Evaluating the efficacy, safety and possible mechanism of action of potassium humate with selenium

by

Tsakani Locrecia Chauke

Submitted in partial fulfilment of the requirements for the degree

Magister Scientiae in Pharmacology

in the

DEPARTMENT OF PHARMACOLOGY
FACULTY OF HEALTH SCIENCES

UNIVERSITY OF PRETORIA

2013

Acknowledgments

First of all I would like to thank God almighty who made it possible for me to be here today, who gave me the strength and ability to do this study and write this dissertation. Without Him I would be nothing

It is with sincere gratitude that I acknowledge the following people:

My supervisor Dr AD Cromarty, “Dr D” for his technical brilliance, relentless ability to solve problems, endless patience, great sense of humour and believing in me
Professor C Medlen for initiating the study.
Ms Isle Janse van Rensburg at UPBRC for her assistance with the animal study.
Dr JJ van Tonder for his assistance with the flow cytometer, antioxidant assays and statistical analysis.
Ms June Serem from the Department of Anatomy for her assistance with the antioxidant assays and statistical analysis.
Prof P Becker for assistance with statistical planning.
My mother "Mastene" and sister for their support and for believing in me, you guys are the best.
Last but not least my wonderful husband Joseph for the love, patience and support.

Declaration

The work described in this dissertation was carried out at the Department of Pharmacology, University of Pretoria, Pretoria, South Africa, under the supervision of Dr AD Cromarty. I declare that this dissertation is my own work submitted for the degree of Masters of Science and that this dissertation does not incorporate, without written acknowledgement, any material that has previously been submitted for the award of any other degree or diploma in any university, college, or other educational institution and to the best of my knowledge, this dissertation does not contain any material previously published or written by another person except where due reference is made in the text, including the disclosure of contributions for any work based in joint research or publications.

…… ……..

Signed **Date**

Abstract

Aim. The aim of the study was to evaluate the efficacy, safety and possible mechanism of action of potassium humate loaded with selenium

Objectives. The objectives of the study were to evaluate the possible *in vitro* cytotoxic effect of potassium humate loaded with selenium (Phse) on the growth of primary cell cultures (lymphocytes), to evaluate the *in vitro* antioxidant activity of Phse, to evaluate the *in vitro* effect of Phse on CR3 expression using mixed leukocytes, to evaluate the anti-inflammatory properties of Phse using the carrageenan-induced paw oedema rat model and finally to evaluate the effect of Phse on acute phase proteins in the rat model

Methods. For the cytotoxicity effects on lymphocytes, the MTT assay was used where lymphocytes were isolated and divided in to two groups, one group was stimulated with PHA and the other not, then the cells were treated with different concentrations of the test compounds. The evaluation of the antioxidant activity was done using the ORAC and DCFH-DA assays with the DCFH-DA assay also done using the $HepG_2$ cell line. The expression of CR3 by mixed leukocytes was quantified by flow cytometry. The evaluation of the anti-inflammatory properties of Phse was done using the carrageenan-induced paw oedema rat model. The rats were randomly assigned to six groups, the negative control, positive control, experimental group 1, 2, 3 and sham group. A once daily dose by gavage for five consecutive days of their respective treatment was administered. Prior to the assay the rats were dosed according to the experimental group to which they were assigned. On the fifth day of the experiment, 50 µl of λ- Carrageenan was injected subplanter into the right hind paw of the rats and 50 µl of saline into the left hindpaw. The right hind paw volume of each rat was measured hourly from the time of injection for seven consecutive hours with a water displacement plethysmometer. At the end of the 7 hours the rats were anaesthetised and approximately 5 ml of blood was collected via cardiac puncture. The blood was centrifuged, the plasma removed and frozen at -80°C until assayed. For evaluating the effects of the test compounds on acute phase proteins ELISA was used according to the manufacturer's protocols.

Results. None of the test compounds were toxic to lymphocytes but rather caused cell proliferation. The test compounds demonstrated no antioxidant activity, with Phse showing pro-oxidant activity. All the test compounds inhibited the expression of CR3 significantly with selenium free Ph being the most potent inhibitor. Ph reduced the carrageenan induced paw

oedema volumes in a similar manner to indomethacin and Phse had an insignificant effect. Ph decreased SP slightly but the results were not statistically significant whereas Se and Phse had no effect. All the test compounds statistically significantly decreased plasma CRP levels with Se showing the greatest effect.

Conclusion. Phse is safe but not more effective than Ph as an anti-inflammatory agent.

Table of Contents

List of Figures

Table of Tables

Abbreviations

AAPH	2,2´-azobis-2-2methyl-propanimidamide dihydrochloride
Ag	Antigen
NH_4Cl	Ammonium chloride
APR	Acute phase response
ºC	Degree Celcius
Ca^{2+}	Calcium
CD	Cluster of differentiation
CO_2	Carbon dioxide
COX	Cyclo-oxygenase enzyme
CR3	Complement receptor 3
CRP	C-reactive protein
DCF	2´-7´-Dichloroflourescein
DCFH DA	2´-7´-Dichlorodihydroflourescein diacetate
DCFH	2´-7´-Dichlorodihydroflourescein
dH_2O	Deionised water
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EMEM	Eagle's Minimum Essential Medium
GPx	Glutathione peroxidase
GSH	Plasma glutathione
GSH-Px	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
HBSS	Hank's Balanced Salt Solution
HepG2	Hepatocellular carcinoma (human)
HI-FCS	Heat inactive foetal calf serum
HIV	Human Immunodeficiency virus
iC3b	Inactive complement cleavage fragment
ICAM-1	Intercellular Adhesion molecule

IgG	Immunoglobulin G
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IL	Interleukin
IL2R	Interleukin 2 receptor
MoAbs	Monoclonal Antibodies
MPO	Myeloperoxidase
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl-2Htetrazolium)
MTT	3-[4, 5 dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide
NEAA	Non-Essential Amino Acids
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Neurokinin
NK1R	Neurokinin-1 Receptor
NO	Nitric oxide
ORAC	Oxygen Radicals Absorbance Capacity
PBS	Phosphate buffered saline
PE	Phycoerythrin
Pen/Strep	Penicillin/Streptomycin
PG2	Prostaglandin 2
PHA	Phytohaemaglutinin
Phse	Potassium humate with selenium
PMA	Phorbol 12-myristate 13-acetate
ROS	Radical Oxygen Species
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
Se	Selenium
SOD	Superoxide dismutases
TH_1	T-helper cell
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylate
UPBRC	University of Pretoria Biomedical Research Centre

Chapter 1

Introduction

The principal function of the immune system is to defend the host from infectious microbes in its environment (Chaplin., 2006). The immune system is highly evolved to protect its environment that is rich in resources that foreign organisms attempt to attack in an effort to make use of these resources (Farmer and Packard., 1986). If the host becomes exposed to foreign pathogens an acute inflammatory response is triggered. This process involves a synchronized delivery of blood components (plasma and leukocytes) to the site of infection or injury. This initial recognition of infection is mediated by tissue resident macrophages and mast cells, leading to the production of a variety of inflammatory mediators, including chemokines, vasoactive amines and bioactive products of proteolytic cascades (Medzhitov., 2008). When tissue homeostasis is disturbed chronically, interactions between immune cells can be altered leading to inflammatory disorders such as atherosclerosis and reperfusion reactions. Inflammation is also associated with many other disorders like acne vulgaris, autoimmune diseases, hypersensitivity, rheumatoid arthritis, amongst others (de Visser *et al*., 2006).

Humate have been used for the last 3000 years as folk remedies for a broad diversity of illnesses. Their therapeutic properties include antibacterial, anti-inflammatory, pulmorary tuberculosis, antitoxic, they have also been used for chronic radiculites, plexitises, rheumatoid arthritis, rhinitis and to increase the capacity of human mononuclear leukocyte from patients with coronary artery disease to induce neovascularisation in the local graft-versus-host reactions (Schepetkin *et al*., 2002).

Extensive research has been carried out on humates at the University Pretoria, Department of Pharmacology (South Africa), where studies have focused on the immunostimulatory properties of synthetic humates where it was found that oxihumate possesses both immunostimulatory and anti-viral activity (Jooné *et al*., 2002), the *in vitro* anti-inflammatory properties, where it was shown that potassium humate (Ph) exerts it anti-inflammatory properties either by direct blocking of an adhesion molecule, by

deactivating phagocytic stimulants such as bacterial peptides or inhibiting the complement receptor 3 (Jooné and van Rensburg., 2004). A study done by van Rensburg *et al* (2007) showed that Ph is effective in the treatment of contact hypersensitivity in rats. Badenhorst *et al* (2008) proved that Ph is effective in the treatment of osteoarthritis of the knee in humans, another study showed that Ph inhibits the production of TNF-α, IL-1β and IL-6 by stimulated mononuclear lymphocytes and increased lymphocyte proliferation (van Rensburg and Naudé., 2009) and a study done by Naudé and Cromarty (2010) found that when given by gavage reduced the paw volume of carrageenan induced oedema in rats similar to indomethacin.

Local inflammation is often a significant part of an effective immune response. The resolution of the inflammatory state can result in significant tissue damage and fibrosis, generally this damage is physiological and tolerated, however organ dysfunction and scarring can occur if inflammation is intense and chronic (Chaplin., 2006). Non-steroidal drugs are commonly used as anti-inflammatory agents and mediate their effect through the inhibition of cyclooxygenases activity. Even though the therapy is effective it is associated with many side effects such as gastro-intestinal irritation, ulceration and kidney impairment (Nantel *et al*., 1999)

This study focused on evaluating the efficacy, safety and possible mechanism of action of Ph combined with selenium (Se) by determining the cytotoxicity on lymphocytes using the MTT assay and the antioxidant properties using both ORAC and DCFH-DA assays. The DCFH-DA assay was done using human hepatocarcinoma cells ($HepG_2$). The $HepG_2$ cell line was chosen for this assay because the cells retain their morphology and most of their function in culture and because the cell line is a reliable model used to assay many dietary antioxidants and conditions with only minor interassay variations (Cuello *et al*., 2007). The quantification of the expression of surface complement receptor 3 receptors (CR3) was done using flow cytometry, with the investigation of the *in vivo* anti-inflammatory properties carried out using the carrageenan induced rat paw oedema as a model and lastly the evaluation of the acute phase proteins, substance P (SP) and C-reactive protein (CRP) using ELISA.

Literature review

Potassium humate

1.1 Characteristics of humates

Humic substances (HS) are natural organic compounds consisting of about 50 to 90 % of the organic matter of peat, liginites, sapropels and the non-living organic matter of soil and water ecosystems (Kulikova *et al*., 2005) . HS differ with respect to their origin and consist of a variety of molecular structures, depending on the source of organic material involved in the humification process. The synthesis of biopolymers is achieved in accordance with the genetic code in living cells. There is no established program of any kind in the process of humification, therefore any substance can materialize, both simple and complex than the than the initial biomolecules (Schepetkin *et al*., 2002, Steinberg *et al*., 2003). The secondary product again undergo the synthesis reaction or decomposition and this process is continueous, only the most stable compounds are accumulated, as a result of the numerous reactions in the humus matter. Examples of biogenic sediments formed mainly from plants include peat, sapropel and mumie. Peat is the organic soil produced as a result of partial disintegration and humification of dead marsh plants in conditions of high humidity. The organic matter of peat in 90% consists of humin, humic and fulvic acid (up to 40%), lignin, polysaccharides, lipids, pectines, hemicelluloses and cellulose (Schepetkin *et al*., 2002).

According to the classical definition, HS are naturally occurring heterogeneous organic substances that are generally characterised as being yellow to black in colour, with high molecular weight and refractory. Based on their aqueous solubility, these mixtures of complex organic compounds that are usually split into three fractions, humic acid (HA), the fraction of HS that is insoluble in water under acidic conditions (pH<2) but soluble at higher pH values, fulvic acid (FA), the fraction of HS that is soluble in water under all pH conditions, humin the fraction of HS that is insoluble in water at any pH value(Cheng *et al*., 2002, Jooné *et al*., 2003, Jooné *et al*., 2004, Kulikova *et al*., 2005).

Humic acid (HAs) are subclass of HS, consisting of high molecular polymeric aromatic substances with complex structures of polyaromatic and heterocyclic chemicals, they are most abundant in the top 1-2 ft of earth's crust, where they intermingle with air and water. There are several functional groups found on the surface of HAs, these groups are amines, carboxylic, carbonyl, phenol, catechol and quinine and are chemically combined to cross link the molecular backbone. HAs can be soluble and chemically active at neutral or alkaline pH (Paciolla., 1999). These substances possess medium molecular size and their molecular weight is about 5000 to 100000 Dalton. They contain 33-36 % oxygen and 4 % nitrogen. Soluble HAs are available as either sodium humate or potassium humate (Islam *et al*., 2005).

Fulvic acids (FA) have the lowest molecular size of about 2000 Daltons and are soluble under all pH conditions, they possess the lowest nitrogen content (less than 4%) and the highest oxygen content (around 45-48%), as well as higher acid functional group content. These substances dissolve in dilute alkaline solutions and will not precipitate even if the solution turns slightly acidic (Islam *et al*., 2005).

Humins have the highest molecular size and a molecular weight of about 300 000 Dalton. They have the highest nitrogen content (around 4%) and the lowest oxygen content (32-%) (Islam *et al*., 2005).

1.2 The therapeutic properties of humates

Humates have been reported to possess a variety of therapeutic properties, these include antibacterial, antitoxic, antiulcerogenic, antiarthritic, antiallergic, immunomodulatory and anti-inflammatory (Jooné *et al*., 2004, Schepetkin *et al*., 2002, van Rensburg *et al*., 2002, van Rensburg and Naudé., 2009). In Europe humic acids are used as active substances in prophylactic and as therapeutically drugs in veterinary practice (Vašková *et al*., 2011). With regards to volume, therapeutic spectrum and tradition, the balneotherapeutic use of peat represents most significant medical application of HS. Long ago in Babylonia and the Roman Empire, the heavily degraded high moor peat, which is abundant in HS has

been used therapeutically, the healing effects of mud were already recognised by the inhabitants. In Europe in the early 19th century health clinics' specialities, mud baths were offered. In addition mud baths which consist of peat pulp, baths with suspended peat materials as well as drinking cures were also offered, the latter especially in case of gastric, intestinal or hepatic diseases. Mud therapy was traditional indicated for gynaecological and rheumatic diseases (Klöcking and Helbig., 2005). Studies done on peat extracts such as Torfot (a patented drug in Russia) and Tolpa Peat Preparation (TTP) (patented in Poland) indicate that Torfot which is a product of distillation of specific peat layers is useful in the treatment of patients with keratitis chorioretinitis and vascular and degenerative processes in the retina when administered in the form of hypodermic or subconjunctival injections. Torfot improves blood circulation and tissue regeneration; it also possesses antibacterial and anti-inflammatory properties. TTP was found to inhibit interleukin-1 release from patient with rheumatoid arthritis and suppressed lipid peroxidation in the human placenta's mitochondria (Schepetkin *et al*., 2002).

HS have the ability to inhibit mutagenesis and possess photoprotecting, free radical scavenging, anti-inflammatory and toxic compound removing properties that can inhibit cancer development (Pant *et al*., 2012). Mumie (Shilajit) is a semi-hard black resin; a product of long term humification of lichen as well as *Euphorbia* and *Trifolium* (clover) plants in mountain areas. In the water soluble fractions of mumie the main group of organic substances is fulvic acid. For almost 3000 years mumie has been used in folk medicine in a number of countries, this substance in commonly prescribed for diabetes, digestive disorders, nervous diseases, chronic bronchitis, genitourinary diseases, tuberculosis, asthma, bone fracture, anaemia and eczema (Schepetkin *et al*., 2003). Mumie has also been shown to possess anti-viral and bacteriostatic properties. The bacteriostatic and anti-inflammatory actions of this substance decrease the period of wound healing, granulation and epithelisation, as well as facilitates the process of wound cleaning from necrotic wounds (Schepetkin *et al*., 2002).

1.3 Anti-inflammatory properties of humates

The anti-inflammatory properties of humates have been extensively studied and are well documented in literature (Kühnert *et al.*, 1982, Ye *et al.*, 1985, Klöcking *et al.*, 1968, Salz, 1974, Klöcking 1994b, Schewe *et al.*, 1991). The anti-inflammatory properties of HA were described as being effective as that of dimethyl sulfoxide (DMSO), a well known drug carrier and anti-inflammatory agent by Kühnert *et al* (1982). Based on the lignin derived humic acid theory Klöcking *et al* (1968) attributed the anti-inflammatory action of HA to the polyphenolic structure and Salz (1974) ascribed it to the fact that it stimulated higher blood flow. A study by Gau *et al* (2000), revealed that the activation of NFκB by lipopolysaccharide (LPS) was inhibited by HA and that HA slightly reduced NFκB binding to DNA, but at a concentration of 100 μg/ml completely inhibited the degradation of IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha). By masking the nuclear localization signals (NLS) of NF-κB proteins and keeping them sequestered in an inactive state in the cytoplasm IκBα inhibits NF-κB. It also blocks the ability of NF-κB transcription factors to bind to DNA, which is required for NF-κB's proper functioning. NF-κB is a protein complex that controls the transcription of DNA, it is involved in cellular responses to stimuli such as stress, cytokines, free radicals, and bacterial or viral antigens. Incorrect regulation of NF-κB is associated with cancer, inflammatory and autoimmune diseases, septic shock, viral infection, and improper immune development.

Work done by Klöcking (1994b) showed that synthetic humic acids strongly inhibited the lipoxygenase enzyme from rabbit reticulocytes, while HA weakly inhibited prostaglandin H synthase from sheep vesicular glands. Klöcking later provided a feasible biochemical explanation of the anti-inflammatory actions of humic acids. In a study by Jooné and van Rensburg (2004) it was shown that humate block the expression of complement receptor 3 (CR3) by activated neutrophils. The production of many reactive oxygen species, cytokines, nitrogen intermediates and proteolytic enzymes that can cause tissues damage and even lead to autoimmune disease is as a result of the over expression of CR3. These authors go further and show that the anti-inflammatory properties of humate may also be attributed to the ability humate to also inhibit both eosinophil cationic protein (ECP) and

Myeloperoxidase (MPO) by stimulated neutrophils and eosinophils. MPO play a vital role in inflammation, it is responsible for amplifying the oxidizing potential of H_2O_2, utilising it as a co-substrate to generate pro-inflammatory free radical species. ECPs are cytotoxic proteins that accumulate in tissues involved in allergic reactions, e.g. in asthmatic patients ECP infiltrate the bronchial tissues of the lungs, causing inflammatory damage to the adjacent tissues (Venge 1990). In recent studies Ph has been shown to suppress ear swelling in contact hypersensitivity animal model which was comparable to prednisolone (Cromarty, 2003). A study by van Rensburg et al (2007) showed that brown coal derived humate reduced DNBF-induced contact hypersensitivity in rats at a dosage of 61 mg/kg BW, administered by gavage directly after the sensitization step and continued for 8 days. The authors attributed these results to either an inhibition of the sensitization step or an inhibition of the challenge step or both. In another study potassium humate (Ph) (60 mg/kg BW) and indomethacin (10 mg/kg) administed by gavage in rats significantly decreased the carrageenan induced inflammation over the 300min period from as early as 60min after the carrageenan injection (Naude *et al.*, 2010). van Rensburg and Naude (2009) demonstrated that Ph inhibited the classical and alternative complement pathways in a dose related manner from 10 µg/ml and higher, these authors went further and showed that Ph also blocks the production of pro-inflammatory cytokines, Tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) by Phytohaemaglutinin (PHA) stimulated mononuclear lymphocytes (MNL)

1.4 Negative aspects of potassium humate

Humic acids have been shown to suppress the activation of NF-κB by LPS, they also have been associated with the complete *in vitro* inhibition of the degradation of IκBα at a concentration of 100 µg/ml and the slight reduction in the binding of NF-κB to DNA (Gau *et al.*, 2000). These authors also suggest that the possible explanation for the causes of the infection and inflammation observed in patients with Blackfoot disease may be due to humic acid suppression of the immune or inflammatory reactions of human vascular endothelial cells to endotoxins. In a study by Hseu *et al* (2000) humic acid was shown to induce echinocyte transformation through oxidative stress and damage to cytoskeletal proteins. Another study by these authors concluded that humic acid induced apoptotic

cell death of endothelial cells using Ca^{2+} and oxidative stress as mediators (Hseu *et al*., 2002)

Selenium

1.1 Characteristics of selenium

Selenium (Se) is a trace element that was discovered in 1817 by Jons Berzelius, who named it after the Greek goddess of the moon Selene (McKenzie *et al*., 1998, Pappa *et al*., 2007). Se in now acknowledged as a trace element of fundamental importance to human health, animals and some bacteria (Pappa *et al*., 2007, Valdiglesias *et al*., 2010). Se is most abundant in soil, entering the food chain through integration into vegetable protein as the amino acids selenocysteine and selenomethionine (McKenzie *et al*., 1998). It was discovered to be a vital cofactor of the enzyme gluthathione peroxidase in the 1970's and is present in one of three forms in the body; firstly as selenoproteins, secondly bound to non-specific plasma proteins and thirdly in organic forms such as selenites, alkyl selenides and elemental Se which are in transit thoughtout the body (Calello., 2010). Se is utilised in more than 25 selenoproteins to exert biological functions in humans, each categorised by the integration of Se into the primary protein sequence as the amino acid; selenocysteine. Some of these selenoproteins are selenoenzymes for example thioredoxin reductase and glutathione peroxidase and iodothyronine 5-deiodinases (Valdiglesias *et al*., 2010, Wu *et al*., 2009).

Glutathione peroxidises (GPx), the best studied of these enzymes is glutathione peroxidase (GPxs) which catalyses the elimination of hydrogen peroxide and organic peroxides (R-O-OH) using the oxidation of the reduced glutathione (GSH) as a substrate (Calello, 2010, Micke *et al*., 2009). The enzymatic activity of GPx has been shown to be dramatically reduced by the substitution of selenocysteine with normal cysteine at its active site. There are four characterised forms of GPx containing selenium. GPx-1 is found in the cytosol, GPx-2 which is gastrointestinal spefic, GPx-3 in the plasma and in the metabolism of phospholipid hydroperoxides GPx-4 performs special functions (Micke *et al*., 2009, Fairweather-Tait *et al*., 2011). GPx1-3 catalyses the reduction of

organic hydroperoxides and hydrogen peroxide, whereas GPx-4 can directly reduce cholesterol hydroperoxides and phospholipid hydroperoxides. GPx-1 and GPx-2 have well characterised antioxidant properties (Fairweather-Tait *et al*., 2011).

Thioredoxin reductases (TXNRDs), are involved in the control of cellular proliferation, cell survival and apoptosis through the control of thioredoxin (Trx) activity and redox state and plays a crucial role in biological response to oxidative stress. Three TXNRDs have been identified in mammals, TXNRD1 in the cytosol/nucleus, TXNRD2 in mitochondria, and thioredoxin glutathione reductase in the testis (Fairweather-Tait *et al*., 2011, Papp *et al*., 2007).

1.2 Therapeutic properties of selenium

In human health Se functions as a redox centre, as an antioxidant, in thyroid hormone metabolism, in reproduction and in immune function (Rayman., 2000). GPxs are scavengers of ROS, which implies that Se protects DNA from oxidative damage (Wu *et al*., 2009). The most researched and well documented example of this redox function to date is the reduction of hydrogen peroxide and phospholipid hydroperoxidases to non-toxic products (water and alcohols) by GPxs. This function aids to preserve membrane integrity, prevent prostacyclin production and decreases the likelihood of spreading of further oxidative damage to biomolecules such as unsaturated lipids, lipoproteins and DNA, each implicated in conditions such as atherosclerosis and cancer respectively (Calello., 2010, Rayman., 2000, Kiremidjian-Schumacher *et al*., 1994).

Se has been comprehensively studied for its antioxidant properties, studies show that Se compounds also possess pro-oxidant properties; they cause DNA damage and cell death, an activity that plays a vital role in the treatment of cancer (Ramoutar and Brumaghim 2010). Se supplementation, appears to have significant effects even in selenium-replete individuals, it has distinct immunostimutant effects, including an augmentation of proliferation of activated T cells, amplified cytotoxic lymphocyte-mediated tumor cytotoxicity and natural killer cell activity. Se supplementation seems to endorse

differentiation of CD4+ T-helper cells into T-helper-1 (Th1) rather than T-helper-2 (Th2) effector cells which has been shown to be beneficial in patients with allergic asthma (Rayman., 2012, Rayman., 2000, Hoffman *et al*., 2010, Wood *et al*., 2000, Kiremidjian-Schumacher *et al*., 2000, Hawkes *et al*., 2001, Broome *et al*., 2004).

The highest concentration of Se in found in the thyroid gland, where selenium-dependent iodothyronine deiodinases activates triodothyronine (T3) from its inactive precursor thyroxine (T4). Thyroid generate hydrogen peroxidise to be utilised by thyroid peroxidise in the synthesis of T_3 and T_4 from iodine and thyroglobulin. Se in the form of GPx-3 protects thyroid cells from hydrogen peroxidase, a role which is consistent with the inverse relationship detected between Se status and thyroid volume, thyroid tissue damage and goiter in French women and the positive association between low prediagnostic serum-selenium concentration and the incidences of thyroid cancer in Norway (Rayman., 2012).

1.3 Anti-inflammatory properties of selenium

Se can influence inflammatory response in a number of way, one being the inhibition of the NF-κB cascade, which is responsible for the production of interleukins and tumor necrosis factor-α (TNF-α) (Kertz-Remy *et al*., 2001). The is a link between decreases Se levels and chronic inflammatory disorders, and cross-sectional case-control studies indicate that patients with inflammatory disorders such as cystic fibrosis, acne and inflammatory bowel disease may be as a result of lower selenium status than healthy controls, therefore through increasing antioxidant activity and suppressing inflammatory conditions supplementation with Se could possibly improve some of the symptoms of such disorders (Michalke., 2004). Selenium has also been identified as a potential therapy for rheumatoid arthritis (RA) patients, because of the low Se status in RA patients (Tarp., 1995). In a study by Desai *et al* (2010) where Se was incorporated into two derivatives of celecoxib (selenocoxib-2 and selenocoxib-3) it was shown that selenocoxib-2 had a more pronounced anti-inflammatory response in macrophages than celecoxib in terms of the

inhibition of NF-κB activation and consequent downregulation of expression of a number of downstream pro-inflammatory genes at the transcription level.

1.4 Negative aspects of selenium exposure

Despite the requirement for Se in the diet, excess Se can elicit adverse reactions. Although these adverse events due to Se exposure are uncommon, chronic exposure to Se compounds is associated with several adverse health effects in humans. Following dietary exposure of around 300 µg/day, toxicity is seen on the endocrine system especially on the synthesis of thyroid hormones; toxicity is also seen in the metabolism of growth hormone and insulin-like growth factor-1 (Vinceti *et al*., 2001). There have been several claims that Se could be a teratogen, several miscarriages and an infant born with bilateral clubfoot were reported amongst female technicians exposed to selenite powder. (Fan and Kizer., 1990). In laboratory animals, livestock and humans, selenosis can occur following long-term exposure to Se concentrations as low as 5 mg Se/kg of diet (5 ppm) (Koller and Exon., 1986)

Inflammation

The inflammatory process is a response that encompasses a complex sequence of tissue reactions. Inflammation is an innate and adaptive reaction that is initiated by noxious stimuli and conditions such as infection and tissue injury. A controlled inflammatory response is beneficial in providing protection against infection but can become harmful if disregulated or inappropriate (Medzhitov *et al*., 2008). It is a highly coordinated multiphase process triggered by infections or tissue injury and is the product of a sequence of tightly regulated overlapping events characterized by several checkpoints and numerous mediator molecules (Barton., 2008, Bei *et al*., 2008, Medzhitov., 2008). The reaction to tissue injury is a multifunctional network of chemical signals which will initiate and maintain the host response selected to heal the afflicted tissue. This involves activation and recruitment of leukocytes from the vascular system to the site of damage (Coussens and Werb., 2002). Leukocyte recruitment into tissues is the hallmark of all

types of inflammatory responses (Graça-Souza *et al*., 2002). The migration of leukocytes through the vascular endothelium and their accumulation in the damaged tissue together with protein-rich plasma is initiated by the local production of inflammatory mediators that cause blood vessel dilation and amplifies endothelial permeability. These mediators include vasoactive amines such as serotonin and histamine, bradykinin as well as prostaglandins and other arachidonic acid derivatives (Bei *et al*., 2008). Initially neutrophils accumulate into the damaged tissues and are believed to be the cells coordinating the recruitment of other inflammatory cells to these sites of injury (Coussens and Werb., 2002) through extensive degranulation and significant respiratory burst processes that causes the extracellular production of new soluble mediators, such as proteinases and reactive oxygen (Bei *et al*., 2008).

There are other steps involved in the initiation of inflammation; these steps include the activation of L-, P- and E-selectins which are members of the selectin family of adhesion molecules responsible for a number of responses 1) the triggering of signals that stimulate and upregulate leukocyte intergrin expression mediated by cytokines and leukocyte-activating molecules, 2) the attachment of neutrophils on the surface of the vascular endothelium using tight adhesion through $\alpha_4\beta_1$ intergrins binding to endothelial vascular cell-adhesion molecule (VCAM-1) and MadCAM-1, 3) transmigration through the endothelium to sites of injury, thought to be facilitated by extracellular proteases such as matrix metalloproteinases (MMPs) (Coussens and Werb., 2002, Furie and Randolph., 1995).

At this early stage of inflammation, both cytokines and chemokines direct the advancement of the inflammatory reaction. Tissue damage stimulates the synthesis of the major inflammatory and immunomodulatory cytokines, including interleukin 1 (IL-1), tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ), IL-6, IL-13 and IL-17. These cytokines then stimulate the release of different chemokines by stromal and inflammatory cells, thereby activating a polarized response (Bei *et al*., 2008, Mantovani *et al*., 2007). Localised stimulated lymphocytes will induce the recruitment of monocytes, the monocytes in turn differentiate into macrophages in tissues and once triggered the

macrophages will produce proteinases, eicosanoids, cytokines, reactive oxygen (ROSs), and reactive nitrogen species (RNSs) (Coussens and Werb., 2002, Louis *et al*., 2005). Another key mediator in inflammation is nitric oxide (NO). NO is produced via the oxidation of the terminal guanidine nitrogen atom of L-arginine by the enzyme nitric oxide synthase (NOS). During an inflammatory response it increases vascular permeability and oedema by changes in the local blood flow as well as increase the release of pro-inflammatory prostaglandins (Salvemini *et al*., 1996). It is commonly understood that controlled inflammatory response is beneficial, for example in providing protection against infection, but can become harmful if disregulated for example in septic shock (Medzhitov., 2008).

The immune system

The immune system is an extremely evolved biological system which identifies and eliminates foreign pathogens and damaged cells (Farmer and Packard., 1986). A successful immune response eradicates the assaulting antigen, and once clear of this stimulus, the response returns to near basal level. In addition to eliminating the antigen the immune system uses other mechanisms, initially to increase and later to down-regulate its activity (Delves and Roitt., 2000). The immune system is composed of two distinct compartments 1) the innate immune responses which are hard-wired responses that are encoded by genes in the host's germline, and 2) the adaptive immune responses that demonstrate post exposure specificity for selected target antigens through the antigen-specific receptors expressed on the surfaces of T and B lymphocytes (Chaplin., 2006).

The first line of defence against foreign materials is made up of the innate immune cells such as, dendritic cells (DCs), natural killer cells (NK), neutrophils, basophils, macrophages and mast cells. DCs, macrophages and mast cells are pre-stationed in tissues and function as sentinel cells that continuously monitor their microenvironment for signs of distress (Visser *et al*., 2006). When tissue homeostasis is disturbed, sentinel macrophages and mast cells immediately produce soluble mediators like cytokines,

chemokines, matrix metalloproteases and reactive oxygen species (ROS), as well as bioactive mediators like histamine, that initiates the cell mobilisation and infiltration of additional leukocyte into injured tissues (Chaplin., 2006, Saadi *et al*., 2002) (Visser *et al*., 2006). The innate immune cells are unique because they possess the ability to promptly respond when tissue injury occurs without memory of prior assault or antigen specificity. (Visser *et al*., 2006)

Acute activation of these diverse immune-response pathways efficiently eliminates assaulting pathogens, damaged cells and extracellular matrix (ECM). Moreover, once invading agents are removed, the process of normalising cell-proliferation and cell death pathways is governed by immune cells, this is meant to enable re-epithelialisation and new ECM synthesis. Once inflammation resolves and wound healing is complete, tissue homeostasis returns. Immune cells exert multiple effector functions that are continually fine-tuned as tissue microenvironments change and because of their enormous plasticity. The immune system is intrinsically involved in retaining tissue homeostasis but has been implicated in the pathogenesis of many chronic diseases, such as arthritis, heart disease, Alzheimer disease and cancer (Chaplin., 2006, Delves and Roitt., 2000, Visser *et al*., 2006),

Motivation for the Study

In the quest to establish a safe and effective treatment for inflammation, various reports have been published on the safety and possible anti-inflammatory properties of potassium humate (Ph) as well as the antioxidant activity of Se, but the mechanism of action of neither of these compounds is well understood. The safety and efficacy when used in combination has not been established and there is no evidence suggesting that the combination of Ph with Se will yield a synergistic effect when administered orally or that it is an effective and safe treatment for inflammation.

This study assessed the combination of Ph with 0.2% Se AAC. These products are already available in health shops, are relatively cheap and are registered as a food supplement.

Aim

To evaluate the efficacy, safety and possible mechanism of action of potassium humate loaded with selenium (Phse)

Study objectives

- To evaluate the possible *in vitro* cytotoxic effect of Phse on the growth of primary cell cultures (lymphocytes).
- To evaluate the *in vitro* antioxidant activity of Phse
- To evaluate the *in vitro* effect of Phse on CR3 expression using mixed leucocytes
- To evaluate the anti-inflammatory properties of Phse using the carrageenan-induced paw oedema rat model.
- To evaluate the effect of Phse on acute phase proteins in the rat model.

Chapter 2

Introduction of techniques

Al l the solutions and recipes are given in Appendix I

2.1 Cytotoxicity of potassium humate with selenium

In vitro Cytotoxicity assessment is widely used by the pharmaceutical industry to screen for toxicity in potential therapeutic compounds. The development of *in vitro* Cytotoxicity assays has been driven by the need to rapidly evaluate the potential toxicity of compounds that are proposed for use as therapeutic agents at an early stage of development and whenever possible to limit animal experimentation, and to carry out numerous different tests using applicable concentrations with limited quantities of therapeutic agent as test compound.

Ph has been shown previously to have insignificant *in vitro* cytotoxic effects on resting lymphocytes but does cause proliferation of PHA- stimulated lymphocytes at concentrations of 20 µg/ml to 100 µg/ml in a dose related manner (van Rensburg and Naudé., 2009). This effect is similar to that reported for Se that increases proliferation of lymphocytes in response to mitogenic stimulation (Gill and Walker., 2008).

The MTT cell enumeration assay was used to evaluate the cytotoxicity of Ph loaded with Se by comparing treated to untreated lymphocyte populations in resting and PHA stimulated conditions. The MTT assay measures the relative reducing potential of the mitochondria of living cells using a colorimetric reaction. Viable cells with active mitochondria reduce the soluble, membrane crossing MTT reagent to form an insoluble highly coloured formazan product that is quantitated by spectrophotometric means. Comparison to control treatments has been proven to be an accurate assessment of cell numbers used to assess relative cell population growth or growth inhibition due to toxicity.

2.2 Anti-oxidant properties of potassium humate with selenium

In all respiring cells, oxidative stress can occur if high levels of ROS such as superoxide, hydrogen peroxide and hydroxyl radicals are formed. When activated, phagocytic leukocytes such as macrophages and neutrophils demonstrate a rapid increase in ROS due to the oxidative bust. Although ROS formation is a normal function of these phagocytic cells where the ROS play an important role in microbicidal activity, differentiation and for cell to cell communication, it is normally well regulated. (Hoffman., 2007). However, high levels ROS are cytotoxic capable of damaging lipids, proteins and nucleic acids of both target cells as well as in the host cell.

One of the major causes of intracellular injury is thought to be the excessive accumulation of ROS within several cellular regions that leads to cell aging and to age-related degenerative diseases such as cancer, brain dysfunction and coronary heart disease (Cuello *et al*., 2007).This study investigated whether Ph in combination with Se could improve both anti-inflammatory and anti-oxidant effects compared to Ph on its own. To evaluate the antioxidant potential of these compounds two assays were used, the ORAC assay and the 2´-7´-Dichlorodihydrofluorescein diacetate (DCFH-DA) assay.

The Oxygen Radical Absorption Capacity (ORAC) is a reliable, simple and sensitive method to quantitate the peroxyl radical absorbing capacity of antioxidants. The assay measures antioxidant inhibition of peroxyl radicals formed by oxidation and thus imitates classical radical chain breaking antioxidants activity propagated by H atom transfer. In the assay, the peroxyl radical reacts with a fluorescent probe (fluorescein) to produce a non-fluorescent product. The fluorescent probe reaction with peroxyl radicals results in the loss of fluorescence over time. To evade underestimation of antioxidant activity and to account for potential effects of secondary antioxidant products, the ORAC assay follows the reaction for extended periods. The assay delivers a controllable source of peroxyl radicals that models reactions in physiological systems and can be manipulated to detect both hydrophilic and hydrophobic antioxidants by changing the radical source and solvent (Cao *et al*., 1993, Dávalos *et al*., 2004, Prior *et al*., 2005)

2´-7´-Dichlorodihydrofluorescein diacetate (DCFH-DA) is a membrane permeable, relatively non-fluorescent molecule widely used for directly measuring the redox status of cells. It is extremely sensitive to changes in the redox state of the cell and can be used to track changes in ROS over time. After entry into the cell, intracellular esterase enzymes cleave DCFH-DA to form 2´-7´-dichlorodihydrofluorescein ($DCFH_2$) which is poorly membrane permeable and therefore trapped in the cells and has a low fluorescence. In the presence of hydrogen peroxide or nitrogen oxides, several enzymes like peroxidases, cytochrome c as well as ions like Fe^{2+} can oxidise $DCFH_2$ to 2´-7´-dichlorofluorescein (DCF) which is highly fluorescent. The oxidative increase of DCF in cells may be measured by an increase in fluorescence at 530 nm when the sample is excited at 485 nm. Fluorescence at 530 nm is anticipated to be proportional to the concentration of hydrogen peroxide in the cell (Cuello *et al*., 2007, Curtin *et al*., 2002).

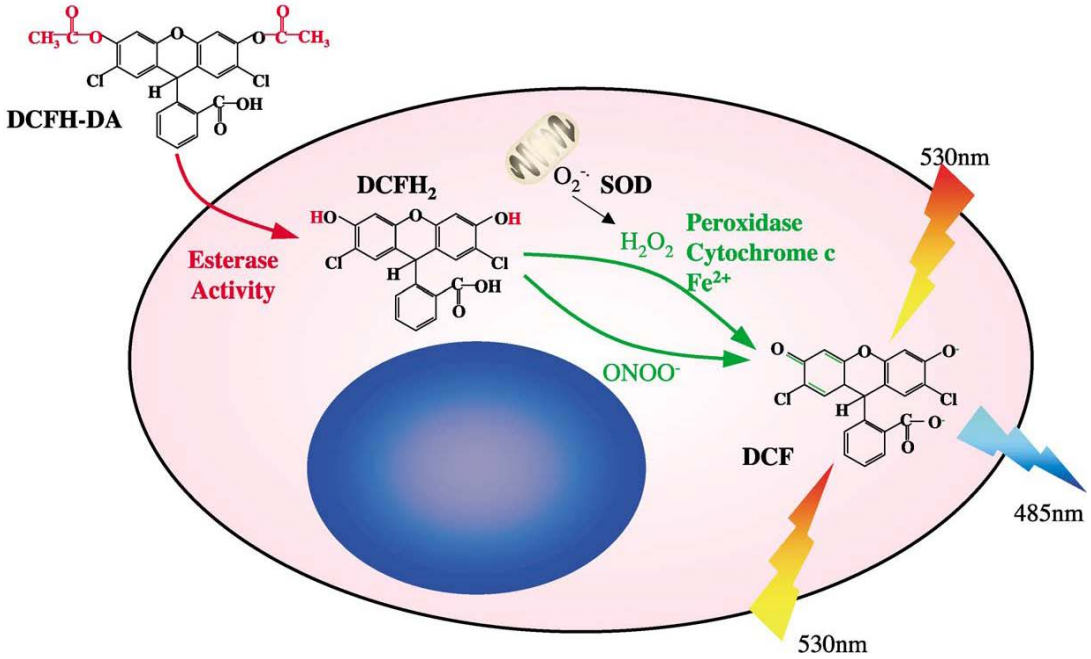

Figure 2.1: A diagram illustrating the mechanism of action of DCFH-DA in the assessment of intracellular oxidative species (Taken from Curtin et al., 2002 with permission)

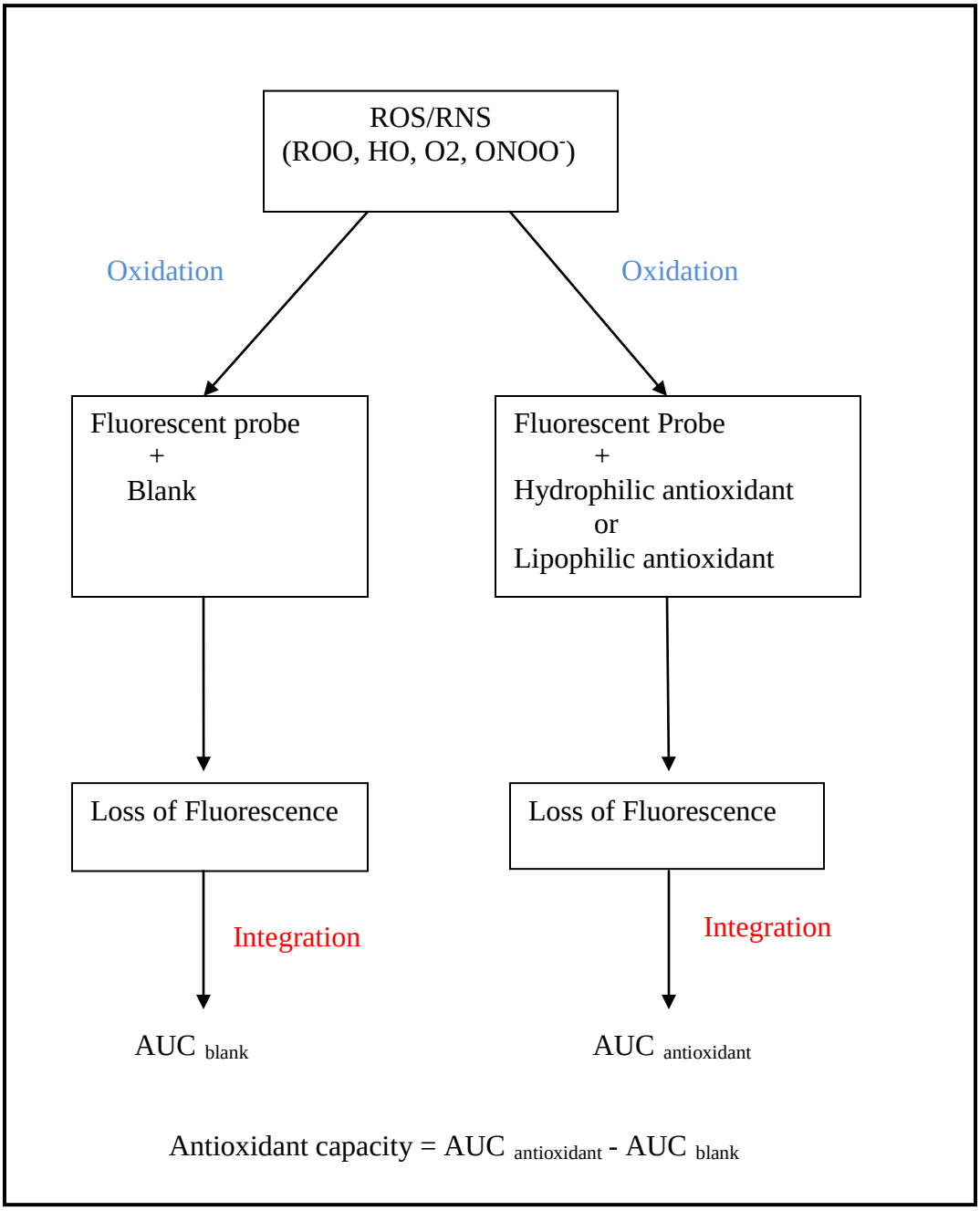

Figure 2.2: Schematic illustration of the principles of ORAC assay based on the descriptions by Haung et al., 2002 with slight modifications

2.3 Complement receptor 3 (CR3) expression

During the process of inflammation, neutrophils leave the vasculature to reach the region of inflammation using a process referred to as diapedesis. One of the early events during diapedesis is the adhesion of neutrophils to the endothelium. Cell-surface glycoproteins including the iC3b receptor (CR3) mediate this process of adhesion. CR3 is made up of a 165 kDa alpha chain (CD11b) that is non-covalently bound to a 95 kDa beta chain (CD18). CR3 is reported to be the most abundant adhesion molecule on mature granulocytes and literature suggests that it is the dominant adhesion-related molecule of human neutrophils (Phillips *et al*., 1988).

In phagocytic cells, CR3 is responsible for their ability to adhere to endothelial cells and migrate out of the vascular system to sites of infection. In neutrophils not all CR3 is expressed on the membrane but large quantities are stored in specific intracellular granules near the cell membrane. When neutrophils are exposed to phagocyte activating agents such as FMLP and PMA a rapid large increase in the expressed CR3 on the external membrane surface occurs due to surface expression of CR3 that is derived from the CR3 stored in the granules (Muto *et al*., 1993).

CR3 is the major adhesion-promoting receptor found on the cell surface of activated phagocytes, by inhibiting the recruitment of leukocytes into tissues. Agents that block CR3 are beneficial in the treatment of inappropriate inflammation (Jooné *et al*., 2004). In 2001, Jooné *et al* showed that a semi synthetic humate derived from bitumous coal, named oxihumate, inhibits the binding of treated neutrophils to transfected baby hamster kidney cells *in vivo* that over-express human E-selectin and ICAM-1 adhesion molecules. Jooné *et al*., (2004) went further and showed that the anti-inflammatory effects of oxihumate could be as a result of its inhibitory effects on the expression of CR3 by activated neutrophils as well as the adhesion of these cells to ICAM-1. Cromarty (2003) showed that the expression of CR3 molecule was intensely inhibited by refined water-soluble subfactions of oxihumate.

In this study the synergistic effect of Phse on the *in vitro* cellular expression of CR3 by neutrophils was quantified.

2.4 The carrageenan induced paw oedema rat model.

Carrageenan induced paw oedema rat model established in 1962 by Winter *et al* is a very useful model for the assessment of anti-inflammatory agents. Carrageenans are a family of linear sulfated polysaccharides that are extracted from red seaweeds. There are three types of carrageenan, which differ in their degree of sulfation, Kappa-carrageenan has one sulfate per disaccharide, Iota-carrageenan has two sulfates per disaccharide and Lambda carrageenan has three sulfates per disaccharide (Tateda *et al*., 1998).

In host biological system, this polysaccharide exerts a variety of activities, such as activation of Hageman factor, inhibition of complement and cytotoxicity to macrophages. In experimental conditions, carrageenan has been generally used as a tool to induce inflammation in animals (Tateda *et al*., 1998). After the subplater injection of 0.50 mg of carrageenan in rats, the accumulation of oedema fluid as a function of time is biphasic. The first phase starts immediately after injection of the irritant and diminishes in an hour. The second period characterised by accelerated oedema formation starts at the end of the 1st hour and last for about three hours (Vinegar *et al*., 1969).

Intraplantar injection of carrageenan produces peripheral inflammation leading to oedema which is reportedly accompanied by a marked accumulation of COX-2 mRNA and thromboxane in the affected paw, and also produces hyperalgesia with a fairly rapid onset (3-4 h) (Hay and Belleroche., 1997) but relatively short duration of about 9 hours. Studies have revealed that carrageenan has the ability to initiate the production of inflammatory cytokines such as interleukin-1 (IL-1), IL-6 and tumour necrosis factor (TNF) (Ogata *et al*., 1991, Tateda *et al*., 1995, Utsunomiya *et al*., 1991)
In the *in vivo* study reported in this dissertation, the anti-inflammatory properties of Phse were investigated using the carrageenan-induced paw oedema rat model of inflammation.

2.5 Acute phase proteins

Acute- phase proteins are defined as proteins whose plasma concentration surges (positive acute-phase) or declines (negative acute-phase) by at least 25 percent during inflammatory responses or disorders. The concentrations of acute-phase proteins change due to rapid changes in their production by hepatocytes. Infections, trauma, surgery, burns, tissue infarction, various immunologically mediated and crystal-induced inflammatory conditions or advanced cancer can lead to significant fluctuations in the plasma concentrations of acute-phase proteins (Gabay *et al*., 1999).

When the body experiences an immunological challenge the first reaction is the innate, non-specific response followed by specific immune reactions. The initial systemic reaction of the body to local or systemic disturbances in homeostasis is called the acute-phase response; this response can be triggered by an infection, tissue injury, trauma or surgery. The affected tissue itself will initiate a number of responses at the site of invasion by a micro-organism or the place of injury. The vascular system and inflammatory cells are initially activated by the release of pro-inflammatory cytokines. These reactions are accompanied by the production of other cytokines and other inflammatory intermediaries which diffuse to the extracellular fluid compartment and circulate in the blood (Gruyse *et al*., 2005). In response to these circulating pro-inflammatory cytokines distant tissue such as the liver produce large quantities of other inflammatory mediators, amongst which are the acute phase protein C reactive protein (CRP) A non-hepatic acute phase peptide is Substance P (SP) which is expressed by eosinophils, endothelial cells and macrophages.

CRP was the first described acute phase protein, and was discovered due to its ability to bind to the C-polysaccharide of pneumococci. It is a ring of five 23000 Da units (Gruyse *et al*., 2005). CRP is part of the pentaxin family of calcium-dependent ligand-binding plasma proteins. It is produced as part of the non-specific acute phase reaction to most forms of inflammation, tissue damage and infection (Pepys and Gideon., 2003). It is synthesised by the liver and has a normal plasma level of less than 1 μg/ml but can increase up to 1000-fold following tissue damage or infection during the first 24 to 48

hours, a response shown primarily to be due to the stimulation by interleukin-6 (IL-6) (Cermak *et al*., 1993, Padilla *et al*., 2003). An important function CRP is thought to be the amplification of the host defence system by increasing its recognition capacities, and activating the classical complement system C_{1q} pathway. It also binds to phosphocholine in bacterial cell walls as well as other anionic substrates and appears to stimulate removal of assaulting organisms and damaged cells (Cermak *et al*., 1993, Padilla *et al*., 2003, Blackburn., 1994). The circulating CRP value in most, though not all diseases is thought to reflect on going inflammation and/or tissue damage more precisely than other laboratory parameters of acute phase proteins (Pepys and Gideon., 2003).

SP was first discovered in the early part of the last century, originally isolated as a crude extract from equine brain and gut and found to have potent hypotensive, smooth muscle contractile and vasodilatory properties (Harrison and Geppetti., 2001, Goetzl *et al*., 1985). It is a neuropeptide with 11-amino acids that is produced by nerve endings in tissues and selected immune cells. Substance P expression is restricted to central nervous system and the peripheral nervous system. It is located in high concentrations in the dorsal root of the spinal cord; hence it is classified as a mediator of pain which plays a vital role in inflammatory states (Harrison and Geppetti., 2001, Bhatia *et al*., 2000, Tissot *et al*., 1988). The undecapeptide substance P is a member of the tachykinin family and acts via membrane bound NK-1 receptor (NK-1R) to elicit its effects (Bhatia *et al*., 2000, Hökfelt *et al*., 2001). The production of substance P from peripheral endings of C nerve fibres results in neurogenic inflammation, vasodilation and/or plasma protein extravasation (Tissot *et al*., 1988). Substance P has been shown to augment the severity of experimental adjuvant arthritis and to induce secretion of prostaglandin E_2 from synovocytes, cytokines from monocytes and histamine and leukotrienes from serosal mast cells and macrophages (Gilligan *et al*.,1994, Raychaudhuri *et al*., 1991). The levels of substance P were found to be high in experimental pleurisy rat models and in IL-1 induced inflammation in rabbit knees. Substance P has also been associated with the pathogenesis of rheumatoid arthritis. In man, the elevation of NK-1 receptors in the intestinal blood vessels and lymphoid tissues has been associated with inflammatory bowel disease (Harrison and Geppetti., 2001). NK-1 receptor antagonist (MK-869) was

reported to have antidepressant activity as effective as paroxetine which was the reference drug used (Hökfelt *et al*., 2001).

The evaluation of SP antagonists as potential therapeutic agents in the setting of clinical trials further emphasizes the need for accurate measurements of serum or plasma SP that have high levels of specificity, sensitivity, and reproducibility.

It has been shown that intraplantar administration of carrageenan increases prostaglandins (PGs) in the spinal cord which adds to the development of altered pain sensitivity and also prompts changes in the levels of mRNA and proteins encoded for by immediate early genes (IEGs) (Hay and Belleroche., 1997). IEGs are genes which are activated transiently and rapidly in reply to a broad variety of cellular stimuli, these genes are sometimes referred to as the gateway to genomic response. In their role as the gateway they generate transcription factors or other DNA binding proteins (Davis *et al*., 2003)

Chapter 3

Materials and Methods

3.1 Cytotoxicity assay

3.1.1 Isolation of a mixed lymphocyte population from human blood for cytotoxicity assay

This procedure was performed under sterile conditions in a laminar flow cabinet and is based on a method by Bøyum 1976.

Blanket ethical approval was obtained from the University of Pretoria Research Ethics Committee for the collection of blood from healthy volunteers. Venous blood was collected from healthy consenting adults into evacuated potassium EDTA tubes by venepuncture if small volumes (less than 50 ml) of blood were used. For larger volumes (more than 50 ml) blood was collected into FENWAL Blood–Pack blood bags to which 5 units of sterile preservative free heparin per ml blood had been added prior to collection.

Fifteen millilitres of Histopaque-1077 was poured into 50 ml sterile graduated plastic centrifuge tubes. A volume not exceeding 35 ml of the freshly collected blood was carefully layered onto the Histopaque. The tubes were centrifuged at 520 x *g* for 30 minutes at 20ºC after which the portion of the top layer containing the plasma and platelets was removed and kept for a source of plasma or discarded. The lymphocyte/monocyte layer was carefully removed and added into a new sterile tube and diluted to at least six times the volume with Roswell Park Memorial Institute Medium without deactivated foetal calf serum (RPMI-) medium, then centrifuged at 240 x *g* for 10 minutes. The supernatant was discarded and the tubes filled with cold 0.83% ammonium chloride to lyse any contaminating red blood cells and put on ice for 10 minutes. The tubes were centrifuged at 240 x *g* for 10 minutes and the supernatant discarded and the tubes filled with RPMI- and centrifuged again at 240 x *g* for 10 minutes. The supernatant was discarded and the cells resuspended in 1 ml of Roswell Park Memorial Institute Medium with 10% deactivated foetal calf serum (RPMI+). The cells were manually

counted (50 µl cell suspension added to 450 µl counting solution and allowed to stand for at least two minutes before counting in a Neubauer cell counting chamber at 400 x magnification) and the cell suspension further diluted to a concentration of approximately $2x10^6$ lymphocytes per millilitre of medium.

Two sterile 96 well plates were generally used per assay. They were divided into positive control wells, negative control wells and experimental wells, one plate was simulated with Phytohaemagglutinin (PHA) and the other was left unstimulated. From the prepared lymphocyte suspension, 100 µl was pipetted into the wells and incubated for an hour before adding 20 µl of different concentrations of Ph, Se and Phse respectively to the experimental wells. The negative control wells received 20 µl of medium, the stimulated wells 20 µl of PHA solution and to the resting cells 20 µl of medium. RPMI+ medium was added to the wells to bring the volume to 200 µl in each well. The plates were incubated for 3 days at 37°C and 5% CO_2 in a CO_2 incubator.

3.1.2 MTT assay

After the incubation period twenty microliters of a 5 mg/ml MTT in PBS solution was added to each well. The plates were re-incubated for 3-4 hr at 37°C in a $C0_2$ incubator. After the incubation period the plates were centrifuged at 520 x *g* for 10 min, the supernatant was carefully removed and the pellets washed with 150 µl PBS. After the wash step the pellets were treated with 100 µl dimethyl sulfoxide (DMSO) per well and the plates were gently shaken for 1 hr on a plate shaker. The plate was then read on a plate reader that spectrophotometrically measured each well individually at a wavelength of 570 nm (reference 630 nm). The data was corrected by subtracting the blank readings and expressed as a percent of the average mean of the untreated control cell readings.

3.2 Antioxidant assays

3.2.1 Kinetic evaluation of intracellular ROS concentration by fluorometery

To determine whether any intracellular ROS generation occurred over longer humate exposure periods, kinetic intracellular oxidant concentration assay experiments were conducted.

Intracellular ROS was detected according to the method of Zhang *et al*., 2009, with slight modifications.

After the 48 hr seeding incubation of HepG2 cells, 40 µl of 20µM 2´-7´-Dichlorodihydroflourescein diacetate (DCFH-DA) in PBS was added to each well and incubated for 1 hr at 37°C. The loading medium was then carefully removed and cells washed with 200 µl of PBS. Cells were kept hydrated by the addition of 50 µl of PBS followed by the addition of 50 µl of either PBS, 2,2´-azobis-2-2 methyl-propanimidamide dihydrochloride (AAPH) (300 µM) or test compounds (Ph 6.25 µg/ml - 100ug/ml, Se 0.096 µg/ml - 1.53 µg/ml, Phse 6.25 µg/ml - 1.53 µg/ml). After addition of the test compounds and controls, fluorescence was determined over a period of 3 hr at 37°C. Fluorescence was measured on a FluoroStar Optima fluorescence plate reader using λ_{ex}= 492 nm and λ_{em}= 525 nm at a gain setting of 750. AAPH without further treatment was used as a positive control (Ximenes *et al*., 2009)

3.2.2 Oxygen radical antioxidant capacity (ORAC) assay

This procedure is based on the method by Ou *et al* (2002) with slight modifications.

AAPH was used as a peroxyl radical generator, trolox as an antioxidant standard (0 - 1000 µM), fluorescein as a fluorescent probe and PBS as a blank. To 160 µl of 0.139 mM fluorescein working solution, 40 µl of PBS, or Trolox or different concentrations of Se (0.01 µg/ml-1.53 µg/ml) were added. This was followed by the addition of 40 µl of 0.11

µM AAPH. The plate was read for four hours the FluoroStar Optima fluorescent plate reader and the excitation wavelength of 485 nm and emission wavelength of 520 nm.

3.3 Complement receptor 3 (CR3) expression

3.3.1 Isolation of Neutrophils from human blood

The method used for the isolation of neutrophils is based on the Ficoll-Hypaque method that was first described by Böyum (1968). Briefly the method is as follows.

Venous blood was collected from healthy consenting adults into evacuated potassium EDTA tubes by venipuncture. Fifteen millilitres of Histopaque-1077 was poured into sterile 50 ml graduated plastic centrifuge tubes. A volume of 35 ml of freshly collected blood was carefully overlaid onto the Histopaque. The tubes were centrifuged at 520 x *g* for 30 minutes at 20ºC after which the portion of the top layer containing the plasma and platelets was removed and discarded or kept for further use of the plasma. The lymphocyte/monocyte layer was carefully removed together with the bulk of the Histopaque layer. The neutrophil layer which is directly above and in direct contact with the red blood cell concentrated below the Histopaque layer The neutrophils were gently removed with minimal inclusion of red cells and diluted to at least six times the volume with ice cold 0.83% ammonium chloride solution, allowed to stand on ice for 10 minutes during which time the red blood cells haemolysed, before centrifuging at 480 x *g* for 10 minutes at 10ºC to sediment the granulocytes. If there was still evidence of red blood cells in the pellet the ammonium chloride haemolysis step was repeated. The pellet was washed with RPMI supplemented with10% FCS, centrifuged at 480 x *g* for 10 minutes at 10ºC and resuspended in cold medium to approximately $1/10^{th}$ of the original volume of blood.

The cells were manually counted (50 µl cell suspension added to 450 µl counting solution and allowed to stand for at least two minutes before counting in a Neubauer cell counting chamber at 400 x magnification) and the cell suspension further diluted to a concentration of approximately 10 x 10^6 neutrophils per ml with RPMI+ medium.

3.3.2 Relative quantitation of complement receptor 3 (CR3) expression

Expression of CR3 by neutrophils was quantified by flow cytometric method based on the method originally reported by Rabinovitch and June (1990), adapted by Jooné (2002) and Cromarty (2003) with slight modifications.

Isolated neutrophils were made up to 1 x 10^7 cells per millilitre as described above. Two 5 ml plastic test tubes for each test compound, one for resting cells and one for stimulated cells, as well as equivalent control tubes were prepared by dispensing 50 µl of this cell suspension into 400 µl aliquots of RPMI 1640 medium containing 10% HI-FCS per tube and pre-incubated in a water bath at 37ºC for 15 minutes. The test compounds were replaced by HI-FCS fortified RPMI in the two negative control tubes; otherwise all additions were identical to the test compounds. After the pre-incubation period, 50 µl isolated various concentrations of test compound were added to both the tubes of cell suspension per paired set, gently mixed and incubated at 37ºC for a further 20 minutes. After the second incubation time the stimulated tube from each pair was treated with 50 µl of a 1.0 µg/ml solution of freshly prepared PMA in RPMI. To the paired resting cell tubes 50 µl RPMI was added alone. After a further 20 minutes incubation time the tubes were quickly vortex mixed and transferred to an ice bath and 100 µl aliquots removed and added to corresponding clean counting tubes to which 5 µl of PE conjugated anti-CD11b antibody had been added. Isotypic background controls were provided by the addition of 100 µl aliquots from the resting or stimulated control tubes to 5 µl PE conjugated isotypic anti-mouse IgG. After 10 minutes incubation time in a dark cupboard the suspensions were diluted to 600 µl with Isoflow® and the suspensions were analysed within an hour on a FC-500 flow cytometer (Beckman Coulter) equipped with an air cooled Argon ion laser. Time per analysis was set to 100 seconds or 50000 events. The neutrophil population in the scattergram was gated and this region analysed for mean fluorescent intensity. Relative quantitation of the surface exposed CR3 is directly related to the fluorescent signal measured and the mean peak position was used for the calculations.

3.4 An in vivo investigation of the anti-inflammatory properties of potassium humate with selenium

Localised oedema develops when carrageenan is injected into the foot pad of the rat due to the release of histamine and serotonin, which both play a major role in the initial development of the inflammatory response (Howland 2006).

3.4.1 Animal Study

Animal studies were conducted using the South African National Standard SANS 10386:2008 (The care and use of animals for scientific purposes). Ethical clearance for the use of the Sprague Dawley rats was obtained from the University of Pretoria Animal Use and Care Committee (H010-10).

In this study fifty four female Sprague Dawley (SD) rats, 12 weeks old, weighing 150 g - 200 g were used. The rats were housed at the UPBRC for at least one week to acclimatise before the study was initiated with *ad lib* access to drinking water and standard rat chow. Twelve hour day/night light cycles, with an environment set at 45 – 65% relative humidity and 20˚C were applied. All procedures were according to the standard operating procedures used at the University of Pretoria Biomedical Research Centre (UPBRC).

The rats were randomly allocated into weight matched groups into one of the following 6 groups:

1. Negative control group, ten SD rats.
2. Experimental group 1, ten SD rats.
3. Experimental group 2; ten SD rats.
4. Experimental group 3; ten SD rats.
5. Positive control group, ten SD rats.
6. Control group (no treatment), 4 SD rats

3.4.2 Experimental design and drug administration

A carrageenan-induced paw oedema model was used according to a method described by Naudé *et al* (2010) with slight modifications.

The rats were weighed prior to dosing on Day 1 to ensure that the correct dose was administered. A once daily dose by gavage for five consecutive days was dosed according to the experimental group to which the rats were assigned. The negative control group received 1 ml of water, the experimental group 1 received approximately 1 ml of 50 mg/kg body mass Ph, the experimental Group 2 received approximately 1ml of 0.92 mg/kg body mass Se, the experimental Group 3 received approximately 1 ml of a combination, Phse (50 mg/kg + 0.92 mg/kg) body mass. On the fifth day of the experiment, the initial right hind paw volume of each rat was measured with a water displacement plethysmometer and the submersion level marked on the skin. The positive control group received indomethacin (10 mg/kg) by oral gavage.

λ-Carrageenan (50 µl of a 2% solution in saline) was injected subplantar into the right hind paw and 50 µl saline into the left hind paw an hour after administration of the test compounds. The hind paw volumes were measured hourly from the time of injection for seven consecutive hours with a water displacement plethysmometer. At the end of the 7 hours the rats were anaesthetised using isofluorane and approximately 5 ml of blood was collected via cardiac puncture into evacuated potassium EDTA tubes from all fifty four rats (including the non-treatment control group). The blood was quickly centrifuged at 4˚C for 10 minutes at 520 x *g*, the plasma removed and frozen at -80°C until assayed. The blood cells were discarded. The rats were euthanized using an overdose of isoflurane and the rat cadavers disposed of using SOPs from UPBRC.

3.4.3 Plasma levels of acute phase proteins

The high-sensitivity rat C-reactive protein (CRP) ELISA kit was purchased from Kimaya Biomedical Company, Seattle, WA, USA, cat no: KT-099. The rat substance P ELISA kit

was purchased from Enzo Life Sciences, cat no: ADI-900-018. Both of these ELISA kits were used according to their manufacturer's protocols.

Plasma samples were thawed, vortex mixed, briefly centrifuged at 2000 *g* for 5 minutes and aliquots analysed for the two acute phase proteins using the respective ELISA kits using the enclosed manufacturer's protocols for plasma concentration determination. Results were calculated from calibration curves fitted using the supplied calibration standards then compared between the different treatment groups.

3.5 Statistical analyses

All experiments were performed in triplicates on three separate days. The results were expressed as mean ± SEM as determined by Graphpad Prism 5 software, unpaired t-test was used for the cytotoxicity assay and CR3 expression and the results from the antioxidant assay, animal study and acute phase proteins were attenuated through the use of one way analyses of variance (ANOVA) with a post-hoc Dunnetts test. Significance was noted as $p<0.05$

Chapter 4

Results

4.1 Cytotoxicity assay

Ph had no cytotoxic effect on resting lymphocytes up to a concentration of 100 µg/ml, but increased the proliferation of PHA-stimulated lymphocytes at concentrations above 10 µg/ml in a dose-related manner (Fig. 4.1A).

Similar results were observed with Phse (Fig. 4.1C).

Se showed no effect on the resting lymphocytes but did show a small proliferative response at the lowest concentrations from 0.1 µg/ml to 1.53 µg/ml (Fig. 4.1B) for the PHA stimulated lymphocytes.

A: Effect of Potassium humate on lymphocyte viability

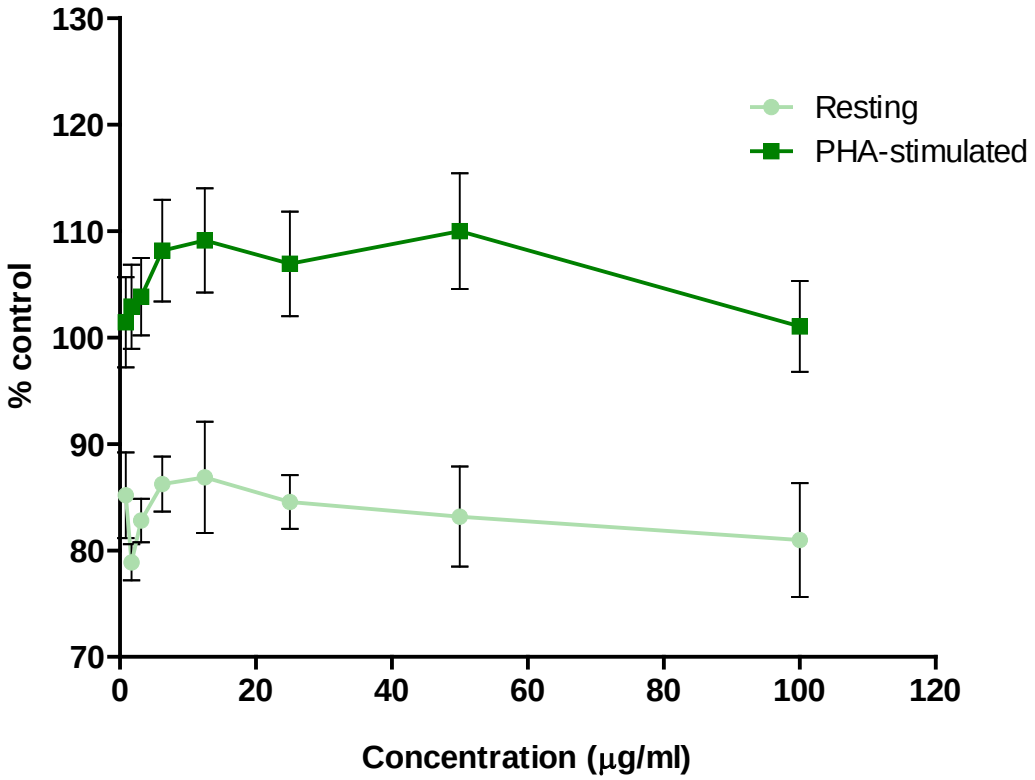

Figure 4.1A: The effects of 72 hr treatment with various concentrations of Ph on resting and PHA-stimulated lymphocytes. The results are expressed as percentage of control ± SEM. n = 30

B: Effect of Selenium AAC on lymphocyte viability

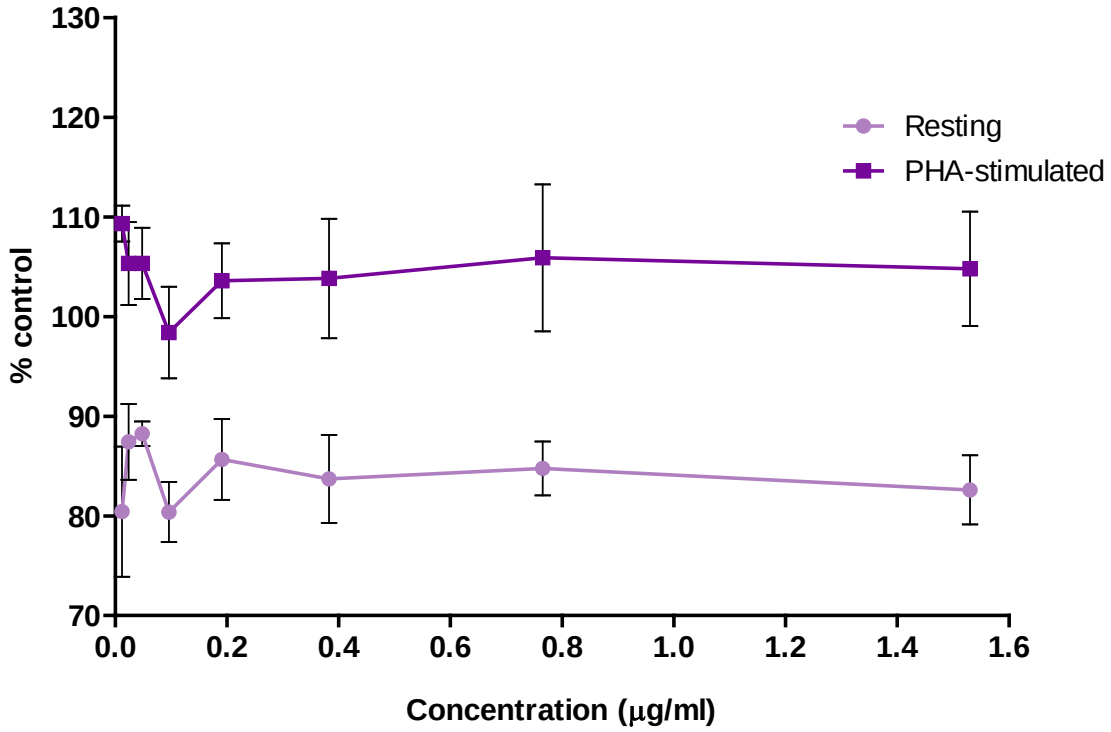

Figure 4.1B: The effect of 72 hr treatment with various concentrations of Se amino acid chelate on resting and PHA-stimulated human lymphocytes. The results are expressed as percentage of control ± SEM. n =30

C: Effect of Potassium humate plus Selenium on lymphocyte viability

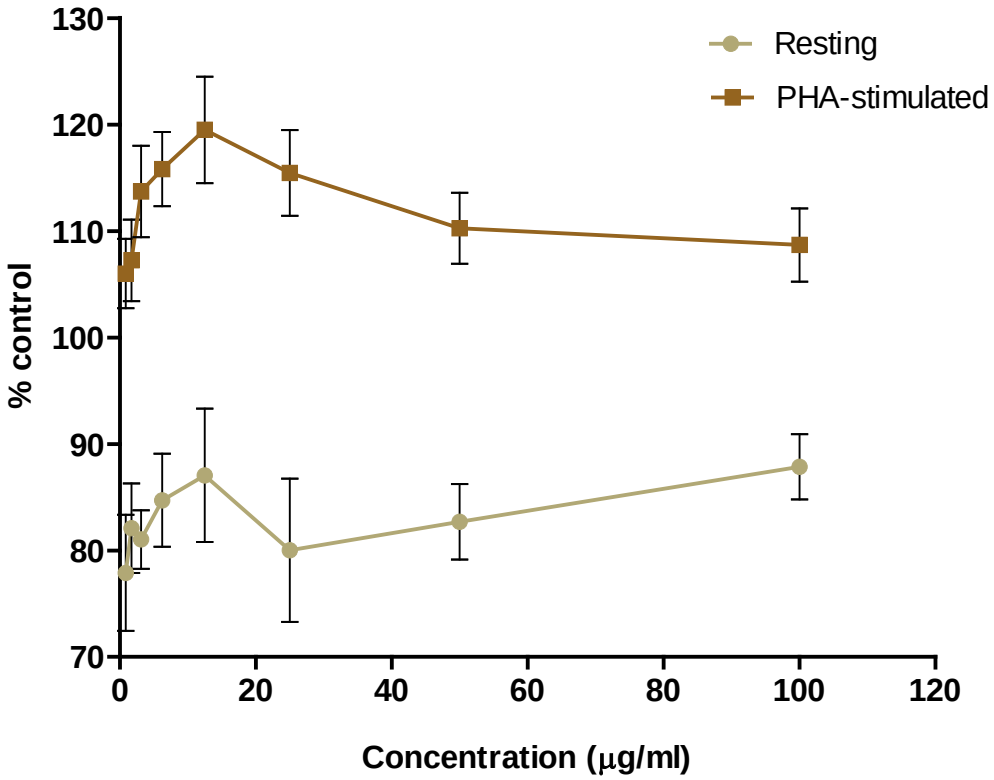

Figure 4.1C: The effects of 72 hr treatment with various concentrations of Phse on resting and PHA-stimulated human lymphocytes. The results are expressed as percentage of control ± SEM. n = 30

4.2 Antioxidant activity

The results of the DCFH-DA assay showed that Ph, Se and Phse showed no significant radical scavenging against AAPH at any of the tested concentrations, instead at concentrations of 50 µg/ml and 100 µg/ml Ph showed a slight pro-oxidant effect when compared to the oxidant (AAPH) used as the positive control (Fig 4.2A).

At concentrations of 0.191 µg/ml and 0.383 µg/ml Se showed slight protection against AAPH but this was not statistically significant ($p>0.05$) (Fig 4.2B).

The results observed with Phse were similar to those observed with Ph alone, where a pro-oxidant effect was seen at the two highest concentrations tested (Fig 4.2C). The radical scavenging capacity of each concentration of the test compounds was tested and the means were found not to be statistically significant. The results are expressed as relative fluorescence intensity.

The results of the ORAC assay (Fig 4.3) indicate that at the tested concentrations Se does not show protection against ROS generation when compared to Trolox. The results are expressed as net area under the curve.

The colour intensity of humic acid alone and in combination with selenium makes the ORAC assay invalid because of the quenching effect of potassium humate, hence the antioxidant activity of selenium alone was tested with this assay.

A: Kinetic assay of intracellular oxidant in HepG2 cells treated with Potassium humate and AAPH

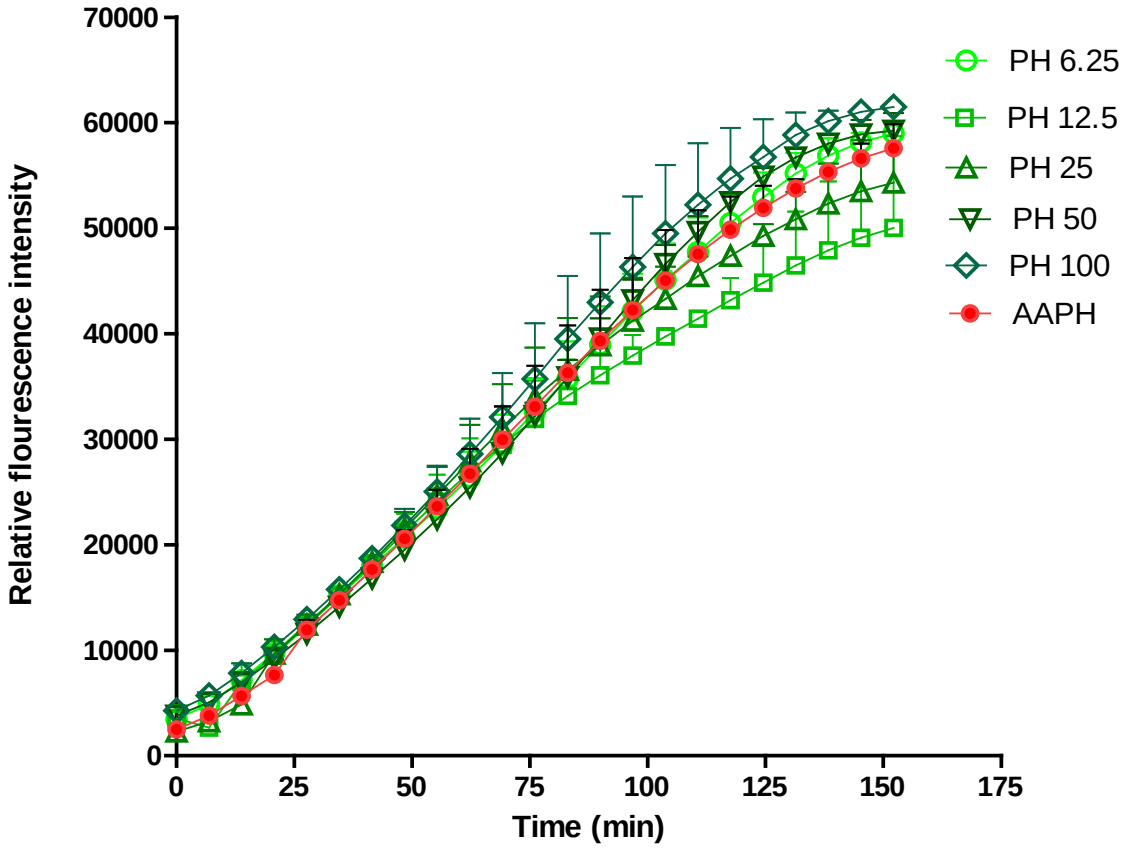

Figure 4.2A: The intracellular antioxidant activity of Ph in HepG2 cells using DCFH-DA assay (kinetic), expressed as relative fluorescence intensity over 2.5 hours. n = 12

B: Kinetic assay of intracellular oxidant in HepG2 cells treated with Selenium AAC and AAPH

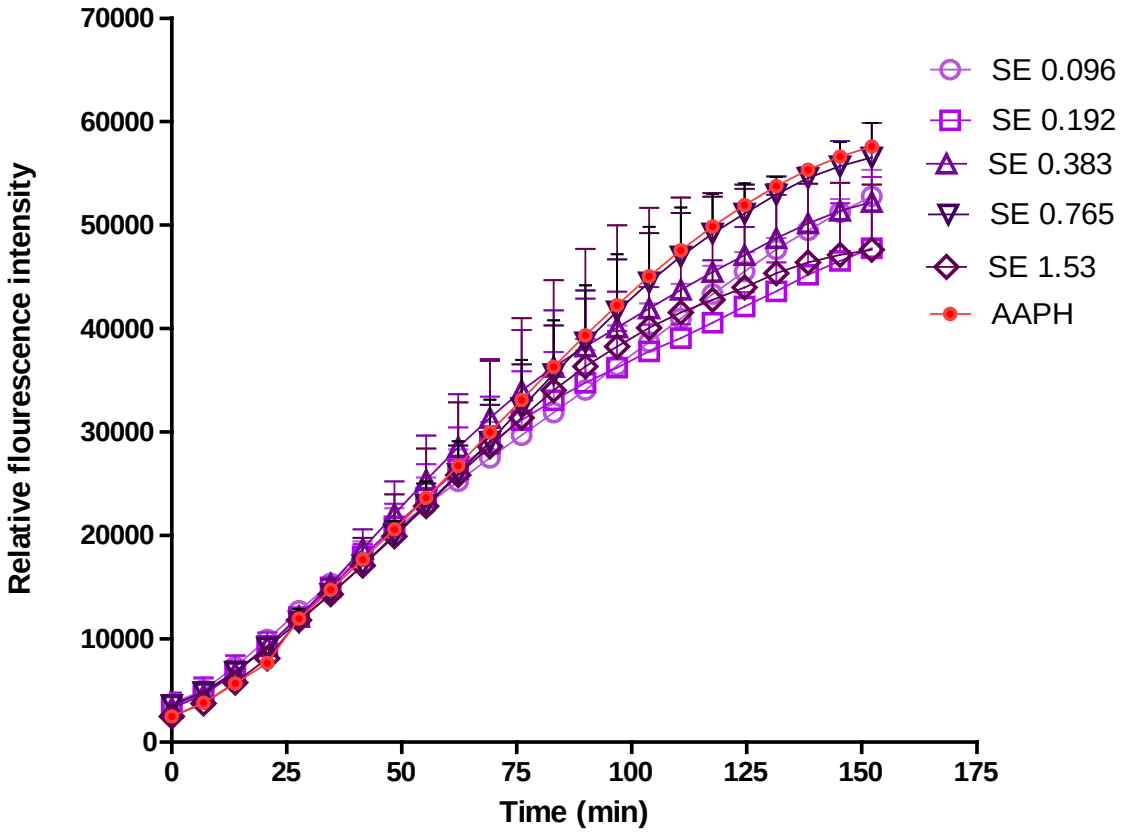

Figure 4.2B: The intracellular antioxidant activity of Se in HepG2 cells using DCFH-DA assay (kinetics), expressed as relative fluorescence intensity over 2.5 hours. n=12

C: Kinetic assay of intracellular oxidant in HepG2 cells treated with Potassium humate and Selenium AAC with AAPH as radical generator

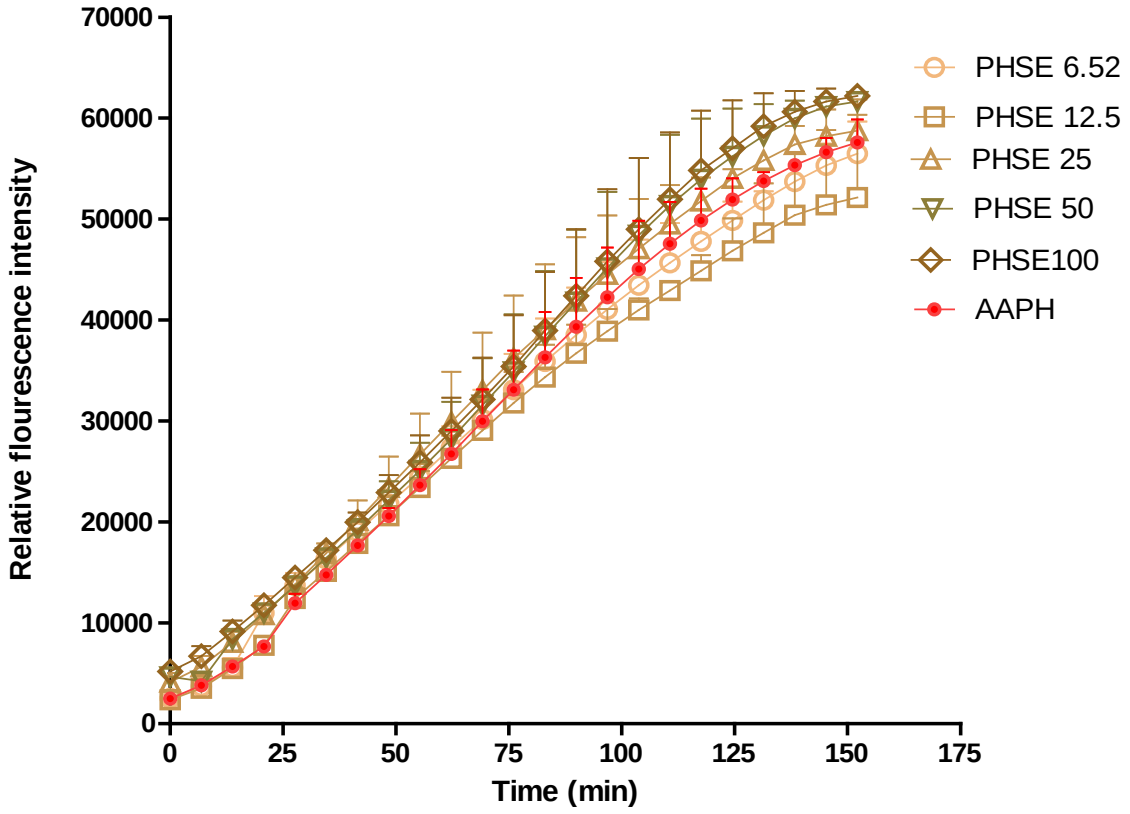

Figure 4.2C: The intracellular antioxidant activity of Phse in HepG2 cells using DCFH-DA assay (kinetic), expressed as relative fluorescence intensity over 2.5 hours. n = 12.

D: ORAC

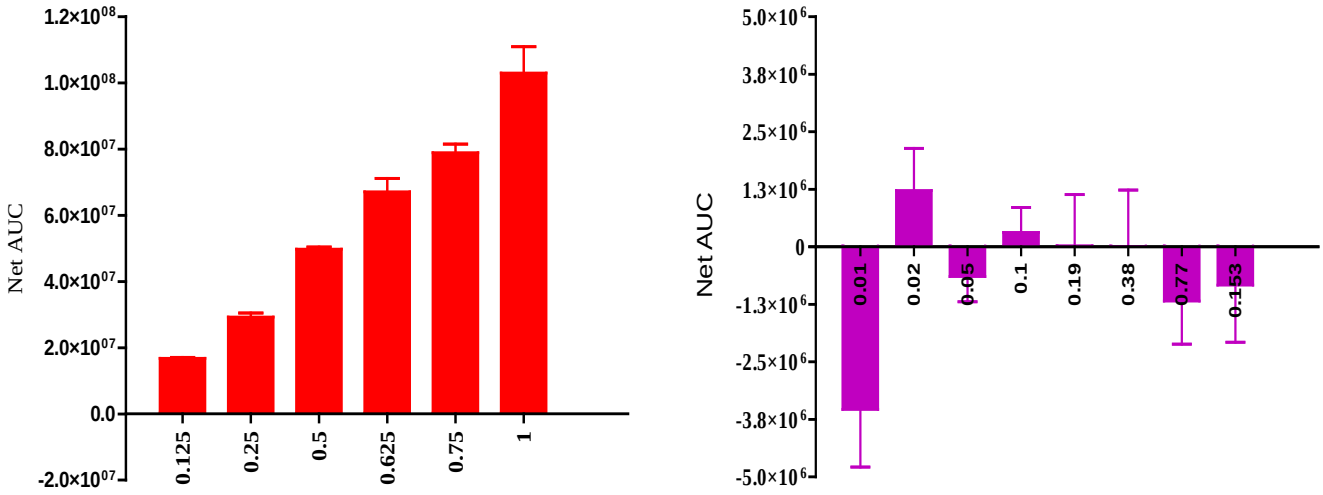

Figure 4.3: The antioxidant activity of various concentrations of Se AAC using the ORAC assay. The results are expressed net area under the curve (AUC). n = 12

4.3 CR-3 Expression

Ph caused a dose-related inhibition of CR3 expression by PMA stimulated neutrophils from 25 µg/ml and higher, but showed no effect on resting neutrophils. The inhibition was statistically significant when compared to the positive controls (Fig. 4.4A).

Similar results were observed with Phse (Fig. 4.4B) where an inhibition of CR3expression was observed for stimulated neutrophils from a concentration of humate above 12.5 µg/ml.

Se on its own also showed a statistically significant inhibition of CR3 from a concentration of 0.77 µg/ml and higher on PMA stimulated and had no effect on resting neutrophils at the tested concentrations (Fig 4.4C). The results are expressed as mean fluorescence intensity ± SEM of four different experiments.

A: Changes in CR3 expression by resting and PMA stimulated neutrophils after Potassium humate pre-treatment

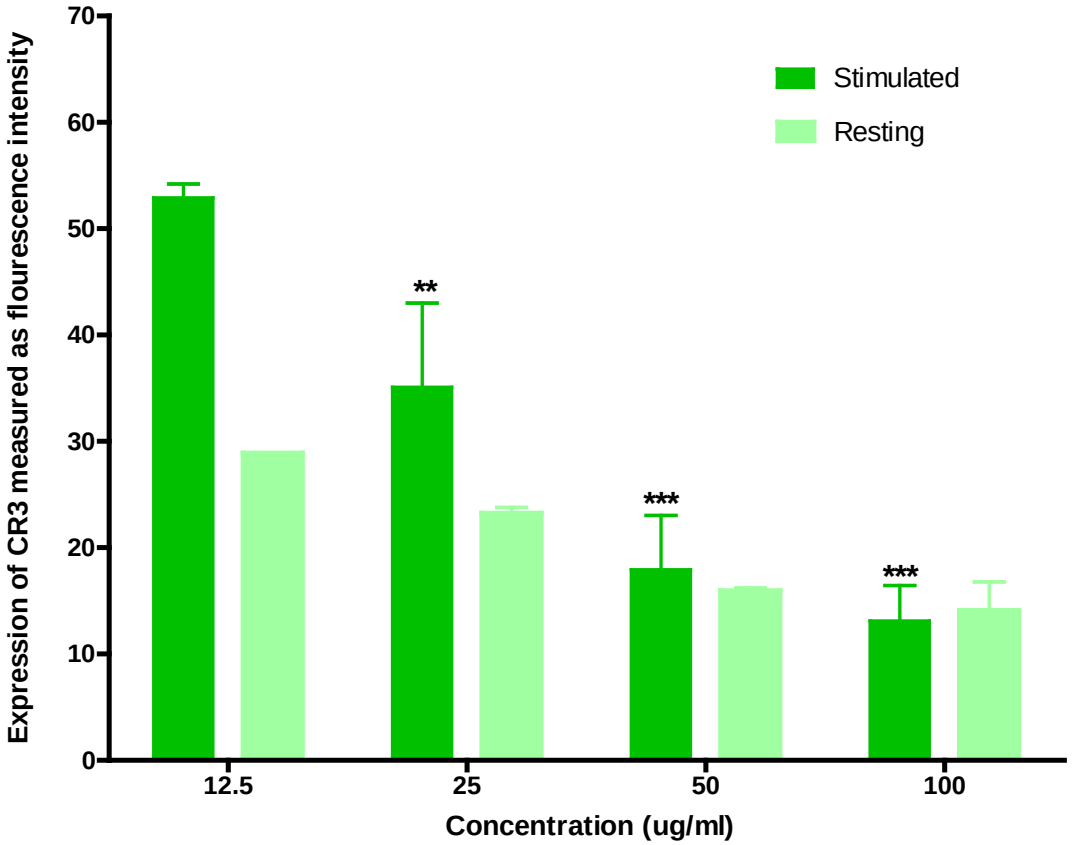

Figure 4.4A: The effects of 20-min treatment with various concentrations of Ph on the expression of CR3 on resting and PMA-stimulated neutrophils, determined by flow cytometer. The results are expressed as the mean fluorescence intensity ± SEM of four different experiments. n = 12. *p<0.05; **p<0.01; ***p<0.001

B: Changes in CR3 expression by resting and PMA stimulated neutrophils after Selenium AAC pre-treatment

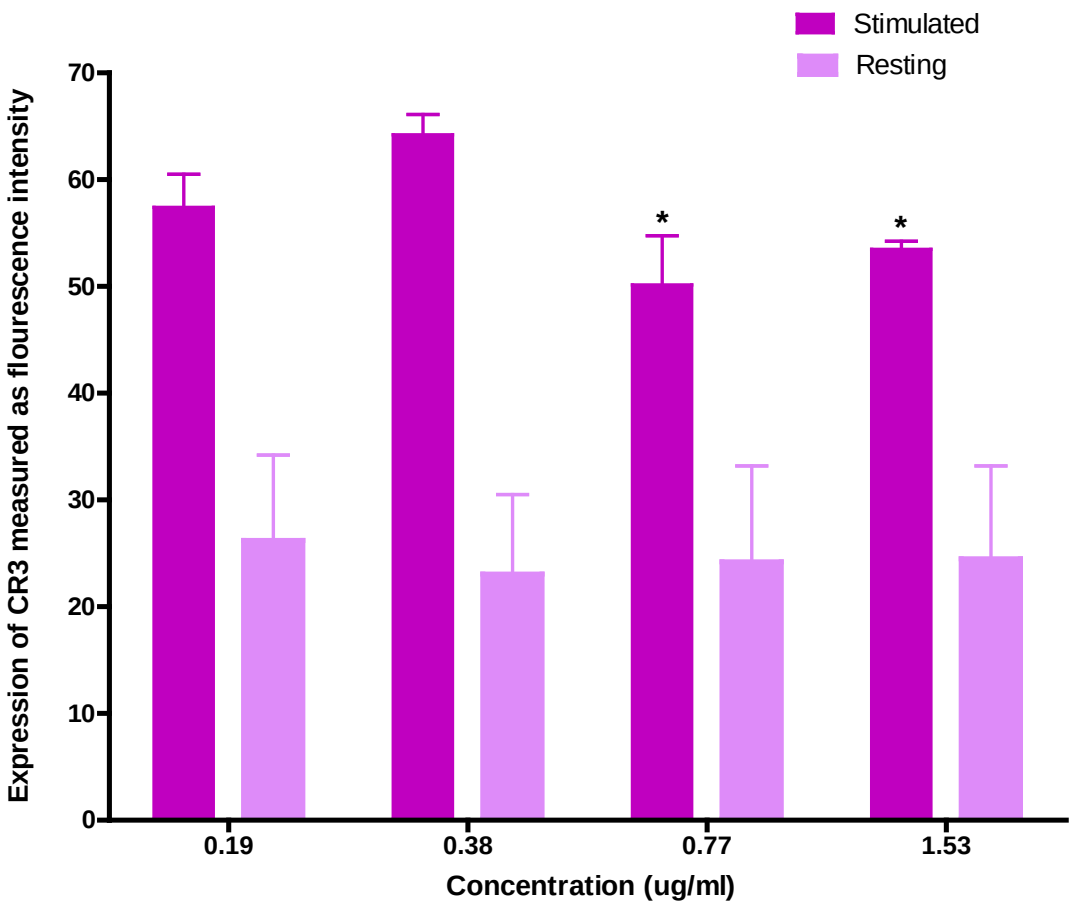

Figure 4.4B: The effects 20-min treatment with various concentrations of Se on the expression of on resting and PMA-simulated neutrophils determined by flow cytometer. The results are expressed as the mean fluorescence intensity ± SEM of four different experiments. n = 12. *p<0.05

C: Changes in CR3 expression by resting and PMA stimulated neutrophils after Potassium humate plus Selenium AAC pre-treatment

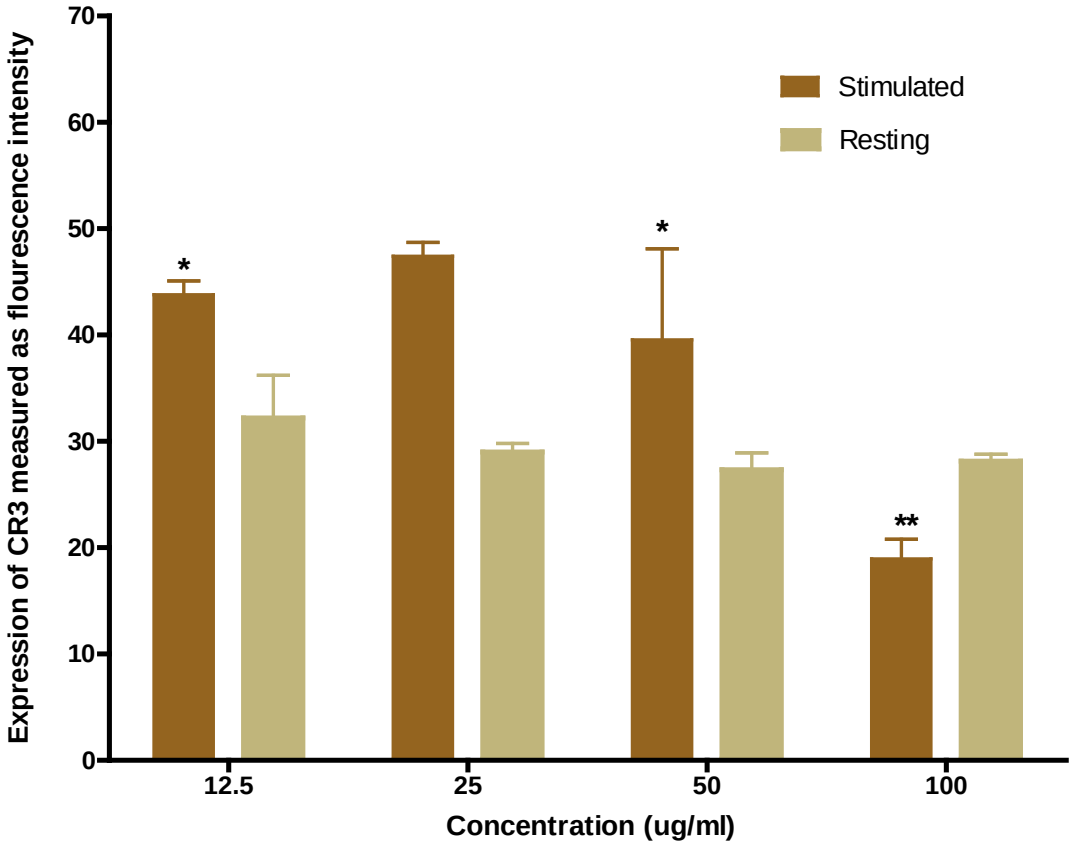

Figure 4.4C: The effects of a 20-min treatment with various concentrations of Phse on the expression of CR3 on resting and PMA-stimulated neutrophils, determined by flow cytometer. The results are expressed as the mean fluorescence intensity ± SEM of four different experiments. n = 12. *<0.05; **p<0.01

4.4 Animal study

To ensure that the reading of the water plethysmometer was consistent and acurate both the hind paws of the rats were marked just below the ankles. Those markings were used as levels to which the paws were plunged into the water for the volume measurements.

The results of the study show that indomethacin (positive control) caused a statistically significant decrease ($p<0.0001$) in the oedema volumes compared to untreated animals from Hour 1 through to Hour 7. At Hour 1 there was a 17% decrease, at Hour 2 a 20% decrease, at Hour 3 a 25% decrease, at Hour 4 a 14 % decrease, at Hour 5 a 21% decrease, at Hour 7 a 21% decrease. Ph statistically significantly decrease the paw oedema volume in a similar manner to indomethacin from Hour 2 to Hour 7, at Hour 2 it showed a 21% decrease in paw oedema volumes, at Hour 3 a 15% decrease ($p<0.001$), at Hour 4 a 10%, at Hour 5 a 16%, at Hour 6 a 14% and at Hour 7 a 15% ($p<0.05$)

Se only caused a 10% decrease in paw oedema at Hour 5, while at all other time points treatment with selenium AAC caused an increase in paw oedema volume which was even greater than that of the untreated negative control.

Phse's effect was inconsistent at Hour 1 it caused a statistically significantly 10% ($p<0.05$) decrease in paw edema volume but from Hour 2 to 4 it shows an increasing trend, then a 14% ($p<0.05$) decrease again at Hour 5 then increasing again at the end of the study

The results of this study are illustrated as paw volume differences between the left and right paw volumes (delta values). The respective experimental groups were compared to the negative control group.

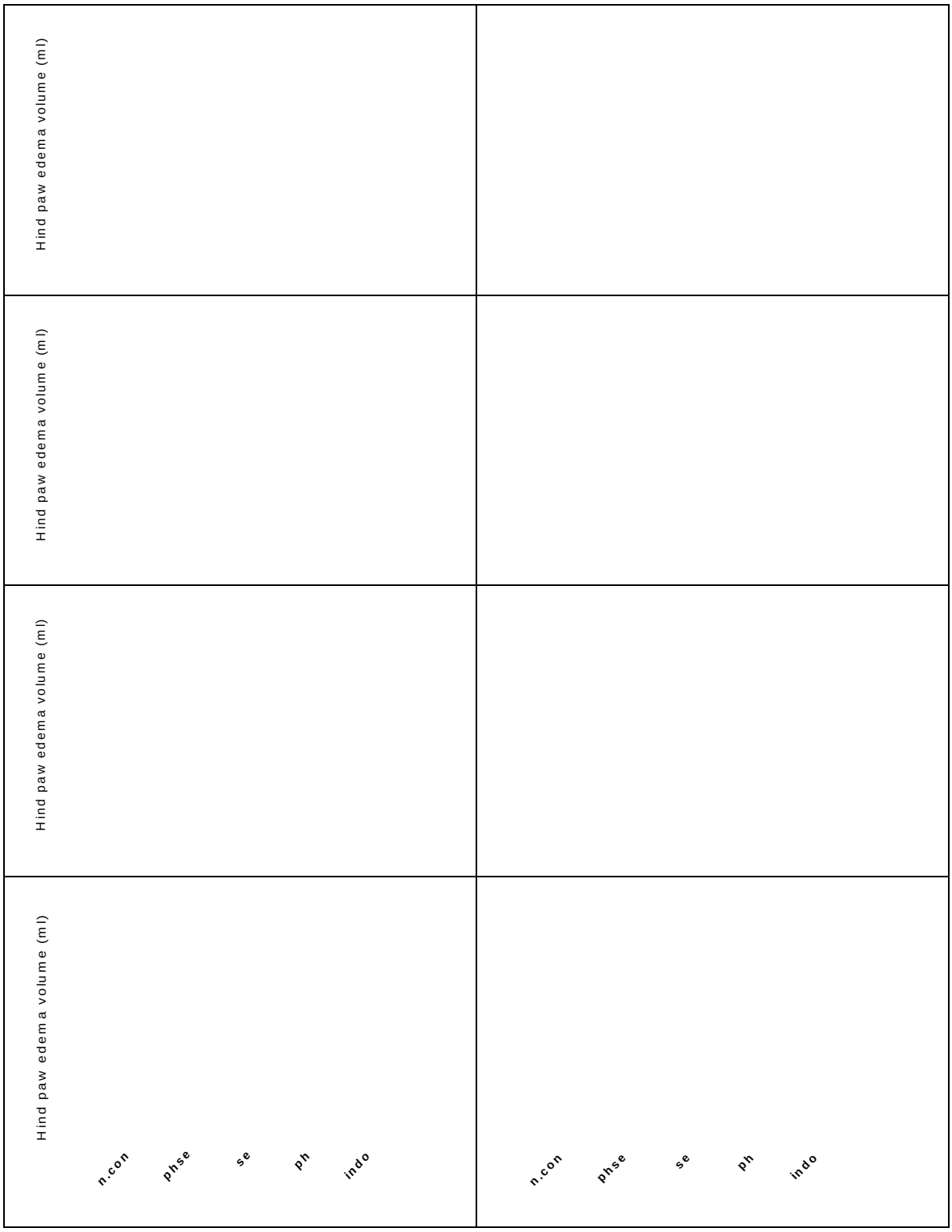

Figure 4.5: The effects of PH, Se, PHSe on paw oedema of carrageenan-induced inflammation in rats, recorded hourly over 7 hr. Data are expressed as delta volume changes of values (ml) of the rat paw volumes expressed as means ± SEM n=10 * $p<0.05$; ** $p<0.01$; *** $p<0.001$. n con = negative control; Phse = potassium humate with selenium; Se = selenium; Ph potassium humate alone; Indo = indomethacin used as positive control

Table 4.1: The results showing the delta values (ml) of the paw oedema in the carrageenan-induced inflammation in rats using a water displacement plethysmometer.

Time	Test compound	Δ paw volume (ml)	95% Cl of difference
Hour 0	N.control vs Phse	0.024	-0.070 to 0.118
	N. control vs Se	0.079	-0.015to 0.172
	N. control vs Ph	0.069	-0.025 to 0.163
	N. control vs Indo	0.049	-0.045 to 0.142
Hour 1	N. control vs Phse	0.105	0.001 to 0.210
	N. control vs Se	0.026	-0.074 to 0.127
	N. control vs Ph	0.096	-0.001 to 0.194
	N. control vs Indo	0.172	0.068 to 0.276
Hour 2	N. control vs Phse	0.088	-0.062 to 0.238
	N. control vs Se	0.108	-0.042 to 0.258
	N.control vs Ph	0.212	0.051 to 0.374
	N. control vs Indo	0.198	0.048 to 0.348
Hour 3	N.control vs Phse	0.084	-0.018 to 0.187
	N.control vs Se	0.041	-0.061 to 0.144
	N.control vs Ph	0.159	0.056 to 0.261
	N. control vs Indo	0.259	0.146 to 0.371
Hour 4	N.control vs Phse	0.035	-0.033 to 0.103
	N.control vs Se	0.075	-0.001 to 0.150
	N.control vs Ph	0.105	0.021 to 0.190
	N. control vs Indo	0.145	0.066 to 0.224
Hour 5	N.control vs Phse	0.146	0.034 to 0.258
	N.control vs Se	0.108	0.002 to 0.215
	N.control vs Ph	0.164	0.045 to 0.284
	N. control vs Indo	0.212	0.108 to 0.315
Hour 6	N.control vs Phse	0.032	-0.102 to 0.166
	N.control vs Se	-0.008	-0.133 to 0.118
	N.control vs Ph	0.149	0.009 to 0.289
	N. control vs Indo	0.144	-0.033 to 0.322
Hour 7	N.control vs Phse	0.045	-0.049 to 0.139
	N.control vs Se	0.007	-0.081 to 0.094
	N.control vs Ph	0.156	0.057 to 0.254
	N. control vs Indo	0.212	0.088 to 0.336

4.5 Acute phase proteins

The results show that the positive control (indomethacin) and Ph caused a decrease in the plasma level of SP in carrageenan challenged rats compared to the untreated control animals, but this change was not statistically significant. Se and Phse demonstrated no effect on the level of SP in the rat plasma (Fig 4.6A). The sham treatment group represent the baseline

Ph, Se and Phse caused a statistically significant decrease in plasma CRP levels of carrageenan challenged rats. Se on its own showed the greatest decrease in the CRP concentrations having the most positive effect $p<0.001$. The positive control indomethacin showed only a slight decrease in plasma CRP that was not statistically significant (Fig 4.6B)

Differences in plasma levels of Substance P in carrageenan treated rats after 7 hours after selected pretreatment.

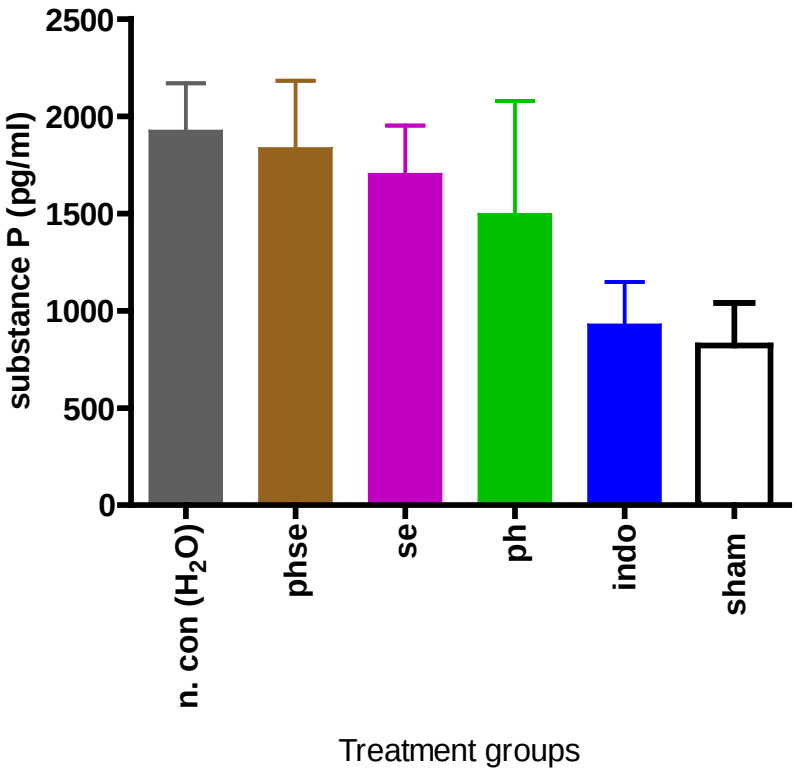

Figure 4.6: The effects of Ph, Se, Phse pretreatment on the plasma levels of substance P in rats 7 hr after carrageenan induced inflammation, determined by ELISA. Data expressed as pg/ml of plasma. n = 20.

Differences in plasma levels of C-reactive protein in carrageenan treated rats after 7 hours after selected pretreatment.

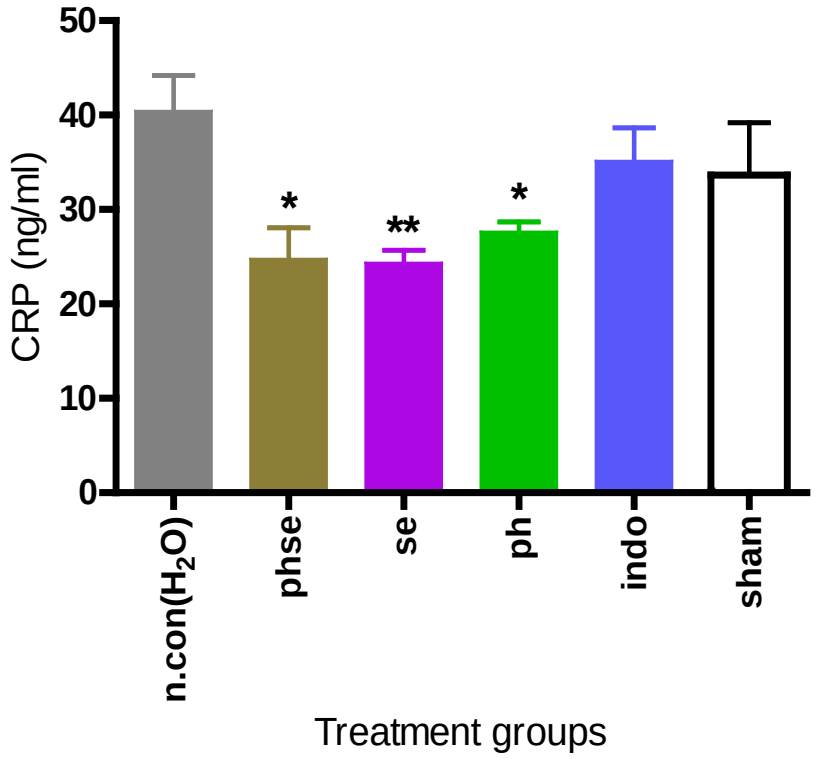

Figure 4.7: The effects of pretreatment with Ph, Se, Phse on the plasma levels of C-reactive proteins in rats 7 hr after carrageenan induced inflammation, as determined by ELISA. Data expressed as ng/ml of plasma. n = 20. *p<0.05; **p<0.01; ***p<0.001

Table 4.2: The statistical analysis of CRP results measured in pg/ml using a one way ANOVA and Dunnett's multiple comparison test as a post hoc test

Test compounds	Mean difference	95% Cl of difference
n.control vs Ph	12.80	0.12820 to 25.48
n.control vs Se	16.09	5.232 to 26.94
n.control vs Phse	15.67	2.997 to 28.34
n.control vs indo	5.299	-7.375 to 17.97
n.control vs sham	6.794	-7.841 to 21.43

n.control: negative control

Chapter 5

Discussion

This is the first study to document the effects of potassium humate loaded with selenium on the proliferation of both resting and PHA stimulated human lymphocytes *in vitro*.

In the case of Ph the results of this study correlate with the results obtained by van Rensburg and Naudé (2009), where Ph increased the proliferation of PHA stimulated lymphocytes and had an insignificant effect on resting lymphocytes. Jooné and van Rensburg (2004) reported a similar result with lymphocytes isolated from HIV-infected patients, indicating that this response is not limited to the *in vitro* setting. The central feature of the immune response is the proliferation of Ag-reactive lymphocytes. This proliferation is facilitated by the interaction of cytokines with their receptors on the surface of activated lymphoid cells (Gately *et al*., 1991). The function of IL-2, a T-cell-derived cytokine, is to promote T-lymphocyte growth and maturation (Joone *et al*., 2003). The interaction between IL-2 and its specific receptors following T lymphocyte activation triggers the T lymphocytes proliferation (Minami *et al*., 1993). In a study by Jooné *et al*., 2003 where a mixed lymphocyte suspension was stimulated with PHA and treated with various concentrations of oxihumate, the oxihumate was shown to have significantly increased the IL-2 production by PHA-stimulated MNL. These authors went further and showed that at a concentration of 100 µg oxihumate not only increased the expression of IL-2 but also its surface receptor, CD25, leading them to make the suggestion that humates appear to augment the actions of TH1 cells which produce IL-2.

Selenium supplementation has been reported to have many positive effects. Amongst others, it has been found to increase T cell proliferation, an effect which was also observed in this study, with no observed cytotoxicity at any of the tested concentrations. Literature states that Se escalates the expression of IL-2 receptors and increases the percentages of activated T cells (Gill and Walker., 2008). In selenium-deplete individuals supplementation has marked immuno-stimulant effects including an enhancement of proliferation of activated T cells by clonal expansion (Rayman., 2000). It

is possible that Se exerts its effects by modifying the redox status of the cell or by providing the requirements for the upregulation of selenoproteins for the activated immune cells. Se can also increase the activity of selenophosphate synthetase which directly affects the synthesis of selenocysteine (a key constituent of selenoproteins), in activated T cells and an increase in the biosynthesis of several proteins in the lymphocytes has been reported (Gill and Walker., 2008). Rayman (2000) suggested that the capacity of Se to up-regulate the expression of receptors for the growth-regulatory cytokines IL-2 on the surface of activated lymphocytes might be the mechanism of action and Hoffman (2007) reported that supplementation with Se results in increased expression of both the α (CD25) and to a lesser extent β/γ (CD 122/CD 132) subunits of the IL-2R on stimulated lymphocytes. This results in a greater number of high-affinity IL-2R per cell and enhances proliferation and differentiation on lymphocytes.

The combination of potassium humate with selenium had the greatest proliferative effect on stimulated lymphocytes at concentrations between 10 - 50 µg/ml. The mechanisms of action of the two substances in combination is unknown, but this study demonstrated that the combination is non-toxic to lymphocytes. More studies would need to be done to investigate whether an additive or synergistic effect is taking place.

An interesting observation was made where there was a reduction in cell proliferation at a concentration of 50 µg/ml or greater with both Ph and the Phse combination, while selenium on its own showed an increase in proliferation. Previous studies done on Ph showed lymphocyte proliferation for stimulated lymphocytes in a dose-related manner (Jooné *et al*., 2003), which was not observed in this study despite working in the same concentration range. Humic acids contain many aromatic rings and often complex metals such as chrome, manganese, arsenic, lead, zinc, cadmium and nickel. The presence of phenolics, hydroxyl, phenolic hydroxyl, ketone, quinine, semiquinone, carboxyl, carbonyl and alkoxyl groups have been revealed in humic acid using chemical and infrared analysis (Yang *et al*., 2004). It can be expected that two separate natural sources of humic acids probably do not contain identical molecules. Humic acid from one source may have a positive influence, while the influence of those from another source can be an

adverse one (Yang *et al*., 2004). This might due to the fact that the Ph used in this study and the one used in Jooné *et al* (2003) study are from completely different sources, and therefore could have different properties.

Humic acids can strongly interact with both inorganic and organic agents due to their chemical structure and thus might play a role in redox regulation. Humic acids are involved in the generation of ROS, such as the superoxide anion and deplete glutathione and several antioxidant enzymes (Vašková *et al*., 2011). Reactive oxygen species are typically defined as oxygen-containing radicals which can exists independently with one or more unpaired electrons (Halliwell and Gutteridge., 1984, Halliwell and Cross., 1994). A similar observation was made in this study where Ph showed no protective effect against ROS at any of the tested concentrations up to 100 µg/ml.

A study using humic acids extracted from podzol soils stimulated rat liver mitochondria respiration that resulted in the production of measurable intracellular reactive oxygen species (Visser., 1987). Another study done on cultured rabbit articular chondrocytes indicated that humic acid induced oxidative injury may be due to superoxide anion production (Liang., 1999) Oxidative stress generated by humic acid is implied in the process leading to apoptosis of endothelial cells (Hseu *et al*., 2002 and Vašková *et al*., 2011). Following oxidative stress, protein kinases may be activated and these enzymes may play a vital role in the steps leading to apoptosis (Brawn *et al*., 1994). According to Yang *et al* (1994) treating HUVECs with humic acid significantly amplified the protein kinase activity and in a study done by Hseu *et al* (2002) they demonstrated that humic acid induced apoptosis requires activation by a protein kinase.

Se as part of selenoproteins plays an important role in the maintenance of proper balance between negative and positive effects of ROS (Hoffman., 2007). Selenoproteins exists in every cell type and their function is to catalyse oxido-reductive reactions. There are twenty five known eukaryotic selenoproteins which are organised into groups based on their location and functional properties of selenium. Selenoprotein P, type I iodothyronine 5´-deiodinase, GSH-Px and thioredoxin reductase have been characterized

in animals and humans. According to literature, half of the characterized selenoproteins have been associated with antioxidant functions (Battin *et al.*, 2009 and Tapiero *et al.*, 2003). They also play a vital role in the immune system because they protect the host from oxidative stress.

During the metabolism of xenobiotics and exposure to ultraviolet radiation, oxidative species can be generated (McKenzie *et al*., 1998). ROS generation and the antioxidant defence activity appear more or less balanced *in vivo*. For continuous low-level oxidative damage in the human body, the balance may be slightly tipped in favour of the ROS. This generates a need for repair systems that can adequately deal with oxidatively damaged molecules. However, the result of a greater imbalance in favour of the ROS is oxidative stress (Halliwell and Cross., 1994). A lot of research has been done on the antioxidant activities of Se and many researchers have proven that Se is a potent antioxidant, however Se showed essentially no antioxidant activity in this study. A small antioxidant effect was observed using the DCFH-DA assay, but only at the lowest concentrations of Se (0.096 µg/ml and 0.191 µg/ml) and these effects were not found to be statistically significant. The DCFH-DA kinetic assay shows the antioxidant activity of compounds over a period of time, in this case 2.5 hours. According to the results obtained in this study Se does not show significant protection against ROS generated by AAPH at any measured time point although the effect did appear to be maximal at the final 2.5 hour time point.

With the ORAC assay Se showed activity which was equivalent to that of Trolox. According to Valdiglesias *et al* (2010) the optimal concentrations and the appropriate form of Se supplementation that provides protection against ROS and genetic damage with the least toxicity continues to be debated in spite of the many reported antioxidant properties and its known necessity in human and animal nutrition. The concentration of Se used in this study was very low, perhaps even too low to have an effect on the redox status of the cells. A similar study done by Cuello *et al* (2007) where $HepG_2$ cells were exposed to 0.01 µM – 10 µM of Se for 2 hr and 20 hr showed no toxicity and Se seemed to reduce the progressive formation and accumulation of oxygen radicals. The highest

concentration of Se used in this study was $1.9x10^{-5}$ µM which is approximately 500 times less than the Cuello *et al* study concentration used. This could be one of the reasons why Se did not demonstrate suppression of the generation of ROS.

The combination of Ph with Se exhibited a pro-oxidant effect in a dose dependent manner, as the concentration of the Phse increased the ROS generation increased. This might be due to the fact that humic acids are known to induce oxidative injury mediated by superoxide anion production and apoptosis induction. Experiments would need to be conducted to investigate this result, but it can be assumed that aromatic rings of humic acid lack sufficient phenolic groups to give antioxidant properties or because these rings are not in favourable positions or are blocked, e.g. by methyl groups (Vašková *et al.*, 2011).

Considering this fact and the low concentration of Se used in combination with Ph in this study, it is possible that the Se could not protect the cells against the ROS generated by the much higher concentrations of Ph.

In this study Ph significantly inhibited the expression of CR3 by activated neutrophils in a dose-related manner, from a concentration of 25µg/ml and higher. The results of this study correlate with the results observed by Jooné and Jansen van Rensburg (2004), Cromarty (2004) and Jansen van Rensburg and Naudé (2009). CR3 inhibition might explain some of the anti-inflammatory properties referred to in the literature. Overexpression of CR3 by phagocytic cells has been implicated in tissue injury and can lead to inappropriate immune responses, because of the link to the production of many pro-inflammatory cytokines, reactive oxygen species and nitrogen intermediates as well as proteolytic enzymes.

In a study by Jaeschke *et al* (1993) anti-CR3 antibodies were shown to decrease ischemia/reperfusion injury, suggesting that these antibodies can be effective in the treatment of endotoxic challenges and haemorrhagic shock (Jaeschke *et al.*, 1991). Another study showed that neutrophil infiltration into the inflamed intestine of

indomethacin-treated rats was to some extent blocked by anti-CD11b antibody treatment and completely blocked by anti-CD18 treatment (Stadnyk *et al*., 2002). A study done by Palmen *et al* (1995) showed that anti-CD11b/CD18 mAbs reduced the number of infiltrating granulocytes and macrophages into the colon in rats with 2,4,6-trinitrobenzene sulfonic acid induced colitis, and also observed a reduction in MPO activity and tissue injury.

The results observed in this study confirmed results from previous studies that indicate that Ph not only inhibits the expression of CR3 in stimulated neutrophils, but it is also non-toxic to the cells. Also, other researchers reported that Ph inhibited the adhesion of stimulated neutrophils to cells expressing ICAM-1, the ligand of CR3 (Jooné and Jansen van Rensburg., 2004).

Pharmacological agents such as antioxidants have been assumed to inhibit the cytokine-induced expression of ICAM-1, mainly by blocking activation of the NFκB (Horváthová *et al*., 1999). The initial stage of neutrophil diapedesis is the binding of CR3 to fibrinogen, ICAM-1 or E-selectin, the latter two being present on the surface of activated endothelial cells of the vascular system, (Kishimoto and Baldwin., 1999, Rasool., 2008). In this study Se significantly reduced the expression of CR3 on PMA stimulated neutrophils from a concentration of 0.77 µg/ml Se and higher, and in a study done by Horváthová *et al* (1999) where HUVECs were incubated with or without PBMCs isolated from EDTA-blood of asthmatics to assess the expression of adhesion molecules. The HUVECs were treated with either the medium alone or medium containing recombinant human gamma interferon together with various concentrations of Se (0, 0.02, 0.2 and 10 µg/ml). The ability of Se to inhibit the expression of adhesion molecules was analysed using flow cytometry, and it was found that Se did show an inhibitory effect on the expression of ICAM-1, P-selectin and ELAM-1 from 0.1 µg/ml to 10 µg/ml with the IC_{50} of 0.5 µg/ml for ICAM-1.

Another study done by Jehovah *et al* (2002) where 20 corticoid-dependent asthmatics were enrolled into a study, they received Se supplementation of 200 µg daily for 6

months, blood was collected before supplementation and after 3 and 6 months of Se supplementation. PBMCs were isolated from the blood and stimulated with INF-γ and probed with monoclonal antibodies conjugated with fluorescein or Phycoerythrin directed against antigens CD11a, CD11b, CD18, CD49d, CD62L. ELISA was used to assess the expression of ICAM-1, VCAM-1, P-selectin and E-selectin. Se was shown to decrease the expression of CD11b and there was a significant decrease in VCAM-1 and P-selectin after 3 months of Se supplementation.

When used in combination, potassium humate and selenium had the same effect as when Ph was used alone. Potentially this was because of the low concentration of Se which rendered it ineffective in the presence of high concentrations of Ph, hence the dose related response with no statistical significance.

Initiation of an acute inflammatory response is associated with several relatively fast vascular changes (Salvemini *et al.,* 1996). The carrageenan-induced paw oedema is a useful model to evaluate the initial phases of the inflammatory response including the mediators involved in these changes. The development of oedema in the rat paw resulting from a challenge with carrageenan has been defined as a biphasic episode. During the very early phase of oedema (0 - 1 hr), non-steroidal anti-inflammatory drugs like aspirin and indomethacin would have no effect due to the involvement of short term mediators like histamine, 5-hydroxytryptamine (5-HT) and bradykinin. The late phase is the accelerating phase of swelling (1- 6 hr), and this phase is associated with increased production of prostaglandins and has been attributed to the initiation of inducible cyclo-oxygenase (COX2) in the paw tissue exposed to the initial oedema and cellular infiltration. The infiltration and activation of local neutrophils leads to the production and release of ROS which also contributes to the inflammatory response (Salvemini *et al.*, 1996).

A study done by Naudé *et al* (2010) used female Sprague-Dawley rats that were dosed with 60 mg/kg bodyweight humic acid once daily by oral gavage for five consecutive days and where 10 mg/kg body weigh indomethacin was used as a single dose positive

control. Ph and indomethacin significantly reduced the carrageenan-induced inflammation over a period of 300 min. Another study done by van Rensburg *et al* (2007) showed that a brown coal derived humate was safe up to 1000 mg/kg bodyweight per day when administered by oral gavage for 12 days That study also showed that Ph had no teratogenic effect as no adverse effects on newborn pups was observed when administered to pregnant female rats.

The results of this current study concur with the results of the above mentioned studies in that, no rats showed any signs of adverse effects (loss of weight, loss of appetite, loss of fur, decreased activeness) during the course of the study. Similar to the study done by Naudé *et al* (2010), Ph decreased the carrageenan-induced oedema significantly from hour two to seven even though the effect was not equivalent to the positive control where indomethacin was administered once off.

Indomethacin is a non-selective inhibitor of COX1 and COX2 enzymes that participate in prostaglandins synthesis from arachidonic acid. When activated these enzymes lead to the production of prostaglandins, prostacyclins and thromboxanes which are mediators for inflammation (Seibert *et al*., 1994). The results of this study show that Ph reduces the carrageenan-induced rat paw oedema to a similar extent to that of a once off dose of indomethacin. This might be due to the fact that Ph like indomethacin inhibits COX 1 and 2, or that Ph inhibits both the alternative and classical complement pathways in addition to inhibiting the production of TNF-α, IL-1β and IL-6 by PHA stimulated MNL (van Rensburg and Naudé., 2009). It has also been shown that humic acids significantly decrease lipopolysaccharide-induced adhesion molecules like ICAM-1,VCAM-1 and E-selectin expressed by cultured human umbilical vein endothelial cells which might be a possible mechanism of action by which Ph inhibits initial inflammatory reactions (Naudé *et al*., 2010).

Se is a nutritionally crucial trace element essential for the creation of redox-active selenoenzymes such as glutathione peroxidase (GSH-Px) and thioredoxin reductase. The need for Se as an antioxidant is increased by inflammation. It is assumed that by blocking the activation of NF-κB, antioxidants can inhibit the expression of cellular adhesion

molecules (Horváthová *et al*., 1999). The results of this study correlates with the results found by Parnham *et al* (1987) where it was observed that *in vivo* ebselen (a seleno-organic compound with glutathione peroxidase-like activity *in vitro*) is a weak, oral inhibitor of carrageenan paw oedema in the rat, distinguishing it from classical NSAID's such as indomethacin. Leyck and Parnham (1990) went further and found out that *in vivo* ebselen is an acute anti-inflammatory compound with a non-NSAID profile. The weak inhibitory effect on carrageenan-induced rat paw oedema mirrors the weak inhibition of PGE_2 production *in vitro* and the carrageenan-induced model is PGE_2-dependent to a substantial extent (Rovenský *et al.,* 2005). The fact that ebselen is inactive against carrageenan- induced pleuritis in rats while other NSAIDs are effective inhibitors of exudation and leucocyte recruitment supports the observations made in this study. Supplementation with Se increases the concentration of Se in the serum and erythrocytes but only weakly increases its concentration in polymorphonuclear leucocytes and this might be the reason for its inadequate anti-inflammatory effect in arthritis (Rovenský *et al*., 2005). This could also explain the finding of this current study bearing in mind the low concentration of Se used in this study which might add to the lack of activity of Se.

Destruction of tissue or cells elicits both a local and systemic acute phase response (Schreiber *et al*., 1989). In this study Ph, Se and Phse showed no effect on substance P plasma concentration but showed a statistically significant decrease in the plasma levels of CRP. In the rat substance P (SP) appears to be involved in regulating hyper inflammation and immune responses to amplify the pain signal, initiate peripheral vasodilatation and plasma extravasation and homing of leukocytes to the area of injury. SP is released centrally in the spinal cord. (Mantyh., 1991) and preferably binds to the neurokinin 1(NK-1) receptors. NK-1 receptor is the endogenous receptor for SP (Gerard *et al*., 1991, Maggi and Schwartz., 1997) and is a member of the tachykinin receptor sub-family of GPCRs (Maggi., 1995). The binding of SP to NK-1 receptors results in the transmission of pain and stress signals, the contraction of smooth muscles and promotion of inflammation (Seto *et al*., 2004). The general characteristic of NK-1 activation is neurogenic inflammation which results in plasma leakage which is accompanied by increased blood flow, associated with dilation of the arterioles and manifesting as typical

wheal and flare reactions (Harrison and Geppeti., 2001). In another study where SP was injected in the rat paw at a concentration of up to 500 pmol (an amount that was two folds greater than that measured in the plasma of the rats treated with 50 µg of carrageenan); SP only produced moderate oedema (Gilligan *et al*., 1994). This lead the investigators to suggest that SP itself is not a powerful inflammatory agent, but in conjunction with mediator expressing cells attracted by an irritant, such as carrageenan, this neuropeptide may encourage the release of various inflammatory mediators which in turn will amplify the inflammatory response (Gilligan *et al*.,1994). The primary action of SP may be to promote and orchestrate the inflammatory and immune responses in the afflicted tissue, because for tissue restoration to occur, there has to be an appropriate inflammatory and immune response (Mantyh., 1991).

During inflammation, CRP is produced by hepatocytes in response to IL-1, IL-6 or TNFα and serum levels of CRP are positively linked with the serum levels of IL-6 (Gabay and Kushner., 1999, Kabutomori and Kamakura., 2002). CRP activates the complement system and enhances phagocytosis, mainly through the C3 convertase. The activation of complement cascade results in opson activity and release of anaphylatoxins (Cermak *et al*., 1993, Blackburn., 1994). In monocytes CRP also induces the expression of inflammatory cytokines and tissue factors (Ceciliani *et al*., 2002).

The results of this study show that Ph, Se and the combination caused a statistically significant decrease in the plasma levels of CRP. Amongst biomarkers of the acute-phase response in almost all diseases, increased circulating concentrations of CRP reflects ongoing inflammation and/or tissue damage most accurately (Pepys and Hirschfield., 2003). Very few drugs and no other current therapies reduce CRP values, unless they also affect the underlying pathology responsible for the acute-phase stimulus. Thus CRP is a very useful nonspecific, biomarker of the extent of inflammation which contributes to monitoring the response of inflammation and infection to treatment and detection of co-infection in immuno-compromised individuals (Pepys and Hirschfield., 2003). In the study by Jansen van Rensburg and Naudé (2009), Ph was shown to decrease the production of pro-inflammatory mediators like TNFα, IL-1β and IL-6 by PHA stimulated

MNL. It is possible that CRP reduction in the rat plasma CRP observed in this study was also due to TNFα, IL-1 and IL-6 reduction resulting from Ph and Phse treatment. Se on its own caused a more significant decrease in rat plasma CRP, which correlates to the results of Zhang *et al* (2002) where it was demonstrated that selenite significantly inhibited TNFα-induced expression of adhesion molecules and with the study by Hassanzadeh *et al* (2006), where it was demonstrated that patients suffering from acute myocardial infarction presented with lower plasma concentration of Se and higher concentrations of pro-inflammatory cytokines; viz. TNFα and IL-6.

In the present study it was observed that the CRP plasma concentration of the sham treatment group were also high which was a surprising result. Literature suggest that CRP is not a typical acute phase protein in the rat and under basal conditions rats have much higher plasma CRP concentrations in contrast to humans, about 300-500 mg/l, which is 100 times higher than the concentrations in humans (Padilla *et al*., 2003). This could explain the high concentration of CRP in the sham treatment group. The Phse combination did not show an additive or synergistic effect and indomethacin did not have any effect on plasma levels of CRP.

The aim of this study was to evaluate the efficacy, safety and possible mechanism of action of Phse. This study proved that Phse is not cytotoxic when tested *in vitro* and causes stimulated lymphocytes to proliferate in a similar manner to the much studies Ph. The study also showed that the combination does not possess antioxidant activity; Se at the concentrations used in the study had no antioxidant effect. CR3 expression by stimulated neutrophils was significantly inhibited by all the test compounds but Ph was the most potent inhibitor. Ph also reduced the carrageenan induced paw oedema volumes in manner similar to indomethacin whereas Phse had no effect. Ph caused a slight decrease in plasma concentrations of SP but the decrease was not statistically significant, while both Se and Phse had no effect. All the test compounds decreased the concentration of CRP significantly with Se showing the greatest effect. Phse did show some anti-inflammatory properties by inhibiting CR3 expression and the acute phase protein CRP. The results of this study suggests that it is not a potent anti-inflammatory compound and

that the addition of Se to Ph at the concentrations suggested does not add any value to the already well documented properties of Ph.

Future research

The effect of Ph and Phse on other acute phase proteins should be investigated.

Phse showed pro-inflammatory activity in the carrageenan induced oedema model and this should also be further investigated.

Criticism of the study

- The animal study should have been designed to investigate other safety parameters e.g. dissecting the rats to check the effect of the test compounds on gross anatomy.
- A higher concentration of Se should have been tested to determine the concentration at which Se starts showing its antioxidant effects

References

Badenhorst BE, CEJ van Rensburg, JR Snyman and PJ Becker (2008). Humic acid reduces inflammation in patients with osteoarthritis. South African Society for Basic and Clinical Pharmacology Congress Rhodes, 5-8 October 2008

Battin EE and Brumaghim JL. (2009). Antioxidant activity of sulphur and selenium: A review of reactive oxygen species scavenging, glutathione peroxidase, and metal-biding antioxidant mechanisms. *Cell Biochem Biophys*, 55: 1-23

Barton GM. (2008) A calculated response: control of inflammation by the innate immune system. *J. Clin. Invest.* 118:413–420

Bei R, Masuelli L, Palumbo C, Tresoldi I, Scardino A, Modesti A. (2008) Long-lasting tissue inflammatory processes trigger autoimmune responses to extracellular matrix molecules. *Int Rev Immunol*, 27: 137–175

Bhatia M, Brady M, Shokuhi S, Christmas S, Neoptolemos JP, Slavin J. (2000). Inflammatory mediators in acute pancreatitis. *J Pathol*, 190:117-125

Blackburn WD. (1994) Validity of acute phase proteins as markers of disease activity. *J Rhematol*, 21:9-13

Böyum A. (1968). Isolation of leucocytes from human blood futher observations methylcellulose, dextran and ficoll as erythrocyte aggregation agents. *Scand J Clin Lab Inv Suppl*, 97, 31-50

Bøyum A. (1976) Isolation of lymphocytes, granulocytes and monocytes. *Scand J Immunol*, 5(s5): 9-15

Brawn MK, Chiou WJ and Leach KL. (1994) Oxidant-induced activation of protein Kinase C in UC11MG cells. *Free Rad Res*, 22(1): 23-37

Broome CS, McArdle F, Kyle JA. (2004) An increase in selenium intake improves immune function and poliovirus handling in adults with marginal selenium status. Am J Clin Nutr, 80: 154-213

Callelo DP. (2010) Clinical basis of medical toxicology: Selenium, Ninth Ed, Goldfrank's Toxicologic Emergencies, pp 1317-1320. New York

Cao G, Alessio HM, Cutler RG. (1993) Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Bio Med*, 14: 303-313

Ceciliani F, Giordano,A, Spagnolo V. (2002) The systemic reaction during inflammation: the acute phase proteins. *Protein Pept Lett*, 9(3): 21-23

Cermak J, Key NS, Bach RR, Balla J, Jacob HS, Vercellotti GM. (1993) C-reactive protein induces human peripheral blood monocytes to synthesize tissue factor. *Blood*, 82(2): 513-520

Chaplin DD, (2006) Overview of the human immune response. *J Allergy Clin Immunol* 117:S430-5

Cheng ML, Ho HY, Huang YW, Lu FJ, Chiu DTY. (2002) Humic acid induces oxidative DNA damage, growth retardation and apoptosis in human primary fibroblast. *Exp Biol Med*, 228: 413-423

Coussens LM and Werb Z. (2002) Inflammation and cancer. *Nature*, 420(6917): 860–867

Cromarty AD. (2004) The identification of bio-available and active components in oxihumate. Pretoria: University of Pretoria (PhD in Pharmacology)

Cuello S, Ramos S, Mateos R, Martin MA, Madrid Y, Cámara C, BravoL, Goya L, (2007). Selenium methylselenocysteine protects human hepatoma HepG2 cell against oxidative stress induced by tert-butyl hydroperoxide. *J. Clin. Invest*, 82: 495-501

Curtin J, Donovan M, Cotter TG. (2002) Regulation and measurement of oxidative stress in apoptosis. *J Immunol Methods*, 265:49-72

Dávalos A, Gomenz-Cordoves C, Bartolome B, (2004) Extending applicability of the oxygen radical absorbance capacity (ORAC-flourescein) assay. *J Agr Food Chem*, 52(1), 48-54

Delves PJ and Roitt IM. (2000) The immune system. *New Engl J Med*, 343(2): 108-117,

Desai D, Kaushai N, Gandhi U, Arner R, D'Souza Chen G, Vunta H, EI-Bayoumy K, Amin S, Sandeep K. (2010) Synthesis and evaluation of the anti-inflammatory properties of selenium-derivaties of celecoxib. *Chem. Biol Interact*, 188(3): 446-456

de Visser KE, Eichten A, and Coussens LM. (2006) Paradoxical roles of the immune system during cancer development. *Nat Rev*, 6: 25-37

Fairweather-Tait SJ, Bao Y, Broadley MR, Collings R, Ford D, Hesketh JE, Hurst R. (2011) Selenium in human health and disease. *Antioxid Redox Signal*, 14: 1337-1383

Fan AM, Kizer KW (1990). Selenium. Nutrition, toxicology and clinical aspects. *West J.Med*, 153(2): 160-167

Farmer JD and Packard NH. (1986) The immune system, adptation and machine learning. *Physica* 22D:187-204

Furie MB and Randolph GJ. (1995) Chemokines and Tissue Injury. *Am J Pathol*, 146(6): 1287-1301

Gabay C, Kushner I. (1999). Acute-phase proteins and other systemic response to inflammation. *New Engl J Med*, 340(6): 448-454

Gately MK, Desai BB, Wolitzky AG, QuinnPM, Dwyer CM, Podlaski FJ, Familletti PC, Sinigaglia F, Chizonnite R, Gubler U, Stern AS. (1991) Regulation of human lymphocyte proliferation by a factor heterodimeric cytokine IL2 cytotoxic lymphocyte maturation. *J Immunol*, 147: 874-882

Gau RJ, Yang HL, Chow SN, Suen JL, Lu FJ. (2000) Humic acid suppresses the LPS-induced expression of cell-surface adhesion proteins through the inhibition of NF-kB activation. *Toxicol Apple Pharm*, 166: 59-67

Gerard NP, Garraway LA, Eddy RL, Shows TB, Iijima H, Paquet J and Gerard C.(1991) Human substance P receptor (NK-1): Organization of the gene, chromosome localization, and functional expression of cDNA clones, *Biochemistry*, 30(44): 1064-10646

Gill, H., Walker, G., (2008). Selenium, immune function and resistance to viral infestions. *Nutrition Dietics*, 65(3), 41-47

Gilligan JP, Lovato SJ, Erion M, Jeng AY. (1994) Modulation of carrageenan-induced hind paw oedema by substance P. *Inflammation*, 18(3):285-292

Goetzl EJ, Chernov T, Renold F, and Payan DG. (1985) Neuropeptide regulation of the expression of immediate hypersensitivity. *J Immunol*, 135(2): 802s-805s

Graça-Souza AV, Arruda MAB, de Freitas MS, Barja-Fidalgo C and Pedro LO. (2002) Neutrophil activation by heme: Implications for inflammatory processes. *Blood* 99: 4160-4165

Gruys E, Toussaint MJM, Niewold TA, Koopmans SJ. (2005) Acute phase protein reaction and acute phase protein. *Journal of Zhejiang University Science*, 6B (11):1045-1056

Halliwell B and Cross CE. (1994) Oxygen-derived Species: Their relation to human disease and environmental stress. *Environ Health Perspect* 102 (Suppl 10):5-12

Halliwell B and Gutteridge JMC. (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J*, 219:1-14

Harrison S and Geppeti P. (2001) Substance P. *Int J Biochem Cell B*, 33: 555-576

Hassanzadeh M, Faridhosseini R, Mahini M, Faridhoseini F, Ranjbar A. (2006) Serum levels of TNFα, IL-6 and selenium in patients with acute and chronic coronary artery disease. *Iran Immunol*, 3(3): 142-5,

Hawkes WC, Kelly DS, Taylor PC. (2001) The effects of dietary selenium on the immune system in healthy men. *Biol Trace Elem Res*, 81: 189-213

Hay C, de Belleroche J. (1997) Carrageenan-induced hyperalgesia is associated with increased cyclo-oxygenase-2 expression in spinal cord. *NeuroReport*, 8: 1249-1251

Haung D, Ou B, Hampsch-Woodill M, Flanagan JA, Prior R. (2002) High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *JAgr Food Chem*, 50:4437-4444

Hoffman P. (2007) Mechanisms by which selenium influences immune responses. *Arch. Immunol. Ther. Exp*, 55: 289-297

Hoffman FW, Hashimoto AC, Shafer LA, Dow S, Berry MJ, Hoffman PR. (2010) Dietary selenium modulates activation and differentiation of CD4+ T cells in mice through a mechanism involving cellular free thiols. *J Nutr* , 140: 1155-61

Hoèkfelt T, Pernow B and Wahren J. (2001) Substance P: a pioneer amongst neuropeptides. *J Intern Med*, 249: 27-40.

Horváthová M, Jahnova E, Gazdik F. (1999) Effects of selenium supplementation in asthmatic subjects on the expression of endothelial cell adhesion molecule in culture. *Biol Trace Elem Res*, 69: 16-26

Howland RD. (2006) Anti-inflammatory drugs and Autacoids. Pharmacology. Philadelphia: Lippincott Willliams and Wilkins, 495-499

Hseu YC, Lu FJ, Engelking LR, Chen CL, Chen YH, Yan HL. (2000). Humic acid-induced echinocyte transformation in human erythrocytes: Characterisation of morphological changes and determination of the mechanism underlying damage. *J Toxicol Env Heal A*, 60: 215-230

Hseu YC, Huang HW, Wang SY, Chen HY, Lu FJ, Gau RJ, Yang HL. (2002) Humic acid induces apoptosis in human endothelial cells. *Toxicol Appl Pharm*, 182: 34-43

Hseu YC, Wang SY, Chen HY, Lu FJ, Gau RJ, Lui TZ, Yang HL. (2002) Humic acid induce the generation of nitric oxide in human umbilical vein endothelial cells: stimulation of nitric oxide synthase during cell injury. *Free Radical Bio Med*, 32(7): 619-629

Islam KMS, Schumacher A, Gropp JM. (2005) *Pakistan Journal of Nutrition*, 4(3): 126-134

Jaeschke H, Farhood A and Smith CW. (1991) Neutrophil-induced liver cell injury in endotoxin shock is a CD11b/CD18-dependent mechanism. *Am J Physiol Gastrointest Liver Physiol* 261:G1051-G1056

Jaeschke H, Farhood A, Bautista AP, Spolaris Z, Spitzer JJ, Smith CW.(1993) Functional inactivation of neutrophils with Mac-1 (CD11b/CD18) monoclonal antibody protects against ischaemia-reperfusion injury in rat liver. *Hepatology*, 17:915-923

Jahnova E, Horvathova M, GazdikF, Weissova S. (2002) Effects of selenium supplementation on expression of adhesion molecules in corticoid-dependent asthmatics. *Bratisl Lek Listy*, 103(1), 12-16

Jooné GK, DekkerJ, and van Rensburg CEJ. (2001) An in vitro investigation of the anti-inflammatory properties of oxihumate. 11th International Congress of Immunology, Stockholm, Sweden 22/07/2001

Jooné GK, Dekker J, van Rensburg CEJ. (2003) Investigation of the immunostimulatory properties of oxihumate. *Z Naturforsch*, 58: 263-267

Jooné GK, van Rensburg CEJ. (2004) An in vitro investigation of the anti-inflammatory properties of Ph. *Inflammation*, 28(3): 196-174

Kabutomori O, Kanakura Y. (2002) Movement of toxic granulation neutrophils and C-reactive protein in inflammatory processes. *Am J Med*, 112: 595-596

Kishimoto TK, Baldwin ET. (1999) The role of β2 integrins in inflammation: Basic principles and clinical correlates. Philadelphia, Lippincott Williams and Wilkins

Kiremidjian-Schumacher L, Roy M, Wishe HI, Cohen MW, Stotzky G. (1994) Supplementation with selenium and human immune cell functions. *Biol Trace Elem Res*, 41:115-127

Kiremidjian-Schumacher L, Roy M, Glickman R. (2000) Selenium and immunecompetence in patients with head and neck cancer. *Biol Trace Elem Res*, 73: 97-111

Klöcking R, Helbig B. (2005) Medical aspects and applications of humic substances. *Biopolymers for Medical and Pharmaceutical Applications*, 1-16

Klöcking R. (1994b) Humic substances as potential therapeutics. Humic Substances in the Global Environment and Implication on Human Health (ed. by N. Senesi & T. M. Miano), pp. 1245-1257. Elsevier Science, Amsterdam

Klöcking R, Hofmann R, Mücke D. (1968) Tierexperimentelle Untersuchungen zur entzündungshemmenden Wirkung von Humaten. Arzneim Forsch., 18, 941-942.

Koller LD, Exon JH, Talcott PA, Osborne CA, Henningsen GM. (1986) Immune responses in rats supplemented with selenium. *Clin.exp.Immunol*, 63: 570-576

Koller LD, Exon JH (1986). The two faces of selenium-deficiency and toxicity are similar in animals and man. *Can J Vet Res*, 50(3): 297-306

Kühnert M, Golbs S, Fuchs V. (1982) Chemische charakterisierung und besondere pharmakologisch-toxikologische eigenschaften von huminasäuren. *Arch Exper Vet Med*, 36(2): 169-177

Kulikova NA, Stepanova EV, Koroleva OV. (2005) Mitigating activity of humic substances direct influence on biota.

Kertz-Remy C, Arrigo AP. (2001) Selenium: a key element that controls NF-kappa B activation and I kappa B alpha half life. *Biofactors*, 14: 117-125

Leyck S and Parnham MJ. (1990) Acute anti-inflammatory and gastric effects of the seleno-organic compound ebselem. *Agents Action*, 30(3/40: 426-431

Liang HJ, Tsa CL, Chen PQ and Lu FJ. (1999) Oxidative injury induced by synthetic humic acid polymer and monomer in cultured rabbit articular chondrocytes. *Life Sci*, 64(11): 1163-1173

Louis NA, Hamilton KE, Colgan SP. (2005) Lipid mediator networks and leukocyte transmigration. *Prostag, Leukotr Ess* 73: 197–202

Lu FJ, Yamamura Y,and Yamauchi H. (1988) Studies on fluorescent compounds in water of a well in Blackfoot disease endemic areas in Taiwan: Humic substances. *J Formosan Med Assoc*, 87: 66-71

Maggi CA. (1995) The mammalian tachykinin receptors. *Gen Pharmacol*, 26(5): 911-944

Maggi CA, Schwartz TW. (1997) The dual nature of the tachykinin NK_1 receptor. *TiPS*, 18: 351-355

Mantovani A, Garlanda C, Locati M, Rodriguez TV, Feo SG, Savino B, Vecchi A. (2007) Regulatory pathways in inflammation. *Autoimmunity Rev* 7:8–11

Mantyh PW. (1991) Substance P and the inflammatory and immune response. *Ann NY Acad Sci*, 632: 263-271

McKenzie RC, Rafferty TS, Beckett GJ. (1998) Selenium: an essential element for immune function. *Immunol Today*, 19(8): 342-345

Medzhitov R. (2008) Origin and physiological roles of inflammation. *Nature*, 454: 428-435

Michalke B. (2004) Selenium speciation in human serum of cystic fibrosisatients compared from healthy persons. *J Chromatogr* A, 1058: 203-8

Micke O, Schomburg L, Buentzel J, Kisters K, Muecke R. (2009) Selenium in oncology: From chemistry to clinics. *Molecules*, 14: 3975-3988

Minami Y, Kono T, Miyazaki T, Taniguchi T. (1993) The IL-2 receptor complex: Its structure, function and target genes. *Annu Rev Immunol*, 11:245-268

Muto S, Vétvička V, Ross GD. (1993) CR3 (CD11b/CD18) expressed by cytotoxic T cells and natural killer cells is unregulated in a manner similar to neutrophil CR3 following stimulation with various activating agents. *J Clin Immunol*, 13(3): 176-184

Nantel F, Denis D, Gordon R, Northey A, Cirino M, Metters KM, Chan CC (1999). Distribution and regulation of cyclooxygenase-2 in carrageenan-induced inflammation. *Brit J Pharmacol*, 128: 853-859

Naudé PJW, Cromarty AD, van Rensburg CEJ. (2010) Potssium humate inhibits carrageenaan-induced paw oedema and a graft-versus-host reaction in rats. *Inflammopharmacol*, 18: 33-39

Ogata M, Yoshida S, Kamochi M, Shigematsu A, Mizuguchi Y. (1991) Enhancement of lipopolysaccharide-induced tumor necrosis factor production in mice by carrageenan pretreatment. *Infect Immun*, 59: 679-683

Ou P, Wolff SP. (1996) A discontinuous method for catalase determination at near physiological concentrations of H2O2 and its application to the study of H_2O_2 fluxes within cells. *J Biochem Bioph Meth*, 31: 59-67

Paciolla MD, Davies G, Jansen SA. (1999) Generation of hydroxyl radicals from metal-loaded humic acids. *Environ Sci Technol*, 33: 1814-1818

Padilla ND, Bleeker WK, Lubbers Y, Rigter CMM, Van Mierlo GJ, Daha MR, Hack CE. (2003) Rat C-reactive protein activates the autologous complement system. *Immunology*, 109: 564-571

Palmen MJHJ, Dijkstra CD, van der Ende MB, Pena AS, van Rees EP. (1995) Anti-CD11b/CD18 antibodies reduced inflammation in acute colitis in rats. *Clin Exp Immunol*, 101: 351-356

Pant K, Singh B, Thakur P. (2012) Shilajit: A humic matter panacea for cancer. *International Journal of Toxicological and Pharmacological Research*, 4(2): 17-25

Papp LV, Lu J, Holmgren A, Khanna KK. (2007) From selenium to seleproteins: synthesis, identity and their role in human health. *Antiox Redox Sig*, 9(7):775-806

Pepys MB and Hirschfield GM. (2003) C-reactive protein: a critical update. *J.Clin.Invest*, 111:1805-1812

Phillips MR, Buyon JP, Winchester R, Weissmann G, Abramson SB. (1988) Up-regulation of the iC3b receptor (CR3) is neither necessary nor sufficient to promote neutrophil aggregation. *J. Clin*. Invest, 82: 495-501

Prior RL, Wu X, Schaich K. (2005) Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem*, 53: 4290-4302

Rabinovitch PS, June CH. (1990) Intracellular ionized calcium, membrane potential and pH, Flow Cytometry: A clinical approach. (ed. by M. G. Ormerod), pp. 161-185. IRL Press, Oxford University Press,

Ramoutar RR, Brumaghim JL. (2010) Antioxidant and anticancer properties and mechanisms of inorganic selenium, oxo-sulphur and oxo-selenium compounds. *Cell Biichem Biophys*, 58: 1-23

Rasool Y. (2008). An evaluation of the anti-inflammatory activity and mechanism of action of three novel auranofin derivatives. Pretoria: University of Pretoria, (Masters in Pharmacology)

Raychaudhuri A, Colombo C, Pastor G, Wong M and Jeng AY. (1991) Effects of capsaicin on carrageenan-induced inflammation in rat pleurisy and exudate substance P level. *Agents Action*, 31(1/2): 251-253

Rayman MP. (2000) The importance of selenium to human health. *Lancet*, 356: 233–41

Rayman MP. (2012) Selenium and human health. *Lancet*, 379: 1256-1268

Rovenský J, Svík K, Matha V, Ištok R, Kamarád V, Ebringer L, Fereník M,and Iková MS. (2005) Combination treatment of rat adjuvant-induced arthritis with methotrexate, probiotic bacteria *Enterococcus faecium*, and selenium. *Ann. N.Y. Acad. Sci*, 1051: 570–581

Saadi S, Wrenshall LE, and Platt JL. (2002) Regional manifestations and control of the immune system. *FASEB J.* 16: 849–856

Salvemini D, Wang ZQ, Wyatt PS, Bourdon DM, Marino MH, Manning PT & Currie MG. (1996) Nitric oxide: a key mediator in the early and late phase of carrageenan-induced rat paw inflammation. *Brit J Pharmacol* 118: 829-838

Salz,G. (1974) Salhumin-Gel, ein Lokaltherapeutikum mit hyperämisierender, entzündungshemmender und analgetischer Wirkung. *Med Monatsschr*, **28**, 548-550.

Seibert K, Zhang Y, Leahy K, Hauser S, Masferrer J, Perkins W, Lee L, and Isakson P. (1994) Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. *Proc. Nati. Acad. Sci*, 91:12013-12017

Seto S, Tanioka A, Ikeda M, Izawa S. (2004) Design and synthesis of novel 9-substituted-7-aryl-3,4,5,6-tetrahydro-2H-pyrido[4,3-b]- and [2,3-b]-1,5-oxazocin-6-ones as NK1 antagonists. *Bioorg Med Chem Lett*, 15:1479–1484

Schewe C, Klocking R, Helbig B, Schewe T. (1991) Lipoxygenase-inhibitory action of antiviral polymeric oxidation products of polyphenols. *Biomed. Biochim.* Acta, 50, 299-305.

Schreiber G, Tsykin A, Aldred AR, Thomas T, Fung W, Dickson PW, Cole T, Birch H, De jong FA, Milland J. (1989) The acute phase response in the rodent[a]. *Ann NY Acad Sci*, 557: 61-85

Schepetkin I, Khlebnikov A, Kwon BS. (2002) Medical drugs from humus matter: Focus on Mumie. *Drug Develop Res* 57:140–159

Schepetkin I, Khlebnikov A, Ah SY, Woo SB, Jeong CS, Klubachuk ON, Kwon BS. (2003) Characterization and biological activities of humic substances form mumie. *J Agric Food Chem*, 51: 5245-5254

Stadnyk AW, Dollard C, Issekutz TB, Issekutz AC. (2002) Neutrophil migration into indomethacin induced rat small intestinal injury is CD11a/CD18 and CD11b/CD18 co-dependent. *Gut*, 50:629–635

Steinberg CEW, Paul A, Pflugmacher S, Meinelt T, Klöckling R, Claudia W. (2003) Pure humic substances have the potential to act as xenobiotic chemicals- A review. *FEB*, 12(5): 391-401

Tarp U. (1995) Selenium in rheumatoid arthritis. A review. *Analyst*, 120: 877-81

Tateda K, Matsumoto T, Yamaguchi K. (1998) Acute induction of interleukin-6 and biophasic changes of serum complement C3 by carrageenan in mice. *Mediators Inflamm* 7: 221-223

Tateda K, Irifune K, Shimoguchi K. (1995) Potential of carrageenan to enhance antibacterial host-defence system in mice. *J Infect Chem-other*, 1 59-63

Tapeiro H, Townsend DM, Tew KD. (2003) The antioxidant role of selenium and seleno-compounds. *Biomed and Pharmacother*, 57: 134-144

Tissot M, Pradelles P, and Giroud JP, (1988) Substance –P-like levels in inflammatory exudates. *Inflammation*, 12(1): 25-35

Utsunomiya I, Nagai S, Oh-Ishi S. (1991) Sequential appearance of IL-1 and IL-6 activation in rat carrageenan-induced pleurisy. *J Immunol*, 147: 1803-1809

Valdiglesias V, Pásaro E, Méndez J, Laffon B. (2010) *In vitro* evaluation of selenium genotoxic, cytotoxic, and protective effects: a review. *Arch Toxicol*, 84: 337-351

van Rensburg CEJ, Malfeld SCK, and Dekker J. (2002) Topical application of oxifulvic acid suppresses the cutaneous immune response in mice. *Chemotherapy*, 48: 138-143.

van Rensburg CEJ and Naudé PJ. (2009) Potassium humate inhibits complement activation and the production of inflammatory cytokines in vitro. *Inflammation*, 32(4): 270-276

Van Rensburg CEJ, Snyman JR, Mokoele T, and Cromarty AD. (2007) Brown coal derived humate inhibits contact hypersensitivity; an efficacy, toxicity and teratogenicity study in rats. *Inflammation*, 30(5): 148-152

Vašková J, Veliká B, Pilátová M, Kron I, Vaško. (2011) Effects of humic acids *in vitro*. *In Vitro Cell.Dev.Biol.-Animal*, 47(5-6): 367-82

Venge P, Henriksen J, Dahl R, Hakansson LC. (1990) Excercise-induced asthma and the generation of neutrophil chemotatic activity. *J Allergy Clin Immunol*, 85(2): 498-504

Vinceti M, Wei ET, Malagoli C, Bergomi M, Vicoli G (2001). Aderse health effects of selenium in humans. *Rev Environ Health*, 16(4): 233-251

Vinegar R, Schreiber W, Hugo R. (1969) Biophasic development of carragenaan edema in rats. *J Pharmacol Exp Ther*, 166: 96-103

Visser SA. (1987) Effects of humic substances on mitochondrial respiration and oxidative phosphorylation. *Sci Total Environ*, 62: 347-354

Winter CA, Rilsley EA, Nuss GW. (1962) Carrageenan-induced oedema in hind paw of rat an assay for anti-inflammatory activity. *Proc Soc Exp Biol*, 111: 544-547

Wood SM, Beckham C, Yosioka A, Darban H, Waston RR. (2000) β-Carotene and selenium supplementation enchances immune response in aged humans. *Integr Med*, 2: 85-92

Wu J, Lyons GH, Graham RD, and Fenech MF. (2009) The effect of selenium, as selenomethionine, on genome stability and cytotoxicity in human lymphocytes measured using the cytokinesis-block micronucleus cytome assay. *Mutagenesis* 24(3): 225-232

Ximenes VF, Pessoa AS, Padoran CZ, Abrantes DC, Gomes FH, Maticoli MA, de Menezes ML. (2009) Oxidation of melatonin by AAPH-derived peroxyl radicals: evidence of a pro-oxidant effect of melatonin. *Biochim Biophys Acta*, 1790(8), 787-92

Yang HL, Hseu YC, Hseu YT, Lu FJ, Lin E, Lai JS. (2004) Humic acid induces apoptosis in human premyelocytic leukemia HL-60 cells. *Life Sci*, 75: 1817-1831

Ye SB, Chen JY, Zeng ZX. (1985) *Ssu Chuan I Hsueh Yuan Hsueh Pao*, 16, 127-129

Zhang F, Yu W, Hargrove JL, Greenspan P, Dean RG, Taylor EW, Hartle DK. (2002) Inhibition of TNFα-induced ICAM-1, VCAM-1 and E-selectin expression by selenium. *Atherosclerosis* 161: 381–386

Zhang L, Seitz LC, Abramczyk AM, Chan C. (2009) Synergistic effect of cAMP and palmitate in promoting altered mitochondrial function and cell death in HepG2 cells. *Exp Cell Res* 316: 716-727

Appendix 1

6.1 Reagent protocols

AAPH

α,α′-Azodiisobutyramidine dihydrochloride was obtained from Sigms-Aldrich (St. Louis, USA) in powder form. A 300 µM solution was prepared in EMEM prior to use.

λ- carrageenan

λ-carrageenan was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A 2% solution was prepared by dissolving100 mg of λ-carrageenan in 5 ml of dH_2O

Crystal violet

Crystal violet solution was prepared by adding 50 µl of 0.1% crystal violet to 2 ml of acetic acid (both from Sigma-Aldrich, St. Louis, USA) and making them up to 100 ml with dH_2O.

DCFH-DA

DCFH-DA was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A 2 mM solution was prepared by dissolving 4.9 mg DCFH-DA in 5 ml ethanol and stored at -20°C. A 50 µM working solution was prepared by diluting 100 µl stock solution with 3.9 ml PBS prior to use.

EMEM cell culture medium

EMEM was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A solution of 1.04% was prepared in autoclaved, ultra-pure, pyrogen-free, deionized water and adjusted to pH 7.4 using sodium hydrogen carbonate obtained from Merck Chemicals (Darmstadt, Germany) in powder form. The solution was filtered *in vacuo* thrice (Sartorius, 0.22 µm), then supplemented with the addition of 1% penicillin/streptomycin and stored at 4°C.

Fluorescein

Fluorescein was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A stock solution of 75 µg/ml was prepared by dissolving 3.76 mg of fluorscein in 50 ml PBS, a working solution was prepared by dissolving 140 µl of stock solution in 5 ml of PBS then diluting to 45 ml with d H_2O

Indomethacin

Indomethacin was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A 2 mg/ml solution was prepared by dissolving 2 mg in 1 ml of dH_2O. This was used to dose the positive control rats.

MTT

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A 5 mg/ml solution was prepared using PBS. The solution was filtered using a syringe filter thrice (Sartorius, sterile 0.22 µm)

0.83% NH_4Cl lysing buffer

0.83% NH_4Cl was prepared by adding 74 mg EDTA, 1 g $NaHCO_3$, 8.3 g NH_4Cl (all from Merck Chemicals Darmstadt, Germany) to a litre of dH_2O. For sterile applications the solution was filter sterilised 3 times through a 0.22µm filter.

PBS

BBL™ FTA haemagglutination buffer was obtained from BD in powder form. A 0.92% solution was prepared in distilled water and stored at 4°C. For sterile applications the solution was filter sterilised 3 times through a 0.22µm filter.

PHA

Phytohaemagglutinin was obtained from Remel in powder form in sealed vials, 45 mg per vial. The powder of a vial was dissolved in 5 ml of sterile dH_2O. Aliquots of 400 µl were made and frozen in -20°C.

PMA

Phorbol myristate acetate was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A solution of 1 mg/ml was prepared in absolute ethanol and stored at -80 in 500 µl aliquots. Prior to use 1 µg/ml working solution was prepared by diluting 1.5 µl stock solution in 1.5 ml RPMI-1640 .

RPMI-1640

RPMI-1640 was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A solution of 1.04% was prepared in autoclaved, ultra-pure, pyrogen-free, deionied water and adjusted to pH 7.4 using sodium hydrogen carbonate (Merck Chemicals Darmstadt, Germany) in powder form. The solution was filtered in *vacuo thrice* (Sartorius, 0.22 µm), supplemented with 1% penicillin/streptomycin and stored at 4°C. RPMI+ was prepared by adding 50 ml of heat inactivate foetal calf serum to 450 ml of RPMI

Trolox: ((+)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid)

Trolox was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A 1 mM solution was prepared by dissolving 2.5 mg Trolox in 10 ml PBS prior to use. Sonication was used to assist in dissolution.