamethasone, and their respective recoveries of 102 – 108 % and 99 – 107 %. Furthermore, *Phela* was screened against cyclosporine, cyclophosphamide and dexamethasone to test for potential interactions using slide-a-lyzer technique. Plasma spiked with immunesuppresants and *Phela* was dialyzed against 0.1M Sodium phosphate buffer over 24 hours. The results indicated that the above-mentioned drugs can be co-administered with *Phela* without interference.

A rat model of by which to characterise *Phela* was developed in a 3-part experiment. Firstly, the dose of *Phela* for immune stimulation in healthy rats was determined.

Here, 5 groups were treated with either saline, levamisole, 5, 15.4 or 75 mg/kg of *Phela.* Physiological function tests and selected immune markers (CD_4, CD_8, IgG, IgM, IL-2 and IL-10) concentration was determined. *Phela* led to ample stimulation of the immune system as indicated by increased in white cells, CD_4 and IL-2 count at doses of 5 and 15.4 mg/kg.

Thereafter, the optimum dose and time of immune suppression by known immune suppressants; cyclosporine, cyclophosphamide and dexamethasone was established. Different groups of twelve rats each were treated with either immunesuppresant-only, along with a control group in each case. As expected, the animals exhibited abnormal physiological function tests in association with progressive immunesuppression. Cyclosporine inhibited the cell-mediated immunity, while cyclophosphamide suppressed the humoral immunity and the effect of dexamethasone was multi-systemic.

Furthermore, the rat model was applied to determine the mechanism of immunomodulation of *Phela*. Different groups of 15 rats each were pre-treated with immunesuppresant-only to induce immunesuppression. Thereafter, the control-groups continued on the immunesuppresant-only and the test groups we co-treated with an immunesuppresants and *Phela* for 21 days. *Phela* stopped the progression of immunesuppression in rats treated with cyclosporine as indicated by the reversal and/or resistance to CsA induced changes in the WCC, neutrophils, lymphocytes, CD_4, CD_8, and IL-2.

Furthermore, *Phela* prevented progression of CP-induced body and thymus weight loss, suppression of IgG and IgM, with minimal effect on CD_4 and CD_8 count. This implies that the mechanism of immunomodulation of *Phela* is cell-mediated in rats. Therefore, *Phela* would be a candidate for testing against diseases or disorders associated with suppressed CMI, such as HIV/AIDS and Tuberculosis. In conclusion a rat immunesuppression model has been successfully developed and applied to characterise the mechanism of immunomodulation of *Phela*.

OPSOMMING

Sleutel: Phela, Rotmodel, meganisme van immuunmodulasie, sel-bemiddeld, *Tradisionele-kruie-medisinale-produkte*, TH_1 *sitokiene*

Tradisionele medisyne word toenemend gesien as ‘n bron van behandeling; nogtans bly daar ‘n gaping tussen die tradisionele gebruik en die potensiële kliniese gebruik. *Phela,* ‘n Suid Afrikaanse tradisionele kruie medisyne word tans ontwiklel vir pasiënte met immuunonderdrukking. Die effektiwiteit daarvan het aanvanklik bekend geword uit anektodiese vertellings van tradisionele genesers. Daar is egter geen ondersteunende wetenskaplike inligting oor die meganisme van werking op die beweerde vermoëns. Ongelukkig bestaan daar geen toepaslike dieremodel vir die toetsing van immuun-versterkende middels nie. Gebaseer op die begrip van die model van immuunrespons in spesifieke siektes, sal ‘n *in vivo* model waarin the sel-bemiddelde, humorale of nie-spesifieke immuunresponse bestudeer kan word,meer toepaslik wees. Hier maak ons ‘n studie bekend vir die ontwikkeling van ‘n rotmodel waardeur die effek van *Phela* op geselekteerde immuunmerkers in immuunonderdrukte rotte gekarakteriseer kan word.

Phela kwaliteitskontrole analises is gedoen deur middel van HPLC-DAD en HPLC-FLD metodes voor die aanvang van alle eksperimente en het onderskeidelik 2 en 4 merkpieke getoon wat ooreenstem met vorige waarnemings. Daarna is ‘n eenvoudige en akkurate HPLC-UV metode vir die gelyktydige waarneming van siklofosfamied en deksametasoon gevalideer en toegepas op rotplasma. Die metode was liniêr met regressie en korrelasie koëffisiënte van y = 0.04x+0.11 en 0.999 vir siklofosfamied, en y = 0.32x–1.52 en 0.998 vir deksametasoon; en herwinnings van 102 – 108 % en 99 – 107 %, onderskeidelik. Verder is *Phela* saam met siklosporien, siklofosfamied en deksametasoon getoets vir potensiële interaksies met behulp van ‘n slide-a-lyzer tegniek. Plasma gemerk met immuunonderdrukkers; en *Phela* is gedialiseer teen 0.1M natriumfosfaat bufferoplossing oor 24 uur. Die resultate het getoon dat bogenoemde middels saam met *Phela* toegedien kan word sonder inmenging.

‘n Rotmodel waardeur *Phela* gekarakteriseer kon word is ontwikkel in ‘n eksperiment met drie dele. Eerstens is die dosis van *Phela* vir immuunstimulasie in gesonde rotte bepaal. Hier is 5 groepe behandel met óf soutoplossing; óf levamisool, 5, 15.4 of 75 mg/kg; óf

Phela. Fisiologiese funksie is getoets en die konsentrasies van geselekteerde immuunmerkers (CD_4, CD_8, IgG, IgM, IL-2 en IL-10) is bepaal. *Phela* het beduidende stimulasie van die immuunsisteem veroorsaak, soos aangedui deur verhoogde witselle, CD_4 en IL-2 tellings teen dosisse van 5 en 15.4 mg/kg. Hierna is die optimale dosis en tyd vir immuunonderdrukking deur bekende immuunonderdrukkers; siklosporien, siklofosfamied en deksametasoon bepaal. Verskillende groepe van twaalf rotte elk is behandel met óf immuunonderdrukker alleen, saam met 'n kontrolegroep in elke geval. Soos verwag, het die diere abnormale fisiologiese toetsuitslae saam met progressiewe immuunonderdrukking getoon. Siklosporien het sel-bemiddelde immuniteit geïnhibeer; siklofosfamied het die humorale immuniteit onderdruk en die effek van deksametasoon was multi-sistemies.

Verder is die rotmodel gebruik om *Phela* se meganisme van immuunmodulasie te bepaal. Verskillende groepe van 15 rotte elk is vooraf behandel met immuunonderdrukkers alleen om immuunonderdrukking te induseer. Hierna is die behandeling van die kontrolegroepe voortgesit met die immuunonderdrukker alleen en die toetsgroepe is behandel met die immuunonderdrukkers saam met *Phela* vir 21 dae. *Phela* het die progressie van die immuunonderdrukking in rotte wat behandel is met siklosporien gestop soos aangedui deur die omkering en/of weerstand teen CsA geïnduseerde veranderinge in die witseltelling, neutrofiele, limfosiete, CD_4, CD_8, en IL-2 bepalings.

Verder het *Phela* ook die progressie van CP-geïnduseerde liggaams- en timus-massaverlies voorkom, asook onderdrukking van IgG en IgM, met minimale effek op CD_4 en CD_8 tellings. Hierdie impliseer dat die meganisme van immuunmodulasie deur *Phela* in rotte sel-bemiddeld is. Hiervolgens behoort *Phela* 'n kandidaat te wees vir toetsing teen siektes of toestande geassosiëer met onderdrukte selbemiddelde immuniteit, soos MIV/VIGS en Tuberkulose. In konklusie is 'n rotmodel suksesvol ontwikkel en toegepas om die meganisme van immuunmodulasie deur *Phela* te karakteriseer.