Efficacy and mechanistic study of a carbohydrate derived fulvic acid with anti-inflammatory properties

by

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Abstract

FAs (FA) are naturally occurring, refractory, complex organic acids that are reported to have several medicinal properties and are normally found in soils and water. They are available from health shops and pharmacies in several formulations with a number of claimed benefits but are not registered medicines. FAs are a product of the humification process of plant material originating in the environment from which these acids were obtained, but often include other organic compounds that are incorporated into the FA product. FAs have high carboxylic acid and hydroxyl content that enables stable complexation of different mineral ions that could lead to toxic heavy metal content, which can be harmful if consumed. This study assessed a synthetic FA, obtained using a patented controlled wet oxidation method of a metal-free food grade carbohydrate, to confirm biological activity and to profile the mass distribution of different components.

Fulvimed SA (Pty) Ltd use good manufacturing practice and a patented process to synthesise large quantities of pure, high-quality FA in a liquid concentrate termed Carbohydrate-Derived FA (CHD-FA). Initial studies showed that this synthetic CHD-FA has more than 50 individual unidentified compounds, most of which appear to be carboxylic acids and common physiological metabolites, with no evidence of toxic compounds. This synthetic FA is aimed at the medicinal market as the FA that is obtained through this process has been shown to contain no toxic heavy metals.

The main aim of this study was to determine whether a new more concentrated powdered formulation of this CHD-FA possesses similar anti-inflammatory properties to the previously tested less concentrated liquid formulation.

The results from this study indicated that the new formulation of CHD-FA is effective in suppressing acute carrageenan-induced inflammation in the inflammatory rat footpad model. In addition, *in vitro* testing showed that CHD-FA exhibited a dose-dependent partial inhibition of complement receptor-3 (CR3) expression by activated neutrophils. Neutrophil adhesion to vascular endothelium or opsonised particles requires cell surface expression of CR3 receptors that is essential for neutrophil function. If cell adhesion is inhibited by the presence of CHD-FA, then extravascularisation of the neutrophils would

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be suppressed and extravascular inflammatory responses would be expected to be decreased. Further testing showed biologically relevant antioxidant properties using a lucigenin-enhanced chemiluminescence assay of stimulated neutrophils. Scavenging of the 2,2-azinobis (3-ethylbenzothiazoline 6-sulfonate) radical cation ($ABTS^{\cdot +}$) using the TEAC assay confirmed the antioxidant activity of this new formulation of CHD-FA.

Although not regarded as a modern technique, thin layer chromatography (TLC) was used to separate different FA batches to compare product reproducibility and in an attempt to obtain a profile of the compounds within the synthetic CHD-FA product. This was combined with a technique of direct elution from the TLC plates into a tandem -mass spectrometer to assess the mass distribution of the different spots obtained from the FAs.

It has been demonstrated that CHD-FA possesses significant anti-inflammatory properties by *in vitro* and *in vivo* animal studies and confirmed *in vitro* antioxidant properties. These properties suggests that CHD-FA may be an effective immunomodulator for the treatment of diseases associated with an overproduction of reactive oxidants by human phagocytes such as the neutrophils.

In addition, it can be seen that there are a large number of compounds in the FA with many co-eluting under different separating conditions and having a mass range extending beyond those previously reported.

Despite the complex nature of CHD-FA, it has been shown to have several properties that indicate that it would act as an anti-inflammatory compound.

Key-Words: Carbohydrates-Derived FA, neutrophils, inflammation, antioxidants, carrageen-induced oedema

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To My father, the almighty God and my Lord, I give all glory and honour unto you.

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Declaration by candidate

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Abbreviations

ANOVA .... One-way analysis of variance

AUCC .... Animal use and care committee

ABTS................... 2,2-azinobis (3-ethylbenzothiazoline) 6-sulfonate

CHD-FA .... Carbohydrate-derived FA

CR3..................... Complement Receptor-3

CRP .... C-reactive protein

DAF..................... Decay-accelerating factor

ECM.................... Extracellular matrix

ESI QTOF .... Electrospray coupled quadrupole time of flight mass spectrometer

FA .... Fulvic acid

G-CSF................. Granulocytes colony-stimulating factor

HBSS .... Hank's balanced salt olution

H_2O_2.................... Hydrogen eroxide

ICAM-1................ Intercellular adhesion molecule

IL......................... Interleukin

MBL.................... Mannose-binding lectin

MAC.................... Membrane ttack complex

MIF...................... Migration inhibiting factors

MNL .... Mononuclear leukocytes

NADPH .... Nicotinamide adenine dinucleotide phosphate

NMR.................... Nuclear magnetic resonance

OTAU.................. Onderstepoort teaching animal unit

PRR .... Pattern recognition receptor

PAMPs................ Pathogen-associated molecular patterns

PHA..................... Phytohaemagglutinin A

PMA Phorbol-12-myristate-13-acetate

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ROS .... Reactive oxygen species

RNS .... Reactive nitrogen species

q-TOF.................. Quadrupole time of flight tandem mass spectrometer

TLC .... Thin layer chromatography

TOF..................... Time of flight mass spectrometer

Trolox .... 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

UPBRC .... University of Pretoria Biomedical Research Centre

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Chapter 1

1 Literature review

1.1 Inflammation

Inflammation forms part of the complex biological response of tissue to harmful stimuli like pathogens, irritants or damaged cells. Inflammation occurs when an organism protects itself by removing injurious stimuli and initiates the healing process. This is a mechanism of the immediate and general innate immunity as compared to the adaptive immunity which is specific for each pathogen.(1)

These inflammatory responses involve both cellular and humoral components of the immune system and can recruit increasing numbers of both cells or active mediators to increase or shut down the response. Although inflammatory responses generally manifest as acute responses that clear up within hours to days, chronic responses lasting weeks to years are common, exhibiting varying intensity and can become destructive to tissue when uncontrolled for extended periods such as during joint or tissue remodelling diseases, cancers and autoimmune diseases amongst others.(1)

Inflammation is typically characterised by redness, localised swelling, heat, and pain, all existing at the same time. When these parameters occur simultaneously, they indicate that inflammation is present, but not all symptoms are essential to confirm its existence. This implies that inflammation is difficult to recognize where these characteristic symptoms are not simultaneously observed. If these characteristics are all observed, it shows that inflammation has reached a convincing intensity, but smaller degrees of the same disturbances may exist without being obvious.(1)

Pattern recognition receptor (PRRs) activation occurs when microbe derived structures known as pathogen-associated molecular patterns (PAMPs) bind to these cell surface receptors. PAMP’s activate the PRRs expressed on both immune and nonimmune cells thereby triggering the inflammatory response. Some PRRs recognize various endogenous signals activated during tissue or cell damage and

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known as “danger associated molecular patterns” (DAMPS)(2). These are host biomolecules that can initiate and perpetuate a non-infectious initiated inflammatory response. Disrupted or damaged cells can also recruit innate inflammatory cells in the absence of pathogens by releasing DAMPs.(2)

The inflammatory processes are important, normally well controlled immunological responses required for many physiological processes, for example for wound healing, an inflammatory process is needed to remove damaged cells and potential invading pathogens during the second phase of healing. However, continuous progressive destruction of tissue through uncontrolled inflammation would compromise a person’s wellbeing or survival, such as in the cases of allergic reactions or rheumatoid arthritis, where chronic inflammation can be severe. For this reason, inflammation is closely monitored and controlled within the body.(2)

1.2 Cellular components of inflammation

Inflammatory responses require activation of circulating components including leukocytes, especially the granulocytes that contain many enzyme-containing lysosomal granules in their cytoplasm. These cells include:

- Granulocytes

Neutrophils: These act as phagocytes that normally circulate in the bloodstream and are stimulated by pro-inflammatory cytokines to migrate through vessel walls by diapedesis near an inflammatory lesion. They are generally the first leukocytes to arrive at a site of injury, attracted through the release of chemokines from platelets or antigen presenting cells. Vascular permeability close to the site of injury is increased leading to oedema and increased extracellular exudate. Once the leukocytes enter the exudate, other cells are attracted to the lesion by specific chemotactic factors and retained by the meshwork formed by fibrinous exudate.(3)

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Activated neutrophils use cytotoxic mechanism to restrict pathogen replication and engulf invading pathogens by phagocytosis, degrading them by releasing granule derived antimicrobial peptides and reactive oxygen species (ROS).(4)

At the injury site, the neutrophils phagocytose (ingest) foreign or dead cells until its life span is over. The dead phagocyte then becomes part of the purulent exudate, or pus, which leaves the site through the lymphatic system or through the epithelium.(3)

Eosinophils: These cells are more commonly associated with parasitic and allergic responses at the site of infection or intrusion.(5)

Basophils: These cells circulate in the bloodstream and when activated by exposure to immunoglobulin E (IgE), release histamine, bradykinin, and serotonin. These released substances increase capillary permeability and blood flow to the immediate area. Basophils have similar activity to the related tissue-associated mast cells.(5)

Phagocytes: These are group of cells that can engulf and destroy microorganisms, dead cells, and foreign particles. This begins when the phagocytic cells enter the inflammatory site. There are four steps in phagocytosis:(5)

- Recognition of the target and its adherence to the phagocyte
- Engulfment (ingestion, or endocytosis)
- Fusion with lysosomes within the phagocyte
- Destruction of the target by lysosomal enzymes

- Lymphocytes

B-lymphocytes: These comprise the humoral immune system, meaning that they circulate in the blood and B-cell maturity occurs in the bone marrow and during passage through the lymph nodes or spleen. A mature B lymphocyte circulates in the blood in an inactive state, becoming activated after exposure to a specific protein to which it has been genetically programmed during development and differentiation to respond. Upon activation, the B cell releases antibodies to destroy the substance that caused its activation.(5)

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T-lymphocytes

These cells play a central role in the cell-mediated immune system and can be divided into several different cell types according to the surface antigens expressed. These include the CD8-expressing natural killer cells, the CD4-expressing helper T-cells and the CD17-expressing regulatory T-cells. These cells form in bone marrow, but maturation occurs during passage through the thymus gland. When a T-cell encounters a cell presenting specific protein antigens to which it has been programmed to respond during development, it can directly attack the antigen exhibiting cell or trigger the B-cells to respond to the substance depending on the cell phenotype.(5)

Macrophages are important components of the mononuclear phagocyte system, and are critical in inflammation initiation, maintenance, and resolution. Macrophages are unique in that they are involved in all stages of tissue repair. During inflammation, macrophages present antigens, perform phagocytosis of foreign or damaged cells or material, and modulate the immune response by producing cytokines and growth factors. Mast cells, that reside in connective tissue matrices and on epithelial surfaces, are effector cells that initiate inflammatory responses. Activated macrophages release a variety of inflammatory mediators, including cytokines, chemokines, histamine, proteases, prostaglandins, leukotrienes, and serglycin proteoglycans. Macrophages presenting early during inflammation are functionally distinct from those presenting at later stages. According to Smigiel *et al.*,(6) depletion of macrophages in the early phases of wound repair significantly impairs scar formation and depletion of macrophages during later stages leads to an inability to resolve scars. Hence early phase macrophages, which are predominately M1-biased cells, like macrophages expressing TNF, interleukin (IL)-18, IL-12 and IL-23 contribute to extracellular matrix (ECM) deposition and fibrosis likely by producing pro-fibrotic cytokines that promote the activation of resident fibroblasts and pericytes into ECM-producing myofibroblasts.

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Monocytes can leave the circulation permanently where they differentiate into macrophages or dendritic cells and can be recruited via chemotaxis to damaged tissues. Inflammation-mediated immune cell alterations are associated with many diseases, including asthma, cancer, chronic inflammatory diseases, atherosclerosis, diabetes, and autoimmune and degenerative diseases.(7)

Monocytes migrate to the inflamed tissue from the peripheral blood system. These monocytes are mobilized and recruited from the blood by adhesion to the endothelium followed by trans-endothelial migration and locomotion to the target tissue after exposure to soluble immunologic and/or inflammatory stimuli like cytokines and chemokines.(7)

1.2.1 Cytokines

Cytokines are low molecular weight regulatory proteins or glycoproteins secreted mostly by leukocytes, but also by various other cells in the body in response to a number of injury or infection related stimuli. For an effective immune response, lymphoid cells, inflammatory cells and haemopoietic cells must be involved, with the complex interactions amongst these cells being mediated by a group of soluble proteins called cytokines.(8)

Properties of cytokines

Cytokines bind to specific cell surface receptors on target cells triggering intracellular signal-transduction pathways that ultimately alter gene expression within target cells. Cytokines are often pleiotropic, redundant, synergistic, antagonistic and cascade inducing. This permits them to regulate immune cellular activity in a coordinated, interactive way with multiple possible trigger and suppression pathways.(8)

Cytokines incudes proteins secreted by lymphocytes called lymphokines and those secreted by monocytes and macrophages, called monokines. They are generally referred as (IL) and to date more than 50 interleukin genes have been identified yet only about 36 gene products have been identified.(8)

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The specificity of cytokine activity is mostly gained through the selective expression and distribution of cytokine receptors among the different cell types. A number of signal-transducing cytokine receptors, referred to as β-receptors, have a broad and sometimes even ubiquitous expression pattern, and these are the receptors that are usually shared between different cytokines.(9)

The specificity of induced signalling is gained via individual non-signalling receptors, often called α-receptors, and/or the use of a second β-receptor and thus signalling via a heterodimeric receptor complex. Triggering of a specific signal-transducing receptor is one criterion for grouping cytokines into distinct cytokine families.(9)

Another important aspect of cytokine biology are the soluble cytokine receptors, which are non-cellular membrane-anchored receptors. These proteins retain their ability to bind their ligands with similar affinity as their membrane-bound counterparts but fulfil different functions. Some act agonistically when bound to their ligand and are thereby able to activate cells that lack the respective receptor on their cell surface and would usually not respond to the cytokine at all. Others are antagonists and function as soluble decoy receptors that compete with the membrane-bound receptors for ligand binding and thus inactivate the cytokine by binding a portion of the free cytokines.(9)

Each interleukin acts on a specific limited group of cells which express the specific receptors for the corresponding interleukin. During viral infections, interferons are produced to limit the spread of the virus. Differentiation and division of the bone marrow stem cells involves the colony stimulating factors. TNF-α and TNF-β have a variety of functions and play a role of mediating inflammation and cytotoxic reactions.(9)

Several pro-inflammatory cytokines play a key role in mediating acute inflammatory functions namely, IL-1β, TNF-α, IL-6, IL-11, IL-8 and other chemokines, granulocytes colony-stimulating factor (G-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF). TNF-α and TNF-β and IL-1 are extremely potent proinflammatory molecules that primarily mediate acute inflammation.(10). Cytokines involved in

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chronic inflammation include a wide range of cytokines promoting both humoral and cellular inflammatory processes. Chronic inflammation may develop following acute inflammation when the cytokine profile balance is altered to show a higher ratio of proinflammatory cytokines. Chronic inflammation may last for months and in some instances for years. During chronic inflammation, cytokine interactions result in the chemotaxis of monocytes to the site of the infection where macrophage-activating factors such as IFN-γ, MCP-1 and other molecules, activate the differentiation to macrophages, while migration- inhibiting factors (MIF) such as GM-CSF and IFN-γ retain these cells at inflammatory site.(10)

Cytokines () are predominantly released from immune cells, including monocytes, macrophages, and lymphocytes. Pro- and anti-inflammatory cytokines facilitate and inhibit inflammation, respectively.(10)

Cytokines that mediate chronic inflammation are dividing into two groups: Those that participate in humoral inflammation such as IL-3, IL-4, IL-5, IL-6, IL-7, IL-9. IL-10, IL-13 and transforming growth factor-β (TGF-β) and those that contribute to cellular function namely: IL-1, IL-2, IL-3, IL-4, IL-7, IL-9, IL-12 and the interferons (IFN-γ), TGF-β and TNF-α and β.(10)

Cytokines are modulators of inflammation. They participate in acute and chronic inflammation in a complex network of interaction. Understanding of pathways regulated by cytokines will allow developments of agents for improved modulation of the inflammatory response for the treatment of diseases.(11)

A better understanding of inflammatory response pathways and molecular mechanisms will undoubtedly contribute to improved prevention and treatment of inflammatory diseases.(10)

1.3 The complement system

The complement system is a critical part of the innate immune system and consists of a series of nine main and several minor soluble proteins produced mostly in the liver and which circulate freely in the blood system. These proteins can be activated

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through a cascade of proteolytic events through selective serine proteases that ultimately results in a protein complex known as a membrane attack complex (MAC) that can rupture the membrane of bacteria. These proteins also play a role in the activation of the innate immune cell responses of phagocytic cells. Under normal conditions the complement system is inactive but can respond to a stimulus within a very short time with the end result of activation of an inflammatory response.(12)

Activation of the complement system mainly follows three different pathways: classic, lectin, or alternative pathway, depending on the activation triggers. The complement system is activated in a well-defined sequential linear cascade of protease reactions, with activation through cleavage of one component leading to activation of the next component in the sequence. “Alternate pathway” activation occurs when components of microbial cell surfaces activate small amounts of C3. This pathway is regulated by several regulatory proteins such as Factor H, Factor I, properdin, decay-accelerating factor (DAF or CD55), etc. Complement activation products are important markers of activation with CBb a unique marker for the alternative pathway activation.(12)

After initial activation, sequential protease-mediated cleavage of complement proteins leads to activation of the aforementioned complement-mediated functions and downstream activation of adaptive immune responses. Another role for complement is immune homeostasis as clearance of apoptotic cells and debris removes self-antigens from the circulation, which reduces the risk of developing autoimmunity.(12)

Figure 1.1 illustrates the different activation pathways of the complement system with the classic, alternative and lectin as initiation point with the common MAC endpoint.

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Figure 1.1: Overview of the complement system and regulatory proteins. Activation occurs through 3 different pathways: the classical, lectin, and alternative pathways. (12)(with permission)

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The classical pathway is initiated through activation of the C1 complex (C1qrs), and the lectin pathway is triggered by PRRs, including mannose- binding lectin (MBL) and MBL-associated serine proteases (MASPs) 1 and 2.(12)

The alternative pathway can be activated by spontaneous hydrolysis of C3 or by C3 formed via the classical and lectin pathways. Activation of complement results in the production of the classical and lectin pathway C3 convertase (C4b2a) and the alternative pathway C3 convertase (C3bBb), all of which have proteolytic activity for C3 and form their respective C5 convertases by generation of more C3b (C4b2a3b and C3bBbC3b). Cleavage of C5 leads onward to the common terminal pathway, which produces the cytolytic MAC.(12)

Regulatory proteins are present both in the fluid phase or bound to the cell surface. The major fluid phase regulators include the serum C1INH, C4BP, protein S, Factor H, Factor I, and an AI, whereas the major cell-bound regulators include CD55 (DAF), CD59 (MAC-IP, or protectin), MCP (or CD46), CR1 (or CD35), and CRIg.(12)

Surfaces of pathogens that have neutral or positive charge characteristics and do not express or contain complement inhibitors. activates the alternative pathway. This happens due to a process called “tickover” of C3 that occurs spontaneously. According to Holers et al, tick over involves the interaction of conformationally altered C3 with factor B, and results in the fixation of active C3b on pathogens or other surfaces.

The spontaneous activation of the alternative pathway via tickover may play a major role in human pathologies where tissue damage is complement-mediated

.

1.4 Fulvic acid

Fulvic acid (FA) is a refractory, complex organic acid found in many soils and water. They are the acidic water-soluble fraction of humic substances like shilajit and the cause of brown -coloured waters in vegetation -rich areas. Shilajit is a semi-hard brownish black -to- dark brown greasy resin that has distinctive coniferous smell and

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a bitter taste as it is formed through long -term humification of area -prevalent plant material. It is commonly known as mumie, vegetable asphalt, and vegetable mineral in different parts of India where it is commonly used in Ayurvedic medicine. Shilajit has been therapeutically used for centuries as part of traditional medicine systems in many Asian countries, for example, it has been used as a treatment for genito-urinary disease, diabetes, digestive disorders, nerve diseases, tuberculosis, chronic bronchitis, asthma, anaemia, eczema, bone fractures and other diseases.(13)

FA samples from different regions of the world have similar physical properties and gross chemical composition when assayed with bulk property instruments like infra-red and nuclear magnetic resonance (NMR) spectrometers. There are differences in the ratio of the individual chemical components and the associated mineral content. FA is the primary organic substance in acidic aqueous extracts of many soils and lake sediments. FA is soluble in water under all pH conditions. Removal of humic acid by acidification results in relatively pure FA. It has been reported that FA exhibits many biological and medicinal properties.(13)

Although contentious several authors have reported that FAs are supramolecular organic compounds with molecular mass between 2 000 and 50 000 Da that are soluble in acid aqueous solutions and have high proton exchange capacity. They appear light-yellow to yellow brown in colour when in dilute solution. Figure 1.2 below summarises the physicochemical characteristics of different classes of humic substances including FA and humic acid.

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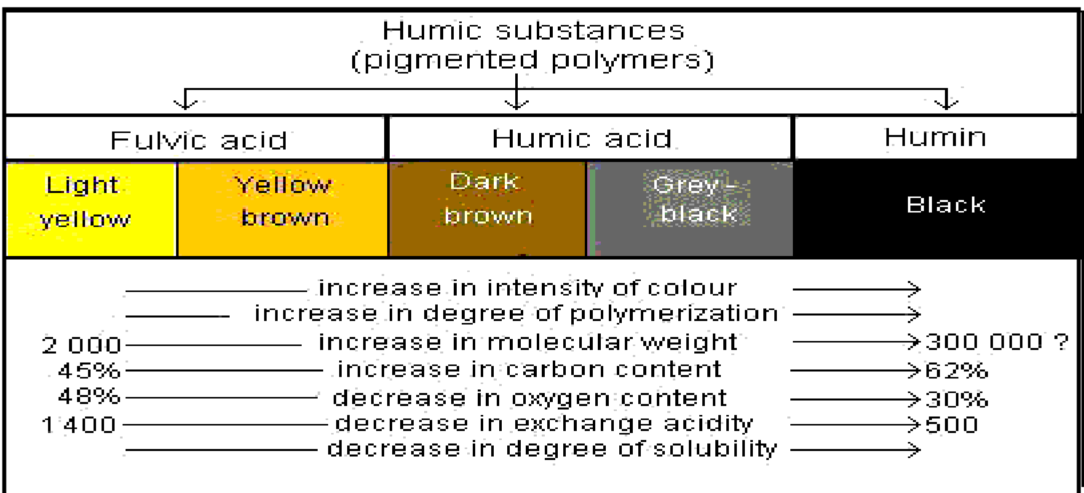

Figure 1.2: Physicochemical characteristics of humic substances. With permission from Stevenson 1982.

Although the exact structure of FA has not been confirmed, there are many common chemical structures present and several authors report that FA is a dynamic supramolecular structure that contains several aliphatic and aromatic structures with a high degree of acid, ketone and hydroxyl substitution. Several researchers have proposed chemical structures for FA from different origins but in general these are not consistently found in randomly selected products.(13)

In recent years the number of papers in which molecular modelling is used to study HS structures has increased. Determining the molecular structure of FA is important for understanding the physicochemical properties and its reactivity.(14) Several individual compounds have been isolated from FAs through various mobile phases using normal and reverse phase chromatography. The molecular structure of FA also contains a variety of other oxygen-containing structures, such as carbonyl groups, ether bonds, alcohol hydroxyl groups, and anhydrides. The molecular structure of FA contains a large number of oxygen-containing functional groups such as carboxyl and hydroxyl groups.(14)

FAs are ubiquitous naturally occurring acidic compounds with reported medicinal properties including anti-microbial, anti-viral and anti-inflammatory properties. It can

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be used for the tropical treatment of psoriasis and eczema. Van Rensburg described the anti-inflammatory properties of FA using a murine model of contact hypersensitivity(15)

Fulvimed (Pty) Ltd, a company operating out of Stellenbosch near Cape Town, South Africa developed a synthetic FA based on chemical modification of a food -grade carbohydrate. This synthetic FA derived from a food source does not contain any traces of toxic metals such as manganese, cadmium, chrome or aluminium compared to FAs derived from coal or natural sources.

Fulvimed has previously subjected this synthetic CHD-FA to a battery of *in vitro* testing, as done on natural FA and coal-derived FAs and found that it has the same antimicrobial efficacy as the coal derived synthetic FA.(16)

Biological functions of FA

FA promotes several nutrients to interact with each other in the GIT, where nutrients will be dissolved into a protected ionic form. There is great benefit if nutrients react with their co-factors, and FA is reported to enhance this association. It also makes vitamins and elemental minerals more absorbable by complexing them into organic/ ionic forms that are easily transported into and through membranes and cell walls. Once this happens, the nutrients become bio-chemically reactive, bio-available, mobile and readily absorbable. This results in improved benefit from the consumed nutrients.(17)

Research on alternative medicinal products has increased in the past three decades. Growing evidence suggests that extracts from peat, sapropel and shilajit may represent a source of novel compounds with medicinal properties. (13) Shilajit has been found to activate phagocytosis and cytokine release by murine peritoneal macrophages, stimulate osteoblastic differentiation of mesenchymal stem cells and induce proliferation of lymphocytes in the cortical thymus layer, and increased migration of these cells into lymph nodes and spleen.(13)

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Several research groups have performed testing on FA from the aqueous extracts of shilajit and these have reported to have many of the claimed biological and medicinal properties of shilajit. FA has been used externally to treat haematoma, phlebitis, desmorrhexis, myogelosis, arthrosis, polyarthritis, osteoarthritis and osteochondrosis. Orally is has been used to treat gastritis, diarrhoea, stomach ulcers, dysentery, colitis and diabetes mellitus. (18)

The anti-inflammatory activity of a topically applied FA cream at 4.5% and 9% was compared with that of diclofenac sodium at 1% and betamethasone at 0.1% in a murine model of contact hypersensitivity.

The FA creams compared favourably with both the control creams in suppressing the cutaneous inflammatory response. The anti-inflammatory properties of FA was also confirmed in a study, where pyotraumatic dermatitis in cats and dogs was successfully treated.(18)

In clinical phase testing, a pilot study was undertaken to establish the safety and efficacy of topically applied Oxifulvic acid cream (4.5%) compared to hydrocortisone cream (0.1%) in healthy volunteers.(19) FA had no significant effect on any of the safety parameters and did not induce sensitization when applied on the skin. The 4.5% FA cream caused inhibition of an elicited inflammatory reaction at 15 min and significantly differing from the 9% cream at 24 h where the 4.5% cream showed superior results. The anti-inflammatory effects of the Oxifulvic acid creams were estimated to be similar to that achieved by the hydrocortisone cream used as a positive control.

Because of an unacceptably high heavy metal content of FA derived from lignic coal, a new product was developed by Fulvimed (Pty) Ltd using a natural food quality carbohydrate source instead of coal resulting in a metal free FA product dubbed CHD-FA that was initially manufactured as a liquid containing 4% dry mass per volume of the active FA compounds.

An initial study done by Sabi (16) showed that this liquid form of CHD-FA was orally bioavailable and showed anti-inflammatory properties using the acute inflammation

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model of carrageenan induced rat paw oedema where the of paw volume showed significant differences between the different treatment groups. In that study, rats were orally dosed daily with diluted formulations of the liquid CHD-FA at concentrations of 50, 100 and 153 mg/kg body weight for a week, prior to inducing inflammatory related oedema by injecting carrageenan into a paw pad.

The CHD-FA treated groups showed dose related anti-inflammatory responses (Figure 5) with the highest doses similar to that of Indomethacin at 10 mg/kg body weight that was used as the positive treatment control, and which inhibited this oedema most although not completely as illustrated in Figure 1.3 (solid red diamonds).

The negative control group was sham treated with water (solid blue circles). FA neutralized to pH 5.5 with sodium acetate, applied by oral gavage at concentration of 153 (open triangles) or 100 mg/kg body weight (open circles), inhibited the inflammation to an extent similar to indomethacin but the 50 mg/kg body weight (open squares) had no observed ani-inflammatory effect.

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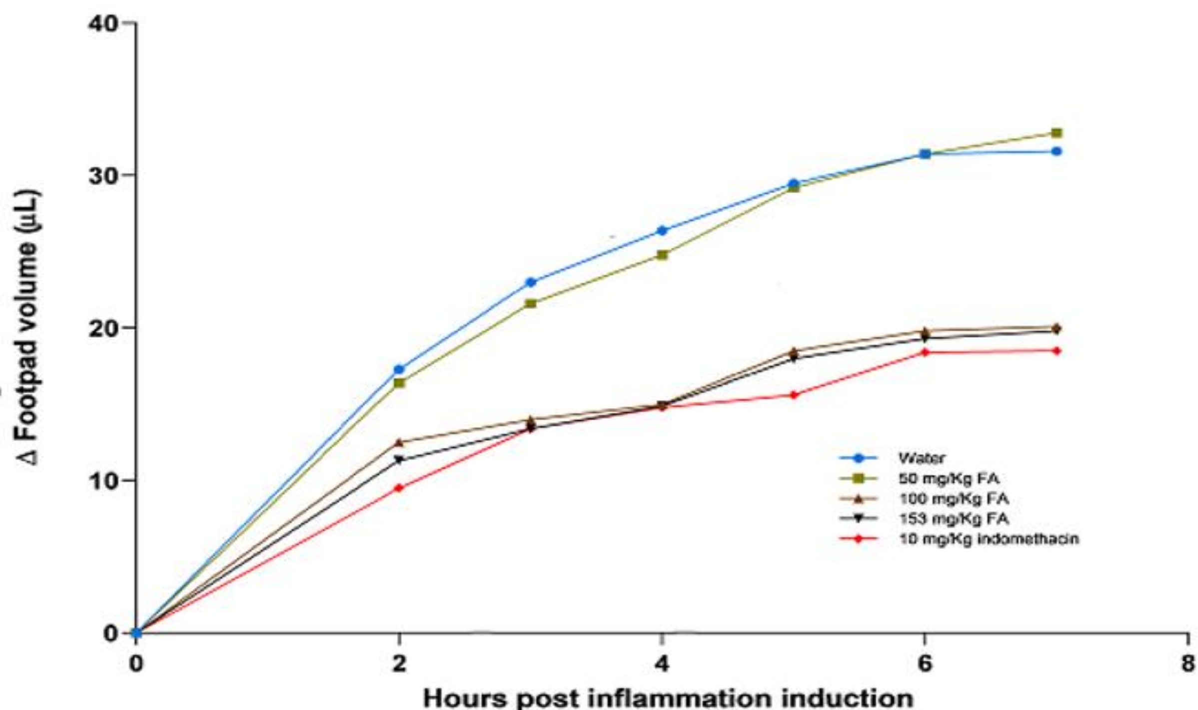

Figure 1.3: Inhibition of carrageenan-induced paw oedema after oral administration of the liquid form of CHD-FA, adapted from Sabi (16) with permission.

It had been determined that it is more practical to administer this product to patients in tablet or capsule form rather than as a calculated dose of 250 mL of a 4% FA solution which has a lasting bitter after taste.

This requirement for a solid dosage form resulted in an additional drying step to concentrate the initial product during the synthetic process and is now designated dry CHD-FA. To confirm that the anti-inflammatory activity against the carrageenan-induced paw oedema in rats had not changed due to the concentration methods this new formulation was investigated with respect to anti-inflammatory aspects such as the carrageenan induced paw oedema and the inhibition of CR3 expression by isolated neutrophils. Further characteristics investigated were the antioxidant activity in solution and oxidative burst limiting ability in neutrophils by chemiluminescence as part of this study.

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1.5 Antioxidants

Antioxidants are a group of substances which, when present at low concentrations, in relation to oxidizable substrates, significantly inhibit or delay oxidative processes of biologically important compounds, while often being sacrificially oxidized themselves. Many studies have revealed that antioxidants may play an important role in preventing diseases caused by free radicals including chronic inflammation.(16),(19)

Antioxidant compounds in food play a major role as health protecting factors. Evidence related to antioxidants suggests that they reduce the risk of chronic disease including cancer and heart related conditions. It is often assumed that antioxidants, including vitamin C, vitamin E, and carotenoids, contribute to this protection by passively reducing the oxidative damage to DNA, lipids, and proteins. This hypothesis is supported by numerous *in vitro* studies in animals and humans.(20)

Antioxidants are obtained primarily from whole grains, fruits and vegetables. Examples of antioxidants are: vitamin C, vitamin E, carotenes, phenolic acids, flavanols, phytate and phytoestrogens. These antioxidants are generally recognized as reducing disease risks when obtained through a diet of food sources that are rich in these antioxidants. Most of the antioxidant compounds are derived from plant sources and belong to different classes of compounds each with a variety of physical and chemical properties. Some have strong antioxidant properties e.g., gallates while others have weak antioxidant properties e.g., monophenols.(20)

A defining characteristic of all antioxidants is the ability to trap and destroy free radicals. Some of the reactive oxygen species (ROS) are free radicals. These are defined as any relatively stable oxygen containing chemical species with one or more unpaired electrons. Although radicals are more reactive than non-radicals, they are capable of existing independently for long times and may even exist long enough to move between cellular compartments. Free radicals are highly reactive compounds, often in the class of ROS and are present in biological system from a number of

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sources due to their function and production as by-products of several biological pathways. They can oxidise nucleic acids, proteins, lipids and can initiate degenerative disease. The antioxidants work by scavenging free radicals including peroxide, hydroperoxide or lipid peroxyl and thus inhibit the unwanted oxidative reactions causing damage that may lead to degenerative disease.(20)

Neutrophil activation results in a process referred to as the oxidative burst during which ROS are formed in high concentrations and released into the surrounding environment to assist in the inactivation and destruction of invading microbes, damaged tissue and foreign material. During inappropriate neutrophil activation under various inflammatory conditions, a chronic excess of the derived ROS, such as hydrogen peroxide and superoxide radicals have been implicated in the associated pathogenesis [23]. The cellular release of these neutrophil derived ROS can be measured *in vitro* using a sensitive chemiluminescence technique.(21)

1.5.1 Antioxidant activity

Antioxidants are important species which possess the ability to protect the body from damage caused by free radical-induced oxidative stress. A variety of free radical-scavenging antioxidants exist within the body, many of which are derived from dietary sources.(22)

The chemical structure of an antioxidant determines its intrinsic reactivity towards free radicals and other ROS and hence the antioxidant activity. They can react in several different ways with other molecules. Living cells can be damaged by free radicals through lipid, protein and DNA oxidation. Free radicals have been implicated in the development of various diseases such as cancer, cardiovascular-, or neurodegenerative-disorders and are reported to accelerate the aging processes. Humans have developed a number of defence mechanisms against free radicals and other oxidizing agents including superoxide dismutase, catalase and glutathione peroxidase enzymes. A second group consists of non-enzymatic factors such as albumin, glutathione, ascorbic acid and alpha tocopherol, uric acid, bilirubin, reduced iron and copper ions.(22)

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An imbalance between oxidizing species and antioxidants can cause oxidative stress that may lead to pathological conditions, but sufficient dietary intake of anti-oxidants can scavenge excess free radicals and oxidants to protect against disease. A study done by Etherton, *et al.*,(23) suggested that consumption of plant derived antioxidants such as flavonoids and phenolic acids prevents various disease. Phenolic acids constitute a large group of organic compounds widely distributed in nature and naturally present in virtually all uncooked plant materials and most plant -related products. They show a broad spectrum of pharmacological activity.(24)

Polyphenols are characterised by multiple phenolic groups in a single molecule and those compounds with catechol like moieties with the ability to delocalise unpaired electrons have the strongest antioxidant activity. Given the role of oxidation in a number of disease pathways and the strong *in vitro* antioxidant activity of many phenolic compounds it was reasonable to assume that antioxidant activity explained the link between dietary polyphenols and disease prevention. The antioxidant systems consist of a network of enzymatic and non-enzymatic antioxidants. Among enzymatic antioxidants, glutathione peroxidase, catalase and superoxide dismutase play a pivotal role. Non-enzymatic antioxidants present in cells are α-tocopherol, ubiquinone, β-carotene, ascorbate and glutathione. Among endogenous antioxidants, α-tocopherol and β-carotene concentrate in cellular membranes and function *in vivo* as protectors against lipid peroxidation, interacting preferentially with free radicals such as lipid peroxyl radicals. Both lipid peroxides and their break-down products, such as malonaldehyde and 4-hydroxy-2-nonenal, can directly or indirectly affect many functions integral to cellular and organ homeostasis. As a result, the increased membrane lipid peroxidation may evoke immune and inflammatory response, activate gene expression and cell proliferation, or initiate apoptosis.(24, 25) Neutrophils boast a potent antimicrobial arsenal that includes oxidants, proteinases and antimicrobial peptides. They are known to produce large quantities of ROS and reactive nitrogen species (RNS) such as O_2^- and NO through activity of oxidant generating systems such as the NADPH oxidase and NO synthase (NOS) respectively. High concentrations of ROS are cell damaging, but moderate amounts

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of ROS are required for cell regulation, acting as mediators and signal transduction molecules.(25)

When pathogens invade tissue,-antigen presenting cells, neutrophils and macrophages phagocytose the pathogen to form a phagosome which merges with a lysosome to form a phagolysosome into which antimicrobial compounds stored in cytoplasmic granules and ROS generated at the phagolysosome membrane are released. This process facilitates cellular killing of the invading microbe. Moreover, neutrophils can release granule proteins and chromatin that form neutrophil extracellular traps (NET's) which degrade virulence factors and bind and kill bacteria in a localised microenvironment within pathological circumstances. Despite the organised mechanism and the built-in safeguards, potent cytotoxic compounds may be released into the extracellular space and damage the surrounding host tissue.

The primary function of ROS and RNS (Reactive Nitrogen Species in host defence is as antimicrobial factors. However, evidence supports the role of ROS and RNS) in the regulation of pivotal cellular signalling events involved in homeostasis, cell proliferation and differentiation as well as in inflammatory and immune responses.(26)

NADPH oxidase

Neutrophils possess an intracellular membrane-bound multi component enzyme complex called NADPH oxidase. When neutrophils are activated, this enzyme complex generates large quantities of ROS.(27)

The NADPH oxidases are a family of multi-subunit enzymes that catalyse the formation of ROS, such as superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2).(28) H_2O_2-induced NADPH oxidase activation in nonphagocytic cells could be an important mechanism by which the degree of oxidative stress, as well as the subsequent cellular damage, is amplified during inflammatory disorders.(26)

NADPH oxidases play important functions in many organ systems and cell types, thereby contributing to normal physiological processes and, when in excess or dysregulated, to the pathogenesis of various human diseases.(28)

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NADPH oxidases are important contributors to oxidant generation in several tissues, including cardiac tissue. However, depending on the phase of ischemic reperfusion injury, NADPH oxidases can be either detrimental or protective, shown to have a double-edged role. Low ROS levels are cardioprotective in pre- and post-conditioning therapies, while high ROS levels are deleterious and lead to cardiomyocyte death.(26)

1.6 Chemiluminescence

Chemiluminescence is the release of energy in the form of emitted photons with almost no release of heat following a chemical reaction. When a chemiluminescence reaction happens, the released energy of the reaction is absorbed by electrons that then enter an excited electron state which then decays to a ground state accompanied by a release of the energy as a photon where the wavelength depends on the spin state of the exited electron.(29)

The difference between chemiluminescence and fluorescence is that the excited electron state is derived from a chemical reaction rather than from absorption of light energy.(29)

Previous studies have shown wide interest on chemiluminometric method where the horse radish peroxidise-luminol-hydrogen peroxide system (HRP-Luminol-H_2O_2) is used as a sensitive assay for monitoring free radicals and reactive metabolites screening antioxidant activity.(15)

The oxidation of luminol involves formation of a complex between the oxidant H_2O_2 and peroxidases to produce luminol radical. The radical then undergoes further reaction resulting in the formation of an electron excited 3-aminophthalate dianion which emits light on return to its ground state.(29)

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1.7 Measurement of the Trolox equivalent antioxidant capacity (TEAC)

Trolox is a water-soluble vitamin E analogue which has been used as an antioxidant standard due to its strong antioxidant properties. The TEAC assay, commercialized by Randox Laboratories, is based on the decrease of the absorbance at 734 nm shown by the highly coloured stable radical cation, 2,2-azinobis (3-ethylbenzothiazoline 6-sulfonate) ($ABTS^{\bullet+}$) but not the reduced parent compound. This reduction of the radical by exposure to antioxidants present in the test sample can be a measure of the antioxidant concentration of the test compound.(30)

The 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox)-equivalent antioxidant capacity (TEAC) assay is based on the decrease in the absorbance of the deep blue coloured radical cations of 2,2-azinobis (3-ethylbenzothiazoline 6-sulfonate) (ABTS) by antioxidants in the test sample (31) Generation of the stable $ABTS^{\bullet+}$ forms the basis of the spectrophotometric methods that have been applied to the measurement of the total antioxidant activity of solutions of pure substances, aqueous mixtures and of beverages. Previously, ABTS assays were based on the activation of the metmyoglobin with hydrogen peroxide in the presence of ABTS to produce the radical cation in the presence or absence of antioxidants.(31)

This technique involves the direct production of the blue green $ABTS^{\bullet+}$ radical chromophore by direct oxidation of ABTS with stoichiometrically less potassium persulfate. When an antioxidant is added to the preformed radical cation solution the radical is reduced back to the almost colourless parent ABTS in a ratio related to the concentration of the antioxidant. The reaction can be performed as an endpoint assay using the final colour intensity or as a kinetic assay where the slope of the change in colour with time indicates the concentration of the antioxidant present. Thus, the extent of decolorization as percentage decrease of $ABTS^{\bullet+}$ radical cation is determined from an appropriate calibration curve as a function of concentration or rate of change in absorbance relative to the reactivity of standard Trolox concentrations under same conditions as the unknown samples.

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1.8 Complement receptor type 3 expression

To perform their role in host defence polymorphonuclear leukocytes (neutrophils) must leave the vascular system through a sequence of adhering to the endothelium, migrate to the site of infection and phagocytise appropriately opsonized particles. CR1 and CR3 play a critical role in the initial cell adherence and phagocytosis. Neutrophils are the first cells that migrate into tissues in response to invading bacteria or other microorganisms, where they act to destroy invading pathogens through an array of microbiocidal mechanisms such as phagocytosis, and production of reactive oxygen species, and release of proteolytic enzymes and cytokines.(32)

Neutrophils play an important system wide protective role in the innate immune system but can also inappropriately cause or amplify an inflammatory process. Neutrophils represent the body's primary line of defence against invading pathogens such as bacteria and they constitute ~40–60% of the leukocyte population. Neutrophils exist in a resting state in the circulation of healthy individuals, which ensures that their toxic intracellular contents are not accidentally released to damage host tissue.(33)

The accumulation of eosinophils in lung tissue are associated with the uncontrolled inflammatory processes responsible for asthmatic diseases. The major cell adhesion receptor on the outer surface of activated phagocytes is the complement receptor type 3 (CR3), which is an αβ integrin. It has been documented that agents that block this CR3 surface receptor are beneficial in the treatment of chronic inflammation by inhibiting the recruitment of leukocytes into tissues.(34)

Neutrophils constitutively express CR3 receptors at which the complement fragment C3bi acts as the ligand. These receptors are heterodimers of a $CD11_{\beta}$ and a CD18 integrin protein which are involved in cell adhesion, migration, extravasation, initiation of immune responses and are highly responsive to external stimuli. CR3 expression is rapidly increased by cell activation by exposure of these cells to chemotactic factors and activation factors such as FMLP or the calcium ionophore

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A23187. It has been suggested that CR3 moieties are stored in the membrane of either the secondary or the tertiary (gelatinase containing) granules.(35)

Neutrophils can be activated by many factors, including cytokines (e.g., tumour necrosis factor alpha (TNF-α) or gamma interferon (IFN-γ), immune effectors (e.g., immunoglobulin or complement), and bacterial components (e.g., lipopolysaccharide (LPS) or peptides containing formyl methionine). Adherence to a solid surface also influences neutrophil activation. Many bacterial pathogens express virulence factors that directly trigger cellular signalling pathways, either positively or by pathway suppression. $CD11_{\beta}$ is a cell surface receptor/antigen that contributes to many cellular processes which are involved in the generation of a protective immune response against pathogenic organisms. $CD11_{\beta}$ belongs to the integrin family of receptors and is expressed on especially neutrophils but also on other leukocytes and spleen cells.(36)

In order for phagocytosis to occur efficiently, opsins, serum proteins such as IgG and the complement fragments C3b & C3bi, are required which bind to the surface of microorganism or foreign particles and these interact with CR3 receptors on phagocytic cells to promote adherence, initiation of phagocytosis and ingestion of the organisms. Increased expression of receptors on the surface of the phagocytes that bind these opsins or proteins would be expected to result in increased avidity for the opsonised particles and enhanced phagocytosis.(37)

It is reported that the exposure of polymorphonuclear neutrophil (PMN) to chemotactic factors causes enhanced rosette formation with complement coated sheep erythrocytes and postulated that this might be important in augmenting the activity of the neutrophils at inflammatory sites *in vivo*. However, rosette assays are influenced by a variety of other factors, so it is not possible to attribute the observed enhancement of rosette formation to only an increased number of receptors.(38)

Anti-$CD11_{\beta}$ monoclonal antibodies used in studies of human neutrophil provides evidence that CR3 receptor numbers are rapidly increased *in vitro* by exposure of cells to stimuli like including N-formyl-methionyl-leucyl–phenylalanine (FMLP) and

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phorbol myristate acetate (PMA) (39) Cells from haemodialysis patients showed an *ex vivo* increase in neutrophil CR3 expression that has been found to involve intra-cellular pool associated receptor mobilization from secondary cytoplasmic granules of neutrophils. This means that an increase in number of CR3 on the surface of neutrophils *ex vivo* reflects a pre-exposure of the neutrophils to stimuli of secretory function.(39)

Characterisation of major components in the CHD-FA

Many attempts have been made to separate fulvic and humic acids using a variety of chromatographic techniques and different stationary phase types including normal phases like pure silica and bonded phases like diol and CN type columns. Many researchers have used reverse phases like C18, C8, phenyl, pentafluorophenyl and even HILIC type separations where the mobile phase has minimal water content. One serious problem associated with all the column based chromatographic separations is the fact that analyte solubility has to be maintained throughout the process otherwise the analyte precipitates within the column and then slowly bleeds off under conditions where the analytes become soluble again in the mobile phase, if ever.

This has proven to be a problem for almost all column-based separations as the supramolecular structure of FA tends to separate during exposure to the stationary phase and alter the solubility of the remaining complex or individual compounds within the supramolecular complex. Another factor is the pH at which the individual compounds will retain solubility and the solvent combinations that would maintain the solubility of all the sub-compounds within the supramolecular structure. These complications related to the process occurring on the column has resulted in many assays being performed in a way that is introducing a bias of the molecules that actually elute from the column, with a strong bias towards smaller molecules and those with high solubility in the mobile phases being used. One chromatographic technique that circumvents some of these problems is thin layer chromatography (TLC) that is based on the same chromatographic distribution process but uses an

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"open column" format. This process involves: a suitable adsorbent (the stationary phase), solvents or solvent mixtures (the mobile phase), and the sample molecules. For thin layer chromatography the adsorbent is coated as a thin layer onto a suitable support (e.g., glass plate, polyester or aluminium sheets) onto which the sample mixture is spotted then separated by "elution" with a suitable solvent.

The advantage of this technique is that compounds that loose solubility or bind irreversibly to the stationary phase can still be visualised by various non-destructive or derivatising methods at the end of the separation.

The dissolved sample mixture is spotted onto the plate near one edge, and the plate is inserted into a sealable tank containing the developing solvent and a filter paper wick to saturate the chamber atmosphere. When the solvent has risen to near the top of the plate by capillary attraction, the plate is removed, dried, and visualized using a number of non-destructive techniques such as UV absorbance, fluorescence, then by minimally reactive methods like iodine vapour, and finally by destructive methods such as strong acid exposure with or without chromophore producing compounds.

In the event of sample components precipitating these can be detected on the plate, at any Rf value even if it has not moved from the origin where the sample was spotted.

Below figure 1.4 illustrates the TLC process.

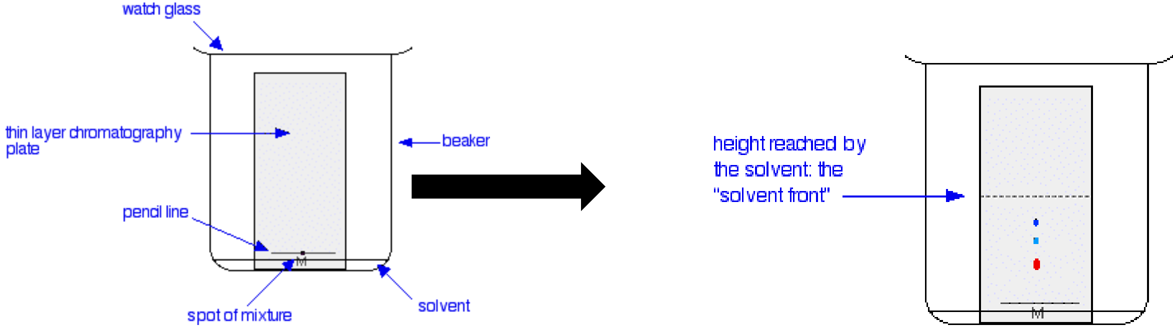

Figure 1.4: Illustration of a typical TLC setup and process followed for separation of CHD-FA compounds

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The speed at which the analyte moves over the stationary phase depends on the mobile phase as it dissolves the solute and moves it up the plate, and the distribution onto the sorbent as it pulls the solute out of solution. Molecules move along the plate relative to the distribution between the mobile and stationary phases as the solute becomes repeatedly absorbed and desorbed. Because of this, only a small fraction of the analyte is moving up the plate at any time, but each compound within the sample mixture will travel a mean distance relative to the solvent front.(40)

The separate zones will spread out because of the random fluctuations and movements of individual particles and variations in the uniformity of the sorbent. Substances that are strongly attracted to the sorbent move more slowly because they spend more time in the sorbent, and those that move more quickly are less attracted to the stationary phase, are more soluble in the mobile phase, and spend more time in the mobile phase.(41)

Therefore, compounds with different solubilities in the mobile phase can be separated from one another by exploiting the diverse interactions of the solutes with the stationary and the mobile phase.

The main advantages of TLC method are high sample throughput and low costs of analysis, despite lower precision and accuracy than column-based chromatography but simultaneous analysis of samples under the same conditions and the short analysis time are advantages.(42)

In normal phase TLC, the sorbent is polar, so the more polar solutes move more slowly and stay closer to the origin, while the nonpolar solutes will move more quickly and closer to the solvent front. By increasing the polarity of the mobile phase, the polar solutes can be drawn farther from the origin to increase separation. The opposite is true for reverse- phase TLC (RP-TLC). A nonpolar sorbent will bind nonpolar solutes better and they will migrate up the plate slowly, and the polar components will move quickly near the polar solvent front. To increase the distance

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travelled by the nonpolar zones, the polarity of the developing solvent must be decreased.(40)

Another factor that is important to maintain solubility of FA can be illustrated by the work of Baglieri (43)who concluded that humic acids are completely soluble at pH 13 and completely insoluble at pH 1. The characteristics of humic substances in a particular sample depends on the extraction procedure and source of the humic substances adopted. This is attributed by the complex nature of soil or water environment from which the substances are being extracted, to the wide range of physical and chemical properties inherent in humic material, and in particular to the association of organic and inorganic soil or water constituents. Therefore, both the type of soil and the extraction pH played a role in the chemical structure and molecular weight of the humic materials extracted and analysed.(43)

Balgieri concluded that, HA were completely solubilised from the environmental sample within less than an hour at pH > 10. At pH between 10 and 9, the dissolution was slower but nearly complete. At pH 8.5 and 8, more than 60% and 40% of the HA respectively were solubilised in the same time. The separation of HA in a soluble and an insoluble phase at these pH values attests for the heterogeneity of the material defined as humic compounds on the basis of its insolubility at pH 1. (43) During chromatography as the different compounds are separated from each other it would be expected that the more acidic compounds would elute slower meaning that the environment within the sample slug is changing and becoming more acidic as the neutral compounds move away. This would have a direct effect of the solubility of the remaining compounds to the extent that some of these compounds may precipitate on the stationary phase.

In this study, TLC was used under a three different mobile phase conditions based on previous work performed in the department and the plates were visualised by non-destructive techniques only, then further analysed by mass spectrometry using a technique of direct spot elution into an ESI based mass spectrometer to determine the mass distribution of the compounds within each spot separate from different

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batches of CHD-FA. The advantage of this approach was that all the visualised spots could be assessed by mass spectrometry and not only the compounds that would typically elute from a chromatography column.

Mass spectrometric analysis is a powerful technique where the mass of ions derived from the compounds can be separated according to their mass- to- charge ratio. In tandem mass spectrometry the precursor ion can be further fragmented to give more information about the structure of the compounds being analysed. Time -of -flight mass spectrometers are accurate mass instruments allowing possible determination of empirical formulas of ions under investigation. As mass spectrometers only separates ions, the first step for analysis of unknown compounds is production of ions from the individual compounds in the source which in this case is a soft ionisation technique called an electrospray ionisation (ESI). The analytes ionise with a charge based on the polarity of the inlet capillary. The molecular ion formed in the source then enters the mass spectrometer and is separated based the mass to charge ratio in the first mass analyser and selected precursor ions from this first mass separation may be fragmented in the second quadrupole. Each precursor ion derived from a molecule then undergoes fragmentation to form a series of product ions. The fragment ions are then separated in the mass spectrometer according to their mass-to-charge ratio and are detected in proportion to their abundance. The typical q-TOF tandem mass spectrometer layout is shown in Figure1.5 below where the first quadrupole and fragmentation cell are shown in the ion flightpath prior to the TOF mass analysis.

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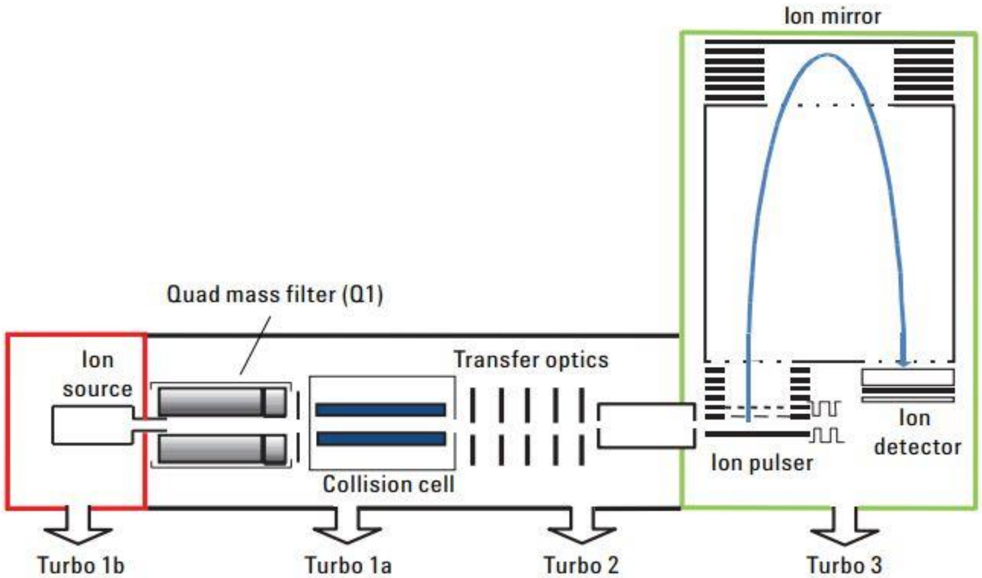

Figure 1.5: A diagram of a quadrupole-TOF mass Spectrometer showing the flight path of the ions from the ionisation source the Q1 quadrupole and collision cell and the final time of flight analyser. Ions move from left to right through the stages of the instrument (Agilent Technologies)

A mass spectrometer generates multiple ions from a mixed sample under investigation that it then separates according to their specific mass-to-charge ratio (m/z) and records the relative abundance of each ion mass. These ions provide information concerning the nature and potentially the structure of their precursor molecule. A typical mass spectrum is shown in Figure 1.6 where the ion intensity at each mass to charge ratio is recorded for a mixture of compounds in an injected sample. Each ion provides information about its relative concentration and the mass of the precursor compound.

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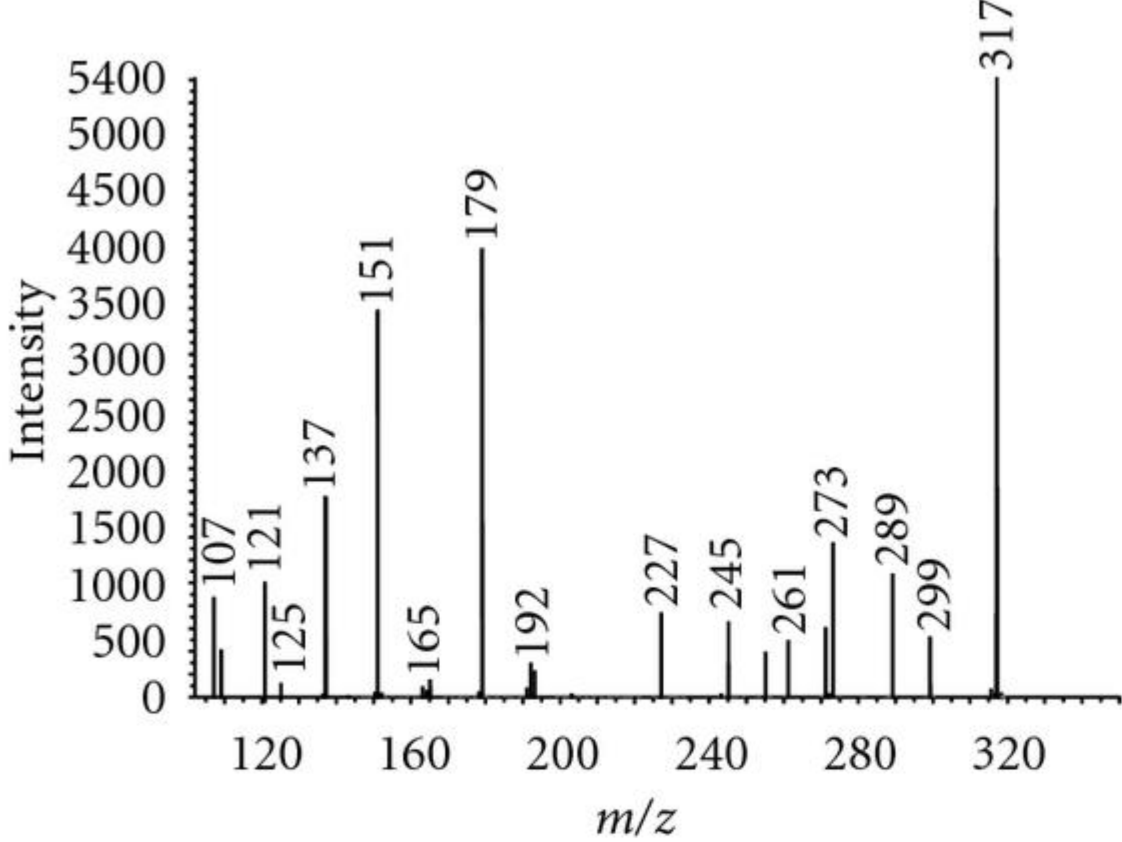

Figure 1.6: An example of a typical mass spectrum showing the intensity of ions at different mass to charge ratios.

This study used a specialised instrument to elute the compounds in a specific spot on the TLC plate directly into the source of the mass spectrometer using a flow of acetonitrile. This provided a complete image of the mass range of the CHD-FA that shows bias to the lower mass ranges when using column chromatography as the technique prior to the mass spectrometer. Using the combination of TLC and mass spectrometry has enabled complete comparison all the compounds in the different formulations of the CHD-FA.

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1.9 Project motivation

In vitro tests done at the Department of Pharmacology have indicated that a new dry form of the CHD-FA that is suitable for formulation in a tablet or capsule format possesses equivalent anti-microbial activities (unpublished results) to the liquid formulation of CHD-FA previously tested in the department for both anti-inflammatory and teratogenicity testing.

A previous investigation of the systemic *in vivo* anti-inflammatory properties of the liquid formulation of CHD-FA, administered by gavage, and using the carrageenan-induced rat paw oedema model (AUCC, Animal Use and Care Committee, protocol number: H19-06) was completed and it was concluded that the CHD-FA was systemically available and was effective in reducing inflammation related oedema at dosages of 100 and 153 mg/kg body weight.

The teratogenicity study was also done (AUCC, Animal Use and Care Committee) protocol number: H26-05), where pregnant rats were treated orally with 175 mg/kg/bodyweight CHD-FA on days five to seven of pregnancy. No clinical signs of toxicity of either the dames or the off-spring were observed during this study.(44)

As the manufacturing process and final concentration technique of the CHD-FA, which is a complex mixture of many different small molecules, could affect the final product, all the pre-clinical testing had to be repeated on the new product to ensure that the biological activity had not been affected. To this end the new product was tested in exactly the same manner as the first liquid form of the CHD-FA to the extent that the concentration of the CHD-FA was made up to be the same concentration as that used in the previous study.

The expectation was that the effects should then be comparable between the two different products and that any differences would then be easy to identify.

In addition, further testing of the antioxidant activity was proposed as one hypothesis of the mechanism of action related to the anti-inflammatory effects is that the FA act as antioxidants. Additional confirmatory tests were also proposed to confirm that the

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same activity seen in relation to the in vitro inhibition of CR3 expression in activated neutrophils was still evident in the dry formulation.

A final question that required answering was whether the mass distribution of the original liquid form and that of the dried form were still equivalent to each other as the drying process may have removed some of the smaller compounds which were more likely to be volatile or pass through the filtration systems. This was to be assessed by a combination of chromatography and mass spectrometry which then used a unique approach of combining TLC with a modern direct elution of all identified spots into a ESI Q-TOF spectrometer.

1.10 Aim

There were three main aims for this study which were firstly related to the systemic *in vivo* anti-inflammatory properties, secondly, to assess characteristics indicating potential mechanisms of action and thirdly to assess whether there were changes in major chemical components between the new dry and previous liquid CHD-FA products.

To test the *in vivo* systemic anti-inflammatory properties of the new dry formulation of CHD-FA suitable for tablet or capsule formulations in a study equivalent to that done with the liquid formulation using the validated carrageenan-induced rat paw oedema model of acute inflammation.

The second aim is to determine the antioxidant capacity of the new dry CHD-FA product and to test the inhibitory effect of the dry CHD-FA on CR3 expression by activated neutrophils.

A final aim was to characterise the major components present in different batches of the CHD-FA and compare these to each other using TLC and mass spectrometry.

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1.11 Objectives

To test the *in vivo* systemic anti-inflammatory efficacy of the new dried form of the CHD-FA product, that can be used in tablet formulations, using the validated rat paw oedema model of acute inflammation.

To assay the antioxidant properties of the dry CHD-FA using the TEAC radical scavenging assay.

To assess the effect of dry CHD-FA on CR3 expression on resting and PMA stimulated neutrophils by flow cytometry and chemiluminescent assays.

To compare the major chemical components of the dry CHD-FA formulation versus previous liquid CHD-FA batches using a combination of TLC and a specialised direct elution into a tandem mass spectrometer technique.

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2 CHAPTER 2:

2.1 Animal study

This was an intervention-based study to measure the systemic anti-inflammatory effect of two different doses of orally gavaged CHD-FA on acute inflammation induced oedema using the carrageenan -induced inflammatory rat paw oedema model where a water displacement plethysmometer was used to measure paw oedema. This study was approved by the University of Pretoria Animal Use and Care Committee with approval number H012-09.

2.1.1 Materials and methods

Healthy female Sprague Dawley (SD) rats of 12 weeks were sourced from a breeding colony at University of Free State Animal Research Centre. High purity carrageenan and indomethacin were purchased from Sigma (St Louis, MO, USA). In this study the carrageenan-induced rat paw oedema assay was used as described in a previous study to allow direct comparison of results between the liquid and dry CHD-FA products.

The methodology is described in detail below.

2.1.2 Carrageenan-induced paw oedema

2.1.2.1 Oedema develops when the linear sulphated polysaccharide, λ-carrageenan, is injected into the hind paw of the rat due to the early inflammatory phase release of histamine and serotonin, which play a major role in the initiation of inflammation [45].

2.1.2.2 Experimental design and drug administration

Forty female Sprague Dawley (SD) rats of 12 weeks old, weighing 150 - 200 g divided into10 rats per treatment group were housed as 5 rats per standard plastic rat boxes with metal grid lids at the UPBRC. Rats were acclimatised at the UPBRC for at least one week before the study is initiated. They had *ad lib* access to water and standard Epol rat chow pellets with a 12-hour day/night light cycles, room temperature of 21 ±1°C and handled according to standard operating procedures

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used at the UPBRC. Extra bedding and enrichment items like paper roll cores and egg boxes were added to the cages. The rats were observed daily for any adverse reactions or stressful behaviour and weighed every second day.

After acclimatisation the rats were ranked according to weight and sequentially allocated into one of 4 groups to ensure weight matched groups. Each rat was then identified by ear clipping. The four groups were randomly assigned to a treatment as follows (summarised in Table 1):

Group 1 Control untreated group; water only dosed by gavage; ten rats.

Group 2 Experimental group (CHD-FA by gavage at 153 mg/kg); ten rats.

Group 3 Experimental group (CHD-FA by gavage at 100 mg/kg); ten rats.

Group 4 Positive control group receiving 10mg/kg indomethacin on Day 5.

The systemic treatment by oral gavage was initiated 6 days prior to the carrageenan challenge where each group was treated by gavage with either sterile tap water (Group I), CHD-FA in sterile tap water with the pH adjusted to 5.5 with potassium acetate on Days 1 – 6 an hour before the carrageenan challenge (Group II and III) or with the sterile water for 4 days followed with 10mg/mL indomethacin on Days 5 and 6 an hour before the carrageenan challenge (Group IV). On Day 6, one hour after the last oral drug administration, the following procedures were followed:

Each rat was weighed, and both the back legs marked with a permanent marker just above the knee to indicate the depth of insertion into the water displacement plethysmometer (Panlab, Barcelona. Spain). The volume of both the back legs were measured before injecting 50 µL of a sterile 2% w/v solution of λ-carrageenan (Sigma Aldrich, St Louis, USA) in saline into the sub-plantar region of the right foot with a short 27 G needle. A volume of 50 µL of sterile saline was injected into the left sub-plantar region of the left paw as a sham treatment. The paw volumes were then measured again every hour by carefully inserting the paw to the mark for both the left and right paw and the volumes of each recorded for each interval. Differential

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paw oedema was calculated by subtracting the volume of the left paw from the right paw for each time point after injection of the carrageenan.

Table 2.1: Summary of experimental procedures

	Day 1 - Day 4	Day 5	Day 6
Control group (I)	Water by oral gavage	Water by oral gavage	Water by oral gavage. Carrageenan induced inflammation. Monitor paw oedema for 7 hours. Termination of rats.
Experimental groups (II-III)	CHD-FA by oral gavage @ 100 or 153 mg/kg	CHD-FA by oral gavage @ 100 or 153 mg/kg	CHD-FA by oral gavage. Carrageenan induced inflammation. Monitor paw oedema for 7 hours. Termination of rats.
Positive control group (IV)	Water by oral gavage	Indomethacin @ 10 mg/kg-bodyweight by gavage	Indomethacin oral gavage. Carrageenan induced inflammation. Monitor paw oedema for 7 hours. Termination of rats.

Daily evaluation of pain and distress. Weighed every second day.

Animals used were not expected to suffer any more than slight pain and discomfort after the carrageenan injection despite the oedema. None of the animals were euthanized before experiment completion. The mean values of the oedema in the treated groups were compared with the mean values of oedema measured in the control group. The differences between the experimental groups and the control group were statistically analysed using the two-way ANOVA test in GraphPad Prism version 5 (GraphPad Software, La Jolla California USA).

2.1.3 Disposal of animals

Animal carcasses were incinerated at Onderstepoort Teaching Animal Unit (OTAU) according to standard procedures at the UPBRC.

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2.1.4 Measured outcomes

The extent of oedema was measured as the difference between left and right foot volumes where only the right foot was injected with carrageenan. The differences in foot volume were assessed for significant differences relative to the treatment using the Kruskal- Wallis statistical assay.

In vitro biological assays of surface adhesion molecules as inflammation markers were done to test CHD-FA on the expression of CR3 on activated neutrophils. This assay was performed using 80, 100 and 150 µg/mL CHD-FA followed by flow cytometric assays with PE-anti-CD11$_{\beta}$ directed monoclonal antibodies using freshly isolated neutrophils from healthy volunteers.

Long standing existing data, large volume of preceding studies that did not need repetition, head-to-head studies were not done It is essential to collect sufficient supporting data before registration of this product as a complementary medicine for the treatment of inflammatory conditions.

2.2 Antioxidant activity

The dry CHD-FA product specially formulated for use in tablets or capsules has been formulated using an additional stage of concentration of the liquid Carbohydrate-Derived FA product previously tested in this laboratory.

In this study, the effects of this new formulation of CHD-FA on the lucigenin enhanced chemiluminescence of neutrophils, in both resting or stimulated state (using phorbol myristate acetate (PMA)) was tested on the cells *in vitro.*

The same CHD-FA solutions were tested for antioxidant ROS scavenging properties in a cell free system using the Trolox Equivalent Antioxidant Capacity (TEAC) assay.

2.2.1 Materials and methods

Trolox, phorbol myristate acetate (PMA), HEPES, Histopaque-1077, lucigenin, 2,2-azinobis 3-ethylbenzothiazoline 6-sulfonate (ABTS) were all purchased from Sigma

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(Sigma, St Louis, MO, USA.). Potassium persulfate and ammonium chloride were from Merck (Darmstadt, Germany). Hanks Balanced Salt Solution (HBSS) was from Highveld Biologicals (Randburg, RSA).

2.2.2 Chemiluminescence Assay

2.2.2.1 Chemiluminescence measurement of the neutrophil generated reactive oxygen species.

Neutrophils were isolated from heparinized venous blood obtained from normal healthy volunteers by density gradient centrifugation on Histopaque-1077 (Sigma, St Louis, MO, USA.) and suspended in phenol red- free buffered Hanks Balanced Salt Solution (HBSS) to a concentration of $1x10^7$ cells/mL following the method described by Fernandes *et al. (45)* Briefly 30 mL whole blood collected into blood bags containing heparin were layered onto 15 mL Histopague-1077 in a 50 mL centrifuge tube and centrifuged at 400 *g* for 25 min at ambient temperature. The neutrophil layer lying just above the red blood cell layer was carefully removed and contaminating erythrocytes lysed using cold 0.84% ammonium chloride for 10 min, then washed with HEPES buffered Hanks Balanced Salt Solution (HBSS) at pH 7.4.(45)

Reactive oxidants generated by PMA-stimulated neutrophils were measured using lucigenin-enhanced chemiluminescence as previously described (46)with minor variations as described below. White 96 well luminescence plates were used for the assay. Briefly, neutrophils at 1×10^7 cells/mL suspended in phenol red- free HEPES buffered HBSS, were incubated at 4°C in the presence of lucigenin (0.5 mg/mL) added for 30 min to preload the cells with the luminescence enhancer.

A homogenous aliquot of 40 µL (400 000 cells) of these lucigenin pre-loaded cells was added to 120 µL of HBSS in a white opaque 96 well luminescence plate. The plate was loaded into an FluoroStar OPTIMA chemiluminometer (BMG Labtech, Offenburg, Germany) equipped with an automatic syringe dispensing system and which is temperature controlled to 37˚C and incubated for 5 min. A volume of 20 µL

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of the relevant concentration of CHD-FA was added and the plate again incubated for a further 15 min at 37°C in the chemiluminometer. The following final concentrations of CHD-FA tested were; 3, 6.3, 12.5, 25, 50 and 100 µg/mL respectively. Both negative and positive control wells had 20 µL HBSS added instead of the CHD-FA product. All wells except the negative controls were then treated while shaking with 20 µL of a freshly prepared solution of PMA (final concentration 10 ng/mL) using the automated syringe dispenser.

The negative controls were treated with 20 µL HBSS. Oxidant production as chemiluminescence was monitored every 15 seconds over a period of 800 seconds as chemiluminescence in mV*sec (area under the curve values). Comparative values were reported for triplicate assays for three independent experiments, using different volunteers for the neutrophil isolation, relative to the positive and negative controls. Results were analysed using Kruskal Wallis statistical test comparing the different treatments based on area under the curve values of individual test wells.

2.2.3 Trolox equivalent antioxidant capacity assay

2.2.3.1 Measurement of the Trolox equivalent antioxidant capacity (TEAC)

Trolox is a water-soluble vitamin E analogue which is used as an antioxidant standard due to its strong antioxidant properties. The TEAC assay, commercialized by Randox Laboratories Ltd., is based on the decrease of the absorbance of the highly coloured radical cation, 2,2-azinobis (3-ethylbenzothiazoline 6-sulfonate) ($ABTS^{•+}$) at 734 nm when reduced to the colourless parent compound.

This reduction of the radical concentration is due to quenching by antioxidants present in the test sample[48]. Briefly, 7 mmoles ABTS and 3 mmoles potassium persulfate ($K_2S_2O_8$) are dissolved in 500 mL water and allowed to stand in the dark at room temperature for 12–16 hrs to allow the formation of the stable and highly coloured ABTS radical cation ($ABTS^{•+}$). Required volumes of this stock solution was diluted with cooled boiled deionised water to give a final absorbance of 0.7 ± 0.02 at 734 nm. The Instrument used was a lambda 25 dual beam uv vis spectrometer,

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perkin ELMIR, waltem, USA The free radical scavenging properties of CHD-FA (at 3, 6.3, 12.5, 25, 50, and 100 µg/mL) was compared to a Trolox calibration curve generated with concentrations from 2 – 16 µM at a fixed reaction time of 5 min. The endpoint readings were analysed to determine the antioxidant capacity of the CHD-FA. In this assay the total antioxidant capacity represents the sum of the antioxidant capacity of all compounds capable of reducing the radical in the CHD-FA.

2.2.4 The anti-inflammatory properties of the dry CHD-FA by measuring inhibition of neutrophil CR3 expression

2.2.4.1 Materials and methods

PMA (phorbol myristate acetate) and Histopaque-1077, RPMI were purchased from Sigma (St Louis, MO, USA). Heat inactivated foetal calf serum was from Gibco (Thermo Fisher Scientific, Edenvale, RSA). Phycoerythrin conjugated anti-$CD11_{\beta}$ monoclonal antibodies and equivalent isotypic IgG, Isoflow® sheath fluid and flow cytometer tubes were purchased from Beckman Coulter (Fullerton, USA).

2.2.4.2 Measurement of inhibition of CR3 expression on activated neutrophils

In this study the cellular expression of CR3 by negative and positive control neutrophils as well as for dose ranging concentrations of CHD-FA were quantitated by a flow cytometric method based on the method by Jooné[54] and used routinely in the Pharmacology Department.

Phycoerythrin (PE) conjugated monoclonal antibodies against the $CD11_{\beta}$ cell surface marker molecules were added to neutrophils in either the unstimulated resting and in the PMA- stimulated state. Adding an excess of fluorescent antibody was to ensure that all the CR3 receptors on the neutrophil membrane surface had been fluorescently labelled.

Isolated neutrophils populations made as described for the chemiluminescence assays above, were made up to 1 x 10^7 cells per millilitre. Two 5 ml plastic test tubes for each test compound concentration, one for resting cells and one for stimulated

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cells, as well as equivalent control tubes were prepared by dispensing 50 µL of this cell suspension into 400 µL aliquots of RPMI 1640 medium containing 10% HI-FCS (heat- inactivated fetal calf serum) per tube and pre-incubated in a water bath at 37^{0}C for 15 min.

The test compounds were replaced by HI-FCS fortified RPMI in the two control tubes; otherwise, all additions were identical to the test compounds. After the pre-incubation period, 50 µL isolated sub-fraction as test compound was added to both the tubes of cell suspension per paired set, gently mixed and incubated for a further 20 min.

After the second incubation time, the stimulated tube from each pair was treated with 50 µL of a 1.0 µg/mL solution of freshly prepared PMA in RPMI. An equivalent 50 µL RPMI alone was added to the paired resting cell tube. After a further 20 min incubation time the tubes were quickly transferred to an ice bath and 100 µL aliquots removed and added to corresponding clean cytometer counting tubes to which 5 µL of PE conjugated anti-CD11$_{\beta}$ antibody had been added. Isotypic background controls were provided by the addition of 100 µL aliquots from the resting or stimulated control tubes to 5 µL PE conjugated anti-mouse IgG. After 10 min of incubation in the dark the suspensions were diluted to 600 µL with Isoflow® sheath fluid.

Analysis of the cellular fluorescence intensity by a flow cytometer provided a quantitative measure of the total CR3 expression on the surface of the neutrophils. An FC 500-MLC flow cytometer (Beckman Coulter, Fullerton, CA) equipped with an air-cooled argon laser operating at 488 nm was used in this study.

The region in the scattergram representing the neutrophils was gated and only the fluorescence within this gated region analysed further.

The mean fluorescent peak intensity in the histogram was used to quantitate the abundance of the expressed CR3 for both resting and stimulated neutrophils in the absence and presence of varying concentrations of the CHD-FA.

The experiment was repeated on neutrophils from four different healthy volunteers with triplicate repeats of each test concentration for each test. Expression of CR3 by

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both resting cells and cells stimulated by the addition of PMA determined the degree of stimulation of CR3 expression in neutrophils from healthy volunteers. The dose response of the CHD-FA was determined from the different concentrations used to pre-treat the neutrophils prior to activation with PMA.

2.2.5 Characterization of major components in the CHD-FA

Thin layer chromatography (TLC) is based on a chromatographic distribution process of the analytes being separated between the stationary and mobile phases. This process involves: a suitable adsorbent (the stationary phase), solvents or solvent mixtures (the mobile phase or eluent), and the sample analyte molecules. For thin layer chromatography the stationary phase is coated as a thin layer onto a suitable support (e.g., glass plate, polyester or aluminium sheet). On this layer the substance mixture is separated by elution with a suitable solvent.

2.2.5.1 Materials and methods

Semi-preparative Silica Gel G TLC plates of 20 x 20 cm with a 250 µm layer impregnated with an inorganic fluorescence indicator were purchased from Merck (Darmstadt. Germany). All solvents, acids and ammonium hydroxide were of analytical grade and purchased from Merck (Darmstadt. Germany). Acetonitrile was mass spectrometric grade Romil solvent purchased from MicroSep (Johannesburg, RSA). Deionised water was produced in-house using an Elga Genetics water purification system. (Veolia Water Technologies, Edenvale, RSA). Standard covered glass TLC tanks were used to run the plates (Camag, Muttenz, Switzerland). The mass spectrometer used was a Xevo G2 qTOF spectrometer from Waters (Milford, MA, USA) and the TLC spot elution system was a Camag TLC-MS Interface 2 (Camag, Muttenz, Switzerland).

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2.2.5.2 *TLC and mass spectrum-based analysis of components of CHD-FA*

Silica based TLC plates were prepared running unspotted plates the full length of the plate in the mobile phase to be used during the development of the plates with the CHD-FA samples. These plates were then oven dried for 24 hrs at 120°C and allowed to cool in a desiccator to avoid water absorption. Plates were then marked by drawing a soft pencil line across the plate about 2 cm from the lower edge of the TLC plate. Samples from each batch of CHD-FA were made up to 4% w/v then spotted along the line at 30 mm centres using a capillary tube delivering 10 µL of each solution, allowed to dry and labelled. Different plates were developed in one of the following mobile phase mixtures;

- acetonitrile:water:ammonium hydroxide in the ratio 6:3:1
- methanol:chloroform:butanol:water:acetic acid in the ratio 10:6:3:3:0.4
- acetone:butanol:water:acetic acid in the ratio 13:4:2:1

When the applied spots had dried, the plate was placed vertically into a shallow layer of one of the above mobile phases in a covered TLC tank fitted with a paper towel saturation wick, to ensure that the atmosphere in the tank is saturated with solvent vapour. It is important that the solvent level is at least 4 mm below the hight of the lowest point of the spotted samples.

As the mobile phase slowly moves up the plate by capillary attraction, the different components of the different CHD-FA samples spotted onto the plate moved at different rates depending on the differential solubility in each phase and become separated or resolved.

UV absorbance and fluorescent colours and intensity were observed under a UV lamp at both 260 nm and 360 nm wavelengths.

Measuring the RF (retention factor) Values

In order to identify the chemical components in the different CHD-FA formulations, the Rf values needed to be measured and this reported together with the masses of the compounds.

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The Rf is the ratio of the distance travelled by the compound relative to the distance travelled by the solvent front and calculated using the following formula.

Rf = Distance travelled by compound/ distance travelled by solvent front.

Different batches of CHDFA were separated and Rf value of each spot for each compound calculated from each plate run under different mobile phase conditions. For the purpose of the Rf value, spots with the highest intensity were identified. To calculate the centre of the spot two measurements were taken, top edge and the bottom edge of the spot. These values were averaged. The compounds with the higher Rf value (closest to 1 and that moved further up the plate) were less polar under the conditions used for the separations.

2.2.5.3 Determination the molecular mass of the compounds separated by TLC

To determine the molecular mass of the different compounds in the CHD-FA products, TLC plates developed using the three different mobile phases were further analysed via mass spectrometry. A Camag TLC-MS Interface 2 system (Camag, Muttenz, Switzerland) was used whereby specific spots on the TLC are clamped under an elution head and a solution of 90% acetonitrile in 0.1% formic acid at 200 μL/min used to elute the compound from the silica directly into the source of a high-resolution Xevo G2 q-TOF mass spectrometer (Waters, Milford, MA, USA). Under special conditions like single compound being introduced into the source, the empirical formula data and fragmentation patterns of different compounds can be obtained.

The Xevo G2 q-TOF was set to scan between 100 and 1200 Da with a cone voltage of 30 V, corona voltage of 3 kV, source temperature of 100°C and desolvation temperature at 350°C. Nitrogen was used for desolvation with cone gas flow rate at 800 L h^{-1}, in both the positive and negative modes, which were used for separate runs on different but equivalently developed TLC plates.

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3 CHAPTER 3: RESULTS

3.1 The anti-inflammatory properties of a new formulation of CHD-FA on carrageenan-induce paw oedema in rats

The acute inflammatory response in the rat footpad was measured using a plethysmometer to show the relative increase in volume of the carrageenan verses the contralateral sham treated paw. Figure 3.1 below illustrates the reduction of paw oedema of two different concentrations of the dry CHD-FA formulation administered orally on a daily basis for one week prior to carrageenan challenge during this study.

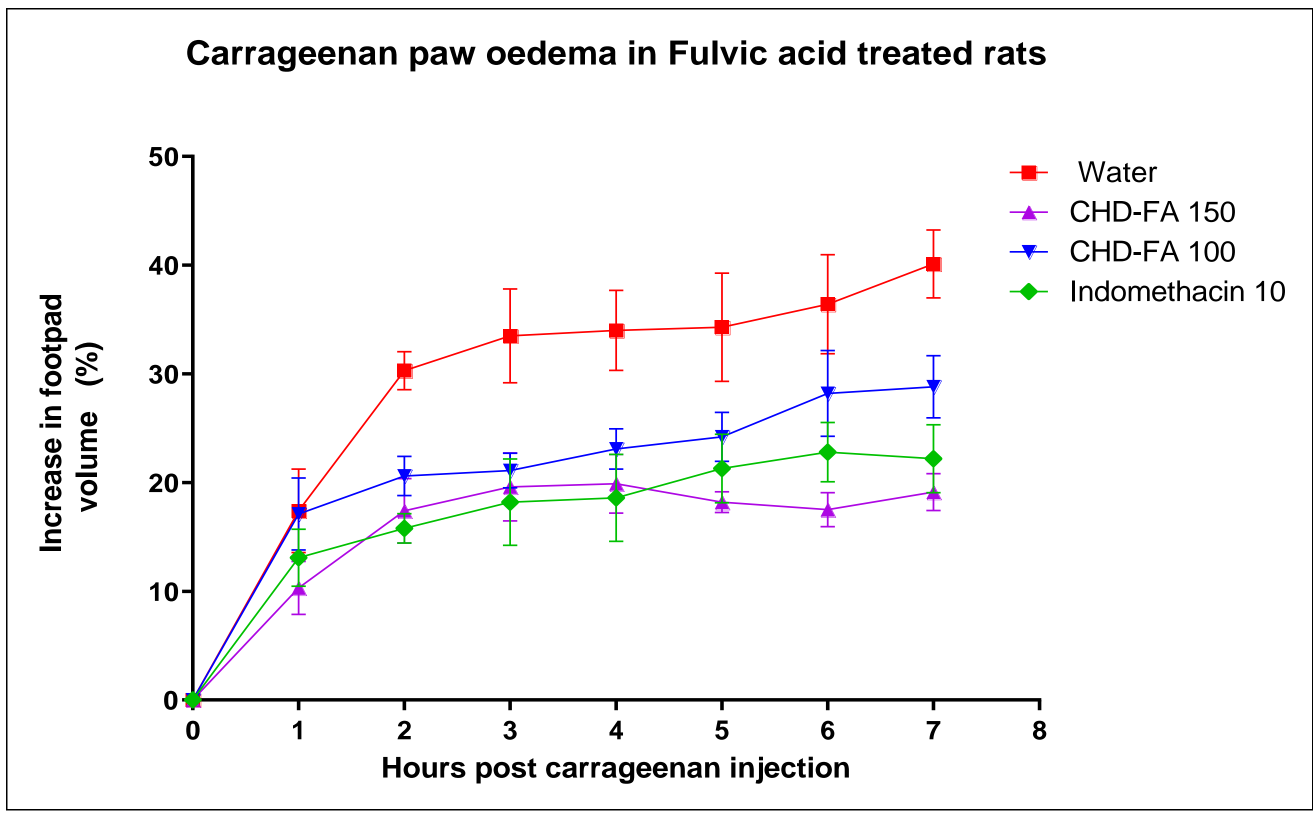

Figure 3.1: The effects of oral administration of CHD-FA (at 100 and 153 mg/kg bw/day) and indomethacin (10 mg/kg bw/day) on a carrageenan induced acute inflammation in rats. Error bars represent SEM and n = 10

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Oral administration of CHD-FA at both dosages, as well as the known anti-inflammatory drug, indomethacin, that was used as the positive control, reduced the inflammation- related oedema ($p<0.01$) between 2 and 7 hours after carrageenan injection compared to the negative control group, that were administered water treatment, and where a increase in paw volume was shown. The higher dose of 153 mg/kg CHD-FA treatment groups appeared to reduce the oedema the most although there was no significant difference when compared to the well documented NSAID, indomethacin at a dose of 10 mg/kg.

3.2 Antioxidant activity of CHD-FA

3.2.1 CHD-FA effect on neutrophil respiratory burst by chemiluminescence assay

The standard method of isolating neutrophils using Histopaque-1077 density media and centrifugation was a quick and effective method for this procedure with approximately 15 x 10^6 neutrophils retrieved per 10 mL whole blood within an hour and a half of drawing the blood and these neutrophils showed limited activation as measured by unchallenged background chemiluminescence.

Exposure to CHD-FA, at both concentrations tested, decreased the lucigenin enhanced chemiluminescence response of PMA stimulated neutrophils to about a fifth of the stimulated control group. The results obtained with 100 µg/mL CHD-FA is shown in Figure 3.2 below. No significant neutrophil chemiluminescence was seen in response of CHD-FA only exposure, indicating that CHD-FA did not act as an activator of the neutrophil oxidative burst.

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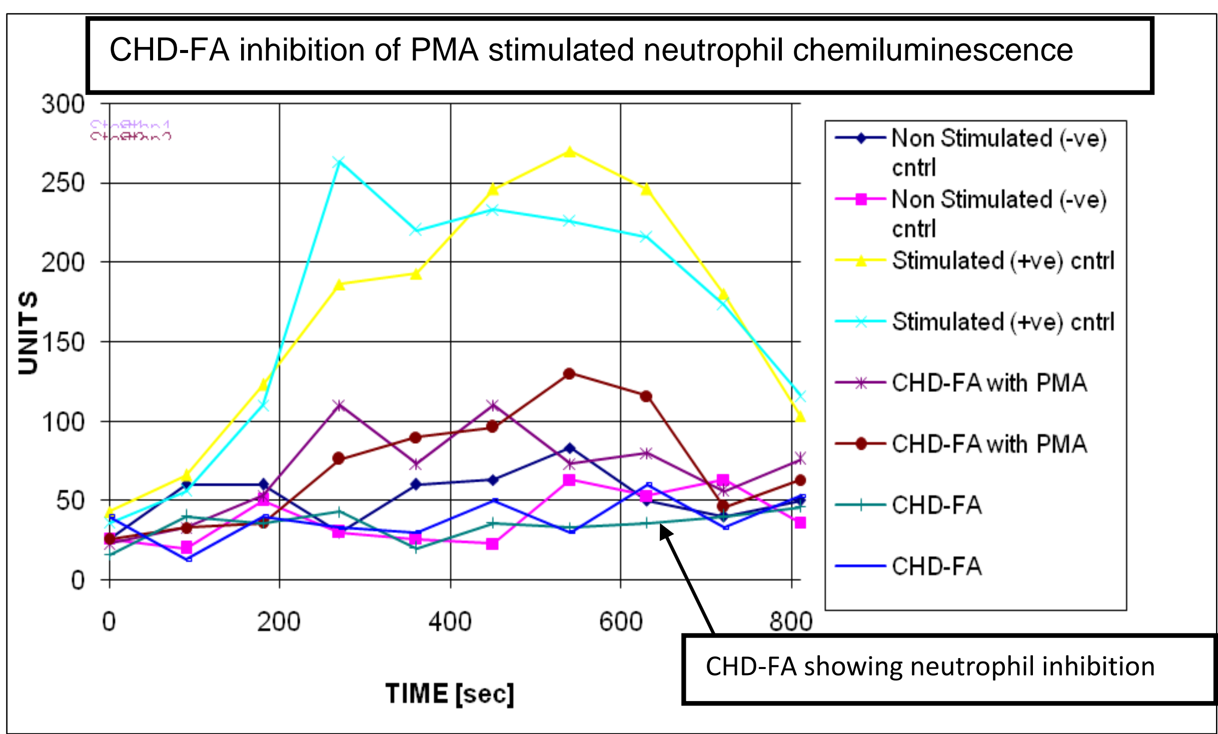

Figure 3.2: Effect of CHD-FA on neutrophil oxidative burst chemiluminescence under different treatments. The non-stimulated neutrophils are negative controls, while the PMA stimulated group represent the positive control showing maximal chemiluminescence. The CHD-FA only treatment shows that the CHD-FA alone does not stimulate the oxidative burst and the CHD-FA + PMA demonstrates an inhibitory effect of the CHD-FA on the PMA stimulation of the neutrophils.

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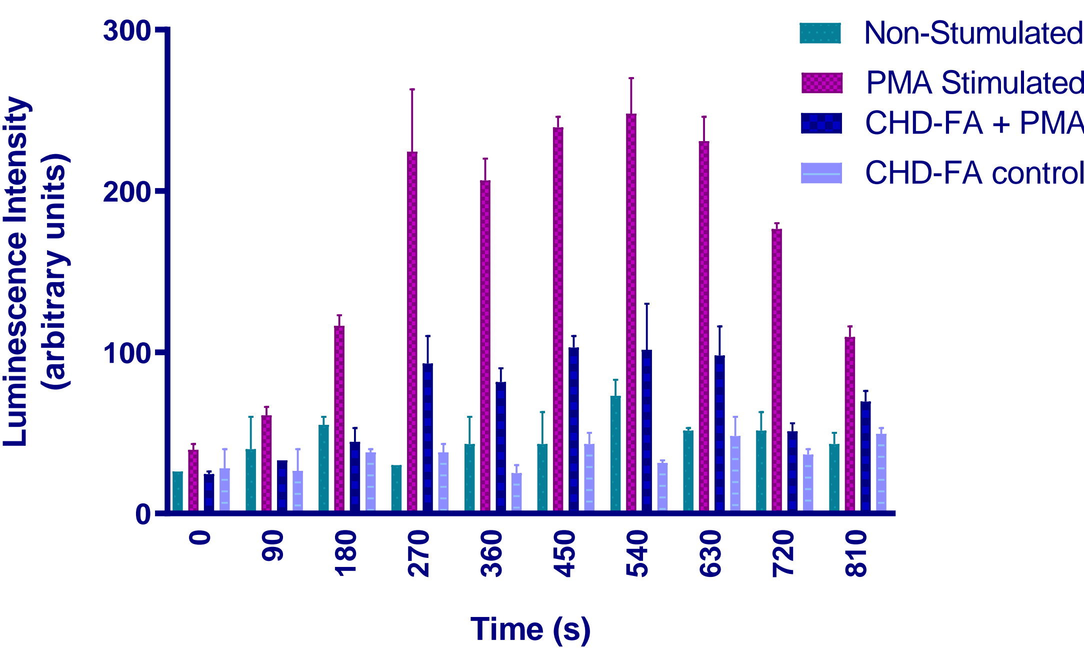

Figure 3.3: Kinetics of the chemiluminescence of neutrophils exposed to 100 µg/mL CHD-FA under different stimulation conditions. The non-stimulated neutrophils are the negative controls, while the PMA stimulated group represent the positive control showing maximal chemiluminescence. The CHD-FA only treatment shows that the CHD-FA alone does not stimulate the oxidative burst while the CHD-FA + PMA demonstrates an inhibitory effect of the CHD-FA on the PMA stimulation of the neutrophils. Mean ± SEM n = 3, replicates

3.2.2 Measurement of the Trolox equivalent antioxidant capacity (TEAC)

ABTS$^{\bullet+}$ radical scavenging was calibrated against the water soluble α-tocopherol analogue, Trolox at different concentrations. CHD-FA displayed antioxidant activity with ABTS$^{\bullet+}$ radical scavenging following a dose dependant scavenging effect. A larger decrease in absorbance at 734 nm would mean greater antioxidant activity [46].

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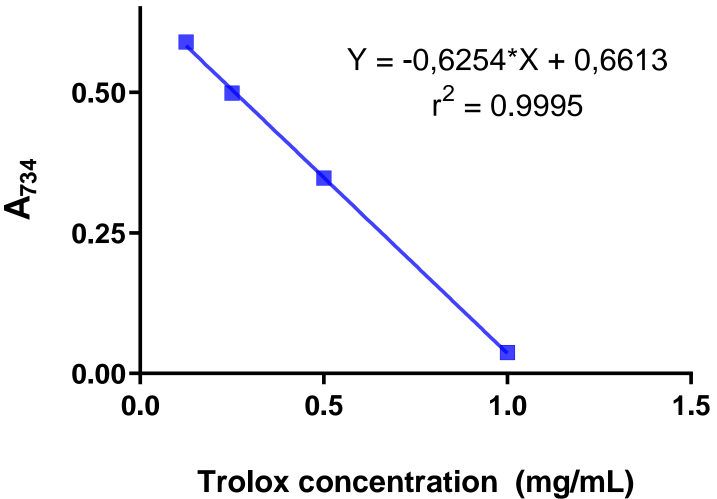

Figure 3.4: ABTS$^{\bullet+}$ radical scavenging of Trolox comparing Trolox concentration to the decrease in absorbance of the highly coloured ABTS$^{\cdot+}$ radical. Note that this is a quenching reaction of the colour produced by the ABTS$^{\bullet+}$ radical.

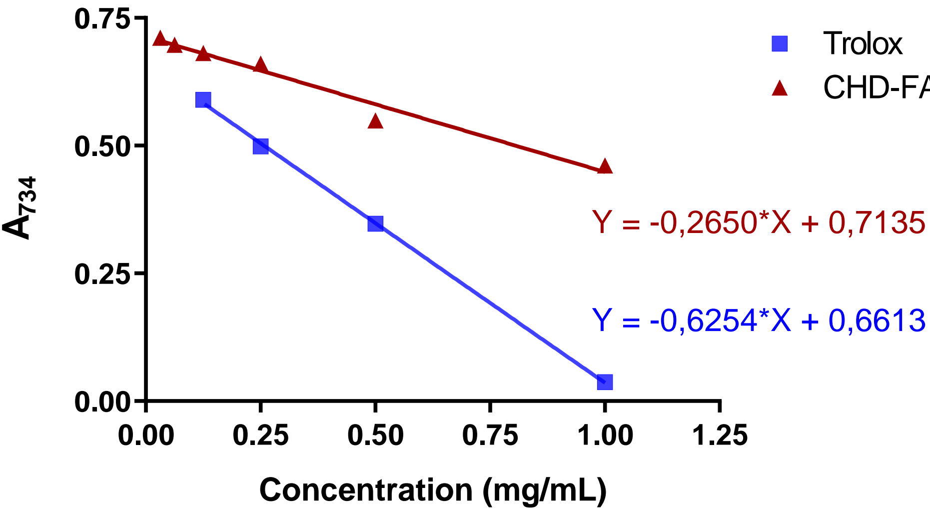

Figure 3.5: ABTS$^{\bullet+}$ radical scavenging by both Trolox and CHD-FA comparing concentrations to the decrease in absorbance of the highly coloured ABTS$^{\cdot+}$ radical. n = 2

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The results from this experiment indicates that CHD-FA has $ABTS^{\bullet+}$ radical scavenging ability at just below half that of Trolox at equivalent concentrations, which would imply significant anti-oxidant activity for the CHD-FA. This antioxidant activity may contribute to the positive effects observed for the reduction in the respiratory burst of the neutrophils as seen when pre-treating neutrophils prior to stimulating with PMA.

3.2.3 CR3 Expression

The use of a mixed leukocyte population for measuring CR3 expression by flow cytometry testing was acceptable because the flow cytometer signal can be gated to specifically monitor the neutrophil population which was then further analysed for fluorescence intensity as an indication of the relative abundance of CR3 on the neutrophil surface in response to the various treatments following activation with PMA(47)

The relative CR3 expression by human neutrophils can be monitored when using the flow cytometry method and fluorescently labelled antibodies raised against the $CD11_{\beta}$ region of this receptor. The neutrophil population is identified and gated in the scattergram of the entire cell suspension and this region then used to assess the fluorescent intensity of the neutrophils due to bound antibody,(47) A fluorescent PE-labelled anti-mouse antibody was used as an isotypic background control.

Figure 3.6 below shows a typical scattergram of a cell suspension of isolated neutrophils with a small population of monocytes visible on the lower left. The left image shows untreated and unstimulated neutrophils that were used to set the gating for comparison to the stimulated neutrophils while the right-hand image shows a histogram indicating the fluorescent intensity of the CR3-bound antibody within the gated area marked as A in the scattergram.

A shift in the position of the histogram to the right indicated increased CR3 abundance due to higher tagged antibody binding. Due to the fact that, different

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donors were used and a variation in the degree of neutrophil stimulation during the isolation procedures, the position of the fluorescence histogram varies on the x-axis to some extent for resting neutrophils.

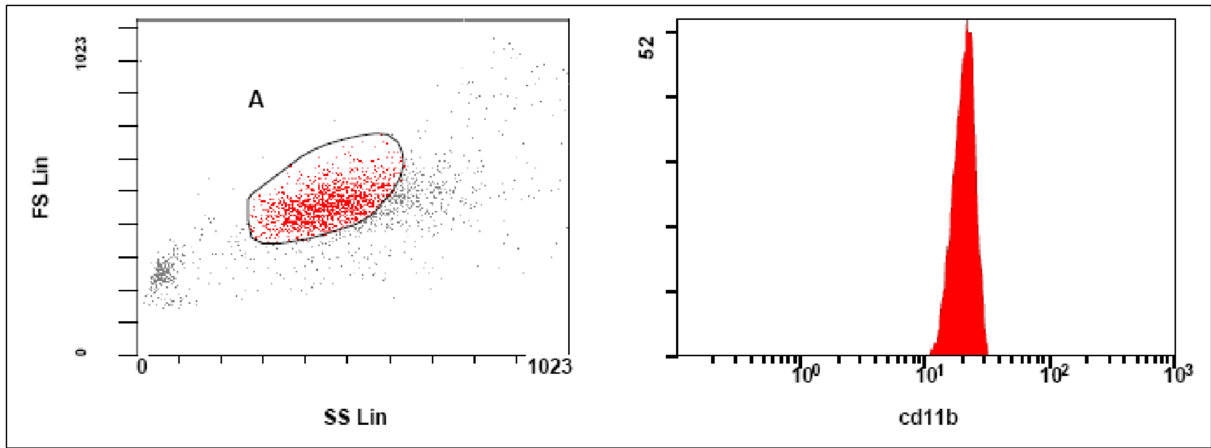

Figure 3.6: A scattergram and histogram from a controlled sample of isolated neutrophils. The histogram on the right indicates the fluorescence intensity distribution of the cells within the gated area on the left (scattergram)

The effects that different concentrations of CHD-FA had on CR3 expression by both non-stimulated and PMA stimulated neutrophils after a 20 min pre-incubated with various concentrations of CHD-FA is shown on Figure 3.7 below. The figure clearly indicates a significant increase in CR3 abundance on the cell membrane of PMA stimulated neutrophils and a significant dose dependant inhibition of CR3 expression by PMA stimulated cells after exposure to various concentrations of CHD-FA.

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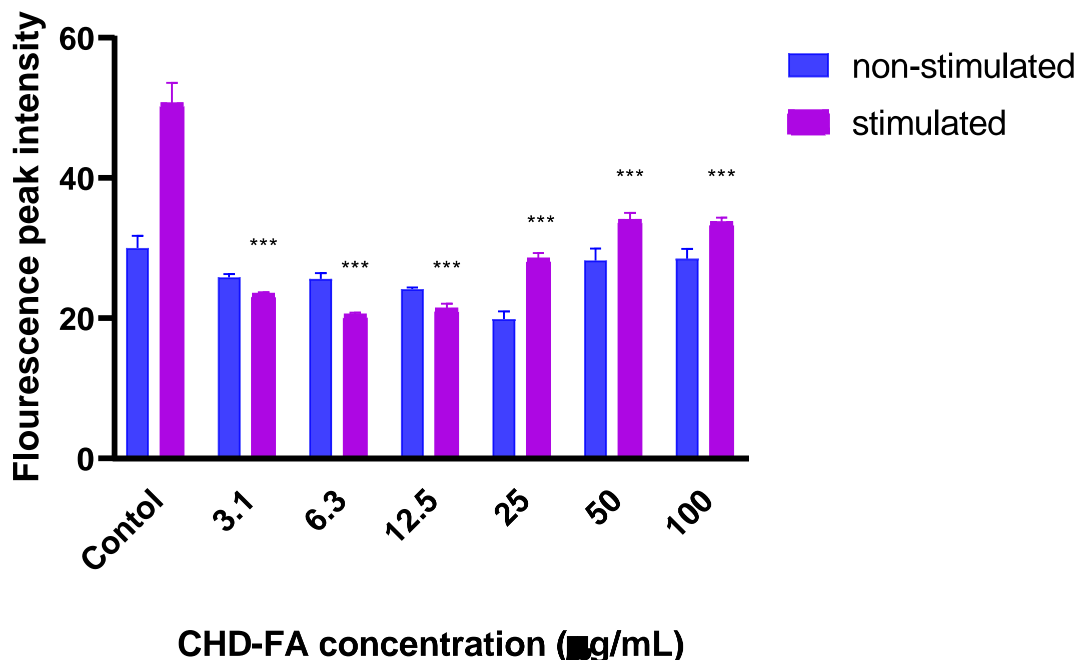

Figure 3.7: Effect of the different CHD-FA concentrations on the cell surface expression of CR3 of neutrophils. Resting cells only contained the CHD-FA test compound whilst the PMA stimulation was initiated after the cells were incubated with CHD-FA at the concentrations indicated. Each bar is an average of 3 different experiments. *** indicates a $p < 0.001$ compared to the untreated stimulated control using the Kruskal Wallis test.

Cell surface CR3 expression following PMA stimulation was increased by almost 60% compared to the resting neutrophils. When the neutrophils had been treated with different concentrations of CHD-FA prior to stimulation with PMA, there was a significant decrease in the CR3 expression compared to the untreated but stimulated neutrophils. Similar effects were seen in studies done by Joone (48) and Cromarty (47) using a coal- derived humic acid product named oxihumate. Adhesion of neutrophils to vascular epithelium requires CR3 expression to enable diapedesis, implying that inhibition of CR3 expression can result in reduced extravascular neutrophils within the tissue that could reduce the acute inflammatory response.

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3.2.4 Characterization of major components in the CHD-FA

It is important to acquire knowledge of the basic structures of humic materials for a full understanding of the role and function of these constituents in any form of medication. However, because of the multiple component supramolecular structure and the possibility of numerous types of linkages that may bind them together, accurate structural formulas have not yet been achieved.(49)

Various techniques have been used to investigate the compositions and structural characteristics, including nuclear magnetic resonance spectrometry, size exclusion chromatography, gas chromatography–mass spectrometry, liquid chromatography–mass spectrometry, UV/visible and infrared spectroscopic analysis, and others (elemental analysis, metal complexation, etc.). According to Frimmel (50)the application of these powerful techniques, alone and in combination have not been successful in identification of humic substances at molecular level.

In this study thin layer chromatography (TLC) was used to separate the components in CHD-FA. All forms of chromatography work on the same principle. They all have a stationary phase (a solid, or a liquid supported on a solid) and a mobile phase (a liquid or a gas) that carries the analyte through the stationary phase.

The solubility of different compounds in the eluting solvent plays an important role in how fast they move up the plate. However, a more important property of the solvent is its ability to itself be adsorbed on the adsorbent. If the solvent is too strongly adsorbed, all the compounds can be displaced causing them to move up the plate together near the solvent front with no separation. And if the solvent is too weakly adsorbed, its solvating power alone may be insufficient to move any compounds fast enough to effect separation.

In order to identify the different components of CHD-FA, this study evaluated three different mobile phases to compare basic (expected high ionisation but increased solubility) verses acidic (suppressing compound ionisation) phases and within the acidic environment two different solvent combinations were compared.

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Low polarity compounds should have higher Rf values than more polar compounds on pure unbonded silica stationary phases. Figures 3.8 to 3.10 below show the different separations achieved by compounds in the different batches of the CHD-FA samples tested showing the many different Rf values that indicates that the samples are complex mixtures. The apparent number of compounds present in the samples differed when comparing the different mobile phases and hints at co-elution of compounds and potential solubility limitations during separation.

3.2.4.1 Measuring the retention factor (Rf) values of the separated spots

In order to identify the components present in the different batches of CHD-FA, the Rf values of each separated spot was calculated. These Rf values are the ratio of the distance travelled from the origin for each spot divided by the distance travelled mobile phase.

The six different batches of CHD-FA were compared and the Rf values of each of the spots derived from the separated compounds calculated for each TLC plate run under the different mobile phase conditions.

For the purpose of calculating the Rf value, the centre of the symmetrical spots with the highest intensity was identified. For spots that were obviously streaking the Rf was calculated as the average Rf of the leading edge and the trailing edge of the streak. Where spots curved the Rf was calculated for the centre of the crescent. All three of the mobile phases appeared to have pseudo-fronts, in that the mobile phase also appeared to be separating on the TLC plate during development and was clearest for the ammonium hydroxide containing mobile phase. It was evident that many of the spots did not represent single well separated compounds but were groups of coeluting compounds, and this could be seen from the UV absorption and the fluorescent colours seen in a large number of the spots as well as the dark brown to black colour appearing near the origin in most batches. Batch 1938 showed a very distinct black residue that remained on the origin for all the mobile phases used.

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Figure 3.8: Silica G60 TLC plate developed with Acetonitrile:Water:Ammonium Hydroxide (6:3:1) as the mobile phase. UV absorbing and fluorescing compounds have been marked. Note the oval slots cut into each spot where the extraction was performed by the Camag TLC MS interface system.

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Figure 3.9: Silica G60 TLC plate developed with Methanol:Chloroform:Butanol:water:Acetic Acid (10:6:3:3:0.4) as the mobile phase. UV absorbing and fluorescing compounds have been marked. Note the oval slots cut into each spot where the extraction was performed by the Camag TLC MS interface system.

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Figure 3.10: Silica G60 TLC plate developed with Acetone:Butanol:Water:Acetic Acid (13:4:2:1) as the mobile phase. UV absorbing and fluorescing compounds have been marked. Note the oval slots cut into each spot where the extraction was performed by the Camag TLC MS interface system.

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Other visualising agents, like phosphomolybdic acid for antioxidant activity (separate TLC plates - data not shown) also showed differing colours indicating interference with the visualising agent reaction.

Batch 390 lacked the spots at the lowest Rf values on all three TLC plates or a large residue on the origin and was determined to have been filtered through a 4000 Da nominal cut-off ultrafiltration membrane. A small low intensity fluorescent spot was left on the origin.

Table 3.1: Rf values of major compounds from different batches of CHD-FA separated by TLC under three different conditions. The colours in the columns matching the batch numbers show the presence of compounds in that batch separating to the Rf value to its left.

Batches tested	Acetonitrile:Water: Ammonium Hydroxide							Methanol: Chloroform: Butanol: water: Acetic Acid							Acetone:Butanol:Water: Acetic Acid						
1395	0.00	X	X	X	X	X	X	0,02	X	X	X	X	X	X	0,0-0.17	X	X	X		X	X
1398	0.19	X	X		X	X	X	0,58	X	X		X	X	X	0,54				X	X	X
390	0.23	X	X		X	X	X	0,75	X	X	X	X	X	X	0,63	X	X	X	X	X	X
391/11	0.27	X	X		X	X	X	0,80	X	X	X	X	X	X	0,81	X	X	X	X	X	X
1226	0,30	X	X	X	X	X	X	0,86	X	X	X	X	X	X	0,90	X	X	X	X	X	X
R72/12	0,43	X	X	X	X	X	X	0,90	X	X	X	X	X	X							
	0.49	X	X	X	X	X	X														
	0.53	X	X	X	X	X	X														
	0.56	X	X	X	X	X	X														
	0.80	X	X	X	X	X	X														
	0.87	X	X	X	X	X	X														

The white blocks indicate the absence of a spot at that Rf value. Compounds with high Rf values (closest to 1) are the least polar.

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3.2.5 Determination the Molecular Mass of the compounds separated by TLC

The compounds separated using the three different mobile phases appeared as spots on the TLC plates when visualised under the UV light at different wavelengths and the fluorescence visible under the 360 nm wavelength also noted. These spots were further individually analysed by mass spectrometry to try identify what the mass of these compounds were. Mass analysis was performed in both the positive and negative mode, using separate TLC plates for each polarity mode and analysed as separate runs.

A graphical representation of many unique masses present in each separated spot collected directly off the silica TLC plates is shown below in Figures 3.11a through to Figure 3.16. These results indicate that each of the separated spots appear to still be complex mixtures of compounds. Each figure showing mass information for individual spots is split into a “gel” view and a spectral view (at the bottom of the figure), where the top “gel” view portion of the figure represents the masses of all the different compounds within the individual spots eluted directly into the mass spectrometer source with no further separation.

The X-axis shows, for all the mass spectra collected, from left to right, the mass range from 100 Da to 1200 Da or part thereof as indicated. Each of the spots were eluted for approximately 1.2 min during which time the spectral intensity dropped due to complete elution of the compounds from the collection zone on the TLC plate. Representative spectra for each spot were extracted from the region of highest spectral intensity and are shown as the spectrum number in brackets of each spot label. The intensity of the different bars in the “gel” view indicates the relative normalised intensity of the individual ions derived from the different compounds in each spot. As the image becomes cluttered when there are many spots within the “gel” view, the image was created for a maximum of only 24 spots per image, therefore the TLC plates developed using alkaline conditions with Acetonitrile:Water:Ammonium Hydroxide were split into two images designated as Figure 3.11a and Figure 3.11b for the positive mode spectra and Figure 3.12a and Figure 3.12b for the negative mode spectra.

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The spectral view at the bottom of each figure is the cumulative overlaid spectra of all the spots shown in the “gel” view above it and this histogram-type layout shows the different masses detected with their intensities.

What is clear from the mass distribution from each spot is that all the spots analysed in positive ionisation mode have at least 20 different masses, some of which are common to all spots, but the ion masses do vary according to the spot Rf value. The majority of compounds show masses of less than 340 Da, implying that these compounds are hydrolysed disaccharides.

A total of 48 spots were identified on the Acetonitrile: Water: Ammonium Hydroxide developed TLC plate with all batches showing compounds with the same Rf values except for Batch 390 that did not have compounds eluting at Rf 0.19 and Rf 0.22.

It should be noted that the apparent intensity is normalised to the most intense ion of all the ions shown within the “gel” view giving a biased impression of ion intensities between figures.

A TLC plate developed using the exact same mobile phase as used for the previous two figures (Acetonitrile: Water: Ammonium Hydroxide - 6:3:1) was analysed in the same way except that the mass spectrometer was operated in a negative mode. Interesting that there were only 42 discernible spots on this TLC plate, with the spots at Rf 0.26 merging with the spots at Rf 0.30. In Figure 3.11c below, the first 21 spots identified are shown. Note that the mass range has been limited to 100 to 900 Da as there are no compounds seen between 900 and 1200 Da. It can be seen that the number of negatively charged compounds per spot is less (about 15) than for the positively charged compounds for the same spot and that all the masses are generally lower than 340 Da similar to the assays for the positive ions. In addition, there is no mass that is common to all the spots, but the spots from the different CHD-FA batches with the same Rf do have the same mass distribution of ions, similar to what was seen for the positively charged compounds.

The last 21 spots from the plate are shown below in Figure 3.11d. Many common masses below 340 Da can be observed across the plate.

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FULVIC ACID-1-POSITIVE [115]
FULVIC ACID-1-POSITIVE [162]
FULVIC ACID-1-POSITIVE [255]
FULVIC ACID-1-POSITIVE [331]
FULVIC ACID-1-POSITIVE [538]
FULVIC ACID-1-POSITIVE [630]
FULVIC ACID-1-POSITIVE [691]
FULVIC ACID-1-POSITIVE [752]
FULVIC ACID-1-POSITIVE [818]
FULVIC ACID-1-POSITIVE [894]
FULVIC ACID-1-POSITIVE [968]
FULVIC ACID-1-POSITIVE [1036]
FULVIC ACID-1-POSITIVE [1103]
FULVIC ACID-1-POSITIVE [1167]
FULVIC ACID-1-POSITIVE [1230]
FULVIC ACID-1-POSITIVE [1306]
FULVIC ACID-1-POSITIVE [1370]
FULVIC ACID-1-POSITIVE [1431]
FULVIC ACID-1-POSITIVE [1510]
FULVIC ACID-1-POSITIVE [1580]
FULVIC ACID-1-POSITIVE [1665]
FULVIC ACID-1-POSITIVE [1731]
FULVIC ACID-1-POSITIVE [1797]
FULVIC ACID-1-POSITIVE [1862]

r. int. (%)
m/z

Figure 3.11a: A graphical representation of the mass spectrometry results for spot numbers 1 to 24 eluted from the Acetonitrile:Water:Ammonium Hydroxide developed TLC plate directly into the mass spectrometer source and analysed in positive mode. The upper part of the image shows the relative intensity, indicated by the intensity of the bars, for all masses detected per spot. The X-axis shows the mass range. The lower histogram shows a mass labelled cumulative overlay of all the masses and the intensities of the spots.

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Figure 3.11b: A graphical representation of the mass spectrometry results for spot numbers 25 to 48 eluted from the Acetonitrile:Water:Ammonium Hydroxide developed TLC plate directly into the mass spectrometer source and analysed in positive mode. The upper part of the image shows the relative intensity, indicated by the intensity of the bars, for all masses detected per spot. The intensity appears higher than in the previous figure due to the normalisation done by the software. The X-axis shows the mass range. The lower histogram shows a mass labelled cumulative overlay of all the masses and the intensities of the spots.

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Figure 3.12a: A graphical representation of the mass spectrometry results for spot numbers 1 to 22 eluted from the Acetonitrile:Water:Ammonium Hydroxide developed TLC plate directly into the mass spectrometer source and analysed in negative mode. The upper part of the image shows the relative intensity, indicated by the intensity of the bars, for all masses detected per spot. The X-axis shows the mass range that is from 100 to 900 Da. The lower histogram shows a mass labelled cumulative overlay of all the masses and the intensities of the spots.

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Figure 3.12b: A graphical representation of the mass spectrometry results for spot numbers 23 to 44 eluted from the Acetonitrile:Water:Ammonium Hydroxide developed TLC plate directly into the mass spectrometer source and analysed in negative mode. The upper part of the image shows the relative intensity, indicated by the intensity of the bars, for all masses detected per spot. The X-axis shows the mass range that is from 100 to 900 Da. The lower histogram shows a mass labelled cumulative overlay of all the masses and the intensities of the spots.

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The TLC plates run under acidic conditions showed fewer separated spots and again showed the same general feature of positive ion masses below 340 Da although the spots from Rf 0.80 and being less polar did show higher masses with some low intensity ions being seen at 911.78 and 918.51 Da. The most intense ions were at 122.10, 357.15 and 413.27 Da that would represent the most likely formulas of $C_4H_{10}O_4$, $C_{17}H_{24}O_8$ and $C_{18}H_{36}O_{10}$ respectively taking the synthetic process and starting material into account. These formulas cannot be confirmed as there are more than 25 possible formulas for the 413.27 Da ion using only the CHNO elements and a 200 ppm mass error. As this compound is only seen in the positive ionisation mode and no equivalent mass seen in the negative ionisation it can be concluded that the compound is not an organic acid.

From mass 463 through to 845 Da is a mass series with increasing mass of 44 Da that is typically a C_2H_4O- or a CO_2- addition series, of which the later would be the most likely due to the oxidation process used in the synthesis of the CHD-FA.

The ions from the negative ionisation with mass greater than 270 Da appear to have a series with increasing mass of 14 Da equivalent to a CH_2- chain length elongation. This same mass difference is not seen in the positively charged ions.

The mass distribution of compounds in spots that separated to specific Rf values from the different CHD-FA batches are very similar, with Batches 1395 and 1398 showing some subtle mass differences relative to the other batches and the absence of the more polar spots from Batch 390 as illustrated in Figures 3.17 and 3.18.

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Figure 3.13: A graphical representation of the mass spectrometry results for all the spots eluted from the Methanol: Chloroform:Butanol:water:Acetic Acid (10:6:3:3:0.4) developed TLC plate directly into the mass spectrometer source and analysed in positive mode. The upper part of the image shows the relative intensity, indicated by the intensity of the bars, for all masses detected per spot. The X-axis shows the mass range that is from 100 to 1200 Da. The lower histogram shows a mass labelled cumulative overlay of all the masses and the intensities of the spots.

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Figure 3.14: A graphical representation of the mass spectrometry results for all the spots eluted from the Methanol: Chloroform:Butanol:water:Acetic Acid (10:6:3:3:0.4) developed TLC plate directly into the mass spectrometer source and analysed in negative mode. The upper part of the image shows the relative intensity, indicated by the intensity of the bars, for all masses detected per spot. The X-axis shows the mass range that is from 100 to 900 Da. The lower histogram shows a mass labelled cumulative overlay of all the masses and the intensities of the spots.

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FULVIC ACID-3-POSITIVE [144]
FULVIC ACID-3-POSITIVE [243]
FULVIC ACID-3-POSITIVE [304]
FULVIC ACID-3-POSITIVE [382]
FULVIC ACID-3-POSITIVE [506]
FULVIC ACID-3-POSITIVE [562]
FULVIC ACID-3-POSITIVE [627]
FULVIC ACID-3-POSITIVE [688]
FULVIC ACID-3-POSITIVE [805]
FULVIC ACID-3-POSITIVE [925]
FULVIC ACID-3-POSITIVE [965]
FULVIC ACID-3-POSITIVE [994]
FULVIC ACID-3-POSITIVE [1078]
FULVIC ACID-3-POSITIVE [1134]
FULVIC ACID-3-POSITIVE [1198]
FULVIC ACID-3-POSITIVE [1274]
FULVIC ACID-3-POSITIVE [1408]
FULVIC ACID-3-POSITIVE [1493]
FULVIC ACID-3-POSITIVE [1585]
FULVIC ACID-3-POSITIVE [1654]
FULVIC ACID-3-POSITIVE [1747]

r. int. (%)
120
90
60
30
0
161.02
138.97
182.96
206.19
299.04
320.99
342.94
387.26
413.26
507.32
552.35-551.35
595.38
596.38
639.40
640.41
683.43
300
600
900
1200
m/z

Figure 3.15: A graphical representation of the mass spectrometry results for all the spots, eluted from the Acetone: Butanol:Water:Acetic Acid developed TLC plate directly into the mass spectrometer source and analysed in positive mode. The upper part of the image shows the relative intensity, indicated by the intensity of the bars, for all masses detected per spot. The X-axis shows the mass range that is from 100 to 1200 Da. The lower histogram shows a cumulative overlay of all the masses and the intensities of the spots. Mass Spectrometry results for the run on a positive mode

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Figure 3.16: A graphical representation of the mass spectrometry results for all the spots eluted from the Acetone: Butanol:Water:Acetic Acid developed TLC plate directly into the mass spectrometer source and analysed in negative mode. The upper part of the image shows the relative intensity, indicated by the intensity of the bars, for all masses detected per spot. The X-axis shows the mass range that is from 100 to 900 Da. The lower histogram shows a mass labelled cumulative overlay of all the masses and the intensities of the spots.

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Figure 3.17: A graphical representation of the mass spectrometry results for the spots with Rf 0.82 from the 6 different CHD-FA batches tested and eluted from the Acetone:Butanol:Water: Acetic Acid developed TLC plate directly into the mass spectrometer source and analysed in negative mode. The upper part of the image shows the relative intensity, indicated by the intensity of the bars, for all masses detected per spot. The X-axis shows the mass range that is from 100 to 600 Da. The lower histogram shows a mass labelled cumulative overlay of all the masses and the intensities of the spots.

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Figure 3.18: A graphical representation of the mass spectrometry results for the least polar spots with Rf 0.88 from the six different CHD-FA batches tested and eluted from the Acetone: Butanol:Water:Acetic Acid developed TLC plate directly into the mass spectrometer source and analysed in negative mode. The upper part of the image shows the relative intensity, indicated by the intensity of the bars, for all masses detected per spot. The X-axis shows the mass range that is from 100 to 600 Da. The lower histogram shows a mass labelled cumulative overlay of all the masses and the intensities of the spots.

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The following eight figures illustrate the similarities and differences in the negative ions resulting from the compounds eluted into the mass spectrometer directly from the TLC plate developed using Acetonitrile: Water:Ammonium Hydroxide. Each figure is a comparison of the spots moving to the same Rf value in the order from top to bottom of the “gel” view: 1395; 1398; 390; 391/11; 1226; and 072/12 at the bottom. Batch 390 was an ultrafiltered product passed through a 4000 Da cut-off membrane that resulted in some of the more polar and potentially polymerised compounds to be removed from the product, which resulted in two spots not appearing on the TLC plate analysed.

In general the spots from the different batches showed the same mass distribution for ions from spots of the same Rf value although there were some intensity differences in these ions. What was noticeable was that there were some ions present in almost all the spots with the exception of the spots at the lowest Rf values of 0.19 where the masses of 297.15; 311.16; 325.18; 339.20 were not present, yet were present in all other spots at all the Rf values from 0.23 to 0.86. Another common mass was 146.96 Da although its intensity was generally lower.

All these masses are less than that of the sucrose used as the starting material and would imply an oxidative hydrolysis takes place during the synthesis of the fulvic acid under the conditions used. The intensities of several of the ions detected in the spots were relatively more intense but this could be due to the actual spectra that were selected from a vast number of spectra collected per spot. Another observation was that the spots that were close to each other appeared to give different masses probably due to the extent of the co-elution of the compounds in two adjacent closely eluting spots. This was especially visible for the spots at Rf 0.23 and Rf 0.56

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Figure 3.19: A graphical representation of the mass spectrometry results for the spots with Rf 0.19 from the different CHD-FA batches tested and eluted from the Acetonitrile: Water: Ammonium Hydroxide developed TLC plate directly into the mass spectrometer source and analysed in negative mode. The upper part of the image shows the relative intensity, indicated by the intensity of the bars, for all masses detected per spot. Note the similarity in the ion masses in the spots from the different batches and that Batch 390 did not have a spot at 0.19 therefore there are only 5 rows in the “gel” view. The X-axis shows the mass range that is from 100 to 600 Da. Note The lower histogram shows a mass labelled cumulative overlay of all the masses and the intensities of the spots.

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Figure 3.20: A graphical representation of the mass spectrometry results for the spots with Rf 0.23 from the six different CHD-FA batches tested and eluted from the Acetonitrile: Water: Ammonium Hydroxide developed TLC plate directly into the mass spectrometer source and analysed in negative mode. The upper part of the image shows the relative intensity, indicated by the intensity of the bars, for all masses detected per spot. Note the similarity in the ion masses in the spots from the different batches. The X-axis shows the mass range that is from 100 to 600 Da. Note that Batch 390 did not have a spot at 0.23 therefore there are only 5 rows in the "gel" view. The lower histogram shows a mass labelled cumulative overlay of all the masses and the intensities of the spots.

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Figure 3.21: A graphical representation of the mass spectrometry results for the spots with Rf 0.3 from the six different CHD-FA batches tested and eluted from the Acetonitrile: Water: Ammonium Hydroxide developed TLC plate directly into the mass spectrometer source and analysed in negative mode. The upper part of the image shows the relative intensity, indicated by the intensity of the bars, for all masses detected per spot. Note the differences in the ion masses in the spots from the different batches due to very closely eluting spots that were eluted into the mass spectrometer source. The X-axis shows the mass range that is from 100 to 600 Da. Note that Batch 390 did not have a spot at Rf 0.3 therefore there are only 5 rows in the "gel" view. The lower histogram shows a mass labelled cumulative overlay of all the masses and the intensities of the spots.

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Figure 3.22: A graphical representation of the mass spectrometry results for the spots with Rf 0.43 from the six different CHD-FA batches tested and eluted from the Acetonitrile: Water: Ammonium Hydroxide developed TLC plate directly into the mass spectrometer source and analysed in negative mode. The upper part of the image shows the relative intensity, indicated by the intensity of the bars, for all masses detected per spot. Note the similarity in the ion masses despite intensity differences in the spots from the different batches. The X-axis shows the mass range that is from 100 to 600 Da. The lower histogram shows a mass labelled cumulative overlay of all the masses and the intensities of the spots.

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Figure 3.23: A graphical representation of the mass spectrometry results for the spots with Rf 0.49 from the six different CHD-FA batches tested and eluted from the Acetonitrile: Water: Ammonium Hydroxide developed TLC plate directly into the mass spectrometer source and analysed in negative mode. The upper part of the image shows the relative intensity, indicated by the intensity of the bars, for all masses detected per spot. Note the similarity in the ion masses despite intensity differences in the spots from the different batches. The spot from Batch 390 appears slightly different due to co-eluting spot at Rf 0.49. The X-axis shows the mass range that is from 100 to 600 Da. The lower histogram shows a mass labelled cumulative overlay of all the masses and the intensities of the spots.

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Figure 3.24: A graphical representation of the mass spectrometry results for the spots with Rf 0.56 from the six different CHD-FA batches tested and eluted from the Acetonitrile: Water: Ammonium Hydroxide developed TLC plate directly into the mass spectrometer source and analysed in negative mode. The upper part of the image shows the relative intensity, indicated by the intensity of the bars, for all masses detected per spot. Although the ion masses are similar there are significant intensity differences in specific masses from the spots from the different batches. The X-axis shows the mass range that is from 100 to 600 Da. The lower histogram shows a mass labelled cumulative overlay of all the masses and the intensities of the spots.

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Figure 3.25: A graphical representation of the mass spectrometry results for the spots with Rf 0.80 from the six different CHD-FA batches tested and eluted from the Acetonitrile:Water:Ammonium Hydroxide developed TLC plate directly into the mass spectrometer source and analysed in negative mode. The upper part of the image shows the relative intensity, indicated by the intensity of the bars, for all masses detected per spot. Although the ion masses are similar there are significant intensity differences in specific masses from the spots from the different batches. The X-axis shows the mass range that is from 100 to 600 Da. The lower histogram shows a mass labelled cumulative overlay of all the masses and the intensities of the spots.

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Figure 3.26: A graphical representation of the mass spectrometry results for the spots with Rf 0.86 from the six different CHD-FA batches tested and eluted from the Acetonitrile:Water:Ammonium Hydroxide developed TLC plate directly into the mass spectrometer source and analysed in negative mode. The upper part of the image shows the relative intensity, indicated by the intensity of the bars, for all masses detected per spot. The ion masses and intensities are very similar with a few specific exceptions for masses from the spots from the different batches. The X-axis shows the mass range that is from 100 to 600 Da. The lower histogram shows a mass labelled cumulative overlay of all the masses and the intensities of the spots.

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The results from this study indicates that although the different CHD-FA products were completely soluble and run under different mobile phase conditions that should have allowed complete separation of compounds, the spots on the developed TLC plates were still complex mixtures of ionisable individual compounds. This finding would support the concept that the fulvic acids are in fact supramolecular structures that stay together even under conditions where they can separate. This supports the hypothesis of Piccolo that fulvic acid is a self-assembled superstructure of relatively small heterogeneous molecules held together primarily by hydrophobic dispersive forces and hydrogen bonds.(51)The fact that under alkaline conditions where fulvic acids are more soluble the separation results in more spots being separated while under acidic conditions fewer spots that run to lower Rf values are seen but that all the spots are still complex mixtures of low mass chemical entities that carry single charges when ionised also points to the supramolecular structure. The fact that there are very few ions that could be paired to the same precursor molecule when comparing positive to negative ions also points to the concept that the ions are probably derived for different components within each of the spots. Another factor supporting the supramolecular structure is that ions of the same mass are derived from spots that have eluted to almost every Rf on the plate which is not logical if the different components of the mixture were not bound to each other in some way. It would be expected that there would be more ions in the negative mode mass spectra due to fulvic acid being high in carboxylic acid moieties which would naturally carry a negative charge, even without ionisation in the mass spectrometer source.

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4 Chapter 4

4.1 Discussions and conclusions

4.1.1 Discussion

Research on alternative medicinal products has increased in the past three decades with much attention given to herbal and traditional type medicines. Unconfirmed anecdotal evidence has suggested that extracts from peat, sapropel and shilajit that contain high concentrations of humic and fulvic acids may represent a source of novel compounds with medicinal properties.(13)

Fulvic acids are naturally occurring, complex, organic acids regarded by some researchers (51) as a supramolecular structure. Fulvic acids are a product of humification of plant material from the environment in which they are found and due to their solubility in water are responsible for the brown colourisation of surface waters. They have a high complexing ability that often results in mineral compounds, including heavy metals and other compounds located at the sites of synthesis, to be incorporated into the fulvic and humic acid products. This can lead to concentrations of toxic heavy metals that could be harmful to the consumer .These acids are reported from various parts of the world to have a number of medicinal properties including; anti-inflammatory, analgesic, antimicrobial, antiviral, allergy suppressing, anti-oxidant and anti-ulcerogenic(17, 19, 48) They are used in Ayurvedic medicine, used in many health spas both topically and as food supplements with many products available through pharmacies and online outlets. This study investigated a synthetic fulvic acid produced through a patented wet oxidation of a metal-free food source carbohydrate at high temperatures. Fulvimed (Pty) Ltd., a South African company, developed an effective large-scale synthetic process, which can reproducibly generate humic and fulvic acids economically in large quantities using a tracible GMP process to ensure pure and high quality fulvic acids suitable for human use. Analysis by various methods has shown more than 50 compounds to be present in this Carbohydrate-derived fulvic acid (CHD-FA), most of which are

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carboxylic acids and common physiological metabolites (acetic acid , pyruvic acid and oxyloacetic acid)with no reported evidence of toxicity.

Fulvic acid that is obtained through this synthetic process contains no toxic heavy metals, which was found to be of concern for many naturally sourced fulvic aids. After several studies were performed on this product a new more concentrated product that was suitable to use in a dry powder formulation was synthesised and this required confirming that the activity of the product matched that of the previously tested liquid product normally supplied as a 4% fulvic acid solution. The main aim of this study was to determine whether the new powdered formulation of this Carbohydrate-derived Fulvic Acid possesses anti-inflammatory properties similar to the previous unconcentrated liquid formulation using comparable techniques in terms of efficacy as used in the previous testing. In addition, it was hoped that specific active compounds could be identified in the fulvic acid product that could account for the medicinal properties observed. This was carried out using thin layer chromatography followed by direct elution from the plate into an accurate mass spectrometer.

The results from this study indicated that the new formulation of CHD-FA is effective in suppressing early inflammatory processes as demonstrated by the significant suppression of the acute carrageen-induced oedema in a challenged rat footpad. This finding supports the work done by Sabi (16) where it was shown that the liquid formulation of the CHD-FA is orally bio-available and showed systemic anti-inflammatory effects that were measured by inhibition of foot pad swelling after carrageenan challenge. Fulvic acid neutralized to pH 5.5 with potassium acetate, applied by oral gavage at concentrations of 100 or 153 mg/kg body weight both inhibited the inflammation to an extent similar to indomethacin that was used a positive anti-inflammatory control treatment. In this study 20 rats were dosed CHD-FA orally at the same concentrations used by Sabi (16) (100 and 153 mg/kg body

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weight) Indomentacin was not used in acute treatment and CHD-FA was given chronically, for prophylactic effect. This will not be acceptable for indomethacin. While a negative and positive control group of 10 rats each received water or indomethacin respectively. The two dry CHD-FA groups demonstrated a dose related anti-inflammatory response that was again comparable to the effect of two doses of indomethacin at the recommended dose of 10 mg/kg body weight. The anti-inflammatory effect was obvious at 2 hours post challenge, indicating early inflammatory suppression and which would be an advantage for treatment of chronic inflammatory processes of conditions. As the safety of the fulvic acid has been established from previous clinical trials that have been performed (52) further clinical trials in human can be done, which could lead to registration of the product for medicinal use.

A common observation that inflammation initiates the release of ROS and other pro-inflammatory mediators at the site of an infection or injury. If a drug has antioxidant properties it would be expected to be able to eliminate excessive ROS, which are often formed by localised neutrophils, and the equivalent nitrogen containing NOS that act as inflammatory mediators. Elimination of these strong oxidative molecules would mean less damage to the surrounding tissues at the site of infection.(21)

It is advantageous to use chemiluminescence as an assay of antioxidant activity because of its high sensitivity and its rapidity. Amplification of light emission can be done by using the lucigenin system due to the high sensitivity and selectivity of the technique[30]. Reduction of light emission by activated neutrophils after treatment with a compound is considered a measure of antioxidant activity.

Antioxidant reactions involve various steps namely initiation, propagation, branching and termination. All these falls into two mechanistic groups, those that inhibit the formation of free radicals from their unstable precursors, this is considered inhibition of the initiation stage, and those that interrupt the radical chain reaction, this step is called the “propagation and branching inhibition”. The first group are called

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preventative antioxidants and the latter group are called chain breaking antioxidants.(41)

Antioxidants are important compounds that possess the ability to protect the body from damage caused by free radical-induced oxidative stress. A variety of free radical-scavenging antioxidants exist within the body many of which are derived from dietary sources.

According to the results obtained from this study, it was found that the dry CHD-FA had strong antioxidant activity , capable of reducing the radical effectively and quickly ,it was half effective as Trolox,, which was detectable at low physiologically attainable concentrations, when using lucigenin enhanced chemiluminescence of stimulated neutrophils. This is because it showed either neutrophil respiratory burst inhibition or direct oxidant scavenging, which means less light emission detected by the fluorometer. This also supports the findings observed by Joone *et al.*(48) when testing a coal-derived humic acid product called “Oxihumate”.

The second antioxidant activity assay that was performed was the *in vitro* chemical scavenging of the 2,2-azinobis (3-ethylbenzothiazoline 6-sulfonate) radical cation ($ABTS^{\bullet+}$) using the TEAC assay to confirm the antioxidant activity of the dry CHD-FA as shown in Figure 3.4 and Figure 3.5. In this assay it can be seen that there is a linear dose response that shows the dry CHD-FA as being approximately half as efficient as the Trolox in scavenging the $ABTS^{\bullet+}$ radical. The fulvic acid is highly water soluble while Trolox is a less soluble molecule in water which would imply taking care in interpreting the results as direct comparisons. The fact that the fulvic acid is water soluble means that it could easily reach any extracellular site in the body easily, making it an effective systemic antioxidant. The caveat is that the compound may not be effectively absorbed from the GIT, but as seen from the carrageenan induced paw oedema experiment with the rats, systemic absorption does appear to be high enough to elicit an effect.

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The expression of CR3 molecules was inhibited by both the tested concentrations of dry CHD-FA. A similar effect was seen on studies done by Joone (48) and Cromarty (47) on the humic acid product, oxihumate. Oxihumate is the potassium salt of humic acid which used a similar wet oxidation process to convert bitiminous coal followed by alkali treatment to form water-soluble humates. Adhesion of neutrophils to vascular epithelium requires expression of CR3 molecules prior to initiation of diapedesis, the first step in neutrophil accumulation in extravascular tissue. Diapedesis is the movement or passage of white blood cells, through intact capillary walls into surrounding body tissue.

Therefore, inhibition of CR3 expression would also result in suppression of diapedesis (47) and the accumulation of high numbers of neutrophils in areas of inflammation or tissue damage. If adhesion is inhibited in the presence of CHD-FA, then extra-vascularisation of the neutrophils would be suppressed and the inflammatory response would be expected to be decreased. This supports the findings that were done on Oxihumate (potassium salt of humic acid), and leads to the conclusion that dry CHD-FA could exhibit neutrophil initiated anti-inflammatory properties.(48)

Natural humification is a gradual process that can take from a few weeks to hundreds of years, giving rise to highly complex humic substances. These humic substances can be extracted based on their solubility, which allows their separation into humic acid (HA), fulvic Acid (FA) and humin (Hu) all of which are still complex mixtures of compounds. Through the controlled synthetic route, a very consistent mixture of compounds appears to be synthesised and it was hoped that through LC-MS/MS these compounds could be easily identified and the active compounds further concentrated to make the fulvic acid more efficient. A problem that was found as a general liquid chromatography problem was that many of the compounds did not elute from the HPLC or UPLC columns and that as much as 50% of the injected sample did not elute from the column. (Personal communications with AD Cromarty and M Stander).this was determined through a mass audit To circumvent this an old approach of using TLC was used where all the spots that separate can still be seen

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and can be individually targeted and directly introduced into the mass spectrometer source using a special instrument designed to extract spots from a TLC plate. Despite this the spot left on the origin of the TLC plates proved to be insoluble in the extraction solvents used and gave very unreproducible results in terns of peak intensities and in the identified masses. For these reasons the mass of the compound spots on the origin are not reported in this study.

Three different TLC methods were used to separate the compounds six different batches of CHD-FA that were spotted on the plate. The three methods involved both acidic and alkaline mobile phases with two methods using the acidic phases used a more lipophilic acetone-based phase and a methanol-based phase. A similar pattern of separation was observed in both the acidic mobile phases while the alkaline phase appeared to separate significantly more compounds from each of the samples. All three mobile phases showed pseudo fronts due to separation of the mobile phases themselves on the TLC plates, and there was a particular spot that moved with this pseudo-front in all the samples run. More distinct spots separated below the pseudo-front with only one spot moving ahead of this clearly defined solvent pseudo-front.

The solubility of different compounds in the eluting solvent plays an important role in how fast they separate while moving up the plate. However, a more important property of the solvent is its ability to itself be adsorbed on the adsorbent. If the solvent is strongly adsorbed, the analyte compounds will be displaced causing them to move up the plate together near the solvent front with little separation from other compounds also highly soluble in the mobile phase. If the solvent interacts too weakly with the stationary phase or does not effectively solubilise the analyte compounds, its solvating power may be insufficient to retain the compounds in solution and the compounds will not be separated but will precipitate onto the stationary phase and not be easily recovered.

The alkaline mobile phase resulted in 8 different spots being separated from the different batches of the CHD-FA with batch 390 showing three missing spots when compared to all the other batches. This batch was subject to a post synthesis

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ultrafiltration process where the product was forced through a 4000 Da ultrafiltration membrane. This would normally imply that the compounds within these three spots are greater than 4000 Da but the equivalent spots from all the other batches tested showed that the majority of ion masses from compounds eluted from these spots were smaller than 340 Da. This was clearly illustrated in Figures 3.19 through to 3.21 where the masses of all the compounds could be seen in the upper gel view part of the figures. The fact that such small compounds were removed by ultrafiltration would imply that the compounds that are within the spots must be bound together in a manner that would promote the formation of large complexes of molecules that would support the hypothesis that fulvic acids are supramolecules made up from a number of different much smaller subunits and held tightly together while in solution but able to be separated into the individual compounds when these are exposed to the ionising forces within the mass spectrometer source where high voltages and high temperatures may disrupt the forces holding these supramolecular structures together.

The range of Rf values for the different spots vary from those left on the spotting origin to a high value of 0.89 which is above the obvious pseudo-front. The spread of Rf values was 0.19; 0.23; 0.30; 0.43; 0.49; 0.56 then a gap to the pseudo-front which ran at Rf 0.82 and the most lipophilic spot at 0.89. These were seen in all the CHD-FA batches, with all these spots showing up under UV light at 260 nm and 360 nm. The spots at Rf 0.3; 0.56 and 0.82 all showed fluorescence under the 360 nm UV lamp. These spots tended to co-elute with other UV absorbing spots and showed different colour development when sprayed with phosphomolybdic acid, a general antioxidant spray reagent or when stained using iodine vapours (unpublished results).

The results from the acid mobile phases showed fewer spots despite the samples coming from exactly the same solution when spotting the samples on the different TLC plates. The more polar methanol based acidic mobile phase showed only 5 spots and a relatively narrow spread of Rf values for the different spots with batch 390 again having the lowest spot missing, yet it was only a single missing spot when

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using this mobile phase. The spots tended to have higher Rf values than for the alkaline mobile phase, varying between 0.57 and 0.88, than the acetone based acidic mobile phase. All the spots separated showed clear co-elution from the UV and fluorescence detected under the 260 nm and 360 nm light. Colour development was also different for the different spots as was seen for the alkaline mobile phase.

The acetone based mobile phase resulted in streaking of compounds from the origin where the spots were spotted up to an Rf of about 0.2, streaks that tended to show a darker colour than the TLC plates themselves. The spots at Rf 0.54 to 0.57 on the two acid mobile phase plates appeared to move with a pseudo-front.

As TLC retains all the non-volatile compounds on the plate after separation, and that these separated compounds can be located using various visualising techniques, the TLC method was an ideal technique to identify compounds in the original sample across all possible masses. This is in contrast to what has been observed with HPLC separations where all the compounds do not elute from the analytical column during chromatography due to solubility concerns and almost irreversible binding of several compounds to the stationary phase of these HPLC columns.

Further analysis of each spot, except those from the origin, was done using a direct extraction LC-MS method from the TLC plates.

What was interesting was that the masses of the positive mode ions and the negative mode ions do not show the classical two Dalton difference which indicates that there are completely different molecules ionising under the ionisation conditions of each mode. Another unexpected observation was that although the fulvic acid appears to be a single large and complex molecule, the majority of the ions formed are below 340 Da which would be just less than the mass of the sugar used during the controlled wet oxidation process. Under the negative ionisation mode there were several mass series with differences of 14 Da that is indicative of a $-CH_2-$ addition as the mass increases through the series. This can be seen from mass 137 through 179 and from 311 to 339 Da. In contrast the positive mode ions showed an increasing series of 28 Da from mass 421 through to 771 Da. This would be a C2H4 molecular

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addition. A classical 44 Da mass difference through addition or removal of a -CO_2- moiety was not evident despite being expected due to the wet oxidation process on the sugars.

In this study it was confirmed that many compounds with masses below 400 Da are present in the different CHD-FA batches, confirming that the spots at specific Rf values contained essentially the same combination of 15 plus different compounds. Similar results showing an abundance of low molecular weight compounds in fulvic acid were obtained by other researchers (53) Those studies could have a probable bias introduced for compounds with low masses due to the extraction procedures used by the authors resulting in few compounds with masses above 500 Da.

The initial intention was to perform LC-MS/MS analysis as each spot was expected to have a single or perhaps two or three compounds which could be individually selected and fragmented to give additional structural data. Unfortunately, none of the spots had fewer than 15 ionic compounds, making the use of tandem mass spectrometry almost completely impossible. This LC-MS analysis, where no compound fragmentation took place, showed that there were numerous compounds found within each spot. This finding supports the findings by Schulten and Schnitzer[55]. that FA is a complex supramolecule. Schulten and Schnitzer (54) proposed that the structure for HA is formed by alkyl benzene moieties attached through covalent bonds and Piccolo *et al.*(51) proposed that HA is a self-assembled supramolecular structure of relatively small heterogeneous molecules held together primarily by hydrophobic dispersive forces and hydrogen bonds. These molecular forces determine the conformational structure in the humic acid molecule.

Due to the long standing existing data, large volume of preceding studies that did not need repetition, head-to-head studies were not done. From the data provided in the different experiments it can be confirmed that the new formulation of dry CHD-FA does compare to the previous liquid formulation of the CHD-FA, produced through the same synthetic methods, in terms of the bioactivity that was monitored

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through an *in vivo* animal study as well as several *in vitro* studies that confirmed inhibition of neutrophil activation using two completely different methods and could demonstrate relatively strong antioxidant activity. The fact that oral dosing of rats for a period of a week prior to carrageenan challenge demonstrates that the active components of the CHD-FA are in fact absorbed and distributed into the systemic system. This week-long exposure to the CHD-FA at 153 mg/kg body weight also showed no signs of toxicity or behavioural changes in the rats. The dry CHD-FA could now be taken forward into real clinical trials to test the efficacy to treat low-grade inflammatory conditions

The synthetic fulvic acid used in this study was assayed for biological activity and results for this study show that it might have good anti-inflammatory properties and could work as an oral formulation with proven anti-inflammatory properties *in vivo*.

4.2 Conclusion

In conclusion, currently marketed anti-inflammatory products exert many unpleasant side effects that can be detrimental to health such as gastro-intestinal bleeds which are common side effects of the non-steroidal anti-inflammatory drugs. In this study it has been shown that dry CHD-FA possesses significant antioxidant properties *in vitro*. This property suggests that this product may be an effective immunomodulator for the treatment of diseases associated with an overproduction of reactive oxidants by human phagocytes. This however needs to be confirmed in *in vivo* systems.(53)

An interesting finding from the TLC and mass spectrometer work is that the fulvic acid does appear to behave as a supramolecular structure despite the fact that it appears to be made up from many small molecules with masses that are generally below 340 Da, which is the approximate mass of the starting sugar used in its synthesis. There is no sugar left in the final product though. Due to the high number of coeluting compounds in each spot it was not possible to find empirical formulae for the different compounds. A further complication is the fact that the formulae are expected to contain only carbon, hydrogen and oxygen which limits the possible

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formulae but also eliminates most of the possible structures. Adduct formation may be a confounding factor in this regard.

The results from this study indicate that the new formulation of dry CHD-FA is systemically effective after oral dosing in suppressing the acute inflammatory response in the carrageenan-induced inflammatory oedema in the rat footpad, proving systemic absorption, and effective acute inflammatory response suppression, which are both characteristics that could recommend the formulation for chronic inflammatory conditions.

The results of this study can be used to motivate for human trials as well as for registration of the CHD-FA for alternative medicine purposes.

These findings are considered to be of technical importance for future studies related to anti-inflammatory properties of CHD-FA in human clinical trials.

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6 Addendum

1. Ethics committe

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through February 28, 2022 and Expires: 03/04/2023.

Faculty of Health Sciences **Research Ethics Committee**

18 March 2022

Approval Certificate
Annual Renewal

Dear Miss S Ndwandwe,

Ethics Reference No.: H012-19 – Line 3
Title: Efficacy and mechanistic study of a carbohydrate derived fulvic acid with anti- inflammatory properties

The **Annual Renewal** as supported by documents received between 2022-02-16 and 2022-03-16 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2022-03-16 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2023-03-18.
- Please remember to use your protocol number (H012-19) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Dr R Sommers
MBChB, MMed (Int), MPharmMed, PhD
Deputy Chairperson *of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

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Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

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2. Research Committee

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

MSc Committee
School of Medicine
Faculty of Health Sciences

MSc Committee
5 April 2017

Prof AD Cromarty
Department of Pharmacology
Faculty of Health Sciences

Dear Prof,

Ms S Ndwandwe, Student no 23321050

Please receive the following comments with reference to the MSc Committee submission of the abovementioned student:

Student name	Ms S Ndwandwe	**Student number**	23321050
Name of study leader	Prof AD Cromarty		
Department	Physiology		
Title of MSc	Efficacy and mechanistic study of a carbohydrate derived fulvic acid with anti- inflammatory properties		
Date of first submission	February 2017		
April 2017	• Thank you for the revised protocol and MSc form.		
Decision	This protocol has been provisionally approved. Please submit the revised protocol to ethics, and supply the MSc committee with proof of acceptance. The internal and external examiners can be nominated and submitted to the MSc Committee six months prior to submission of the dissertation. Please ensure that the CV of the examiners includes: supervision, examination and publication records.		

Yours sincerely

Dr Marleen Kock
Chair: MSc Committee

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3. Statistician letter

BIOSTATISTICS UNIT

LETTER OF STATISTICAL SUPPORT

17 August 2016

To whom it may concern,

This letter is to confirm that Siphephisiwe Ndwandwe from **Department of Pharmacology**, University of Pretoria discussed the Project with the title **"Efficacy and mechanistic study of a new carbohydrate derived fulvic acid with anti-inflammatory properties"** with me.
we therefore confirm that we are aware of the project and also undertake to assist with the statistical analysis of the data generated from the project.

Data Analysis
This is a longitudinal study comparing four treatments, with 8 repeated measures of volume from 10 in each treatment group. Initial analysis of the data will involve graphical displays: plots of individual volumes, possible connected and plots of observed mean response profiles by treatment group. Simple analyses of longitudinal data will be used: time-by-time analysis of volume at each of the 8 times of measurements and time-by-analysis of derived volume gain between successive times using analysis a variance (ANOVA). All statistics will be done using STATA, where p-value<0.05 would be considered statistically significant.

Yours sincerely

Prof Samuel OM Manda
Samuel.manda@mrc.ac.za

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