

**An evaluation of the anti-inflammatory properties
of a brown coal derived potassium humate**

By

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ABSTRACT

Humic substances have been used as folk remedies for the last 3000 years. Recent studies have shown that humates possess anti-inflammatory properties, but the mechanism of how it affects inflammation is still unclear. In this study the anti-inflammatory properties of potassium humate, a water soluble humic acid salt, was investigated on different inflammatory pathways *in vitro* and *in vivo*. The effect of potassium humate on human mononuclear lymphocyte proliferation showed that potassium humate stimulated lymphocyte proliferation of resting-, PHA- and PWM-stimulated lymphocytes *in vitro* from concentrations of 20 to 80 $\mu\text{g/ml}$, in a dose dependant manner, where a maximum proliferation was observed at 80 $\mu\text{g/ml}$ whereas lymphocyte proliferation decreased at 100 $\mu\text{g/ml}$. On the contrary potassium humate, at 40 $\mu\text{g/ml}$, significantly inhibited the supernatant concentrations of the following cytokines; TNF- α , IL-1 β , IL-6 and IL-10 by PHA stimulated lymphocytes.

The effect of potassium humate on the alternative as well as the classical complement pathway was investigated *in vitro* using the haemolytic complement assay. Results indicated that potassium humate inhibits both the alternative and classical complement pathways without affecting the red blood cell membrane stability. Different inflammatory mechanisms were investigated *in vivo*, using the carrageenan-induced paw oedema model and the delayed type hypersensitivity reaction model. The carrageenan-induced paw oedema model was used to determine the effect of potassium humate on acute inflammation in the hind paw. Carrageenan was injected into the right hind footpad of a rat which caused an increase in paw volume due to oedema, which was measured with a plethysmometer. Potassium humate significantly inhibited the oedema at a dose of 60 mg/kg bodyweight and compared favourably with indomethacin at 10 mg/kg bodyweight.

The effect of potassium humate on the delayed type hypersensitivity reaction model was also investigated whereby rats were sensitised with sheep erythrocytes. Potassium humate was administered daily by oral gavage at a dose of 60 mg/kg bodyweight. After 7

days, rats were challenged by injecting sheep erythrocytes into the right hind footpad. The degree of inflammation was determined by measuring the increase of paw volume with a plethysmometer. It was found that potassium humate did not have an anti-inflammatory effect on the delayed type hypersensitivity reaction as opposed to the inhibition caused by dexamethasone at a dose of 30 mg/kg bodyweight. This study showed that potassium humate selectively inhibited the inflammatory pathway of the carrageenan-induced paw oedema as opposed to the delayed type hypersensitivity. The mechanism of the anti-inflammatory property of potassium humate might possibly be due to the inhibition of the complement cascade.

This study clearly shows that potassium humate possesses anti-inflammatory properties that can be utilised in the future as a potential treatment for inflammatory disorders associated with the activation of complement. However further investigation in the mechanism by which potassium humate inhibits complement activation needs to be done.

OPSOMMING

Humien substansie was vir die laaste 3000 jaar as tradisionele medikasie gebruik. Onlangse studies het bewys dat humien substansie anti-inflammatoriese eienskappe besit, maar die meganisme waardeur dit inflammasie beïnvloed, is nog onduidelik. In hierdie studie was die anti-inflammatoriese eienskappe van kalium humaat op verskillende inflammatoriese meganismes *in vitro* en *in vivo* bestudeer. Die effek van kalium humaat op menslike mononukleêre limfosiete het gewys dat dit limfosiet proliferasie van rustende limfosiete sowel as PHA- en PWM-gestimuleerde limfosiete statisties betekenisvol verhoog het in 'n dosis afhanklike wyse teen konsentrasies van 20 tot 80 µg/ml. 'n Maksimale limfosiet proliferasie kan by 80 µg/ml waargeneem word terwyl proliferasie by 100 µg/ml afgeneem het. Aan die ander kant het kalium humaat by 'n konsentrasie van 40 µg/ml die vrystelling van die volgende sitokiene; TNF- α , IL-1 β , IL-6 en IL-10 deur PHA gestimuleerde limfosiete geïnhibeer.

Die *in vitro* effek van kalium humaat op die alternatiewe en klassieke komplement paaie is deur die hemolitiese komplement toets bepaal. Die resultate het gewys dat kalium humaat 'n sterk inhiberende uitwerking op beide die alternatiewe en klassieke paaie het, terwyl dit geen effek op membraan stabiliteit van eritrosiete het nie. Verskillende inflammatoriese meganismes was *in vivo* ondersoek, deur gebruik te maak van die carrageenan-geïnduseerde poot edeem model, asook die vertraagde tipe hipersensitiwiteits reaksie model. Die carrageenan-geïnduseerde poot edeem model was gebruik om die effek van kalium humaat op akute inflammasie vas te stel. Carrageenan was in die regter agterpoot van 'n rot ingespuite, wat 'n vergroting in voet volume as gevolg van edeem veroorsaak het, wat met behulp van 'n plethysmometer vasgestel was. Kalium humaat het by 'n konsentrasie van 60 mg/kg liggaamsmassa edeem van die poot statisties betekenisvol geïnhibeer wat vergelykbaar is met die inhiberende effek van indometasien by 10 mg/kg liggaamsmassa.

Die effek van kalium humaat was ook op die vertraagde tipe hipersensitiwiteits reaksie in 'n dier model ondersoek waardeur die rotte met skaap eritrosiete gesensitiseer was.

Kalium humaat was daaglikks oraal toegedien by 'n dosering van 60 mg/kg liggaamsmassa. Na 7 dae was die rotte getoets deur skaap eritrosiete in die agterpoot in te spuit. Dit was gevind dat kalium humaat nie 'n anti-inflammatoriese effek op die vertraagde tipe hipersensitiwiteits reaksie gehad het nie wat in teenstelling is met die statisties betekenisvolle inhibisie van deksametasoon by 'n dosis van 30 mg/kg liggaamsmassa. Die studie het gewys dat kalium humaat selektief die inflammatoriese pad van die carrageenan-geïnduseerde poot edeem geïnhibeer het, terwyl dit geen effek of die vertraagde tipe hipersensitiwiteits reaksie gehad het nie. Die meganisme van die anti-inflammatoriese eienskappe van kalium humaat mag heel moontlik wees deur die inhibisie van die komplement kaskade.

Die studie het duidelik gewys dat kalium humaat anti-inflammatoriese eienskappe besit wat in die toekoms as 'n moontlike behandeling vir inflammatoriese afwykings geassosieer met die aktivering van komplement gebruik kan word. Verdere studies oor die meganisme waarop komplement inaktivering bewerkstellig, moet egter nog gedoen word.

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ABBREVIATIONS

ANOVA	One-way analysis of variance
APC	Antigen presenting cell
CD	Cluster of differentiation
COX	Cyclo-oxygenase
CR	Complement receptor
CTL	Cytotoxic T lymphocytes
DTH	Delayed type hypersensitivity
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EDTA-VBS	Ethylenediaminetetraacetic acid - veronal buffered saline
EGTA	Ethylene glycol-bis-(β -aminoethyl ether)
EGTA-VBS	Ethylene glycol-bis-(β -aminoethyl ether) - veronal buffered saline
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
HIV	Human immunodeficiency virus
ICAM-1	Intracellular adhesion molecule-1
IFN	Interferon
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
M	Molar
MAC	Membrane attack complex
MHC	Major histocompatibility complex



mM	Millimolar
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF- κ B	Nuclear factor kappa B
NKT	Natural killer T cells
NSAID	Non-steroidal anti-inflammatory drug
PAF	Platelet-activating factor
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinin A
PMA	Phorbol-12-myristate-13-acetate
RE	Rabbit erythrocytes
SD	Sprague-Dawley
SE	Sheep erythrocytes
TNF	Tumour necrosis factor
VCAM	Vascular cell adhesion molecule
VBS	Veronal buffered saline
μ l	Micro litres



CHAPTER 1

INTRODUCTION

1.1 Humates

1.1.1 Characteristics of humates

Humic substances are distributed throughout the environment and are most abundant in the top two feet of the Earth's crust, where they interact with air and water. Humic substances are the most important source of organic carbon in both terrestrial and aquatic environments (Senesi and Loffredo, 1999), and play a key role in nature. They contribute to the growth of plants, are responsible for the structure and physical–chemical properties of soil, and are involved in the majority of surface phenomena that occur in soil (Stevenson, 1994). Many of its properties are known, but its exact structure and function are still in question (Paciolla *et al.*, 2002). Figure 1 gives an illustration of the possible structure and functional groups of humic acid, a subclass of humic substances as suggested by Kleinhempel (1970). Humic substances can be fractionated into fulvic acids, brown humic acids, grey humic acids and humin, as a function of their solubility at different pH values.

Several humic substances have been identified based on solubility, molecular size and composition:

Humic acids: Humic acids are humic substances soluble in water, however are not soluble in water under acid conditions below pH 2. Humic acids are soluble in diluted alkaline solutions and precipitate as soon as the solution becomes slightly acidic. These substances have medium molecular size and their molecular weight is around 5,000 to 100,000 Dalton. Oxygen represents 33-36 %, while nitrogen represents 4 % in humic acids. Due to their medium molecular size, sufficient negative surplus charge on their surfaces for peptising the macromolecules will occur only in a more alkaline medium with a pH over 8 and thus their mobility in the soil is limited in neutral acidic-alkaline conditions (Islam *et al.*, 2005).

Fulvic acids: Fulvic acids are water soluble under all pH conditions. Fulvic acids have the lowest molecular size, as their molecular weight is around 2,000 Dalton. Fulvic acid

is the humic substance with the highest oxygen content (around 45-48 %) and the lowest nitrogen content (less than 4 %). Because of their low molecular weight their surface, negative surplus charge is sufficient to peptise the macromolecules even at neutral or slightly alkaline conditions resulting in significant mobility in the soil (Islam *et al.*, 2005).

Humus: This is the fraction of humic substances that is not soluble in water at any pH value. These substances have the greatest molecular sizes, as their molecular weights can be around 300,000 Dalton. The oxygen content in this substance is the lowest and falls in the range of 32-34 %, while the nitrogen content is the highest, being around 4 %. Because of the high molecular weight, the negative surplus charge on their surfaces is insufficient for peptising the macromolecules even at strongly alkaline pH, and so their mobility in the soil is insignificant when in a coagulated state (Islam *et al.*, 2005).

Phenolic acids: These substances are not defined based on solubility but identified as a component of humic substances (Islam *et al.*, 2005).

The higher soluble humic acids are of biological importance because they participate in many soil-plant processes (Paciolla *et al.*, 2002). Potassium humate, a soluble humic acid salt, is derived from the decomposition of organic matter, especially from plant material. Potassium humate can be derived from bituminous coal or extracted from brown coal or peat (Hartenstein, 1982).

Additionally, humic substances show a strong tendency for interaction with metal cations due to both their colloidal character, and large number of surface functional groups (Alvarez-Puebla *et al.*, 2004) and (Alvarez-Puebla *et al.*, 2004). Because of these characteristics, they play an important role in determining the mobilisation and immobilisation behaviour of metals in the environment. Humic substances show strong retention of atmospheric gases such as O₂, N₂, and CO₂, making them available to micro-organisms and plants, and also for bio-mineralization (Bhardwaj and Gaur, 1970; McMurtrie *et al.*, 2001; Rusina *et al.*, 2003; Stevenson, 1994). It is therefore clear that the

characterisation of the micro structural properties of humic substances is essential to understanding the retention processes that take place in soil.

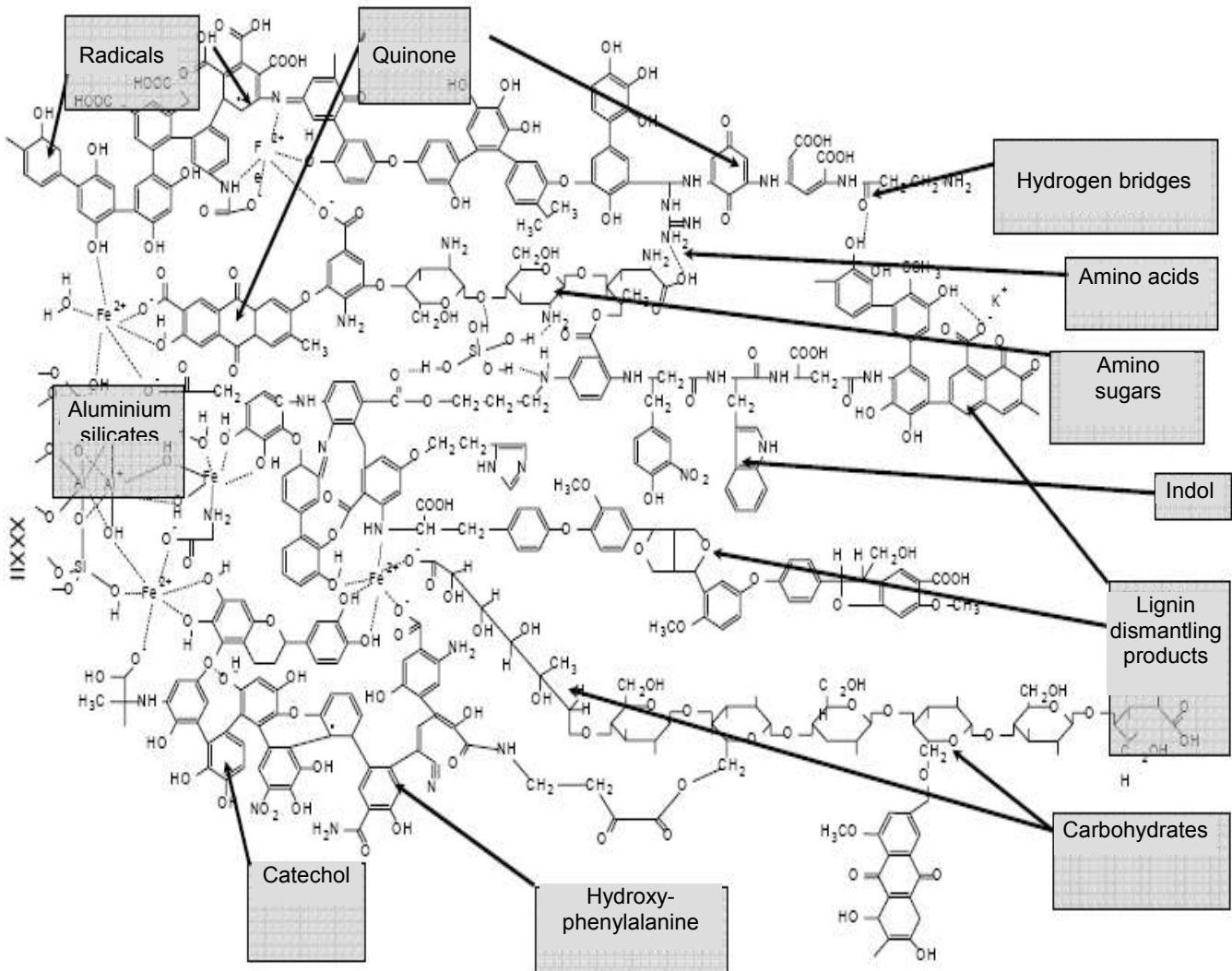


Figure 1: Illustration of the potential humic acid structure (Kleinhempel, 1970).

1.1.2 A brief history of the therapeutic uses of humates

Humates have long been used as folk remedies for a broad diversity of illnesses, for virtually 3000 years (Achard, 1986; Schepetkin *et al.*, 2002). Humates were traditionally

used in Asian herbal medicine to treat injuries, bone fractures, dislocations, diseases of skin and diseases of the peripheral nervous system. Humates were also used by Greek physicians mainly as an anti-inflammatory agent (Schepetkin *et al.*, 2002). In ancient Egypt humates were used to embalm mummies. Humates were used as a youth rejuvenator in traditional Russian and Ayurveda medicine (Khakimov, 1974; Tiwari and Tiwari, 1973).

Mud baths, rich in humic and fulvic acids, were used to treat various ailments, especially inflammatory ailments such as rheumatic conditions, during the 19th century (Batz, 1988; Kleinschmidt, 1988; Kovarik, 1988; Lent, 1988). Peat was applied to wounds and amputations during the First World War to relieve pain, prevent infections and to hasten the healing process (Haanel, 1924; Van Beneden, 1971).

Today several humic acid based medications are commercially available and have beneficial therapeutic effects on thermal burns and anti-inflammatory effects on osteoarthritis, rheumatoid arthritis, ankylosing spondylitis and cervical spondylosis. It has been described to possess antibacterial and anti-ulcerogenic, anti-allergic and anti-inflammatory properties (Schepetkin *et al.*, 2002).

1.1.3 General physiological effects of humates

The environmental and physiological impact that humic acids have on humans and animals has been studied to some degree. Humic acids are known to exist in the gastrointestinal tract of humans and animals (Visser, 1973). They circulate in the blood (Klöcking, 1994) and are metabolised by the liver (Sato *et al.*, 1986). Some reports suggest heparin-like activity for humic acid complexes (Klöcking, 1994). This is probably due to the polyanionic nature of humic acid (Klöcking, 1994).

Guinea pigs which had been exposed to a mud bath for 40 days showed a substantial increase in oxygen uptake by the liver and kidney tissues (Górniok *et al.*, 1967). These

findings correlate well with the findings by Visser, (1987), that humic substances stimulate the respiration of rat liver mitochondria and increase the efficiency of oxidative phosphorylation. *In vitro* humic acids can interact with serum albumin and bonding affinity is increased by the presence of metals such as lead and iron. The immunological activity of albumin is not affected and no interaction takes place between humic acids and β - and γ -globulins (Obenaus, 1965). *In vivo*, 67 % of intracardially injected humic acids were found to occur in a complexed form with albumin after 20 min (Klöcking, 1967).

1.1.4 Humates in the treatment of gastric ulcers and hyperacidity

Humates have been shown to be successful for the treatment of hyperacidity, gastric ulcers and acute gastroenteritis (Gramsch, 1961; Kinzlmeier, 1954; Reichert, 1966; Schlepper, 1960; Weithaler, 1954), possibly by humate calcium exchanged for H^+ in the stomach. Little interaction takes place with mineral acid at higher pH values, thus no alkalosis will take place and no acid rebound will occur. Humates form a bond with the mucous of the stomach wall and is present in the stomach for up to 2 hours, which is an extended time action, compared to most medicines with ion-exchange properties (Gramsch, 1961).

1.1.5 Influence of humates on mineral utilisation

Humic acids act as a dilator, increasing the cell wall permeability. This increased permeability allows easier transfer of minerals from the blood to the bone and cells. Experimental investigations showed that humate extract promoted transport of minerals into muscle and bone tissue (Shvetskii and Vorobeva, 1978). Humates relieved swelling from joint inflammation and it has been shown to bond to the collagen fibres to aid in repair of damaged tendons and bone. Tendon strength has been shown to increase by as much as 75% (Iubitskaia *et al.*, 1999; Kreutz, 1992). Hydroxyapatite is clinically used as a heterologous bone substitute which acts as a “guide line” for the development of the

body's own bone tissue (Kotani *et al.*, 1991). Impregnation of bovine hydroxyapatite with a low molecular humate substance encouraged bone resorption (Schlickewei *et al.*, 1993).

Anaemia and hypercholesterolemia can effectively be treated with peat humic acids, which lead to the increase of the lifespan and percentage of experimental animals' survival (Solovyeva and Lotosh, 1984)

In an *in vivo* study on rats, humates carry life-sustaining minerals to the body but also captures and removes toxic minerals from the body, indicating that humic acid had differentiated effects upon trace elements in laboratory rats (Islam *et al.*, 2005).

1.1.6 Anti-inflammatory properties of humates

Humic acid possesses strong anti-inflammatory properties. In a study, oedema was induced in the legs of rats by a protein injection. Intraperitoneally injected humic acid reduced the paw oedema (Goel *et al.*, 1990). The anti-inflammatory action of humic substances is at least as effective as that of the well-known anti-inflammatory agent dimethyl sulfoxide (DMSO) (Kühnert, 1982). This might possibly be due to the polyphenolic structure of the humic molecule (Klöcking, 1968).

Humic acid has blood flow stimulating properties, which might also contribute to its anti-inflammatory properties (Salz, 1974). Injection of humic acids accelerated wound healing in rabbits (Biber and Bogolyubova, 1952). Contributing factors to the wound healing might be the anti-inflammatory, bacteriostatic action, impact on steroid production and increase in blood flow, caused by humic acid. Humic acid baths taken by humans with polyarthritis showed to lower blood albumin and increase blood globulin levels (Hiller, 1952; Hiller, 1953). Patients suffering from rheumatism and polyarthritis were treated for 20 min, 3 times weekly, over a 5-week period in salicylated humic acid baths at a temperature of approximately 38 °C. These patients experienced a subsidence

of the pain, a relaxation of the tension in the back muscles, and were able to move more freely (Golbs *et al.*, 1982). Humic acid baths have also resulted in an increased serum amino acid level (Hiller, 1953).

An *in vitro* study of oxihumate, a water-soluble humate derived from bituminous coal, increased the proliferation of phytohaemagglutinin A (PHA) stimulated mononuclear lymphocytes as well as monocyte depleted human lymphocytes at concentrations of 20 µg/ml and upwards (Jooné *et al.*, 2003). PHA-stimulated proliferation of mononuclear lymphocytes of human immunodeficiency virus (HIV) infected individuals treated *in vitro* with oxihumate increased significantly. It was also observed *ex vivo* on HIV infected individuals following administration of 4 g oxihumate per day for 2 weeks, compared to placebo-treated individuals. Furthermore, Jooné *et al.*, (2003) found that Oxihumate increased the production of Interleukin-2 (IL-2) as well as the expression of the IL-2 receptor (CD 25) of human lymphocytes *in vitro*. Therefore, the increased lymphocyte proliferation induced by oxihumate *in vitro* can possibly be attributed to the increased production of IL-2 and CD25 (Jooné *et al.*, 2003). Potassium humate decreased complement receptor 3 (CR3) expression of phorbol-12-myristate-13-acetate (PMA), stimulated human neutrophils as well as adhesion of the PMA stimulated human neutrophils to a baby hamster kidney cell line expressing intracellular adhesion molecule-1 (ICAM-1) (Jooné *et al.*, 2004).

Gau *et al.*, (2000) showed that humic acid induced a remarkable reduction of the expression of ICAM-1, vascular cell adhesion molecule-1 (VCAM-1) and E-selectin in lipopolysaccharide (LPS) stimulated human umbilical vein endothelial cells (HUVEC) at a concentration of 100 µg/ml humic acid. However humic acid had no effect on the basal expression of ICAM-1, VCAM-1 and E-selectin on unstimulated cells. The mechanism by which humic acid inhibits ICAM-1, VCAM-1 and E-selectin expression is thought to be through inhibition of nuclear factor kappa B (NF-κB) activation (Gau *et al.*, 2000).

1.1.7 Antibacterial and anti-fungicidal properties of humates

A peat bog mud is well known for their antibacterial properties, and as mentioned, was used during World War I to prevent infection by placing peat directly on the wounds (Haanel, 1924; Van Beneden, 1971). The following pathogens were found to be affected by humic acids at a concentration of 2500 µg/ml: *Stapylococcus epidermis*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Salmonella typhimurium*, *Proteus vulgaris*, *Enterobacter cloaceae*, *Pseudomonas aeruginosa* and *Candida albicans* (Ansorg and Rochus, 1987). The antibacterial and antifungal effects of humic acids might be as a result of their chelating and surface-active properties affecting the nutrient status, and cell wall integrity of the micro-organism (Ansorg and Rochus, 1987). Although humic acid possess anti-bacterial properties, it has been shown to stabilise intestinal flora of animals (Islam *et al.*, 2005).

1.1.8 Antiviral properties of humates

Humic substances possesses antiviral properties against human (Klöcking, 1983), animal and phytopathogenic viruses (Hampton and Fulton, 1961; Thiel, 1981). Ammonium humate has an inhibiting effect on the replication of herpesvirus at concentrations of 0.5 µg/ml and higher (Thiel, 1977). No virus multiplication took place at a concentration of 20 µg/ml (Thiel, 1977).

Viruses found in humans sensitive to humates are herpesvirus hominis, influenza virus and coxsackie virus (Klöcking, 1983). The minimum ammonium humate concentrations needed for the inhibition of the following viruses are as follows: 21 µg/ml for herpes-simplex virus type 1, 40 µg/ml for influenza virus 2 and 100 µg/ml for coxsackie virus A9. A concentration of 1000 µg/ml is needed for several other types of viruses such as echovirus, adenovirus and vaccinia virus (Klöcking and Sprössig, 1975). Klöcking and Sprössig, (1975) reported that humates acts on the multiplication of herpes virus, by

primarily obstructing its absorption on the host cell by forming a physical or chemical barrier between the virus particle and its host (Klöcking and Sprössig, 1975).

Ammonium humate at a concentration of 1 % has been used in the treatment of herpes-induced skin diseases in humans such as herpes facialis, herpes integumentalis and herpes labialis (Klöcking, 1983). Ninety percent of the 78 humans suffering from herpes virus hominis responded well to treatment with a 1 % ammonium humate solution, which has been applied topically several times daily. There were no observable secondary effects (Schiller *et al.*, 1979).

It has been shown that synthetic humate analogues derived from hydroquinone inhibited the infectivity of the HIV virus of MT-2 cells *in vitro* with an IC₅₀ of 50-300 ng/ml (Schneider *et al.*, 1996), similar results were found by Van Rensburg *et al.*, (2002) whereby oxihumate inhibited HIV-1 infection of MT-2 cells with an IC₅₀ of 12.5 µg/ml, in addition no viral resistance to oxihumate developed over a 12 week period *in vitro*. A phase I trial was done to determine the safety and toxicity of oxihumate in HIV-infected patients. Although no significant difference in the viral load or CD4⁺ count between the experimental group that received oxihumate and the placebo group was observed, a significant increase in bodyweight was found in the experimental group compared to the placebo group (Botes *et al.*, 2002).

1.1.9 Humates as a desmutagen

Although humic acid was found not to act as a mutagen or to prevent spontaneous mutations, humic acid still showed to prevent mutagens from acting on cells, thus behaves as a desmutagen. Humic acid acts as a desmutagen by interacting with mutagens in the environment and can play a protective role against certain carcinogens. The efficacy of the binding to certain mutagens was found to be directly related to the molecular weight of the humic acid (Sato *et al.*, 1986).

Humic acid, administered orally to mice during 5 consecutive days at 40 mg/kg bodyweight affected the nucleic acid metabolism in ascites cancer cells and resulted in a 20 % – 25 % decrease of RNA and DNA in the tumour cells. The base composition of the nucleic acids was also found to have changed (Zsindely, 1971). Arrest or regression of the malignant process resulted when a peat preparation was applied orally, rectally or intramuscularly in the region of the tumorous growth area (Adamek, 1976).

1.1.10 Detoxifying properties of humates

Oral doses of humic acid reduce heavy metal absorption in animals and also decrease pesticide toxicity (Lin and Lee, 1992). Humic acid has the ability to interact with various types of organic compounds and will therefore be able to reduce the harmful effect of certain endogenic and exogenic organic toxins and their metabolites in the body (Golbs *et al.*, 1982).

Oxihumate showed a high affinity for mycotoxins such as aflatoxin, zearalenone, ochratoxin A and the ergot alkaloids. Results from a study by Jansen van Rensburg *et al.*, (2006) indicated that humate could be considered as a potential mycotoxin binder.

Mice that received lethal doses of strychnine together with sodium humate had a 70 % higher survival rate (Solovyena and Lotosh, 1984). Sodium humate stimulated the detoxifying function of the liver in the case of a carbon tetrachloride-induced hepatitis (Solovyena and Lotosh, 1984) and reduced the intestinal absorption of parathion when administered together with parathion (Fuchs, 1986).

1.1.11 Toxicity of humic acid

A 30 day toxicity and teratogenicity study was done at the University of Pretoria Biomedical Research Centre on Sprague Dawley rats using potassium humate obtained

from the Latrobe valley in Australia. No toxicity was observed at a dose of 1000 mg/Kg bodyweight and no teratogenicity abnormalities were found at a dose of 500 mg/Kg bodyweight (Van Rensburg *et al.*, in press). This confirms the toxicity results obtained by The European Agency for the Evaluation of Medicinal Products in an identical experiment on humic acids derived from lignite. They also documented an acute toxicity experiment an LD₅₀ greater than 11 500 mg/kg in rats after oral administration [The European Agency for the Evaluation of Medicinal Products, Summary Report, (1999)].

For humans, humic acid at a dose of 100-300 mg/kg bodyweight had no effect on bleeding time, clotting time, thrombin time, plate count, or induced platelet aggregation (Malinowska *et al.*, 1993). Red blood cells and haemoglobin level remained at normal levels under the influence of humate in comparison with control (Lotosh, 1991). Furthermore in a trial whereby HIV-infected patients received oxihumate at a dose of 8 g/day, none of the biochemical and haematological parameters differed significantly from the baseline at the end of the study (Botes *et al.*, 2002)

1.2 Inflammation

Inflammation can be defined as a localised, protective event elicited by injury or other trauma which serves to destroy, dilute, or ward off both the injurious agents and the injured tissue. The inflammatory response is triggered by chemical signals released by tissue and cells of the immune system (Pathak and Palan, 2005).

Celsus in ancient Rome (30–38 B.C.) and Galen (130-200 A.D.) characterised inflammation based on visual observation, using five cardinal signs, namely redness (*rubor*), swelling (*tumour*), heat (*calor*; only applicable to the body's extremities), pain (*dolor*) and loss of function (*functio laesa*) (Hurley, 1972). Although inflammation was recognised as being part of the healing process in the ancient times, inflammation was viewed as being an undesirable response that was harmful to the host, up to the end of the 19th century. The work of Metchnikoff and others in the 19th century contributed new insights in the role inflammation played in the body's defensive and healing process

(Hurley, 1972). Furthermore inflammation is considered the cornerstone of pathology in that the changes observed are indicative of injury and disease. Inflammation is characterised by a number of features that includes; vasodilation leading to redness, increased vascular permeability leading to swelling of tissues (oedema), pain, recruitment of leukocytes into tissues and alterations in tissue function (Page *et al.*, 2006).

1.2.1 Cellular components in inflammation

Inflammatory responses require activation of cellular components: lymphocytes, neutrophils, eosinophils, basophils, mast cells and monocytes, although not all cell types need be involved in an inflammatory episode. These cells migrate from circulation to the area of tissue damage and become activated to assist in the inflammatory response (Bennet and Brown, 2003).

a) Lymphocytes

The lymphocyte is the key cell involved in the immune response, plays a pivotal role in inflammation and represents approximately 20 – 40% of the circulating white blood cells (Rao, 2005). The two major lymphocyte populations are the T- and B- lymphocytes.

T lymphocytes

T cells can only recognise antigens that are associated with the cell-membrane proteins known as major histocompatibility complex (MHC) molecules (Mak and Saunders, 2006). The two well-defined subpopulations of T cells are the T helper cells and the T cytotoxic cells which can be distinguished from one another by the presence of cluster of differentiation 4 (CD4) and cluster of differentiation 8 (CD8) glycoproteins. T cells expressing CD4 on the cell surface (CD4⁺ T cells) generally function as T helper cells and T cells expressing CD8 on the cell surface (CD8⁺ T cells) generally function as cytotoxic T cells (CTL) (Pathak and Palan, 2005).

CD4⁺ T cells

The main function of the CD4⁺ T is predominantly a helper/regulator role. CD4⁺ T cells recognise antigen associated with class II MHC molecules (Rao, 2005). After a T helper cell recognises an antigen-class II MHC complex on an antigen presenting cell (APC) the T helper cell is activated, divides and gives rise to a clone of effector cells, each specific for the same antigen-class II MHC complex (Mak and Saunders, 2006). These T helper cells secrete various growth factors known collectively as cytokines. Secreted cytokines play an imperative part in activating B cells, macrophages, T cytotoxic cells and various other immune cells. Based on cytokine-secreting patterns and function, T helper cells can be categorised into two main subsets – T helper 1 (T_{h1}) and T helper 2 (T_{h2}) cells (Rao, 2005).

T_{h1} cells are the main role players responsible for pro-inflammatory immune responses. However, unregulated T_{h1} cells can also result in delayed-type hypersensitivity and organ-specific autoimmunity. These cells secrete interferon- γ (IFN- γ), tumour necrosis factor- α (TNF- α), tumour necrosis factor- β (TNF- β) and IL-2 (Pathak and Palan, 2005), which promote opsonising of antibody production of B lymphocytes. The most important function of the cytokines produced by T_{h1} cells is to activate macrophages, thus further aiding in the inflammatory response (Mak and Saunders, 2006).

T_{h2} cells are associated with strong antibody and allergic responses, protection of epithelial and mucosal sites against pathogens and elimination of parasites and helminths. Unregulated T_{h2} cells can cause immediate type I hypersensitivity reactions such as asthma and allergies and is associated with an increase in immunoglobulin E (IgE) antibody production. IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 are considered the typical cytokines secreted by T_{h2} cells, of which IL-4 and IL-6 functions as pro-inflammatory cytokines.

CD8⁺ T cells

CD8⁺ T cells selectively respond to MHC class I-associated peptide antigens. Most CD8⁺ T cells are CTL. Activated CTL secrete inflammatory cytokines IL-2, IFN- γ , TNF- α and TNF- β which activates macrophages and induce inflammation (Pathak and Palan, 2005).

B lymphocytes

When B cells first encounter the antigen for which its membrane-bound immunoglobulin is specific, they begin to proliferate and differentiate into plasma cells, which produce large amounts of the receptor immunoglobulin in a soluble form, which is known as antibodies. Antibodies are present in the blood and tissue fluids and bind to specific antigens. IgG antibodies bound to antigens can bind to complement protein, C1 to activate the classical complement pathway. The antibody bound to the antigen activates other parts of the immune system, which help to eliminate the pathogen (Rao, 2005).

b) Neutrophils

The neutrophil is a phagocytic cell characterized by a segmented lobular nucleus and cytoplasmic granules filled with degradative enzymes capable of engulfing macromolecules and particles. Production of neutrophils in the bone marrow is stimulated by granulocyte colony stimulating factor (G-CSF). Newly formed neutrophils are released in circulation and recruited to sites of inflammation by specific chemokines and cytokines i.e. IL-8, C5a, C3a, lymphotoxin, neutrophil-activating protein and platelet factor-4 (Abbas and Lichtman, 2005). Being a highly mobile cell type, neutrophils respond rapidly in great numbers to tissue injury. Specialised constituents of the neutrophil cell surface, cytoplasmic granules, and cytosol mediate ingestion and killing of bacteria. Neutrophils coordinate the attachment and internalisation of the microbial prey by releasing an array of antimicrobial polypeptides and reactive oxidant species into the phagocytic vacuole (Babior, 1999; Klebanoff, 1999; Elsbach and Weiss, 1992). Neutrophils play a major role in complement-mediated inflammatory reactions (Abbas and Lichtman, 2005).

c) Eosinophils

Eosinophils are granulocytes that are abundant in the inflammatory infiltrates of late-phase reactions and contribute to many of the pathological processes in allergic diseases. Granulocyte macrophage colony stimulating factor (GM-CSF), IL-3 and IL-5 promote eosinophil maturation and their numbers can increase by recruitment in the setting of inflammation (Abbus and Lichtman, 2005). Cytokines produced by T_{h2} cells as well as the complement product C5a and the lipid mediators; platelet-activating factor (PAF) and the leukotriene B₄ (LTB₄) promote activation and recruitment of eosinophils to late phase reaction inflammatory sites (Mak and Saunders, 2006). Activated eosinophils produce and release lipid mediators; PAF, prostaglandins and leukotrienes (Page *et al.*, 2006). They also release granule proteins that are toxic to parasitic organisms and may injure normal surrounding tissue (Mak and Saunders, 2006).

d) Basophils and Mast cells

Basophils are present in the body in very low numbers, residing primarily in the blood until they move into the tissues during an inflammatory response, whereas mast cells are found in the tissues throughout the body, predominantly near blood vessels, nerves and beneath epithelia. Both cell types express IgE receptors, which can be triggered by antigen bound to IgE, resulting in cell activation (Mak and Saunders, 2006). Activation of these cells result in the secretion of the preformed contents of their granules i.e. histamine and enzymes by a regulated process of exocytosis. Sensitised cells also secrete lipid mediators; prostaglandins, leukotrienes and PAF as well as the following cytokines; IL-3, IL-4, IL-5, IL-13, TNF- α (Page *et al.*, 2006).

e) Monocytes/macrophages

Monocytes circulate in the blood for approximately 1 day before entering the tissues and serous cavities where they mature into macrophages. Macrophages are large, powerful phagocytes that function primarily in the tissues, engulfing and digesting foreign entities as well as cellular debris. Activated macrophages stimulate acute inflammation through the secretion of cytokines, mainly TNF- α and IL-1, chemokines such as macrophage activating factor, monocyte chemotactic protein-1 and IL-8 and short-lived lipid

mediators such as PAF, prostaglandins and leukotrienes. The collective action of these cytokines and lipid mediators leads to an inflammatory reaction associated with the accumulation of neutrophils (Abbs and Lichtman, 2005; Mac and Saunders, 2006).

1.2.2 Cytokines

Cytokines are polypeptides that are produced by various cell populations involved in the immune response and have a vital role in the initiation and regulation of these immune responses (Page *et al.*, 2006). Although cytokines are structurally different, they share several properties: Cytokine secretion is a brief, self-limited event, the actions of cytokines are often pleiotropic (ability of one cytokine to act on different cell types) and redundant (multiple cytokines having the same functional effects and cytokines often influence the synthesis and actions of other cytokines) (Abbas and Lichtman, 2005; Page *et al.*, 2006).

Interleukins (IL-1 to IL-23) are a large group of cytokines, produced mainly by T cells. Each interleukin acts on a specific, limited group of cells which express the specific receptors for the corresponding interleukin. Interferons are mainly produced early in viral infections and play an important role in limiting their spread. Colony-stimulating factors are involved in directing the division and differentiation of the bone marrow stem cells and the precursors of the blood leukocytes. Other cytokines such as TNF- α and TNF- β , have a variety of functions and are particularly important in mediating inflammation and cytotoxic reactions (Page *et al.*, 2006).

The role of several important cytokines in inflammation

The major cellular sources of IL-1 are activated neutrophils and macrophages. IL-1 β is the principal mediator of the host inflammatory response in natural immunity. T lymphocytes have a higher affinity for IL-1 β . The biological effects of IL-1 β are concentration dependant. At low concentrations it induces the synthesis of IL-6 and IL-1 by vascular endothelial cells and mononuclear phagocytes. Furthermore it acts as

mediator of local inflammation, induces local endothelial cells to secrete adhesion molecules on the cell surfaces to mediate leukocyte adhesion and induces local endothelial cells to promote coagulation and stimulate mononuclear phagocytes and endothelial cells to synthesize chemokines that act as activators of leukocytes. At high concentrations IL-1 causes fever, exert endocrine effects and initiate metabolic wasting by inducing synthesis of amyloid A protein by hepatocytes (Rao, 2005; Mak and Saunders, 2006).

IL-6 is secreted by activated macrophages, endothelial cells and T cells. IL-6 plays a crucial role during acute phase response of inflammation and serves as a co-stimulator of T cells and thymocytes (Rao, 2005; Mak and Saunders, 2006).

IL-8 is released by most cells which encounter TNF- α , IL-1 or bacterial endotoxin. Activated mononuclear phagocytes, fibroblasts and endothelial cells are the primary cellular sources of IL-8. IL-8 is a powerful chemoattractant for neutrophils and promotes inflammatory responses. *In vitro* IL-8 stimulates the activation of endothelial cells and the activation and mobility of T cells, eosinophils, basophils and monocytes (Rao, 2005; Mac and Saunders, 2006).

IL-10 is secreted primarily by activated monocytes, macrophages and T_{h1} and T_{h2} cells. The primary role of IL-10 is to down regulate immunostimulatory effects on various cell types (Rao, 2005; Mac and Saunders, 2006).

The major cellular source of TNF- α is activated mononuclear macrophages as well as antigen-stimulated T cells, NK cells and mast cells. TNF- α is an important inflammatory mediator. The biological action of TNF- α is concentration dependent. At low concentrations TNF- α upregulate the expression of adhesion molecules on vascular endothelial cells, neutrophils, macrophages, and lymphocytes. Furthermore it induces the production of cytokines, particularly IL-1, IL-6 and interferons, chemokines and TNF- α itself by immune cells of various types. Moderate concentrations promote fever response

and increased synthesis of IL-1 and IL-6. At high concentrations, TNF- α secreted during inflammation can be lethal (Rao, 2005; Mac and Saunders, 2006).

The principal sources of IL-12 are activated mononuclear phagocytes and dendritic cells. IL-12p70 is the bioactive form of IL-12 which plays a pivotal role in the regulation of cell-mediated immune response, is a potent stimulator of natural killer (NK) cells and induces the transcription of IFN- γ by NK cells (Rao, 2005; Mac and Saunders, 2006).

IL-2 is primarily produced by CD4⁺ cells and to some extent by CD 8⁺ cells and initiate proliferation and clonal expansion of activated T cells. IL-2 stimulates the growth of NK cells and enhances their cytolytic function, thus converting them into lymphokine activated killer cells. It works synergistically with IL-12 to induce IFN- γ secretion by NK cells. IL-2 acts as a B cell growth factor and stimulates antibody synthesis (Rao, 2005; Mac and Saunders, 2006).

IFN- γ is primarily produced by T_{h1} cells and to a lesser extent by CTL and natural killer cells. IFN- γ is a cytokine closely associated with the inflammatory response and the elimination of intracellularly replicating pathogens (Rao, 2005; Mac and Saunders, 2006).

1.2.3 Complement

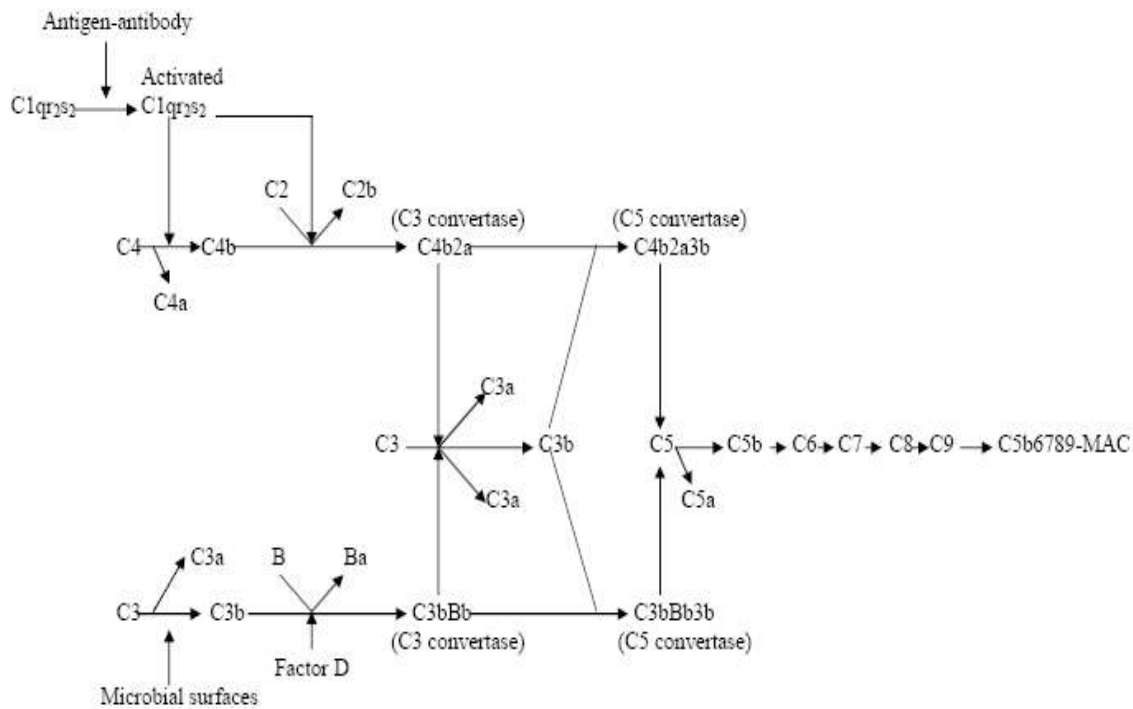
The complement system, the major effector of the humoral and cellular immune system, consists of approximately thirty heat labile, sequentially interacting proteins and glycoproteins found in the blood, plasma, and cell surfaces of all vertebrates that normally exist in an in-active, proenzyme form whose overall function is to destroy cells or invading micro organisms (Mak and Saunders, 2006). Complement activation is a cascading and chronological process. The half-life of each activated complement component is very short. Complement components are synthesised at various sites throughout the body (Pathak and Palan, 2005).

There are two distinct complement pathways of the complement system: the classical complement and the alternative complement pathway (Pathak and Palan, 2005) (Figure 2). The key event in complement activation is the formation of the enzyme C3-convertase. Both pathways share a common terminal reaction sequence that generates a macromolecular membrane attack complex (MAC), which lyses a variety of cells, bacteria and viruses (Rao, 2005). The complement reaction creates a more effective defence mechanism by amplifying the antigen–antibody reaction.

Each complement component is numbered by numerals C1-C9. The peptide fragments formed by the activation of complement are denoted by small letters; smaller fragment designated “a” and the larger fragment “b” (e.g., C5a and C5b). The smaller “a” fragments diffuse in the surrounding area and act as a chemokine, initiating a localized immune response. The larger “b” fragments bind to the target near the site of activation. While these generalizations are not true for every complement component, they should prove a useful guide to the description that follows (Pathak and Palan, 2005; Rao, 2005).

During complement activation, small, diffusible reaction products, C3a and C5a function as chemotactic and anaphylatoxin agents that increases vascular permeability, contract smooth muscle, trigger release of histamin and other substances from mast cells and basophils that induce localised vasodilatation and attract phagocytic cells chemotactically (Mak and Saunders, 2006).

Classical Complement Pathway



Alternative Complement Pathway

Figure 2: Schematic illustration of the alternative and classical complement pathway (Mak and Saunders, 2006; Pathak and Palan, 2005).

Receptors for complement proteins

Many of the biological activities of the complement system are mediated by the binding of complement fragments to membrane receptors expressed on various cell types. Type 1 complement receptor's (CR1) main function is to promote phagocytosis of C3b- and C4b-coated particles and clearance of immune complexes from the circulation. CR1 is a high affinity receptor for C3b and C4b and is mainly expressed in blood cells, including erythrocytes, neutrophils, monocytes, eosinophils, and T and B lymphocytes. Type 2 complement receptor's (CR2) functions are to stimulate humoral immune responses by enhancing B cell activation and promoting the trapping of antigen-antibody complexes in germinal centres. CR2 is present on B lymphocytes, follicular dendritic cells, and some epithelial cells. CR2 specifically binds the cleavage products of C3b.

Type 3 complement receptor (CR3), also known as Mac-1, is an integrin that functions as a receptor for the iC3b fragment generated by proteolysis of C3b. CR3 is expressed on

neutrophils, mononuclear phagocytes, mast cells and NK cells. CR3 on neutrophils and monocytes promotes phagocytosis of microbes, opsonised with iC3b. CR3 binds to ICAM-1 on endothelial cells and promotes leukocyte attachment to the endothelium, even without complement activation, which leads to the recruitment of leukocytes to sites of infection and tissue injury. Type 4 complement receptor (CR4) is structurally similar to CR3 with similar functions (Mak and Saunders, 2006).

1.2.4 Eicosanoids

The activation of phospholipase A₂ in cell membranes initiates a cascade of events that leads to the production of 20-carbon unsaturated fatty acids, which are short-lived, extremely potent and formed in almost every tissue in the body. Eicosanoids can act as local transmitters in many situations including potentiation of pain, control of local blood flow, bronchoconstriction, regulation of platelet function and maintenance of the integrity of the stomach lining. Many families of chemically related eicosanoids are derived from arachidonic acid, the most important ones are the prostaglandins, leukotrienes and thromboxanes (Bennet and Brown, 2003; Page *et al.*, 2006).

Prostaglandins are formed from phospholipase A₂ by the action of cyclo-oxygenase 1 (COX1) and cyclo-oxygenase 2 (COX2). Prostaglandin E₂ are formed by the macrophages and its major functions are protection of gastric mucosa, vasodilation and hyperalgesia. Prostaglandin D₂ is mainly formed by the mast cells and its major functions are vasodilation, inhibition of platelet activation and bronchoconstriction. Prostaglandin F_{2α} and prostacyclin I₂ are produced by endothelial cells and function as vasodilators and inhibitors of platelet activation. Thromboxane A₂ is produced by platelets and functions as a vasoconstrictor, a bronchoconstrictor and a platelet aggregation agent (Bennet and Brown, 2003; Page *et al.*, 2006).

Arachidonic acid can be converted in some cell types by the action of 5-lipoxygenase enzymes into cysteinyl-leukotrienes. The leukotrienes cause increased vascular

permeability, vasoconstriction chemotactic activity of leucocytes and are powerful spasmogens of airway and gastrointestinal smooth muscle (Page *et al.*, 2006).

1.3 Animal models

The first recorded person that employed live animals in research was Erasistratus (304-258 B.C). He studied the cerebrum, cerebellum, nerves, and the valves of the heart. He distinguished between veins and arteries and proposed mechanical explanations for different bodily processes. Animal research has played a vital role in virtually every major medical advance of the last century and will be used as long as medical research continues (Paul and Paul, 2001). Models of human diseases reproduced in animals have long been a requisite for discovering new therapies. Rodents are animals of choice for modern medical researchers because they have a naturally short life span (two to three years) that allows scientists to observe within a short time span what happens during the pathogenesis of a disease. Many animal models have been developed to study the effect of drugs on specific immunological pathways. The carrageenan-induced paw oedema and the delayed type hypersensitivity (DTH) animal models are some of the most popular preclinical models used to investigate the anti-inflammatory properties of drugs.

1.3.1 Carrageenan-induced paw oedema animal model

The carrageenan-induced paw oedema model is very useful for the screening of anti-inflammatory agents and has been used since 1962 (Winter *et al.*, 1962). Carrageenans are a heterogeneous mixture of high-molecular-weight linear sulphated polysaccharides extracted from the cell walls of certain algae of Rhodophyta. The main source of carrageenan is *Chondrus crispus*. It is categorised into kappa-, lambda-, or iota-carrageenan, depending on the degree of sulphation and the nature of the intra-galactan bonding. Carrageenans are widely used in the food industry as a thickening, gelling and protein-suspending agent. Oedema develops when carrageenan is injected in the paw of

the rat and is the result of a biphasic event (Vinegar *et al.*, 1969). Initially the oedema is attributed to the release of histamine and serotonin, whereas the second phase is attributed to the release of prostaglandins (DiRosa and Willoughby, 1971). Tateda *et al.*, (1998) have shown that an intraperitoneal injection of carrageenan enhanced the host serum complement and IL-6 production, as well as IFN- γ and TNF- α levels (Ogata *et al.*, 1996; Tsuji *et al.*, 2004).

The products of the alternative complement pathway attract neutrophils to the injected site. The neutrophils phagocytose carrageenan, forming carrageenan-containing lysosomes that rupture leading to irreversible damage to the neutrophils (Vinegar *et al.*, 1987). The inflammatory process appears to be dependent upon activation of the alternative complement pathway (Di Rosa *et al.*, 1971). The complement system plays a major role as a powerful mediator of inflammation (Turgeon, 1996).

1.3.2 Delayed type hypersensitivity (DTH) animal model

The DTH animal model is commonly used to study leukocyte migration and subsequent macrophage activation. In the classic animal model, a guinea pig is first immunised by the administration of a protein antigen (sheep erythrocytes) in adjuvant; this step is called the sensitisation phase. About 1 to 2 weeks after sensitisation, the animal is challenged subcutaneously with sheep erythrocytes and the reaction is analysed; this step is called the effector phase (Abbas and Lichtmann, 2005).

Contact hypersensitivity is an immune response to a chemically reactive hapten that is bound covalently to self proteins in the uppermost layers of the skin. Contact hypersensitivity in animal models is usually induced by sensitising the animal topically with 2,4-dinitro-fluorobenzene. The animal is then challenged 7 days after sensitising by applying 2,4-dinitro-fluorobenzene topically on the ear. The thickness of the ear is then measured. Langerhans cells serves as APCs (Mak and Saunders, 2005).

1.4 Anti-inflammatory drugs

The two most commonly used anti-inflammatory drugs are non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids.

1.4.1 Non-steroidal anti-inflammatory drugs

NSAIDs were developed to be clinically useful by inhibiting the enzyme COX (Vane, 1971), which was found to also be present in the gastric mucosa (Figure 3). The finding that the COX present in inflammatory lesions (COX2) was distinct from that found in the stomach (COX1) led to the development of selective COX2 inhibitors. These drugs include; celecoxib, rofecoxib, etodolac and nabumetone. COX2 inhibitors provide relief from many of the symptoms of arthritis but have a reduced potential to cause gastric ulceration (Hawkey, *et al.*, 2001) however, selective COX-2 inhibitors still exhibit significant renal toxicity, interfere with the healing of gastrointestinal ulcers, and may exert significant pro-thrombotic and hypertensive side effects (Page *et al.*, 2002).

1.4.2 Glucocorticoids

Similarly, glucocorticoids are widely used in the treatment of inflammation (Figure 3). Examples of corticosteroids are cortisone, prednisolone, dexamethasone and bethamethasone. Unlike the NSAIDs, these agents inhibit transcription of the COX2 genes. Cortisol and synthetic glucocorticoids tend to be active at supra-physiological concentrations. Adverse effects such as suppression of the hypothalamic-pituitary-adrenal axis and Cushingoid changes are inevitable (Sommers, 2000). Many of these adverse effects can be avoided by giving glucocorticoids topically. This has led to the development of inhaled glucocorticoids for the treatment of inflammatory diseases of the respiratory tract and steroid containing creams for the treatment of skin inflammation.

However, applying this approach to the treatment of rheumatoid arthritis requires the undesired use of intra-articular injection.

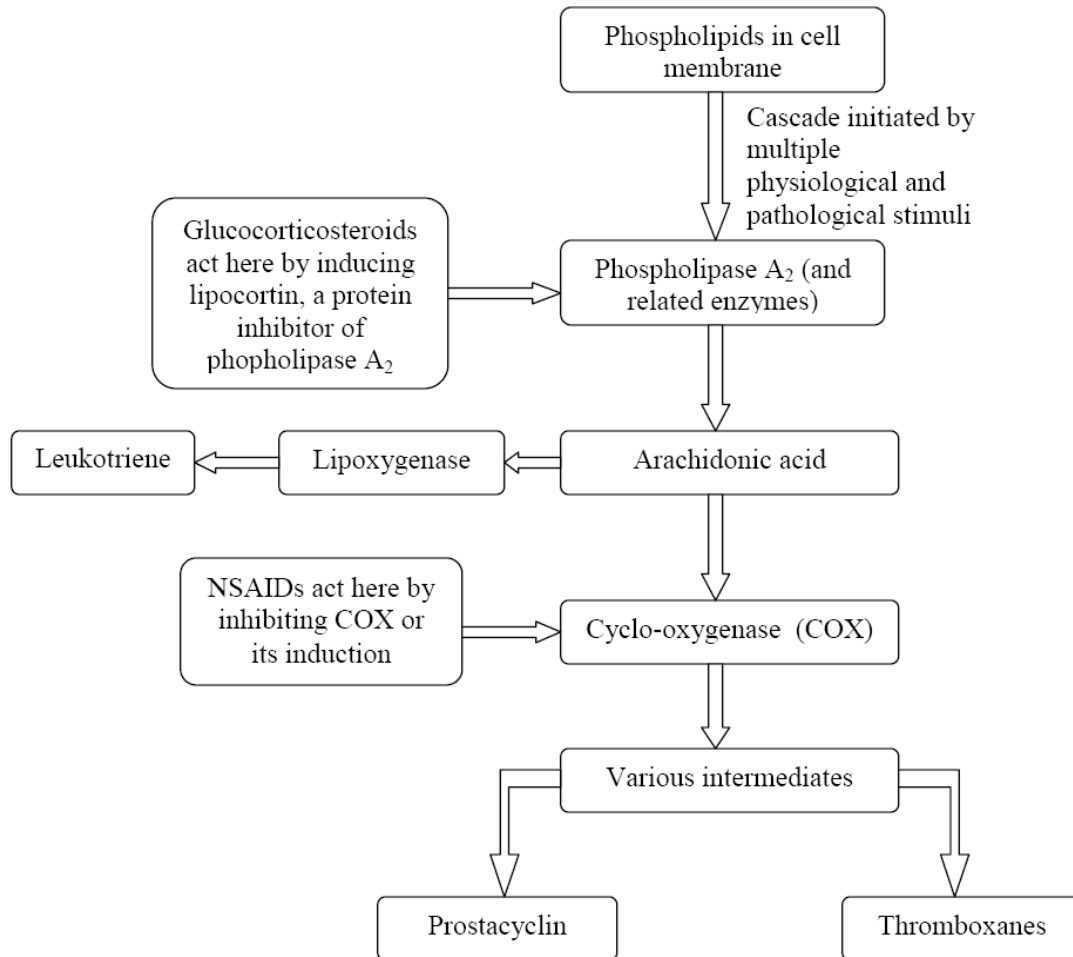


Figure 3: The influence of NSAIDs and glucocorticoids on the release of arachidonic acid and its metabolites (Page *et al.*, 2002).

1.4.3 Disease modifying antirheumatic drugs

Disease-modifying drugs may have the capacity to alter the course of rheumatoid arthritis. These drugs have minimal direct non-specific anti-inflammatory or analgesic effects and usually have a slow onset of efficacy. These drugs include the following:

a) Antimalarials

Hydroxychloroquine and chloroquine are examples of antimalarials used to reduce inflammation of rheumatoid arthritis and are particularly effective in patients with systemic lupus erythematosus. These drugs are taken orally. The exact anti-inflammatory mechanism of hydroxychloroquine and chloroquine is still unclear, but there is some evidence that they interfere with a wide variety of leukocyte functions and that they may inhibit IL-1 production by macrophages and lymphocytes. The major clinical concern regarding the adverse effects of hydroxychloroquine and chloroquine is retinal toxicity (Page *et al.*, 2002).

b) Gold salts

Gold salts have historically been used in the United States for rheumatoid arthritis by injecting the gold salts intramuscularly. Gold sodium thiomalate, and aurothioglucose are two parenteral gold salts and auranofin is a gold salt that can be taken orally. Gold salts are taken up by reticuloendothelial cells (i.e. the bone marrow, lymph nodes, liver and spleen) and impair macrophage function and cytokine activity. These salts can possibly also inhibit prostaglandin synthesis and lysosomal activity and interfere with complement activity. Dermatitis, proteinuria and bone marrow suppression are the adverse effects of gold salts (Page *et al.*, 2002).

c) Penicillamine

Oral penicillamine has been a useful agent in the treatment of rheumatoid arthritis and scleroderma. It suppresses autoantibodies to IgM and has other effects on immune complexes which is still unclear. Penicillamine has many and varied adverse effects (Page *et al.*, 2002).

d) Methotrexate

Methotrexate is effective in the treatment of rheumatoid arthritis, psoriasis, polymyositis, dermatomyositis and systemic lupus erythematosus. Methotrexate is usually administered orally. Methotrexate is an immunosuppressive drug by inhibiting cell

division and suppresses primary and secondary antibody responses. Methotrexate reduces the generation of the 5-lipoxygenase pathway products by leukocytes and may also decrease IL-1 production by macrophages. The main adverse effect of methotrexate is liver damage. Other adverse effects include nausea, oral ulcers, hair loss, acute pneumonitis and bone marrow suppression (Page *et al.*, 2002).

e) Sulfasalazine

Sulfasalazine is used for the treatment of rheumatoid arthritis and inflammatory bowel disease. Sulfasalazine is administered orally. Sulfasalazine reduces natural killer cell activity and alters other lymphocyte functions. The exact mechanism of sulfasalazine is unclear. Adverse reactions include rashes in 20-40 % of patients using sulfasalazine, acute haemolysis in individuals with glucose-6-dehydrogenase deficiency, nausea, fever and arthralgias (Page *et al.*, 2002).

f) Azathioprine

Azathioprine is an immunosuppressive drug used for the treatment of rheumatoid arthritis, systemic lupus erythematosus and polymyositis and to prevent organ rejection after transplantation. Azathioprine is taken orally and is transformed to the active metabolite 6-mercaptopurine in the liver which blocks purine synthesis, cause DNA damage and have cytotoxic actions. Azathioprine suppresses T cell-mediated immune responses and suppresses antibody production. The adverse effects of azathioprine are suppression of the bone marrow and increased risk of malignancy (Page *et al.*, 2002).

g) Cyclophosphamide

Cyclophosphamide is used for the treatment of vasculitis and systemic lupus erythematosus and is also used as chemotherapy of leukemias, lymphomas and other neoplasms. It is administered intravenously and interferes with DNA synthesis and cell division and exerts a strong immunosuppressive activity. Cyclophosphamide inhibits B-cell antibody production and serum immunoglobulin levels, suppresses the antigen-induced proliferative response and cytokine production of T cells, inhibits T cell-mediated activities such as delayed type hypersensitivity reactions and inhibits many of

the inflammatory and immune activities of monocytes. Adverse effects include bone marrow suppression, hemorrhagic cystitis, permanent amenorrhea and azoospermia and increased malignancy (Page *et al.*, 2002).

h) Cyclosporine

Cyclosporine is used as an effective therapy for rheumatoid arthritis, ocular Behçet's disease, psoriasis, atopic dermatitis, aplastic anaemia, transplant rejection, nephrotic syndrome, polymyositis, dermatomyositis and severe glucocorticosteroid-dependant asthma. Cyclosporine has a selective inhibitory effect on T cells by inhibiting T cell receptor-mediated signal transduction pathway. The major adverse effect of cyclosporine is renal toxicity (Page *et al.*, 2002).

i) Leflunomide

Orally administered leflunomide is used for rheumatoid arthritis. Leflunomide is an immunomodulatory agent that has an antiproliferative and anti-inflammatory effect. The major adverse effect of leflunomide is liver toxicity (Page *et al.*, 2002).

TNF- α inhibitors

i) Etanercept

Etanercept is mainly used for rheumatoid arthritis. Etanercept is a genetically engineered protein that binds and inactivates TNF- α and is given as a subcutaneous injection. Allergic reaction is the only serious adverse effect but is uncommon. However, local injection site reactions are a common occurrence (Page *et al.*, 2002).

ii) Infliximab

Infliximab is used together with methotrexate for the treatment of rheumatoid arthritis. Infliximab is injected subcutaneously. Infliximab is an anti-TNF- α monoclonal antibody that binds to soluble cytokine TNF- α and also binds to membrane bound TNF- α . Early hypersensitivity reactions may be observed during or shortly after infusions. Serious infections might also occur (Page *et al.*, 2002).

IL-1 receptor antagonist

i) Anakinra

Anakinra is used in reducing joint pain and swelling of patients suffering from rheumatoid arthritis. Anakinra is a recombinant IL-1 receptor antagonist, administered subcutaneously, which results in a notable reduction of macrophages and lymphocytes in the synovial tissue. Serious adverse effects are rare. Injection site reactions are common (Page *et al.*, 2002).

1.4.4 Complement inhibitors as therapeutic drugs

The concept of developing complement inhibitory drugs is not new but received little scientific attention until recently. There are no inhibitors of complement activation presently available for clinic use (Sahu and Lambris, 2000). An intact complement system is important for protection against infection, but also, for maintaining the internal inflammatory homeostasis. However, improper, enhanced or uncontrolled complement activation is disadvantageous for the host. Undesired activation of the complement system is a major pathogenic factor contributing to various diseases such as ischemia-reperfusion injury, acute graft rejection, sepsis, asthma, allergic reactions, rheumatoid arthritis, Alzheimer's disease, myasthenia gravis and multiple sclerosis (Bureeva *et al.*, 2005). The involvement of this system in the early phases of the inflammatory response, as well as the wide array of pro-inflammatory consequences of complement activation (Morgan *et al.*, 1994) makes the complement system an attractive target for therapeutic intervention and has led to the isolation, design and synthesis of numerous complement inhibitors (Makrides, 1998; Asghar, 1984; Sahul and Lambris, 2000).

A broadly applicable complement inhibiting therapeutic agent, useful in acute and chronic conditions should be highly specific with either a long plasma half-life or active when taken orally and should block pathological activation of complement while causing minimal disruption of systemic complement function (Morgan and Harris, 2003).

a) Natural proteins

C1-inhibitor (C1-Inh) is the only plasma-derived protein that has been comprehensively studied as an *in vivo* complement inhibitor. It is a member of the serine proteinase inhibitor family that inhibits activated components of C1. C1-Inh showed favourable results in diseases such as sepsis (Hack *et al.*, 1992), vascular leak syndrome (Hack *et al.*, 1994) and acute myocardial infarction (Horstick *et al.*, 1997). The drawback of C1-Inh is its susceptibility to be inactivated by neutrophil elastase. To overcome this problem, C1-Inh mutants that are resistant to elastase have been developed (Eldering *et al.*, 1993) however, the therapeutic efficacy of these mutants has not yet been established. Though effective against spontaneous complement activation, these proteins are poor inhibitors of induced complement activation and are therefore unlikely to be useful for therapeutic purposes (Kalli *et al.*, 1994).

b) Recombinant proteins

The first recombinant complement inhibitor made was soluble CR1. CR1 is known to inhibit C3 convertase as well as C5 convertase and to serve as a cofactor for the inactivation of C3b and C4b. In spite of being an effective complement inhibitor, soluble CR1 suffers from a relatively short half-life of 8 hours in humans (Makrides, 1998). The half-life has been increased to 30 hours by modifying the culture conditions (Dellinger *et al.*, 1996). Soluble CR1 has shown encouraging results in Phase II trials in patients with end-stage pulmonary disease undergoing lung transplant surgery. Recently researchers started to experiment with several structural alterations of the soluble CR1 molecule.

c) Antibodies

No natural complement inhibitors of C5 have been identified. Therefore development of mouse antibodies against human C5 became the obvious option, because antibodies have a long half-life and are known to recognize their targets with high specificity. Mouse antibodies have several limitations, such as the problem of immunogenicity and the administration by intravenous perfusion. An antibody called scFv has been tested in cardiopulmonary bypass patients (Fitch *et al.*, 1999). A dose of 2 mg/kg bodyweight of scFv inhibited more than 50 % of the total complement activity for about 14 hours.

Serum from treated patients were tested for soluble C5b to C9 and showed no C5b to C9 generation and a significant reduction in leukocyte activation. Most importantly, these patients showed a significant reduction in cardiopulmonary bypass-induced myocardial damage, cognitive deficits and blood loss (Fitch *et al.*, 1999). Thurmana *et al.*, (2005) generated a novel mouse antibody targeting Factor B, which plays a major role in the activation of the alternative pathway. Preclinical tests have shown that this mouse antibody specifically inhibited the alternative pathway *in vitro* and *in vivo* (Thurmana *et al.*, 2005).

d) Small-molecule inhibitors

Small molecular-weight complement inhibitors possess several advantages over large therapeutic proteins, in that they are cost-effective, have better tissue penetration and can be administered orally (Sahu and Lambris, 2000). The following well-characterized compounds have been described:

i) Anaphylatoxin receptor antagonists

Development of antagonists of the C5a receptor is a relatively old concept. Earlier peptide analogs that were created acted as partial antagonists of the C5a receptor (Mollison *et al.*, 1992). The first full antagonist of C5a receptor was identified by Konteatis *et al.*, (1994). In a porcine myocardial ischemia-reperfusion injury, a C5a receptor antagonist reduced the infarct size markedly (Riley *et al.*, 2000). Ames *et al.*, (2001) demonstrated that a C3a antagonist inhibited neutrophil recruitment in a guinea pig model with LPS-induced airway neutrophilia and decreased paw oedema in a rat adjuvant-induced arthritis model.

ii) Compstatin

A novel 13-residue cyclic peptide that inhibits C3 was identified and later named Compstatin (Sahu *et al.*, 1996). Unlike natural inhibitors of complement that act on C3b, Compstatin binds to native C3 and inhibits its cleavage by C3 convertase. Although the peptide in its current form is effective *in vivo* when injected intra venously, structural

alterations are being considered so that the inhibitor can be administered orally (Sahu and Lambris, 2000).

iii) BCX-1470

Factor D catalyzes the cleavage of factor B bound either to C3(H₂O) or C3b and initiates and amplifies the alternative pathway. The identification of the crystal structure of factor D (Jing *et al.*, 1998) led to the development of BCX-1470, which inhibits factor D in the nanomolar range. BCX-1470 also inhibits C1s, thrombin, factor Xa, and trypsin. Results obtained from *in vivo* tests showed that it inhibited the development of reverse passive Arthus reaction-induced oedema in rats (Szalai *et al.*, 2000). BCX-1470 has also been successfully tested in phase I clinical trials in healthy subjects, in which its safety and pharmacokinetic profile were evaluated.

iv) FUT-175 (Nafamostat)

FUT-175 is a broad-spectrum synthetic serine protease inhibitor that acts as an inhibitor of C1s, factor D, and C3rC5 convertases (Inagi *et al.*, 1991). This inhibitor has been successfully tested in several animal models. It was effective in myocardial ischemia reperfusion (Homeister and Lucchesi, 1994), acute experimental pancreatitis (Araida *et al.*, 1995) and xenotransplantation (Kobayashi *et al.*, 1996). In addition, when administered to glomerulonephritis patients with hypocomplementemia, it improved serum complement levels and reduced proteinuria (Fujita *et al.*, 1993). Patients that suffered subarachnoid hemorrhage were treated with FUT-175. FUT-175 caused a decreased incidence of cerebral infarction resulting from vasospasm (Yanamoto *et al.*, 1992). Since the inhibitor is not specific for complement proteases, it is unclear whether these effects were due to complement inhibition.

v) K-76 monocarboxylic acid

K-76 monocarboxylic acid is a fungal metabolite derived from *Stachybotrys complementi* (Miyazaki *et al.*, 1980). K-76 monocarboxylic acid inhibits both the classical and alternative pathways of complement *in vitro*. It primarily inhibits the complement pathway at the C5 level (Hong *et al.*, 1979), but has also been shown to inhibit Factor I

activity (Hong *et al.*, 1980). K-76 monocarboxylic acid has been tested in several experimental models of complement activation. It reduced complement-mediated leukocyte accumulation in the subcutaneous air pouch of rats (Konno and Tsurufuji, 1983) and prevented complement-mediated injuries in a localised acid aspiration model (Yamada *et al.*, 1997). K-76 monocarboxylic acid failed to prolong the survival of xenografts (Blum *et al.*, 1998).

Possible risks associated with anti-complement therapeutics

Due to a lack of sufficient data the exact risk associated with complement inhibition is not known. Complement deficiencies are associated with increased susceptibility to infection (Wetsel and Colten, 1990), increased susceptibility to endotoxin as a result of impaired clearance (Fischer *et al.*, 1997) and autoimmunity such as that seen in systemic lupus erythematosus and glomerulonephritis (Wetsel and Colten, 1990). These complications would be likely to arise only when total complement inhibition occurs. Studies have shown that a 60% inhibition of complement is sufficient to provide therapeutic benefit in collagen-induced arthritis (Wang *et al.*, 1995). The risks associated with complement inhibitors can therefore be expected to be minimal.

There is thus a clear unmet medical need for drugs that provide relief from the symptoms of inflammation without the undesired side-effects associated with most of the anti-inflammatory drugs currently in use. Ideal anti-inflammatory drugs should be effective, inexpensive and easy to administer with minimal side-effects and be able to modulate the immune system through specific pathways and mediators to inhibit inflammation, rather than to suppress immune cell activity.

1.5 Study aim

The aim of this study was to investigate a possible mechanism of action of potassium humate with special reference to its anti-inflammatory properties by experimenting *in vitro* and *in vivo* on different inflammatory models.

1.6 Study objectives

Mechanistic studies *in vivo*:

- a) To determine whether potassium humate had an anti-inflammatory effect on a delayed type hypersensitivity model.
- b) To determine whether potassium humate had an anti-inflammatory effect on a carrageenan-induced paw oedema model.
- c) To determine whether potassium humate had an effect on serum cytokine concentrations; INF- γ , TNF- α and IL-6 of the serum obtained from the carrageenan-induced paw oedema model.

Mechanistic studies *in vitro*:

- a) To determine the effect of potassium humate on lymphocyte proliferation.
- b) To determine the effect of potassium humate on the release of extra cellular cytokines i.e. IL-1 β , IL-6, IL-8, IL-10, IL-12p70 and TNF- α by stimulated lymphocytes.
- c) To determine the effect of potassium humate on the alternative and classical complement pathways *in vitro*.
- d) To determine the effect of potassium humate on red blood cell membrane stability.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Modified Alsever's solution

24.6 g glucose (BDH Ltd Poole, Engeland), 9.6 g sodium citrate monobasic (Sigma Diagnostics, St Louis, MO, USA) and 5.04 g NaCl (Sigma Diagnostics, St Louis, MO, USA) in dissolved in 1200 ml distilled water. The pH was adjusted to 6.1 with NaOH and sterilised by passage through a 0.22 μm acetate cameo filter (Sartorius, Goettingen, Germany).

2.1.2 Veronal buffered saline (VBS) stock solution

85 g NaCl (Sigma Diagnostics, St Louis, MO, USA) and 2.75g Na-5,5-diethyl barbiturate (The British Drug Houses LTD, Poole, England) dissolved in 1500 ml deionised water.

5.75 g 5,5-diethyl barbituric acid (The British Drug Houses LTD, Poole, England) dissolved in 500 ml hot deionised water.

The two solutions were mixed and allowed to cool off to room temperature.

The pH was adjusted to 7.4.

Just before use, the stock solution was diluted 1:6 with deionised water.

2.1.3 Ethylene glycol-bis-(β -aminoethyl ether) - veronal buffered saline (EGTA-VBS) stock solution

1.0 M MgCl_2 and 0.8 mM ethylene glycol-bis-(β -aminoethyl ether) (EGTA) (Sigma Diagnostics, St Louis, MO, USA) was added to the prepared VBS stock solution.

Just before use, the stock solution was diluted 1:6 with deionised water.

2.1.4 Mg²⁺ and Ca²⁺ VBS stock solution - (VBS²⁺) stock solution

1.0 M MgCl₂ and 0.3 M CaCl₂ was added to the prepared VBS stock solution.

Just before use, the stock solution was diluted 1:6 with deionised water.

2.1.5 Ethylenediaminetetra-acetic acid - veronal buffered saline (EDTA-VBS) stock solution

1 mM ethylenediaminetetraacetic acid (EDTA) was added to the prepared VBS²⁺ stock solution (Sigma Diagnostics, St Louis, MO, USA).

Just before use, the stock solution was diluted 1:6 with deionised water.

2.1.6 Normal human serum

Blood was collected from healthy donors and allowed to clot for 1 hour at room temperature. The blood was then centrifuged at 480 g for 10 min at 4 °C. Serum was collected and stored at – 70 °C.

2.1.7 Ammoniumchloride-solution (0.83%)

8.3 g ammoniumchloride (Merck, Darmstadt, Germany), 1 g sodium carbonate (Merck) and 74 mg EDTA (Sigma Diagnostics, St Louis, MO, USA) was dissolved in 1000 ml distilled water

2.1.8 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution

150 mg MTT-powder (Sigma Diagnostics, St Louis, MO, USA) was dissolved in 30 ml phosphate buffered saline to give a final concentration of 5 mg/ml and was stored at 4°C.

2.1.9 Foetal calf serum (FCS) supplemented RPMI 1640 medium (Complete RPMI)

RPMI 1640 tissue culture medium (Bio Whittaker, Walkersville, Maryland) was supplemented with the following:

- 445 ml RPMI 1640 medium
- 5 ml antibiotics (10 000 U penicillin/ml and 10 000 µg streptomycin/ml) (Bio Whittaker, Walkersville, Maryland)
- 50 ml heat inactivated FCS (Delta Bioproducts, Kempton Park SA)

2.1.10 Phosphate buffered saline (PBS)

The dehydrated FTA-buffer from BBL (BBL Microbiology Systems, Becton Dickenson and Company, VSA) was prepared according to the method supplied by the company.

2.1.11 Heparin solution

Heparin (without preservatives) (Sigma) was dissolved in distilled water to make up a solution of 500 units heparin/ml water and sterilised by passage through a 0.22 µm acetate cameo filter. 0.1 ml of this solution was added to every 10 ml freshly drawn blood to prevent clotting.

2.1.12 Preparation of Phytohaemagglutinin A

PHA (Murex Biotech Ltd., Dartford, England) was dissolved in saline to make up a solution of 20 µg/ml and was stored at -18°C.

2.1.13 Heat inactivated foetal calf serum (FCS)

FCS (Delta Bioproducts, Kemptonpark, South Africa) was incubated for 45 minutes at 56°C.

2.1.14 White cell counting medium

1 ml of a 0.1% crystal violet (Merck) solution and 2 ml acetic acid (Saarchem-Holpro Analytics Ltd) was dissolved in 100 ml distilled water.

2.1.15 Preparation of a potassium humate stock solution

Dissolve 5 g potassium humate in 100 ml of distilled water by using a magnet stirrer for 3 hours. Centrifuged this solution at 3000 g for 30 minutes at room temperature. Keep the supernatant and discard the sediment. Determine the concentration of the supernatant using the gravimetric determination method.

2.2 Methods

In vitro experimental procedures were followed according to the most sterile procedures possible. All of the autopipet tips were autoclaved and stored in sealed containers. Only disposable plastic ware was used (microtiter plates, plastic tubes, etc.). Bottles containing media were kept sterile, stored at 4°C and only opened in a laminar flow cabinet. Microtiter plates were only opened inside the laminar flow cabinet and were tightly sealed when transported and during incubation. When unsterile reagents or experimental solutions were used for experimentation, sterilisation occurred by filtration and then kept sterile in sealed containers and were only opened and used in a laminar flow cabinet. The laminar flow cabinet was sterilised by wiping the workspace continuously with a 70 % ethanol solution.

2.2.1 Gravimetric determination of the concentration of potassium humate

Three 10 ml petri dishes were dried by placing the petri dishes in an oven at 110 °C for 24 hours and then cooled in a desiccator for 10 minutes. The weight of the petri dishes was determined. 5 ml of the potassium humate solution was added to each of the petri dishes and left for 24 hours to dry in an air extraction cabinet. The petri dishes containing the potassium humate were dried by placing the petri dishes in an oven of 110 °C for 24 hours and then cooled in a desiccator for 10 minutes. The petri dishes containing the residues were weighed and the weight of the empty petri dish was subtracted to give the dry mass of the potassium humate. The average dry weight of potassium humate added to the three petri dishes was determined and the potassium humate concentration of the solution prepared in 2.1.14 was calculated. This weight in grams was divided by 5 and then multiplied by 1000 to give the concentration in mg/ml. For *in vitro* experimentation, a stock solution of 3 mg/ml using distilled water was prepared and sterilised by passage through a 0.22 µm acetate cameo filter. The final concentration of the sterile potassium humate solution was determined by repeating the

procedure as described to determine the dry weight. A stock solution with a final concentration of 1 mg/ml using pyrogen-free deionised water was made.

For *in vivo* experimentation, the gravimetric determination of the concentration of potassium humate was followed and a stock solution of 20 mg/ml, using distilled water was prepared.

2.2.2 Delayed type hypersensitivity reaction induced by sensitisation and challenging rats with sheep erythrocytes.

DTH or type IV hypersensitivity reaction is a form of cell-mediated immunity elicited by antigen and is mediated by CD4 T-helper 1 cells. This reaction is called delayed-type hypersensitivity because the reaction appears hours to days after antigen is injected. The DTH reaction is not dependent on complement activation as compared to the carrageenan-induced paw oedema, but is primarily dependant on a cell mediated response. In modern medicine a DTH reaction is used to determine whether an individual has previously been infected with *Mycobacterium tuberculosis*. DTH is mediated by Th₁ cells that enter the site of antigen injection, recognise foreign peptide complexes on the MHC class II molecule and release inflammatory cytokines. These cytokines will then cause local blood vessel permeability, allowing plasma and accessory cells to enter the site, causing visible swelling (Janeway *et al.*, 2005).

Experimental design and drug administration

Rats were housed at the UPBRC for at least one week before the study was initiated with ad lib water and rat chow and a 12 hour day/night light cycle and were handled according to standard procedures used at the UPBRC. A DTH model was used according to a combination of the methods described by Manosroi *et al.*, (2005), Bani *et al.*, (2005) and Sharma *et al.*, (2004). Effects of potassium humate on the antigen-specific cellular immune response in rats were determined from the degree of DTH using a footpad swelling test.

Thirty female SD rats, 12 weeks old, weighing 150g-200g, were used for the main study.

The rats were allocated into one of the following groups:

- Control group (ten SD rats).
- Experimental group (ten SD rats).
- Positive control group (ten SD rats).

Day 1 of the main study:

All the rats were anaesthetised with Isoflurane and 0.5 ml blood was drawn via aseptic cardiac puncture using a 26 gauge needle and collected in 5 ml test tubes. The blood was stored at 4 °C for 2 hours to allow the blood to clot. The blood was then centrifuged for 10 min at 500 g. The serum was collected and stored at -70 °C until use.

Day 2 of the main study:

Each rat was immunised by an intraperitoneal injection of a sheep erythrocyte (SE) suspension (1×10^8 SE in 0.5 ml PBS) as described in 2.2.6.

The experimental group received 0.4 ml potassium humate (60 mg/kg bodyweight) by oral gavage.

Both the positive control (dexamethasone) group and the untreated control group received 0.4 ml water by oral gavage.

Day 3 – day 6 of the main study:

The experimental group received 0.4 ml potassium humate (60 mg/kg bodyweight) by oral gavage.

Both the positive control group and the untreated control group received 0.4 ml water by oral gavage.

Day 7 of the main study:

The experimental group received 0.4 ml potassium humate (60 mg/kg bodyweight) by oral gavage.

The positive control group received a reference drug, i.e. the COX inhibitor dexamethasone (30 mg/kg/bodyweight suspended in 0.4 ml water) by oral gavage (Smith *et al.*, 2000).

The control group received 0.4 ml water by oral gavage.

Day 8 of the main study:

The experimental group received 0.4 ml potassium humate (60 mg/kg bodyweight) by oral gavage.

The positive control group received dexamethasone (30 mg/kg/bodyweight suspended in 0.4 ml water) by oral gavage (Smit *et al.*, 2000).

The control group received 0.4 ml water by oral gavage.

One hour after drug administration the following procedures were followed:

- The volume of each right hind paw was measured with a plethysmometer (Panlab, Barcelona, Spain) directly before the paws were challenged.
- The rats were challenged by injecting the SE suspension (1×10^8 SE in 0.5 ml PBS) subplantar into the right hind footpad.

Day 9 of the main study:

- Footpad swelling of each rat was measured with a water displacement plethysmometer 24 hours after the footpad was challenged, by measuring the paw volume.
- All the rats were anaesthetised via Isoflurane inhalation. While the rats were at the surgical plane of anaesthesia, 2 ml of blood was drawn aseptically via cardiac puncture. The collected blood was stored for 2 hours at 4 °C, allowing the blood to clot. The blood was centrifuged for 10 min at 500 g and serum was collected and stored at -70 °C for use to determine serum inflammatory protein concentrations. The rats were then further exposed to isoflurane until death occurred.

2.2.3 Carrageenan-induced paw oedema

The carrageenan-induced paw oedema is a very useful model for the screening of anti-inflammatory compounds. It has been used since 1962 (Winter *et al.*, 1962) and has been established as the most popular model for evaluation of prospective anti-inflammatory drugs. Oedema develops when carrageenan is injected in the paw of the rat.

Inflammation is determined by the degree of increased paw volume compared to the volume of the unaffected paw which is measured with a plethysmometer over a time period. This is an acute inflammatory reaction which is mainly dependant on alternative complement activation, followed by neutrophil infiltration (Di Rosa *et al.*, 1971).

Experimental design and drug administration

Rats were housed at the University of Pretoria Biomedical Research Centre (UPBRC) for at least one week to acclimatise, before the study was initiated with ad lib water and rat chow and a 12 hour day/night light cycle and was handled according to standard procedures used at the UPBRC. A carrageenan-induced paw oedema was executed according to a method described by Smit *et al.*, (2000), Recio *et al.*, (2000) and Petersson *et al.*, (2001). The carrageenan paw oedema model was used to determine the effects of potassium humate on acute inflammation.

Thirty female Sprague-Dawley (SD) rats, 12 weeks old, weighing 150g-200g, were used.

The rats were allocated into one of the following groups:

- Experimental group (potassium humate); ten SD rats.
- Positive control group (indomethacin); ten SD rats.
- Untreated control group; ten SD rats.

Day 1 of the main study

All the rats were anaesthetised via isoflurane inhalation and 0.5 ml blood was drawn via aseptic cardiac puncture using a 26 gauge needle to be used to test for serum complement proteins and inflammatory cytokines.

Day 2 – day 5 of the main study

The experimental group received 0.4 ml potassium humate (60 mg/kg bodyweight) by oral gavage.

The positive control group received 0.4 ml water by oral gavage.

The untreated control group received 0.4 ml water by oral gavage.

Day 6 of the main study

The experimental group received 0.4 ml potassium humate (60 mg/kg bodyweight) by oral gavage.

The positive control group received a NSAID, i.e. indomethacin (10 mg/kg/bodyweight) by oral gavage (Smit *et al.*, 2000).

The control group received 0.4 ml water by oral gavage.

One hour after drug administration, the following procedures were followed:

- The right hind paw of each rat were measured with a water displacement plethysmometer to measure the paw volume before inflammation was initiated.
- λ -carrageenan was injected sub plantar into the right hind paw to induce inflammation (50 μ l of a 2% solution in saline).
- Paw oedema was measured from the time of injection hourly for 5 hours with a plethysmometer by measuring the paw volume.
- All the rats were anaesthetised via Isoflurane inhalation. While the rats were at the surgical plane of anaesthesia, 2 ml of blood was drawn aseptically via cardiac puncture. The collected blood was stored for 2 hours at 4 °C, allowing the blood to clot. The blood was centrifuged for 10 min at 500 g and the serum collected and stored at -70 °C for use to determine serum inflammatory protein concentrations. The rats were then further exposed to isoflurane until death occurred.

2.2.4 Lymphocyte proliferation assay

PHA and PWM are polyclonal activators of T cells and bind to many T cell receptors and activate T cells in a non-specific manner, similar to peptide-MHC complexes on APCs. PHA and PWM contain carbohydrate-binding proteins, or lectin produced by plants that cross-link human T cell surface molecules, inducing polyclonal activation and agglutination of T cells. PHA is frequently used in experimental immunology to study T cell activation. Mononuclear cells were isolated by a sediment centrifugation as described by Crucian *et al.*, (1996).

Lymphocyte preparation

Blood was drawn by venipuncture of the buccal vein from healthy human volunteers into tubes containing heparin. 30 ml heparinised blood was carefully loaded onto 15 ml Histopaque 1077 (Sigma Diagnostics, St Louis, MO, USA) and centrifuged for 25 min at 420 g at room temperature. The top plasma layer was removed and the lymphocyte/monocyte layer (± 12 ml) transferred to sterile 50 ml tubes. The tubes were then filled with RPMI and centrifuged for 15 min at 180 g at room temperature. The supernatant fluid was discarded and the tubes filled with RPMI and centrifuged for 10 min at 180 g at room temperature. The supernatant was once more discarded and the tubes were filled with sterile, cold ammonium chloride. The tubes with the cells were placed on crushed ice for ± 10 min. The tubes with the cell suspension were centrifuged for 10 min at 180 g at room temperature. The supernatant was then discarded and the tubes filled with RPMI. The tubes were centrifuged for 10 min at 180 g at room temperature, and the supernatant was discarded. The remaining pellets were resuspended in 1 ml complete RPMI. The mononuclear lymphocytes were counted, using a count chamber and suspended at a concentration of 2×10^6 cells/ml in complete RPMI.

Lymphocyte proliferation assay

The lymphocyte proliferation assay was conducted in round bottom 96 well microtiter plates. 100 μ l of a 2×10^6 cells/ml suspension was added to all the wells. 80 μ l complete RPMI was added to all the wells containing the resting control cells. 80 μ l complete

RPMI was added to all the wells containing resting cells to be incubated in potassium humate. 80 μ l complete RPMI was added to all the wells containing control cells to be stimulated with PWM or PHA. 60 μ l complete RPMI was added to all the wells containing cells to be incubated with potassium humate and stimulated with PWM or PHA. The plate containing the cell suspension was incubated for 60 minutes at 37 °C in a 5 % CO₂ incubator. 20 μ l complete RPMI was added to all the wells containing resting control cells. 20 μ l of various concentrations of potassium humate, prepared in distilled water was added to the wells containing resting cells to be incubated with potassium humate as well as to the wells containing cells to be incubated with potassium humate and stimulated with PWM or PHA (final potassium humate concentrations in wells were: 100 μ g/ml, 80 μ g/ml, 60 μ g/ml, 40 μ g/ml, 20 μ g/ml). The plate containing the cell suspension was incubated for 30 min at 37 °C in a 5 % CO₂ incubator. 20 μ l of PWM (20 μ g/ml) or PHA (20 μ g/ml) were added to the wells containing control cells to be stimulated with PWM or PHA and cells incubated with potassium humate to be stimulated with PWM or PHA. The cells were then incubated in a closed container with water for 3 days at 37°C in 5% CO₂ atmosphere.

After the 3-day incubation, the cell viability was assayed with the MTT staining method (Mosmann, T. 1983).

2.2.5 MTT assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay is a colorimetric assay used to quantitate cell proliferation and cell viability. It is a standard assay used to detect viable mammalian cells and can therefore be used to quantify whether a tested compound induce cell proliferation or cytotoxicity.

MTT, yellow in colour, is added to the cells at the end of an experiment after which the cells are incubated for a further 3-4 hours. During this incubation period, MTT is reduced to purple formazan crystals which are insoluble in polar solutions, by the

mitochondrial reductase enzyme (Figure 4). The formazan is then solubilised in DMSO to form a purple solution. The absorbance of this purple coloured solution can then be quantified by a spectrophotometric plate reader, usually at a wavelength of 570 nm.

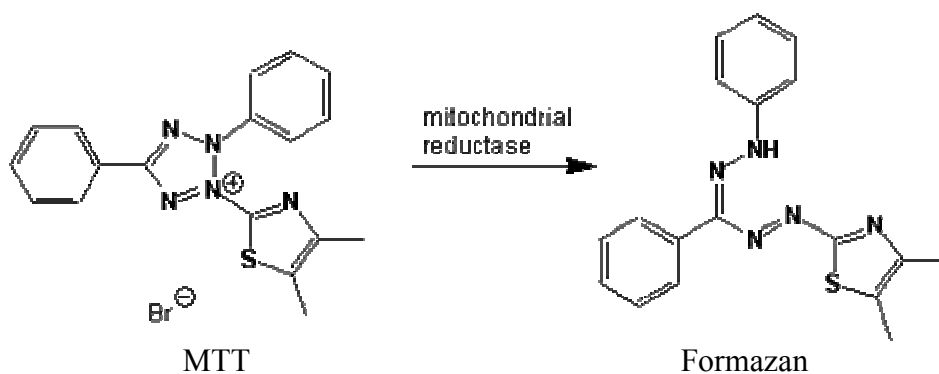


Figure 4: Molecular illustration of the reduction of MTT to formazan by mitochondrial reductase.

MTT can only be reduced to formazan by active reductase enzymes of mitochondria of viable cells. Therefore the intensity of the purple colour of the formazan is directly related to the number of viable cells (Mosman, 1983).

Method

20 μ l of MTT solution (5mg/ml PBS) was added to each well after the 3-day incubation period. The plate was re-incubated for 4 hrs at 37°C in a 5% CO₂ incubator. The plate was then centrifuged at 480 g for 10 min. The supernatant was then removed without disturbing the cell pellet. The pellets were washed with 150 μ l PBS and the supernatant removed without disturbing the cell pellet. The plate was left for 24 hours to dry in a cool dark place. 100 μ l DMSO was added to all of the wells and the crystals solubilised using a plate shaker. The plate was read on a spectrophotometric plate reader (Ceres UV 900 micro-ELISA) at dual wavelength 570 nm and 630 nm as reference wavelength.

2.2.6 Assessment of cytokine concentrations present in the supernatant after a lymphocyte proliferation assay

A Cytometric Bead Array, commonly referred to as a multiplexed bead assay, is a series of spectrally discrete particles that can be used to capture and quantitate soluble analytes such as cytokines (Figure 5). The analyte is then measured by detection of a fluorescence-based emission and flow cytometric analysis.

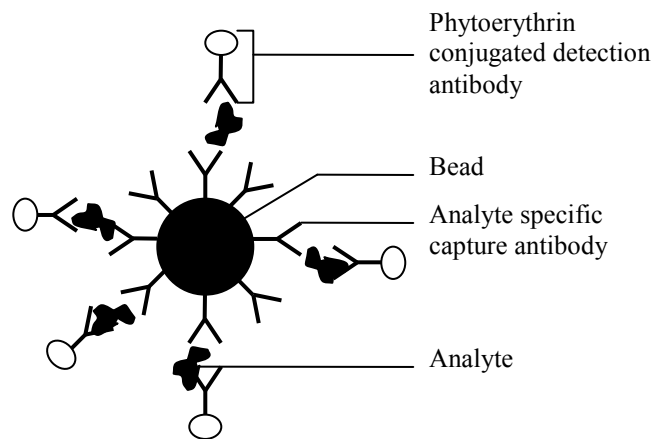


Figure 5: Illustration of a capture bead.

Human lymphocytes were obtained as described in 2.2.2. The lymphocyte proliferation assay was conducted in round bottom 96 well microtiter plates. 100 μl of a 2×10^6 cells/ml cell suspension was added to all the wells. 80 μl complete RPMI was added to all the wells containing the resting control cells. 80 μl complete RPMI was added to all the wells containing resting cells to be incubated in potassium humate. 80 μl complete RPMI was added to all the wells containing control cells to be stimulated with PHA. 60 μl complete RPMI was added to all the wells containing cells to be incubated with potassium humate and stimulated with PHA. The plate containing the cell suspension was incubated for 60

minutes at 37 °C in a 5 % CO₂ incubator. 20 µl complete RPMI was added to all of the wells containing resting control cells. 20 µl of potassium humate (40 µg/ml), prepared in distilled water was added to the wells containing resting cells to be incubated with potassium humate and the wells containing cells to be incubated with potassium humate and stimulated with PHA. The plate containing the cell suspension was incubated for 30 min at 37 °C in a 5 % CO₂ incubator. 20 µl of PHA (20 µg/ml) were added to the wells containing control cells to be stimulated with PHA and cells incubated with potassium humate to be stimulated with PHA. The cells were then incubated in a closed container with water for 36 hours at 37°C in 5% CO₂ atmosphere.

After the 36 hour incubation period, the cells were centrifuged for 10 min at 480 g. The supernatant of each well was transferred into 5 ml test tubes and stored at -70 °C. Cytokine concentrations were determined by a BD FACSArray™ flow cytometer using the BD Cytometric Bead Array Ready-to-Use human inflammation kit to determine concentrations of IL-8, IL-1β, IL-6, IL-10, TNF- α, and IL-12p70 according to the procedures included in the kit.

2.2.7 Microtiter haemolytic assay to determine the effect of potassium humate on the alternative complement pathway

The haemolytic alternative complement assay is a quick and effective *in vitro* method to determine the effects of a drug on the complement cascade. In the haemolytic alternative complement assay, human serum is incubated with rabbit erythrocytes (RE). The complement present in the human serum cause the erythrocytes to rupture, releasing haemoglobin into the supernatant, causing a red solution. A higher concentration of complement in the serum will cause more erythrocytes to rupture which will result in a higher intensity red colour. The absorbance of the red colour caused by haemoglobin in the supernatant can be measured at 412 nm on a spectrophotometric plate reader. Thus the intensity of absorbance measured, is directly related to the alternative complement activity present in the serum. This assay can effectively be used to determine the effects

of experimental compounds on complement activity as the final colour intensity will give an indication of the effect of the experimental compounds on alternative complement activity.

A modified method as described by Klerx *et al.*, (1983), Smit *et al.*, (2000), Mqadmi *et al.*, (2004) and Deharo *et al.*, (2004) was used.

Collection and standardisation of RE

Blood was obtained by puncture of the ear vein of rabbits and collected in equal volumes of modified Alsever's solution. The blood was washed 3 times with EGTA-VBS by centrifuging the blood at 480 g for 10 min at 4 °C, discarding the plasma and buffy coat with each wash. The blood obtained was then stored at 4 °C, suspended in Alsever's solution until used. Before use, blood was washed three times with PBS at 400 g for 10 min and resuspended in the same volume EGTA-VBS as the original volume Alsever's solution. The cells were counted using a cell count chamber and made to a cell suspension of 3×10^8 erythrocytes/ml in EGTA-VBS.

Assay to determine concentration of serum needed for 50% erythrocyte haemolysis (AH₅₀)

The assay was performed in round bottom 96 well microtiter plates. 40 µl of normal human serum (20 %, 15 %, 12.5%, 10 %, 7.5 %, 5 %, 2.5 %, 1.25 %, 0.625 % and 0.313 %) diluted in EGTA-VBS was added to each well. A volume of 10 µl sterile distilled water was then added to each well. 50 µl of RE suspended in EGTA-VBS was added to each well. The plate was then incubated for 60 min at 37 °C. After incubation, a 100 µl of PBS was added to each well. The plate was then centrifuged for 10 min at 500 g. 180 µl of the supernatant were aspirated and the pellet was washed by adding 180 µl PBS and centrifuged once more. Finally 180 µl of the supernatant were then aspirated, leaving a 20 µl pellet. 200 µl of distilled water was added to each well. The plate was then incubated for 60 min at room temperature to lyse the RE. After incubation, 50 µl from each well was transferred to a new microtiter plate and the new plate was read on a spectrophotometric plate reader at 405 nm wavelength. The AH₅₀ (concentration serum

needed for 50% erythrocyte haemolysis) was determined, using the obtained plate readings.

Testing the effects of potassium humate on the alternative complement pathway

A volume of 40 μl of human serum diluted in EGTA-VBS (at the determined AH_{50} serum concentration) was added to all the potassium humate experimental wells. A volume of 10 μl of various concentrations of potassium humate, prepared in distilled water was added to the potassium humate experimental wells (final potassium humate concentrations in wells were: 5 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$, 20 $\mu\text{g/ml}$, 40 $\mu\text{g/ml}$, 60 $\mu\text{g/ml}$, 80 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$). Background haemolysis control wells received 50 μl EGTA-VBS. The total haemolytic control wells each received 50 μl distilled water. The plate containing the different solutions was then incubated for 60 min at 37 °C after which 50 μl of RE suspended in EGTA-VBS was then added to all of the wells. The plate was then incubated for another 60 min at 37 °C. A 100 μl PBS was then added to each well to make up a final volume of 200 μl . The plate was then centrifuged for 10 min at 500 g and washed as described in the previous paragraph. Finally 50 μl from each well was aspirated and transferred to a new microtiter plate and read on a spectrophotometric plate reader at a wavelength of 405 nm.

2.2.8 Microtiter haemolytic assay to determine the effect of potassium humate on the classical complement pathway

The haemolytic classical complement assay is a quick and effective *in vitro* method to determine the effects of a drug on the complement cascade. In the haemolytic classical complement assay, human serum is incubated with SE. The complement present in the human serum cause the erythrocytes to rupture, releasing haemoglobin into the supernatant, causing a red solution. A higher concentration of complement in the serum will cause more erythrocytes to rupture which will result in a higher intensity red colour. The absorbance of the red colour caused by haemoglobin in the supernatant can be measured at 412 nm on a spectrophotometric plate reader. Thus the intensity of

absorbance measured, is directly related to the classical complement activity present in the serum. This assay can effectively be used to determine the effects of experimental compounds on complement activity as the final colour intensity will give an indication of the effect of the experimental compounds on classical complement activity.

A modified method as described by Klerx *et al.*, (1983), Smit *et al.*, (2000), Mqadmi *et al.*, (2004) and Deharo *et al.*, (2004) was used.

Collection and standardization of SE

Blood was obtained from a sheep by jugular vein puncture and collected in an equal volume of modified Alsever's solution. The blood was washed three times with VBS-EDTA (the second and third wash should be colourless) and the plasma and buffy coat discarded. The blood was then stored at 4 °C in the same volume of modified Alsever's solution as used to collect blood. Before use blood was centrifuged at 480 g for 10 min at 4 °C. The blood was washed three times with VBS-EDTA (the second and third wash should be colourless) and resuspended to approximately 1×10^8 cells/ml in VBS-EDTA stock solution. Anti-sheep erythrocyte antibody (Sigma Diagnostics, St Louis, MO, USA) diluted in VBS-EDTA was added to the SE suspension to make a final dilution of 1:2000 to sensitise the SE. This suspension was then incubated for 20 min at 37 °C and then for 20 min at 4 °C. After incubation the SE were washed three times with VBS²⁺.

Assay to determine CH₅₀ (concentration serum needed for 50% erythrocyte haemolysis)

The assay was performed in round bottom 96 well microtiter plates. 40 µl of normal human serum (20 %, 15 %, 12.5%, 10 %, 7.5 %, 5 %, 2.5 %, 1.25 %, 0.625 % and 0.313 %) diluted in VBS²⁺ was added to each well. A volume of 10 µl sterile distilled water was then added to each well. 50 µl of SE suspended in VBS²⁺ was added to each well. The plate was then incubated for 60 min at 37 °C. After incubation, a 100 µl of PBS was added to each well. The plate was then centrifuged for 10 min at 500 g. 180 µl of the supernatant were aspirated and the pellet was washed by adding 180 µl PBS and centrifuged once more. Finally 180 µl of the supernatant were then aspirated, leaving a 20 µl pellet. 200 µl of distilled water was added to each well. The plate was then

incubated for 60 min at room temperature to lyse the SE. After incubation, 50 μ l from each well was transferred to a new microtiter plate and the new plate was read on a spectrophotometric plate reader at 405 nm wavelength. The CH_{50} (concentration serum needed for 50% erythrocyte haemolysis) was determined, using the obtained plate readings.

Testing the effects of potassium humate on the classical complement pathway

A volume of 40 μ l of human serum diluted in VBS²⁺ (the determined CH_{50} serum concentration) was added to all the potassium humate experimental wells. A volume of 10 μ l of various concentrations of potassium humate, prepared in distilled water was added to the potassium humate experimental wells (final potassium humate concentrations in wells were: 5 μ g/ml, 10 μ g/ml, 20 μ g/ml, 40 μ g/ml, 60 μ g/ml, 80 μ g/ml and 100 μ g/ml). Background haemolysis control wells received 50 μ l VBS²⁺. The total haemolytic control wells each received 50 μ l distilled water. The plate containing the different solutions was then incubated for 60 min at 37 °C after which 50 μ l of SE suspended in VBS²⁺ was then added to all of the wells. The plate was then incubated for another 60 min at 37 °C. A 100 μ l PBS was then added to each well to make up a final volume of 200 μ l. The plate was then centrifuged for 10 min at 500 g and washed as described in the previous paragraph. Finally 50 μ l from each well was aspirated and transferred to a new microtiter plate and read on a spectrophotometric plate reader at 405 nm wavelength.

2.2.9 Sheep red blood cell membrane stability

Erythrocytes' main function is to transport oxygen to the body's tissues. It contains about 270 million haemoglobin molecules. The red colour of erythrocytes is due to the haeme group of haemoglobin protein which makes up about 97% of the red cell's dry content, and around 35% of the total content. Erythrocytes are used to test the effect of drugs on the stability of cell membranes. Lysophosphatidylcholine (LPC) is a membrane destabilising bioactive phospholipid that disrupts the erythrocyte membrane, causing

haemoglobin to be released into the supernatant. This property is used to determine the effects of compounds on the stability of membranes. The intensity of the red colour caused by the haemoglobin released by the disrupted erythrocytes can be quantified using a spectrophotometric plate reader at 412 nm wavelength. A modified method as described by Mokgobu *et al.*, (1999) was used to determine the effect of potassium humate on the membrane stability of sheep erythrocytes.

Collection and preparation of SE

Blood was obtained from a sheep by jugular vein puncture and collected in an equal volume of modified Alsever's solution. The blood was washed 3 times with modified Alsever's solution by centrifuging the blood at 480 g for 10 min at 4 °C, discarding the plasma and buffy coat with each wash. The blood was then stored at 4 °C suspended in modified Alsever's solution. Before use blood was centrifuged at 480 g for 10 min at 4 °C. The plasma and buffy coat was discarded and washed 3 times with PBS and the cells resuspended to 3×10^8 erythrocytes/ml in PBS.

Experiment

The assay was performed in 96 well microtiter plates. 40 µl of SE suspended in PBS were added to the experimental wells and control wells followed by 50 µl PBS. 10 µl of different concentrations of potassium humate were then added to the experimental wells (final concentrations in 100 µl: 1,25 µg/ml, 2,5 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml and 100 µg/ml). 10 µl distilled water were added to the control wells. The plate containing SE were incubated for 30 min at 37 °C. 50 µl of LPC solution (1mg/ml) was added to the experimental and control wells and the plate incubated for 5 min at 37 °C where partial haemolysis of the red blood cells took place. After incubation the plate was washed 3 times with PBS. 200 µl of distilled water was added to each well. The plate was then incubated for 60 min at room temperature to lyse the SE. After incubation, 50 µl from each well was transferred to a new microtiter plate and the new plate was read on a spectrophotometric plate reader at a wavelength of 405 nm.

2.2.10 Assessment of serum cytokines

Serum cytokine concentrations were determined using the enzyme-linked immunosorbent assay (ELISA). ELISA is a method for quantifying an antigen immobilised on a solid surface by use of a specific antibody with a covalently coupled enzyme. The amount of antibody that binds to the antigen is proportional to the amount of antigen present and is determined spectrophotometrically measuring the conversion of a clear substrate to a coloured product by the coupled enzyme. The assay generally uses two different types of antibodies. The one type of antibody is specific to the antigen and is immobilised on a solid surface. The second type of antibody reacts to antigen-antibody complexes, and is coupled to an enzyme. This second type of antibody causes a chromogenic or fluorogenic substrate to produce a signal that can be measured spectrophotometrically. The serum cytokine concentrations can be calculated using a standard curve.

Concentration of the cytokines, IL-6, TNF- α and IFN- γ in the serum obtained from the carrageenan-induced paw oedema study, was determined using ELISA. IL-6 was determined using the TiterZyme[®] EIA rat IL-6 ELISA kit whereas TNF- α and IFN- γ concentrations were determined using the eBioscience rat TNF- α and IFN- γ ELISA kits. The methods supplied with the kits were followed.

2.3 Statistical analysis

Data are expressed as means \pm SEM. Statistical significance was calculated using one-way analysis of variance (ANOVA), followed by either Bonferroni test for pair-wise comparisons compared to control or Tukey's multiple comparison test, compared to the equivalent control.



CHAPTER 3

RESULTS

3.1 The effect of potassium humate on the *in vivo* delayed type hypersensitivity model induced by sheep erythrocytes.

The degree of inflammation was measured by the increase in paw volume, using a plethysmometer and is expressed as an increase of the percentage of the initial paw volume before inflammation was induced. The rat was challenged with sheep erythrocytes and increase in paw volume was determined 24 hours afterwards. 24 hours after the footpad was challenged the untreated control group had a paw volume increase of 22.67 % (Figure 6). Potassium humate (at 60 mg/kg bodyweight) had no inhibitory effect on the paw volume. Whereas dexamethasone (at 30 mg/kg bodyweight) treatment reduced the increase in paw volume significantly (Figure 6).

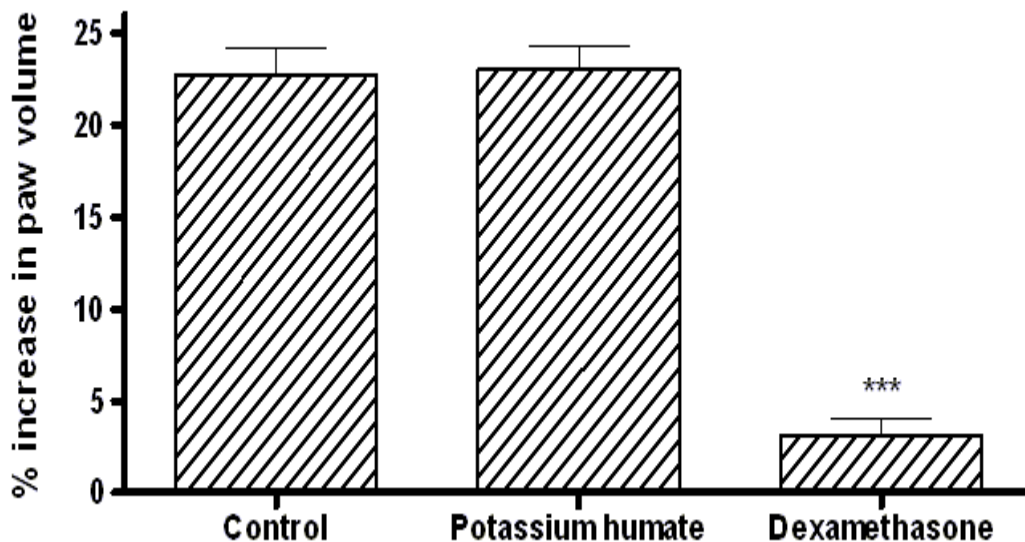


Figure 6. The effect of potassium humate (60 mg/kg bodyweight) and dexamethasone (30 mg/kg bodyweight) on the delayed type hypersensitivity model. Data are expressed as means \pm SEM. Statistical significance was calculated using ANOVA, followed by Tukey's multiple comparison test, compared to the control group.

***P < 0.0001.

3.2 The anti-inflammatory effect of potassium humate on an *in vivo* carrageenan induced inflammation model.

The injection of carrageenan into the hind paw produced an inflammatory response that was quantified by measuring the changes in footpad volume 60, 120, 180, 240 and 300 minutes afterwards. The paw volume of the untreated control rats increased significantly over the 300 minute period to a 31.6 % increase at 300 min (Figure 7). Whereas, this increase was significantly less in both the potassium humate (at 60 mg/kg bodyweight) and indomethacin (at 10 mg/kg bodyweight) treated groups from 60 min onwards (Figure 7).

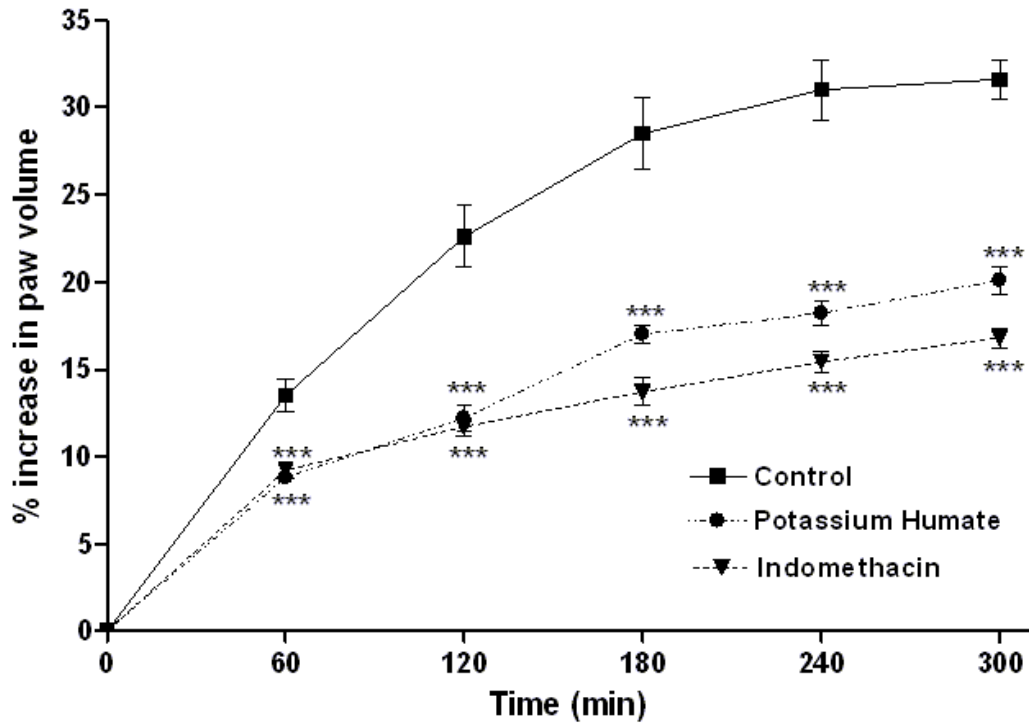


Figure 7. The effect of potassium humate (60 mg/kg bodyweight) and indomethacin (10 mg/kg bodyweight) on an *in vivo* carrageenan induced inflammation model. Data are expressed as means \pm SEM. Statistical significance was calculated using ANOVA, followed by Tukey's multiple comparison test, compared to the untreated control group.

***P < 0.0001.

3.3 The effect of potassium humate on mononuclear human lymphocytes *in vitro*.

Lymphocytes were incubated with a stimulant (PHA or PWM) or without a stimulant for a period of 72 hours. Potassium humate had a slight stimulatory effect on the proliferation of resting lymphocytes. In the case of lymphocytes stimulated with PHA, a significant, dose related increase in lymphocyte proliferation was observed at concentrations $\geq 20 \mu\text{g/ml}$. PWM stimulated lymphocytes also showed a significant increase in lymphocyte proliferation at concentrations $\geq 20 \mu\text{g/ml}$ (Figure 8).

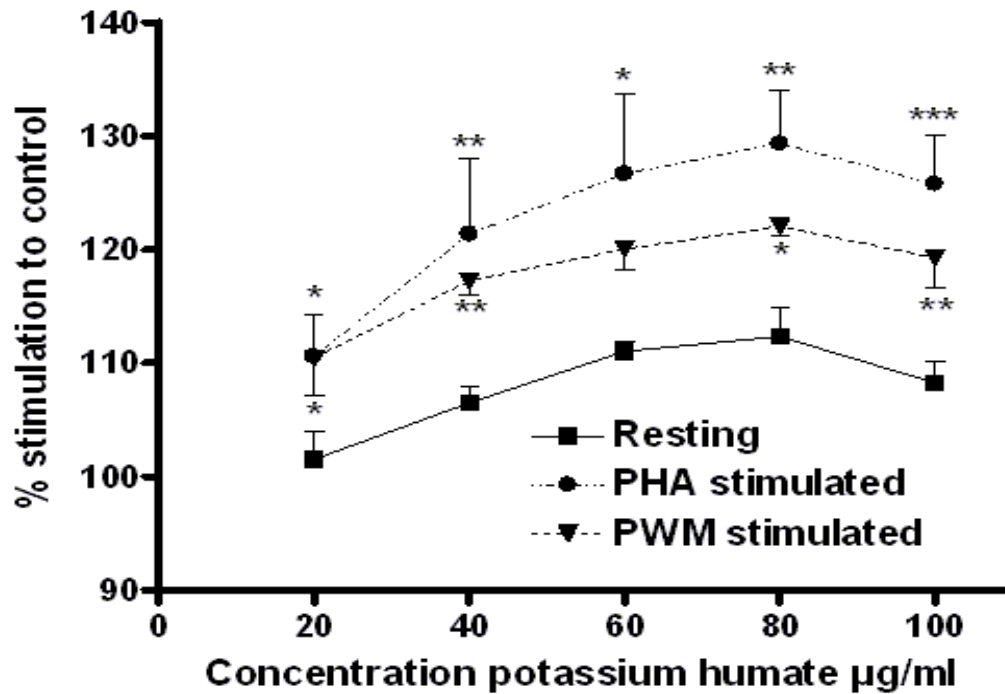


Figure 8. The effect of different concentrations potassium humate on resting and stimulated lymphocyte proliferation *in vitro*. Data are expressed as means \pm SEM. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

*P < 0.05, **P < 0.001, ***P < 0.0001.

3.4 The effect of potassium humate on TNF- α production by resting and PHA stimulated human lymphocytes *in vitro*.

Potassium humate exhibited no effect on the production of TNF- α by resting lymphocytes. However, in the case of PHA stimulated lymphocytes, potassium humate caused a significant decrease on TNF- α concentration (Figure 9).

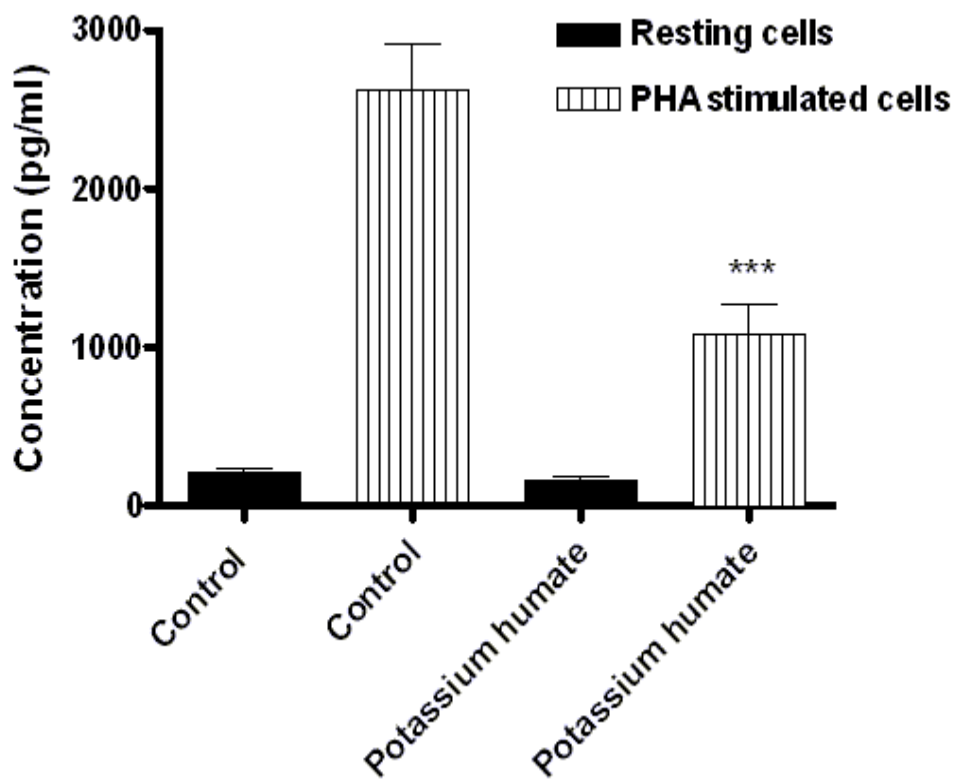


Figure 9. The effect of potassium humate at 40 μ g/ml on TNF- α production by resting and PHA stimulated lymphocytes after 36 hours incubation. Data are expressed as means \pm SEM. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control. ***P < 0.0001.

3.5 The effect of potassium humate on IL-1 β production by resting and PHA stimulated human lymphocytes *in vitro*.

Potassium humate exhibited no effect on the production of IL-1 β by resting lymphocytes. However, in the case of PHA stimulated lymphocytes, potassium humate caused a significant decrease on IL-1 β concentration (Figure 10).

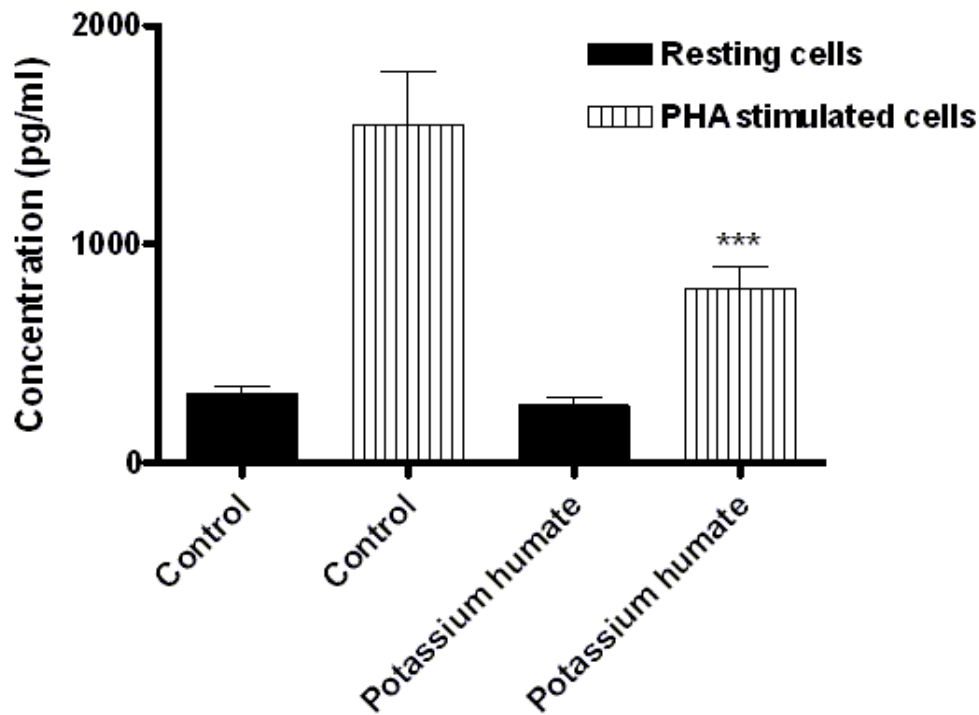


Figure 10. The effect of potassium humate at 40 μ g/ml on IL-1 β production by resting and PHA stimulated lymphocytes after 36 hours incubation. Data are expressed as means \pm SEM. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

***P < 0.0001.

3.6 The effect of potassium humate on IL-6 production by resting and PHA stimulated human lymphocytes *in vitro*.

Potassium humate exhibited no effect on the production of IL-6 by resting lymphocytes. However, in the case of PHA stimulated lymphocytes, potassium humate caused a significant decrease on IL-6 concentration (Figure 11).

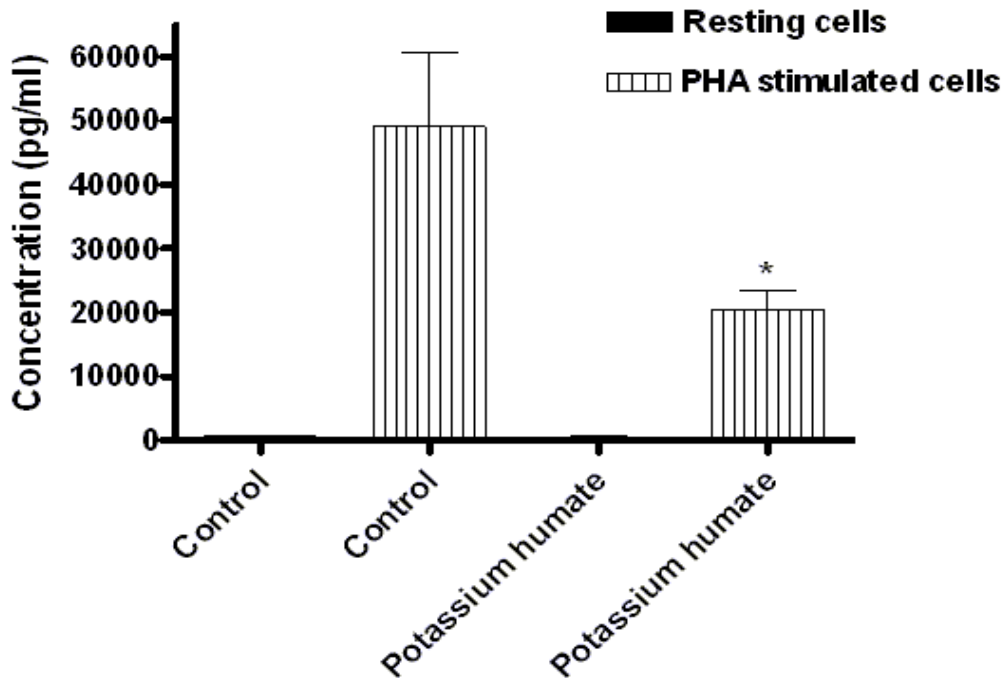


Figure 11. The effect of potassium humate at 40 µg/ml on IL-6 production by resting and PHA stimulated lymphocytes after 36 hours incubation. Data are expressed as means ± SEM. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control. *P < 0.05.

3.7 The effect of potassium humate on IL-10 production by resting and PHA stimulated human lymphocytes *in vitro*.

Potassium humate exhibited no effect on the production of IL-10 by resting lymphocytes. However, in the case of PHA stimulated lymphocytes, potassium humate caused a significant decrease in IL-10 concentration (Figure 12).

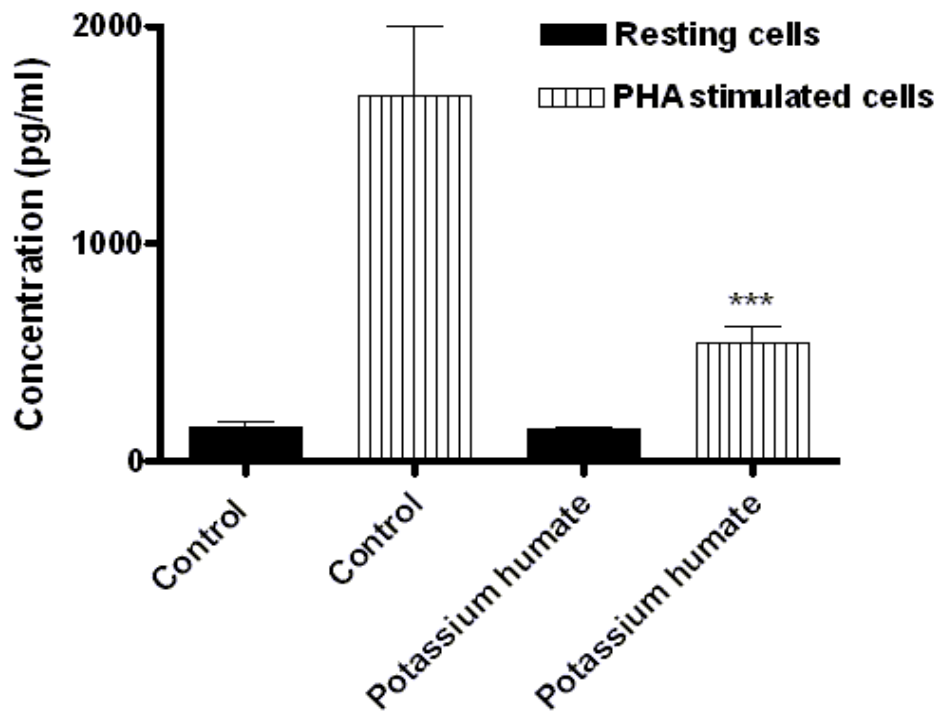


Figure 12. The effect of potassium humate at 40 µg/ml on IL-10 production by resting and PHA stimulated lymphocytes after 36 hours incubation. Data are expressed as means ± SEM. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control. ***P < 0.0001.

3.8 The effect of potassium humate on IL-12p70 production by resting and PHA stimulated human lymphocytes *in vitro*.

No significant alterations of IL-12p70 concentrations were observed (Figure 13).

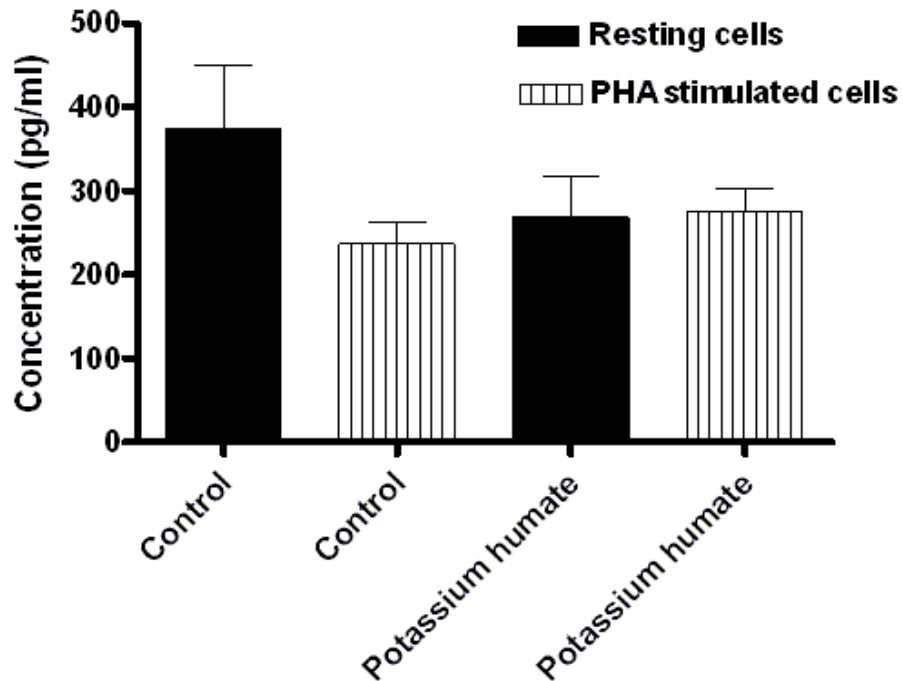


Figure 13. The effect of potassium humate at 40 $\mu\text{g/ml}$ on IL-12p70 production by resting and PHA stimulated lymphocytes after 36 hours incubation. Data are expressed as means \pm SEM. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

3.9 The inhibitory effect of potassium humate on the alternative complement pathway *in vitro*.

Anti-complementary activity expressed as a percentage inhibition of haemolysis (Figure 14). Percent haemolysis was normalised by setting 100 % lysis equal to the lysis obtained by the total haemolytic control and 0 % lysis equal to the lysis obtained by the background haemolysis control. Potassium humate had no effect on alternative complement activity at 5 $\mu\text{g/ml}$, but inhibited haemolysis 31.43 % at 10 $\mu\text{g/ml}$ and 60 % at 20 $\mu\text{g/ml}$. Potassium humate had an observable inhibition on haemolysis of 97.43% at 40 $\mu\text{g/ml}$ and 99.8% at 60 $\mu\text{g/ml}$.

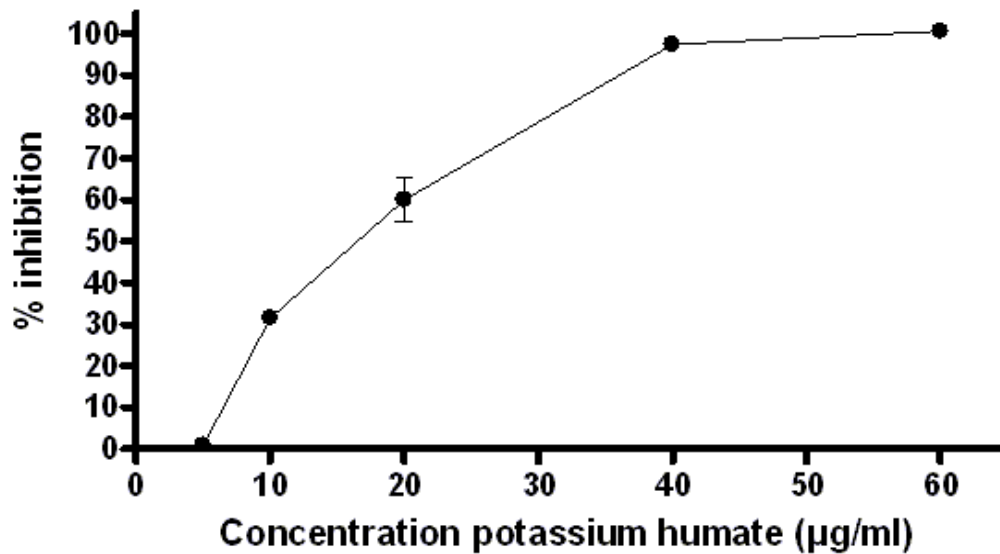


Figure 14. The inhibitory effect of potassium humate on the alternative complement haemolytic assay *in vitro*. Data are expressed as means \pm SEM.

3.10 The inhibitory effect of potassium humate on the classical complement pathway *in vitro*.

Anti-complementary activity expressed as a percentage inhibition of haemolysis (Figure 15). Percent haemolysis was normalised by setting 100 % lysis equal to the lysis obtained by the total haemolytic control and 0 % lysis equal to the lysis obtained by the background haemolysis control. Potassium humate had no effect on alternative complement activity at 5 $\mu\text{g/ml}$, but inhibited haemolysis 17.5 % at 10 $\mu\text{g/ml}$ and 93.75 % at 20 $\mu\text{g/ml}$. Potassium humate had a similar inhibitory effect of 97.25 % at 40 $\mu\text{g/ml}$ and 99 % at 60 $\mu\text{g/ml}$.

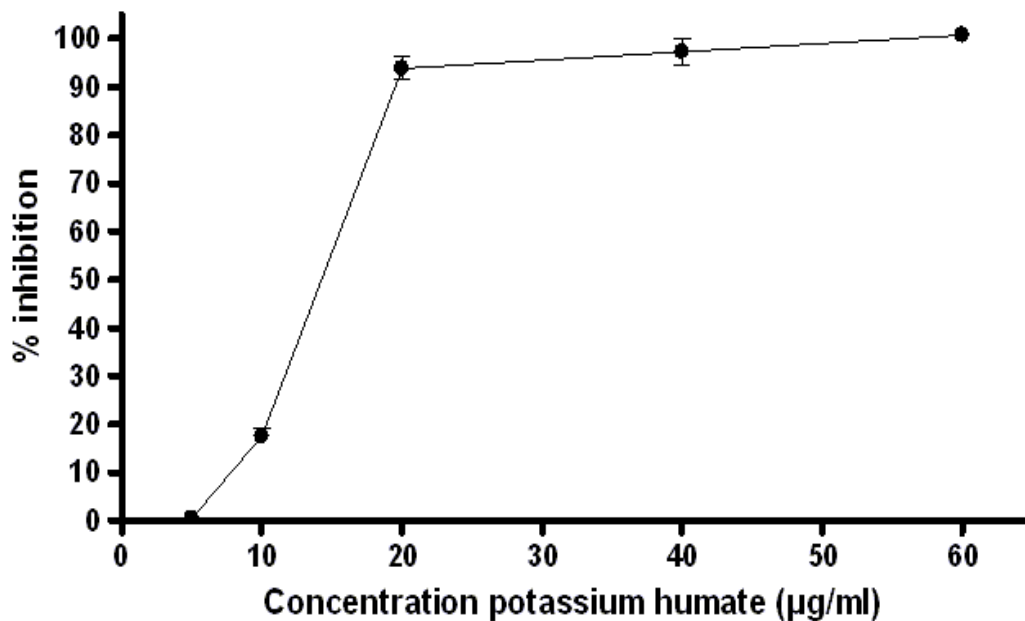


Figure 15. The inhibitory effect of potassium humate on the classical complement haemolytic assay *in vitro*. Data are expressed as means \pm SEM.

3.11 The effect of potassium humate on the membrane stability of red blood cells.

Potassium humate at a concentration of 20 $\mu\text{g/ml}$ and higher had no effect on the haemolysis of red blood cells, exposed to LPC for 5 minutes at a concentration to induce partial haemolysis, indicating that potassium humate had no effect on red blood cell stability (Figure 16).

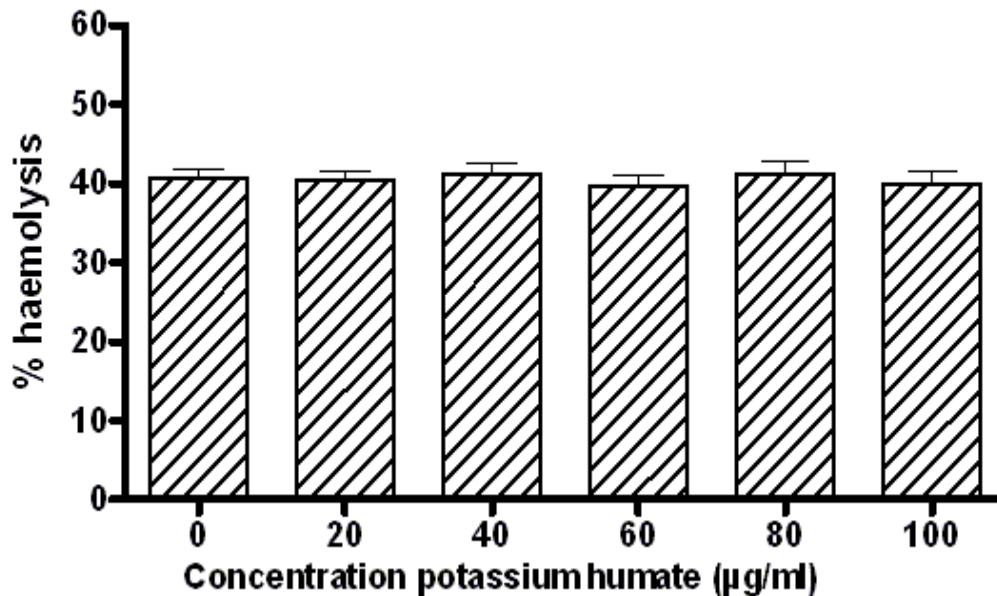


Figure 16. The effects of potassium humate on the lysis of sheep erythrocytes exposed for 5 minutes to lysophosphatidylcholine at a concentration to induce partial haemolysis. Data are expressed as means \pm SEM.

3.12 The effect of potassium humate on rat serum IFN- γ concentration of the carrageenan inflammation model.

Rat serum IFN- γ levels were determined from rat serum that was obtained from untreated rats or rats pre-treated with either potassium humate or indomethacin, 6 hours after subplantar carrageenan injection in the right hind paw. A small but not significant increase of serum IFN- γ levels was observed in the experimental group that received potassium humate and the positive control group that received indomethacin, compared to the untreated control group (Figure 17).

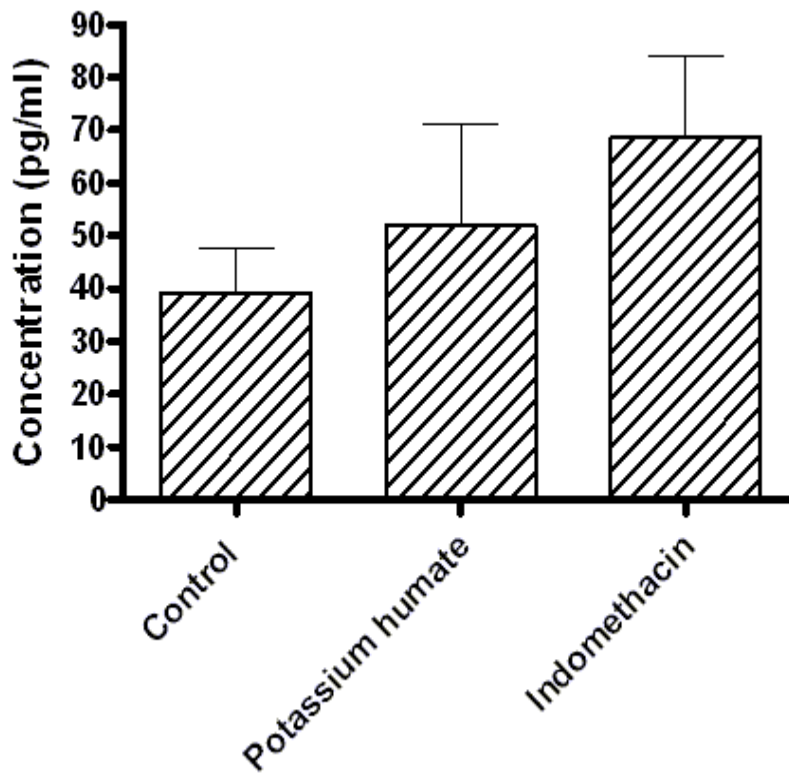


Figure 17. The effect of potassium humate on the concentration of IFN- γ in the serum of untreated rats or rats pre-treated with either potassium humate or indomethacin in the carrageenan-induced inflammation model. Data are expressed as means \pm SEM. Statistical significance was calculated ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

3.13 The effect of potassium humate on rat serum IL-6 concentration of the carrageenan inflammation model.

Rat serum IL-6 levels were determined from rat serum that was obtained from untreated rats or rats pre-treated with either potassium humate or indomethacin, 6 hours after subplantar carrageenan injection in the right hind paw. A small but not significant increase of serum IL-6 levels was observed in the experimental group that received potassium humate and the positive control group that received indomethacin, compared to the untreated control group (Figure 18).

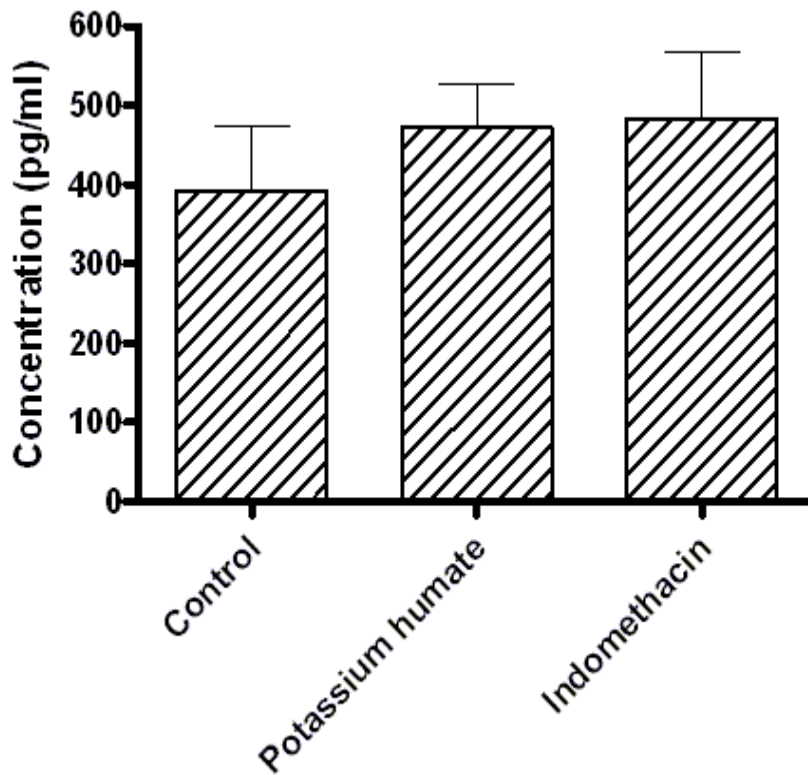


Figure 18. The effect of potassium humate on the concentration of IL-6 in the serum of untreated rats or rats pre-treated with either potassium humate or indomethacin in the carrageenan-induced inflammation model. Data are expressed as means \pm SEM. Statistical significance was calculated ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

3.14 The effect of potassium humate on rat serum TNF- α concentration of the carrageenan inflammation model.

Rat serum TNF- α levels were determined from rat serum that was obtained from untreated rats or rats pre-treated with either potassium humate or indomethacin, 6 hours after subplantar carrageenan injection in the right hind paw. A small but not significant increase of serum TNF- α levels was observed in the experimental group that received potassium humate and the positive control group that received indomethacin, compared to the untreated control group (Figure 19).

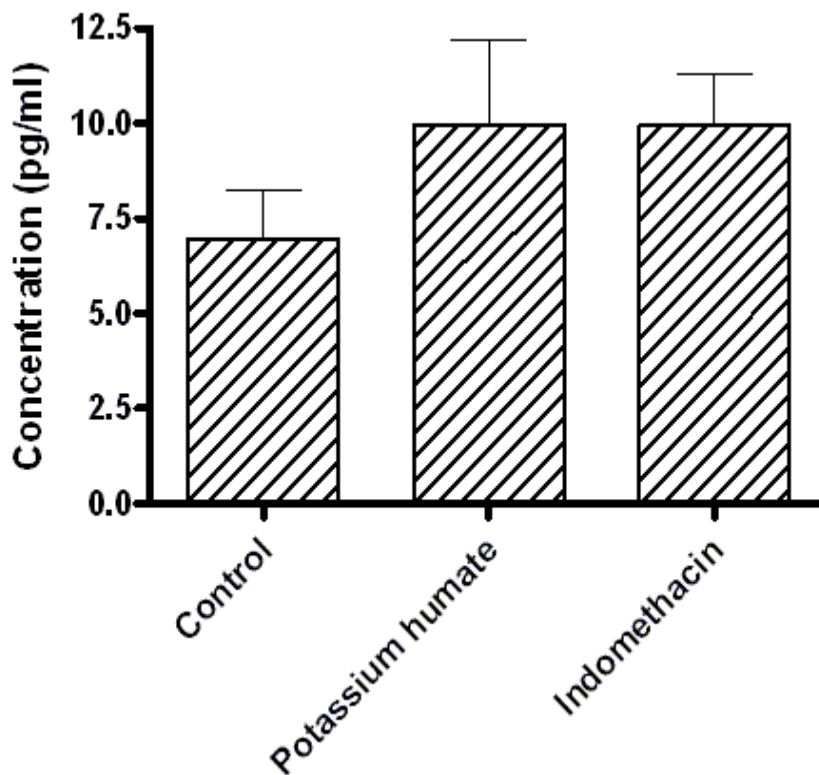


Figure 19. The effect of potassium humate on the concentration of TNF- α in the serum of untreated rats or rats pre-treated with either potassium humate or indomethacin in the carrageenan-induced inflammation model. Data are expressed as means \pm SEM. Statistical significance was calculated ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1 Discussion

Previous studies have indicated that humates possesses anti-inflammatory properties, however no in-depth research has been done to identify a possible mechanism of action.

In a recent study it has been shown that potassium humate suppresses ear swelling in a contact hypersensitivity animal model, comparable to prednisilone (Van Rensburg *et al.*, in press). However, in this study potassium humate had no inhibitory effects on paw oedema on the sheep erythrocyte induced DTH reaction, whereas dexamethasone, the positive treatment drug, had a strong inhibition on this type of inflammation.

According to textbook knowledge (Mak and Saunders, 2006), contact hypersensitivity is mediated by MHC class II-restricted CD4⁺ T cells. Although this may be true for most of the classical DTH responses (elicited by injection of sheep red blood cells), there is accumulating evidence that MHC class I-restricted CD8⁺ T cells play an important role in contact hypersensitivity. It has been showed that contact hypersensitivity could be transferred into both MHC class I- and class II-congenic mice, whereas sheep erythrocyte induced DTH was transferable only into MHC class II-congenic recipients (Minami *et al.*, 1982). Subsequently, MHC class I-deficient mice were found to be unable to develop a potent contact hypersensitivity response, whereas contact hypersensitivity was even exaggerated in MHC class II-deficient mice; however, these latter mice were unable to mount a sheep erythrocyte induced DTH response (Bour *et al.*, 1995).

Moreover, removal of epidermal Langerhans cells and other MHC class II cells before hapten challenge resulted in enhanced contact hypersensitivity but unaltered sheep erythrocyte induced DTH (Grabbe *et al.*, 1995). Similarly, depletion of CD4⁺ T cells enhanced contact hypersensitivity but inhibited the development of sheep erythrocyte induced DTH reaction, whereas depletion of CD8⁺ T cells suppressed contact hypersensitivity but enhanced sheep erythrocyte induced DTH (Gocinski, and Tigelaar, 1995). However, these results were challenged by data demonstrating that priming of naive T cells *in vitro* with haptenated Langerhans cells induces CD4⁺ hapten-specific T

cells (Hauser, 1990), and that CD4⁺-deficient mice have somewhat reduced contact hypersensitivity responses (Kondo *et al.*, 1996). Together, the literature indicate that contact hypersensitivity is associated with activation of a complex set of hapten-specific T cells and, although not fully conclusive, it appears that contact hypersensitivity-mediating effector T cells are of the CD8⁺ phenotype, while CD4⁺ T cells may even have downregulatory effects in contact hypersensitivity responses. By contrast, sheep erythrocyte induced DTH is a response elicited by CD4⁺ T cells with apparent downregulatory effects of CD8⁺ T cells. Because patients with late-stage AIDS can still develop contact dermatitis, but no tuberculin-type DTH responses (Matsushima *et al.*, 1989; Viraben *et al.*, 1994), this concept may also apply to humans.

Thus, by virtue of their specific cytokine secretion pattern, keratinocytes determine the microenvironment in which APCs are activated after epicutaneous hapten application during contact hypersensitivity initiation. By contrast, macrophages may control the cytokine milieu after subcutaneous antigen application during DTH, since young SJL mice that exhibit defective macrophage function do not mount DTH but have normal contact hypersensitivity responses (Finesmith *et al.*, 1995).

Potassium humate might therefore selectively influence CD8⁺ T cell function or the binding of the T-cell receptor to the MHC class I complex, which are mainly responsible for the contact hypersensitivity reaction, without affecting CD4⁺ T cell function, which are mainly responsible for the SE induced DTH reaction. Further investigation is needed to confirm this hypothesis.

Shilajit is a humic acid that exudates from steep rocks of Afghanistan. This compound significantly reduced paw oedema of the carrageenan-induced inflammatory model when administered i.p. at 50 mg/kg bodyweight (Goel *et al.*, 1990). The possible mechanism of the anti-inflammatory effect of shilajit could not be explained. Potassium humate also had a significant inhibitory effect on the carrageenan-induced paw oedema model, similar to indomethacin. Indomethacin, a selective COX-2 inhibitor, reduced paw oedema by inhibiting prostaglandin E₂ (Nantel *et al.*, 1999). Jooné, (2002) showed that oxihumate

had little or no effect on human lymphocyte prostaglandin E₂ activity. All the mediators of the carrageenan-induced paw oedema appear to be dependent upon activating the alternative complement pathway for their activation and release, therefore oedema elicited by carrageenan in the rat footpad requires an intact complement system (Giroud and Willoughby, 1970).

The major difference in the inflammatory pathways between the carrageenan-induced paw oedema and the sheep erythrocyte induced DTH response is that (i) inflammation of the carrageenan-induced paw oedema is mainly dependant on the activation of the complement cascade, followed by infiltration of neutrophils and (ii) inflammation of the erythrocyte induced DTH response is mainly dependant on antigen specific effector T cells that recognise antigen expressed on APCs followed by the secretion of cytokines. For this reason it can be speculated that potassium humate might selectively inhibit the complement cascade with minimal effects on cell mediated immune responses, specifically on CD4⁺ T cell function.

Potassium humate increases lymphocyte proliferation of resting, PHA and PWM stimulated lymphocytes in a dose dependant manner. This result correlates with the results obtained by Jooné *et al.*, (2003). T cell proliferation in response to antigen recognition is mediated primarily by an autocrine growth pathway, in which the responding T cell secretes its own growth-promoting cytokines and also expresses cell surface receptors for these cytokines (Abbas and Lichtman, 2005). During T cell stimulation, the production of the primary autocrine growth factor IL-2 and the expression of its receptor are increased.

Jooné *et al.*, (2003) demonstrated that oxihumate induced the proliferation response of PHA-stimulated human lymphocytes due to an increase of extracellular IL-2 concentrations as well as increased expression of the IL-2 receptor (CD25). In this study increased lymphocyte proliferation was induced from concentrations of 20 µg/ml and higher, with maximum stimulation at 80 µg/ml.

In this study it was found that potassium humate reduced TNF- α levels secreted by PHA stimulated lymphocytes at a concentration of 40 $\mu\text{g/ml}$. This correlates with the observations made by Jooné *et al.*, (2003). TNF- α is the principle mediator of acute inflammatory responses and is responsible for many of the systemic complications of severe infections (Mak and Saunders, 2006) and is a major contributor to autoimmune diseases. TNF- α stimulates the recruitment of neutrophils and monocytes to sites of infection and to activate these cells to eradicate microbes (Abbus and Lichtmann, 2005).

Potassium humate significantly reduced IL-1 β production by PHA stimulated lymphocytes. IL-1 is an inflammatory cytokine similar to TNF- α and is prominent in the innate immune defence system (Abbus and Lichtmann, 2005). Two functionally equivalent isoforms exist: IL-1 α and IL-1 β , but IL-1 β is present at higher concentrations (Abbus and Lichtmann, 2005).

In this study potassium humate significantly inhibited IL-6 production by PHA stimulated lymphocytes whereas Joone (2002) found oxihumate to have no effect on the production of this cytokine. IL-6 is a multi-functional cytokine and plays a role in the synthesis of acute phase proteins by hepatocytes. Its main function is the induction, differentiation and/or maturation of B- and T cells (Mak and Saunders, 2006). Many systemic acute-phase effects are due to the combined action of IL-1, TNF- α and IL-6 (Abbus and Lichtmann, 2005; Mak and Saunders, 2006).

Although IL-10 acts as a brake during inflammatory responses, targeting principally macrophages, neutrophils, eosinophils and mast cells, we found that potassium humate significantly inhibits the production thereof, which is in agreement with the results obtained by Joone (2002) for oxihumate.

Neither PHA nor potassium humate had an effect on IL-12p70 production. IL-12 is a cytokine with a central role in the immune response and plays a crucial part in T_{h1} differentiation and IFN- γ production by activated T_{h1} effector cells (Mak and Saunders, 2006). IL-12p70 is the bioactive form of IL-12.

The *in vitro* investigation of cytokine concentrations of the supernatant of either resting or PHA stimulated mononuclear lymphocytes incubated with potassium humate illustrated that potassium humate inhibited the production of the following pro-inflammatory cytokines; TNF- α , IL-1 β and IL-6 of PHA stimulated cells. Potassium humate decreased IL-10 levels which might have contributed to an increased proliferative effect on lymphocytes *in vitro*. TNF- α and IL-12 are primarily secreted by T_{h1} cells that support the cell mediated immunity whereas IL-6 and IL-10 are primarily produced by T_{h2} cells that support the humoral immunity. Thus potassium humate influence both the cell mediated and humoral immunity *in vitro*. It can be argued that due to the binding properties of humates to various molecules (Corcia *et al.*, 1999), potassium humate might have prevented the binding of interleukins to the antibodies of the BDTM cytometric bead array human inflammation kit. However the possibility that potassium humate interfered with the detection of cytokines *in vitro* can be ruled out because (i) it had no noticeable effects on IL-12p70 levels, (ii) it had no effect on the cytokine concentrations of resting cells.

It has been demonstrated that serum levels of IL-6, TNF- α and IFN- γ increases after carrageenan administration to rats (Cuzzocrea *et al.*, 1999; Ogata *et al.*, 1999; Tateda *et al.*, 1998; Tsuji *et al.*, 2004; Yuan *et al.*, 2006). In this study potassium humate had no effect on IL-6, TNF- α and IFN- γ levels in the serum of rats exposed to carrageenan. Further studies need to be done to confirm this finding.

Potassium humate inhibited both the alternative and classical complement pathways at similar concentrations *in vitro*. The sheep red blood cell membrane stability experiment demonstrated that potassium humate had no effect on cell membrane stability. It can therefore be concluded that potassium humate specifically inhibited the complement pathway without influencing the cell membrane stability. The effects of potassium humate on these pathways can be due to its binding properties which could lead to the inactivation of particular components of the complement system. Further research on the effect of potassium humate on the complement cascade need to be done to identify the exact step that is influenced by potassium humate.

By taking the inhibitory effect of potassium humate on the *in vitro* complement assay into account together with its effects on the production of certain inflammatory cytokines, it can be hypothesised that the mechanism of the anti-inflammatory effect of potassium humate on the carrageenan-induced paw oedema might be due to inhibition of the complement cascade and pro-inflammatory cytokines.

In conclusion, potassium humate can possibly serve as a revolutionary new anti-inflammatory drug. Anti-inflammatory drugs currently available exert many side effects that can be detrimental to health. Potassium humate is safe and inhibits inflammation through specific pathways, thus making it a favourable therapeutic agent to use. This study has shown that potassium humate is a substance that can potentially provide relief from complement and CD 8⁺ T-cell mediated inflammatory conditions such as contact hypersensitivity, acute graft rejection after organ transplant, rheumatoid arthritis, osteoarthritis and myasthenia gravis.

4.2 Conclusion

This research clearly shows that potassium humate, a soluble humic acid salt, possesses substantial anti-inflammatory properties. The findings of this study illustrated that although potassium humate stimulated lymphocyte proliferation, it inhibited the production of certain pro-inflammatory cytokines as well as complement activation. Furthermore it was found that potassium humate inhibited inflammation of the carrageenan-induced paw oedema model but not the sheep erythrocyte induced DTH model. This study has shown that the anti-inflammatory properties of potassium humate can possibly be elucidated by inhibition of the production of pro-inflammatory cytokines and components of the complement cascade. Although the mechanism of action still needs to be explored in depth, these studies paved the way to clinical trials to prove effectiveness in patients suffering from inflammatory related diseases.

REFERENCES

- Abbas, A. K. and Lichtman, A.H. (2005). *Cellular and Molecular Immunology*. W. R. Krehling H. Schmitt (eds). Philadelphia, Elsevier.
- Achard, F.K. (1986). Chemische Untersuchung des Torfs. *Crell's Chem Ann* 2: 391-403.
- Adamek, W. (1976). Intoductory report on oncostatic and therapeutic nature of the peat preparation in human neoplastic disease. Proceedings of the 5th Int'l Peat Congress.
- Alvarez-Puebla, R.A., Valenzuela-Calahorro, C. and Garrido, J.J. (2004). Retention of Co(II), Ni(II), and Cu(II) on a purified brown humic acid. Modeling and characterization of the sorption process. *Langmuir* 20: 3657-3664.
- Alvarez-Puebla, R.A. Valenzuela-Calahorro, C., Garrido, J.J., Alvarez-Puebla, R.A., Valenzuela-Calahorro, C. and Garrido, J.J. (2004). Modeling the adsorption and precipitation processes of Cu(II) on humin. *J Colloid Interface Sci* 277: 55.
- Ames, R.S., Lee, D., Foley, J.J., Jurewicz, A.J., Tornetta, M.A., Bautsch, W., Settmacher, B., Klos, A., Erhard, K.F., Cousins, R.D., Sulpizio, A.C., Hieble, J.P. McCafferty, G., Ward, K.W., Adams, J.L., Bondinell, W.E., Underwood, D.C., Osborn, R.R., Badger, A.M. and Sarau, H.M. (2001). Identification of a selective nonpeptide antagonist of the anaphylatoxin C3a receptor that demonstrates antiinflammatory activity in animal models. *J Immunol* 166: 6341-6348.
- Ansorg, R. and Rochus, W. (1987). Studies on the antimicrobial effect of natural and synthetic humic acids. *Arzeimittelforschung* 28: 2195-2198.

Araida, T., Frey, C.F., Ruebner, B., Carlson, J., King, J. (1995). Therapeutic regimens in acute experimental pancreatitis in rats: effects of a protease inhibitor, a beta-agonist, and antibiotics. *Pancreas* 11: 132–140.

Asghar, S.S. (1984). Pharmacological manipulation of complement system. *Pharmacol Rev* 36: 223-244.

Baatz, H. (1988). Moorthherapie en der frauenheilkunde. In: W. Flaig, C. Goecke, and W. Kauffels (eds). *Wien-berlin: ueberreuter*. p161-168.

Babior, B.M. (1999). NADPH oxidase: an update. *Blood* 93(5): 1464–1476.

Bani. S., Kaul, A., Khan, B., Ahmad, S.F., Suri, K.A., Satti, N.K., Amina, M., Qazi, G.N. (2005). Immunosuppressive properties of an ethyl acetate fraction from *Euphorbia royleana*. *J Ethnopharmacol* 99(2): 185-92.

Bennet, P. N. and Brown M. J. (2003). *Clinical pharmacology*. Smillie, T., Horne, L. (eds). Ninth edition. Edinburg, Churchill Livingstone.

Bhardwaj, K.K. and Gaur, A.C. (1970). The effect of humic and fulvic acids on the growth and efficiency of nitrogen fixation of *Azotobacter chroococcum*. *Folia Microbiol Praha* 15: 364.

Biber, V.A. and Bogolyubova, N.S. (1952). Humic acid of estuarine mud and its biological activity. *Dokl Akad Nauk SSSR* 82: 939-944.

Blum, M.G., Collins, B.J., Chang, A.C., Zhang, J.P., Knaus, S.A., Pierson, R.N. (1998). Complement inhibition by FUT-175 and K76-COOH in a pig-to-human lung xenotransplant model. *Xenotransplant* 5: 35–43.

Botes, M.E., Dekker, J. and Van Rensburg, C.E.J. (2002). Phase I trial with oral oxihumate in HIV-infected patients. *Drug Develop Res* 57: 34-39.

Bour, H., Peyron, E., Gaucherand, M., Garrigue, J.L., Desvignes, C., Kaiserlian, D., Revillard, J.P. and Nicolas, J.F. (1995). Major histocompatibility complex class I-restricted CD8⁺ T cells and class II-restricted CD4⁺ T cells, respectively, mediate and regulate contact sensitivity to dinitrofluorobenzene. *Eur J Immunol* 25: 3006–3010.

Bureeva, S. Andia-Pravdivy, J. and Kaplun, A. (2005). Drug design using the example of the complement system inhibitors' development. *Drug Disc Today* 10(2): 1535-1542.

Corcia, A. D. Costantino, A. Crescenzi, C. and Samperi, R. (1999). Quantification of phenylurea herbicides and their free and humic acid-associated metabolite in natural waters. *J Chromatography A* 852(2): 465-474.

Crucian, B., Dunne, P., Friedman, H., Ragsdale, R., Pross, S. and Wideni, R. (1996). Detection of altered T helper 1 and T helper 2 cytokine production by peripheral blood mononuclear cells in patients with multiple sclerosis utilizing intracellular cytokine detection by flow cytometry and surface marker analysis. *Clin Diagnostic Lab Immunol* 3(4): 411-416.

Cuzzocrea, S. Sautebin, L., De Sarro, G., Costantino, G., Rombola, L., Mazzon, E., Ialenti, A., De Sarro, A., Ciliberto, G., Di Rosa, M., Caputi, A.P. and Thiemermann, C. (1996). Role of IL-6 in the pleurisy and lung injury caused by carrageenan. *J Immunology* 163: 5094-104.

Deharo, E., Baelmans, R., Gimenez, A., Quenevo, C. and Bourdy, G. (2004). In vitro immunomodulatory activity of plants used by the Tacana ethnic group in Bolivia. *Phytomed* 11: 516-22.

Dellinger, R.P., Zimmerman, J.L., Straube, R.C., Metzler, M.H., Wall, M., Brown, B.K., Levin, J.L., Toth, C.A. and Ryan, U.S. (1996). Results of phase I trial of soluble

complement receptor type 1 TP10 in acute lung injury ALI. *Crit Care Med* 24 Suppl 2. A29.

Di Rosa, M., Giroud, J.P. and Willoughby, D.A. (1971). Studies on the mediators of the acute inflammatory response induced in rats in different sites by carrageenan and turpentine. *J Pathol* 104: 15-29.

Di Rosa, M. and Willoughby, D.A. (1971). Screens for anti-inflammatory drugs. *J Pharm Pharmacol* 23(4): 297-298.

Eldering, E., Huijbregts, C.C.M., Nuijens, J.H., Verhoeven, A.J. and Hack, C.E. (1993). Recombinant C1-inhibitor P5rP3 variants display resistance to catalytic inactivation by stimulated neutrophils. *J Clin Invest* 91: 1035–1043.

Elsbach, P. and Weiss, J. (1992). Oxygen-independent antimicrobial systems of phagocytes. In: J.J. Gallin, I.M. Goldstein and R. Snyderman (eds). *Inflammation: Basic Principles and Clinical Correlates*, New York, Raven Press. p 603–636.

Finesmith, T.H., Seaman, S. and Rietschel, R. (1995). Paradoxical coexistence of contact dermatitis and anergy in a man with AIDS. *J Am Acad Dermatol* 32: 526-527.

Fischer, M.B., Prodeus, A.P., Nicholson-Weller, A., Ma, M.H., Murrow, J., Reid, R.R., Warren, H.B., Lage, A.L., Moore, F.D.J., Rosen, F.S. and Carroll, M.C. (1997). Increased susceptibility to endotoxin shock in complement C3- and C4-deficient mice is corrected by C1 inhibitor replacement. *J Immunol* 159: 976–982.

Fitch, J.C., Rollins, S., Matis, L., Alford, B., Aranki, S., Collard, C.D., Dewar, M., Eleftheriades, J., Hines, R., Kopf, G., Kraker, P., Li, L., O'Hara, R., Rinder, C., Rinder, H., Shaw, R., Smith, B., Stahl, G. and Shernan, S.K. (1999). Pharmacology and biological efficacy of a recombinant, humanized, single-chain antibody C5 complement inhibitor in

patients undergoing coronary artery bypass graft surgery with cardiopulmonary bypass. *Circulation* 100: 2499–2506.

Fuchs, V., Kühnert, M. and Golbs, S. (1986). Detoxifizierende wirkung von huminsäuren gegenüber ausgewählten schadstoffen. *Monatsh Veterinaermed* 41: 712-713.

Fujita, Y., Inoue, I., Inagi, R., Miyata, T., Shinzato, T., Sugiyama, S., Miyama, A., Maeda, K. (1993). Inhibitory effect of FUT-175 on complement activation and its application for glomerulonephritis with hypocomplementemia. *Nippon Jinzo Gakkaishi* 35: 393–397.

Gau, R.J., Yang, H.L., Chow, S.N., Suen, J. L. and Lu, F. J. (2000). Humic acid suppresses the LPS-induced expression of cell-surface adhesion proteins through the inhibition of NF-kB activation. *Tox Applied Pharm* 166: 59-67.

Gocinski, B.L. and Tigelaar, R.E. (1990). Roles of CD4⁺ and CD8⁺ T cells in murine contact sensitivity revealed by *in vivo* monoclonal antibody depletion. *J Immunol* 144: 4121–4128.

Goel, R.K., Banerjee, R.S. and Acharya, S.B. (1990). Antiulcerogenic and antiinflammatory studies with shilajit. *J Ethnopharmacol* 29: 95-103.

Golbs, S., Fuchs, V., Kühnert, M. and Polo, C. (1982). Pränataltoxikologische Testung von Huminsäuren an Laboratoriumsratten. *Arc Exper Vet Med* 36: 179 – 185.

Goldsby.R.A., Kindt, T.J. and Osmorne, B.A. (2000). *Kuby Immunology*. Tannenbaum, N., Hadler, J., Kudlacik, G.L. and Folchetti, A. (eds). Fourth edition. New York, W.H. Freeman and Company.

Górniok, A., Majewski, C. and Nowacka, A. (1967). Der einfluss von moorbädern auf die atmungsprozesse in den gewebe von meerschweinchen. *Z. Angew. Baeder Klimaheilkd* 14: 265-270.

Grabbe, S. and Schwarz, T. (1998). Immunoregulatory mechanisms involved in elicitation of allergic contact hypersensitivity. *Immunol Today* 19: 37-44.

Grabbe, S., Steinbrink, K., Steinert, M., Luger, T.A. and Schwarz, T. (1995). Removal of the majority of epidermal Langerhans cells by topical or systemic steroid application enhances the effector phase of murine contact hypersensitivity. *J Immunol* 155(9): 4207-4217.

Gramsch, H. (1961). Ein beitrag zur behandlung der gastropathien. *Med Monatsschr* 15: 658-687.

Haanel, B.F. (1924). Facts about Peat. Mines Branch Publ. No: 614. Ottawa, Canadian Department of Mines.

Hack, C.E., Ogilvie, A.C., Eisele, B., Jansen, P.M., Wagstaff, J. and Thijs, L.G. (1994). Initial studies on the administration of C1-esterase inhibitor to patients with septic shock or with a vascular leak syndrome induced by interleukin-2 therapy. *Prog Clin Biol Res* 388: 335–357.

Hack, C.E., Voerman, H.J., Eisele, B., Keinecke, H.O., Nuijens, J.H., Eerenberg, A.J., Ogilvie, A., Strack van Schijndel, R.J., Delvos, U. and Thijs, L.G. (1992). C1-esterase inhibitor substitution in sepsis. *Lancet* 339(8789):378.

Hartenstein, R. (1981). Sludge decomposition and stabilization. *Science* 212: 743-749.

Hauser, C. (1990). Cultured epidermal Langerhans cells activate effector T cells for contact sensitivity. *J Invest Dermatol* 95: 436-440.

Hawkey, C. J., Jackson, L., Harper, S. E., Simon, T. J., Mortensen, E. and Lines, C. R. (2001). Review article: the gastrointestinal safety profile of rofecoxib, a highly selective inhibitor of cyclooxygenase-2 in humans. *Aliment Pharmacol Ther* 15: 1-9.

Hiller, E. (1952). Experimentelle untersuchungen zum problem der wirkungsweise gewisser huminsäuren aus dem moor bei der behandlung rheumatischer erkrankungen. *Med Monatsschr* 6: 302-306.

Hiller, E. (1953). Untersuchungen über die beeinflussung der oestrogenausscheidung im urin nach bädern mit salizylierten huminsäuren. *Dtsch Med Wochenschr* 78: 691-93.

Hong, K., Kinoshita, T., Kitajima, H. and Inoue, K. (1980). Inhibitory effect of K-76 monocarboxylic acid, an anticomplementary agent, on the C3b inactivator system. *J Immunol* 127: 104-108.

Hong, K., Kinoshita, T., Miyazaki, W., Izawa, T. and Inoue, K. (1979). An anticomplementary agent, K-76 monocarboxylic acid: its site and mechanism of inhibition of the complement activation cascade. *J Immunol* 122: 2418-2423.

Horstick, G., Heimann, A., Goetze, O., Hafner, G., Berg, O., Boehmer, P., Becker, P., Darius, H., Rupprecht, H.J., Loos, M., Bhakdi, S., Meyer, J. and Kempster, O. (1997). Intracoronary application of C1 esterase inhibitor improves cardiac function and reduces myocardial necrosis in an experimental model of ischemia and reperfusion. *Circulation* 95: 701-708.

Hurley, J.V. (1972). *Acute Inflammation*. Edinburg, Churchill Livingstone.

Inagi, R., Miyata, T., Maeda, K., Sugiyama, S., Miyama, A. and Nakashima, I. (1991). FUT-175 as a potent inhibitor of C5rC3 convertase activity for production of C5a and C3a. *Immunol Lett* 27: 49-52.

Islam, K.M.S., Schuhmacher, A. and Gropp, J. M. (2005). Humic acid substances in animal agriculture. *Pakistan J Nutrition* 4(3): 126-134.

Iubitskaia, N. S. and Ivanov, E.M. (1999). Sodium humate in the treatment of osteoarthritis patients. *Vopr Kurortol Fizioter Lech Fiz Kult* 5: 22-24.

Janeway, C.A., Travers, P., Walport, M. and Shlomchik, M.J. (2005). *Immunobiology: The immune system in health and disease*. Lawrence E., Catherall, E., Reandi, S., Bushell, G. and Morales, M. (eds). Sixth edition. New York, Garland Science Publishing.

Jansen van Rensburg, C., Van Rensburg, C.E.J., Ryssen, J.B.J., Casey, N.H. and Rottinghaus, G.E. (2006). Assessment of a humic acid as an aflatoxin binder *in vitro* and in broiler chickens. *Poultry Sci* 85:1576-1585.

Jing, H., Babu, Y.S., Moore, D., Kilpatrick, J.M., Liu, X.Y., Volanakis, J.E. and Narayana, S.V. (1998). Structures of native and complexed complement factor D: implications of the atypical His57 conformation and self-inhibitory loop in the regulation of specific serine protease activity. *J Mol Biol* 282: 1061–1081.

Jooné, G. K. (2002). Die Immuunmodulerende eienskappe van oxihumaat. PhD Thesis. University of Pretoria.

Jooné, G. K., Dekker, J. and Jansen van Rensburg, C.E. (2003). Investigation of the immunostimulatory properties of oxihumate. *Z Naturforsch* 58: 263-267.

Jooné, G.K. and Jansen van Rensburg, C.E. (2004). An *in vitro* investigation of the anti-inflammatory properties of potassium humate. *Inflammation* 28(3):169-174.

Kalli, K.R., Hsu, P. and Fearon, D.T. (1994). Therapeutic uses of recombinant complement protein inhibitors. *Springer Semin Immunopathol* 15: 417–431.

Khakimov, Z.N. (1974). Mumie as a kind of natural resources and its signs. PhD thesis. Tashkent University.

Kinzlmeier, H. (1954). Die beeinflussung der superazidität durch ionenaustauscher. *Arzneim Forsch.* 4: 394-96.

Klebanoff, S.J. (1999). Oxygen metabolites from phagocytes. In: J.I. Gallin and R. Snyderman (eds). *Inflammation: basic pinciples and clinical correlates*. Philadelphia, Williams & Wilkens. p 721–768.

Kleinhempel, D. (1970). *Albrecht Thaer Arch* 14: 3-11

Kleinschmidt, J. (1988). Moorthherapie bei rheumatischen erkrankungen. Flaig, W., Goecke, C. and W. Kauffels (eds). Wien-Berlin, Ueberreuter.

Klerx, J. P.A.M., Beukelman, C. J., Van Dijk, H. and Willers, J.M.N. (1983). Microassay for colorimetric estimation of complement activity in guinea pig, human and mouse serum. *J Immunol Methods* 63: 215-220.

Klöcking, R. (1994). Humic substances as potential therapeutics. Senesi, N. and Miano, T.M. (eds). Amsterdam, Elsevier.

Klöcking, R., Friemel, H. and Mücke, D. (1967). Zur biochemie der huminsäuren die bindung der huminsäuren an serumproteine in vivo (Qualitative und halbquantitative elektrophoretische untersuchungen). *Acta Biol Med Ger* 18(1): 9-13.

Klöcking, R., Hofmann, R. and Mücke, D. (1968). Tierexperimentelle untersuchungen zur entzündungshemmenden wirkung von humaten. *Arzneim Forsch* 18: 941-942.

Klöcking, R. and Sprössig, M. (1975). Wirkung von ammoniumhumat auf einige virus-zell-systeme. *Z Allg Mikrobiol* 15: 25-30.

Klöcking, R. *et al.* (1983). Antiviral wirksam huminsäureähnliche polymere. *Z Physiother* 33: 95-101.

Kobayashi, T., Neethling, F.A., Taniguchi, S., Ye, Y., Niekrasz, M., Koren, E., Hancock, W.W., Takagi, H. and Cooper, D.K.C. (1996). Investigation of the anti-complement agents, FUT-175 and K76COOH, in discordant xenotransplantation. *Xenotransplan* 3: 237–245.

Kondo, S., Beissert, S., Wang, B., Fujisawa, H., Kooshesh, F., Stratigos, A., Granstein, R.D., Mak, T.W. and Sauder D.N. (1996). Hyporesponsiveness in contact hypersensitivity and irritant contact dermatitis in CD4⁺ gene targeted mouse. *J Invest Dermatol* 106: 993-1000.

Konno, S. and Tsurufuji, S. (1983). Induction of zymosan-air-pouch inflammation in rats and its characterization with reference to the effects of anticomplementary and anti-inflammatory agents. *Br J Pharmacol* 80: 269–277.

Kotani, S., Fuyita, Y., Kitsuge, T., Nakamura, T., Yamamuro, T., Ohtsuki, C. and Kokubo, T. (1991). Bone bonding mechanism of α -tricalcium phosphate. *J Biomed Mater Res* 25: 1303-1315.

Konteatis, Z.D., Siciliano, S.J., Vanriper, G., Molineaux, C.J., Pandya, S., Fischer, P., Rosen, H., Mumford, R.A. and Springer, M.S. (1994). Development of C5a receptor antagonists - differential loss of functional responses. *J Immunol* 153: 4200–4205.

Kovarik, R. (1988). Über die anwendung von präparaten aus torf, bzw. *Huminstoffen bei gynäkologischen erkrankungen*. Flaig, W., Goecke, C. and Kauffels, W. (eds). Wien-Berlin, Ueberreuter. P. 177-197.

Kreutz, S.W. (1992). Effects of implanted bovine calcium hydroxyapatite with humate. *Arch Orthop Trauma Surg* 111(5): 259-264.

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

Lent, W. (1988). Bericht über die moorforschung und anwendung in der DDR, Polen, Tschechoslowakei Und UdSSR. In moorthérapie: grundlagen und anwendungen, Flaig, W. Goecke, C. and W. Kauffels (eds). Wien-Berlin, Ueberreuter. p 169-176.

Lin, J. K. and Lee S. F. (1992). Enhancement of the mutagenicity of polyphenols by chlorination and nitrosation in Salmonella typhimurium. *Mutat Res* 269(2): 217-224.

Lotosh, T. D. (1991). Experimental basis and prospects for the use of humic acid preparations from peat in medicine and agricultural production. (Translated from Russian). *Nauchnye Doki Vyss Shkoly Biol Nauki* 10: 99-103.

Mak, W.M. and Saunders, M.E. (2006). *The Immune Response*. Lebedeva, T, and Picknett, V. (eds). London, Elsevier.

Makrides, S.C. (1998). Therapeutic inhibition of the complement system. *Pharmacol Rev* 50: 59-87.

Malinowska, M.H., Pietraszek, D. and Chabielska, E. (1993). Influence of tolpa peat preparation on haemostasis in rats. *Acta Pol Pharm* 50: 507-511.

Manosroi, A., Saraphanchotiwitthaya, A. and Manosroi, J. (2005). In vivo immunomodulating activity of wood extracts from *Clausena excavata* Burm. *J Ethnopharmacol* 102(1): 5-9.

Matsushima, G.K., Gilmore, W., Casteel, N., Frelinger, J.A. and Stohlman, S.A. (1989). Evidence for a subpopulation of antigen-presenting cells specific for the induction of the delayed-type hypersensitivity response. *Cell Immunol* 119: 171-181.

McMurtrie, R.E., Medlyn, B.E. and Dewar, R.C. (2001). Increased understanding of nutrient immobilization in soil organic matter is critical for predicting the carbon sink strength of forest ecosystems over the next 100 years. *Tree Physiol* 21: 831-839.

Minami, M., Okuda, K., Sunday, M.E. and Dorf, M.E. (1982). *H-2K-*, *H-2I-* and *H-2D-* restricted hybridoma contact sensitivity effector cells. *Nature* 297:231-233.

Miyazaki, W., Tomaoka, H., Shinohara, M., Kaise, H., Izawa, T., Nakano, Y., Kinoshita, T., Hong, K. and Inoue, K. (1980). A complement inhibitor produced by *Stachybotrys complementi*, nov. sp. K-76, a new species of fungi imperfecti. *Microbiol Immunol* 24: 1091-1108.

Mokgobu, I., Theron, A.J., Anderson, R. and Feldman, C. (1999). The ketolide antimicrobial agent HMR-3004 inhibits neutrophil superoxide production by a membrane-stabilizing mechanism. *Int J Immunopharmacol* 21(6): 365-77.

Mollison, K.W., Krause, R.A., Fey, T.A., Miller, L., Wiedeman, P.E., Kawai, M., Lane, B., Luly, J.R. and Carter, G.W. (1992). Hexapeptide analogues of C5a anaphylatoxin reveal heterogeneous neutrophil agonism/antagonism. *FASEB J.* 6, A2058.

Morgan, B.P. (1994). Clinical complementology: Recent progress and future trends. *Eur J Clin Invest* 24: 219-228.

Morgan, B.P. and Harris, C.L. (2003). Complement therapeutics; history and current progress. *Molecular Immunol* 40: 159-170.

- Mosmann, T.R. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* 65: 37-64.
- Mqadmi, A. Zheng, X., Song, J., Abramowitz, S., Giclas, P. and Yazdanbakhsh, K. (2004). Prevention of complement-mediated immune hemolysis by a small molecule compound. *Biochem Biophysical Res Commun* 325: 1465-1471.
- Nantel, F., Denis, D., Gordon, R., Northey, A., Cirino, M., Metters, K.M. and Chan, C.C. (1999). Distribution and regulation of cyclooxygenase-2 in carrageenan-induced inflammation. *British J Pharmacol* 128: 853-859.
- Obenaus, R., Friemel, H. and Mücke, D. (1965). Zur biochemie der huminsäuren IV. Die bindung von huminsäuren an serumproteine in vitro (ein- und zweidimensionale agarelektrophoretische untersuchungen). *Acta Biol Med German* 15: 14-19.
- Ogata, M., Matsui, T., Kita, T. and Shigematsu, A. (1999). Carrageenan primes leukocytes to enhance lipopolysaccharide-induced tumor necrosis factor alpha production. *Infect and Immun* 67(7): 3284-3289.
- Page, C.P., Curtis M.J., Walker, M.J.A. and Hoffman, B. B. (2006). *Integrated Pharmacology*. Stibbe, A. (ed). Third edition. Philadelphia, Elsevier.
- Page, C.P, Curtis, M.J, Sutter, M., Walker, M. J.A and Hoffman, B. (2002). *Integrated Pharmacology*. Updated second ed. Edinburgh, Elsevier.
- Pathak, S. and Palan, U. (2005). *Immunology Essential and Fundamental*. Dunn, S. and Cameron, C. (eds). Second edition. Enfield, Science Publishers.
- Paul, E.F. and Paul, J. (2001). *Why Animal Experimentation Matters. The Use of Animals in Medical Research*. Miller, F.D. (ed). Transaction.

- Petersson, M., Wiberg, U., Lundeberg, T. and Uvnas-Moberg, K. (2001). Oxytocin decreases carrageenan induced inflammation in rats. *Peptides* 22(9): 1479-1484.
- Piccolo, A., P. Conte, and A. Cozzolino. (2000). *Differences in high performance size exclusion chromatography between humic substances and macromolecular polymers*. E. A. Ghabbour and G. Davies (eds). Cambridge, UK: Royal Society of Chemistry.
- Rao, C.V. (2005). *Immunology a textbook*. Harrow, Alpha Sciences.
- Recio, M.C., Giner, R.M., Uriburu, L., Manez, S., Cerda, M., De la Fuente, J.R. and Rios, J. L. (2000). In vivo activity of pseudoguaianolide sesquiterpene lactones in acute and chronic inflammation. *Life Sci* 66(26): 2509-2518.
- Reichert, B. (1966). Huminsäuren und ihre derivate in der modernen therapie. *Dtsch Apoth* 18: 658-87.
- Riley, R.D., Sato, H., Zhao, Z., Thourani, V.H., Jordan, J.E., Fernandez, A.X., Ma, X., Hite, D.R., Rigel, D.F., Pellas, T.C., Peppard, J., Bill, K.A., Lappe, R.W. and Vinten-Johansen, J. (2000). Recombinant human complement C5a receptor antagonist reduces infarct size after surgical revascularization. *J Thorac Cardiovasc Surg* 120: 350–358
- Rusina, O., Linnik, O., Eremenko, A. and Kisch, H. (2003). Nitrogen photofixation on nanostructured iron titanate films. *Chemistry* 9: 561-565.
- Sahu, A., Kay, B.K. and Lambris, J.D. (1996). Inhibition of human complement by a C3-binding peptide isolated from a phage displayed random peptide library. *J Immunol* 157: 884–891.
- Sahu, A. and Lambris, J.D. (2000). Complement inhibitors: a resurgent concept in anti-inflammatory therapeutics. *Immunopharmacol* 49:133–148

Salz, G. (1974). Salhumin-gel, ein lokaltherapeutikum mit hyperämischer, entzündungshemmender und analgetischer Wirkung. *Med Monatsschr* 28: 548-550.

Sato, T., Ose, Y. and Nagase, H. (1986). Desmutagenic effect of humic acid. *Mutation Res* 162(2): 173-178.

Schepetkin, I., Khlebnikov, A. and Kwon, B.S. (2002). Medical drugs from humus matter: focus on mumie. *Drug Develop Res* 57(1): 140-159.

Schiller, F., Klöcking, R., Wutzler, P. and Färber, I. (1979). Tropical ammoniumhumat treatment of human HSV infections-preliminary clinical results. *Dermatol Monatsschr* 165: 505-509.

Schlepper, G. (1960). Betrachtungen zur Frage der Übereinstimmung experimenteller Ergebnisse mit klinischen Resultaten bei Superacidität. *Med Klin* 55: 263-266.

Schlickewei, W., Riede, U.N., Yu, J., Zeichmann, W., Kuner, E.H. and Seubert, B. (1993). Influence of humate on calcium hydroxyapatite implants. *Arch of Orthop and Trauma Surg* 112: 275-279.

Schneider, J., Weis, R., Manner, C., Kary, B., Werner, A., Seubert, B.J. and Riede, U.N. (1996). Inhibition of HIV-1 in cell culture by synthetic humate analogues derived from hydroquinone: mechanism of inhibition. *Virology* 218: 389-395.

Senesi, N. and Loffredo, E. (1999). *Soil Physical Chemistry*. Sparks, D.L. (ed). Boca Raton, CRC Press.

Sharma, K.K., Mediratta, P.K., Reeta, K.H. and Mahajan, P. (2004). Acute and delayed restraint stress-induced changes in nitric oxide producing neurons in limbic regions. *Neurosci* 125(4): 981-993.

Smit, H.F. Kroes, B.H., Van den Berg, A. J., Van der Wal, D., Van den Worm, E., Beukelman, C. J., Van Dijk, H. and Labadie, R. P. (2000). Immunomodulatory and anti-inflammatory activity of *Picrorhiza scrophulariiflora*. *J Ethnopharmacol* 73(1-2): 101-109.

Solovyeva, V.P. and Lotosh, T.D. (1984). Biologically active peat substances body resistance stimulators. Dublin, Ireland. *Proceedings of the 7th International Peat congress* 4: 428-434.

Sommers, DeK. (2000). *Sommers' Pharmacology*. Seventh edition. Pretoria, UP drukkers.

Stevens, C.D. (1996). *The Lymphoid System*. McNichol, C.S. and Fithian, M. (eds). Philadelphia, F.A. Davis Company.

Stevenson, F.J. (1994). *Humus chemistry genesis, composition, reactions*. John Wiley & Sons, Inc. New York.

Stevenson, F. J. and Chen, Y. (1991). Stability contents of copper(II)-humate complexes determined by modified potentiometric titration. *Soil Sci Soc Am J* 55(6): 1586-1591.

Szalai, A.J., Digerness, S.B., Agrawal, A., Kearney, J.F., Bucy, R.P., Niwas, S., Kilpatrick, J.M., Babu, Y.S., Volanakis, J.E. (2000). The arthus reaction in rodents: species-specific requirement of complement. *J Immunol* 164: 463–468.

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

Thiel, K.D., Klocking, R., Schweizer, H. and Sprossig, M. (1977). In vitro studies of the antiviral activity of ammonium humate against herpes simplex virus type 1 and type 2. *Zentralbl Bakteriol* 239: 304-321.

Thiel, K.D., Helbig, B., Klöcking, R., Wutzler, P., Sprössig, M. and Schweizer, H. (1981). Comparison of the in vitro activities of ammonium humate and of enzymically oxidized chlorogenic and caffeic acids against type 1 and type 2 human herpes virus. *Pharmazie* 36: 50-53.

Thurmana, J.M., Krausb, D.M., Girardic, G., Hourcaded, D., Kangb, H.J., Royerb, P.A., Mitchelld, L.M., Giclase, P.C., Salmonc, J., Gilkesonf, G. and Holersb, V.M. (2005). A novel inhibitor of the alternative complement pathway prevents antiphospholipid antibody-induced pregnancy loss in mice. *Molec Immunol* 42: 87–97.

Tiwari, V.P., Tiwari, K.C. and Joshi, P. (1973). An interpretation of Ayurvedic findings on shilajit. *Res Indigen* 8(3):53-60.

Tsuji, R.F., Hoshino, K., Noro, Y., Tsuji, N.M., Kurokawa, T., Masuda, T., Akira, S. and Nowak, B. (2003). Suppression of allergic reaction by lambda-carrageenan: toll-like receptor 4/MyD88-dependent and -independent modulation of immunity. *Clin Exp Allergy* 33(2): 249-258.

Turgeon, M.L. (1996). *Soluble Mediators of the Immune System*. Shanahan, J. (ed). Second edition. Missouri, Mosby.

Van Beneden, G. (1971). Les matières organiques dans les eaux et les agents de balnéothérapie. *Presse Therm Clim* 108: 195-204.

Van Rensburg, C.E.J., Dekker, J., Weis, R., Smith, T.L., Janse van Rensburg, E. and Schneider, J. (2002). Investigation of the anti-HIV properties of oxihumate. *Chemotherapy* 48: 138-143.

Van Rensburg, C.E.J., Snyman, J.R., Mokoele, T. and Cromarty, A.D. Brown coal derived humate inhibits contact hypersensitivity; an efficacy, toxicity and teratogenicity study in rats. *Inflammation in press*.

Vane, J.R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol* 231(25): 232-235.

Vinegar, R., Truax ,J.F., Selph, J.L., Johnston, P.R., Venable, A.L. and McKenzie, K.K. (1987). Pathway to carrageenan-induced inflammation in the hind limb of the rat. *Federation Proc* 46: 118-126.

Visser, S.A. (1973). Some biological effects of humic acids in the rat. *Acta Biol Med Germ* 31: 569-581.

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

Viraben, R., Aquilina, C., Cambon, L. and Bazex, J. (1994). Allergic contact dermatitis in HIV-positive patients. *Contact Dermatitis* 31: 326-327.

Wang, Y., Rollins, S.A., Madri, J.A. and Matis, L.A. (1995). Anti-C5 monoclonal antibody therapy prevents collagen-induced arthritis and ameliorates established disease. *Proc Natl Acad Sci U.S.A* 92: 8955–8959.

Weithaler, K. (1954). Experimental-klinischer beitrage zur anwendung von kationenaustauschern bei hyperazidität. *Medizinische* 17: 625-628.

Wetsel, R.A. and Colten, H.R. (1990). The molecular biology of complement deficiency syndromes. In: Spitzer, A., Avner, E.D.(eds). *Inheritance of kidney and urinary tract diseases*. Kluwer Academic Publishers, Norwell, MA, p. 401–429.

Winter, C.A., Risley, E.A. and Nuss, G.W. (1962). Carrageenan-induced edema in the hind paw of rat as an assay for anti-inflammatory activity. *Proc Soc Exp Biol* 111: 544-547.

Yamada, H., Kudoh, I., Nishizawa, H., Kaneko, K., Miyazaki, H., Ohara, M. and Okumura, F. (1997). Complement partially mediates acid aspiration-induced remote organ injury in the rat. *Acta Anaesthesiol Scand* 41: 713–718.

Yanamoto, H., Kikuchi, H., Sato, M., Shimizu, Y., Yoneda, S., Okamoto, S. and Findlay, J.M. (1992). Therapeutic trial of cerebral vasospasm with the serine protease inhibitor, FUT-175, administered in the acute stage after subarachnoid hemorrhage. *Neurosurgery* 30: 358–363.

Yuan, H., Song, J., Li, X., Li, N. and Dai, J. (2006). Immunomodulation and antitumor activity of kappa-carrageenan oligosaccharides. *Cancer Letters* 243(2): 228-234.

Zsindely, A., Hofmann, R. and Klöcking, R. (1971). Über den einfluss oral applizierter huminsäuren auf en nukleinsäurestoffwechsel von ascites-tumorzellen bei mäusen. *Acta Biol Debrecina* 9: 71-77.