CHAPTER 1

1.1 INTRODUCTION

Mankind has been using plants for food, shelter and clothing since antiquity. It is now becoming increasingly evident that man also relies on plants for health purposes. Undoubtedly, a large percentage of the South African population depends on traditional medicine and most of these medicines are plant-based (Mander and Le Breton, 2006). Information on the healing properties of plants has been passed down through generations and the increasing use of plants as medicines has now evolved to include various areas of research.

Current research in medicinal plants focuses mostly on the isolation and characterization of bioactive compounds from these plants. Efforts continue to identify bioactive compounds from various plants that can be used for the treatment of ailments, or as parent compounds for the generation of conventional drugs. Plants remain an attractive source for obtaining bioactive compounds of medical importance as they are cheap, easily accessible and offer variety due to genetic, ecological and environmental differences (Bopana and Saxena, 2007). There is also a need to study and document the pharmacology and toxicology of traditional herbal remedies. This information is necessary, not only to educate the scientific community in understanding the complete effect of these plants, but also to assist traditional medical practitioners in providing remedies that are safe and of high quality.

There is a fast-growing interest in the anti-inflammatory activity of medicinal plants used for the treatment of pain-related ailments (Fawole *et al.*, 2010). Numerous degenerative diseases, e.g. arthritis, cardiovascular diseases and various forms of cancer are often a result of dysregulated inflammation, and one way to alleviate these disorders is to regulate the inflammatory response through the introduction of exogenous anti-inflammatory agents. Isolation of novel anti-inflammatory compounds from plants is important for drug development as well as for validation of the use of plants for the treatment of inflammatory diseases.

1.2 LITERATURE REVIEW

1.2.1 The Inflammatory Response

Inflammation is one of the first responses of the immune system against infection and its primary function is to establish a barrier against infectious agents as well as to assist with tissue healing. In his only surviving work, Celcus (1935) stated that inflammation is characterized by redness, pain, high temperature and swelling of the affected area. These signs of inflammation are mainly due to vasodilation, increased blood flow, fluid build-up, release of soluble mediator molecules, e.g., hydrolytic enzymes, and a boost in cellular metabolism at the inflamed area (Ferrero-Miliani *et al.*, 2006). This inflammatory response allows tissues to respond to injury or infection in a highly coordinated manner.

During bacterial-mediated inflammation an assortment of immune cells, e.g., polymorphonuclear neutrophils (PMNs) and phagocytes are recruited to the site of infection as a host defense mechanism against the invading microorganisms. The host's immune cells synthesize and release a variety of bactericidal molecules including defensins, bactericidal permeability-increasing protein, lysozyme and reactive oxygen species (ROS) (Burg and Pillinger, 2001). Generation of ROS is mediated chiefly by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, through transfer of an electron from NADPH to molecular oxygen (O_2) outside the cell or in phagosomes containing ingested microbes (Dusi *et al.*, 1995).

1.2.2 NADPH Oxidase

NADPH oxidase (phagocyte oxidase) is a superoxide-generating enzyme found in many cells that originate from the mesoderm, including neutrophils, eosinophils and mononuclear phagocytes (Babior *et al.*, 2002). It has a high affinity for NADPH as an electron donor, transferring an electron from NADPH to molecular oxygen and converting it to its one-electron reduced product, the superoxide anion (O_2^-) (Fialkow *et al.*, 2007). This enzyme consists of two complexes, one located in the cytosol and the other on the plasma membrane or on membranes of secretory vesicles (phagosomes) within the cell. As depicted in Fig. 1.1, the cytosolic complex consists of three protein subunits:

$p40^{PHOX}$, $p47^{PHOX}$ and $p67^{PHOX}$; and the membrane complex, also known as cytochrome b_{558}, has two subunits, $gp91^{PHOX}$ and $p22^{PHOX}$ (PHOX = phagocyte oxidase) (Babior, 1999; Banerjee *et al.*, 2000; Renwick *et al.*, 2007).

In the resting cell the heterodimeric cytochrome b_{558} is detached from the cytosolic complex to ensure that the enzyme remains inactive until stimulation (Babior, 1999). Stability of each subunit of cytochrome b_{558} depends on formation of the heterodimer; and mutations in either one of the subunits leads to both being absent from the cell surface (Babior *et al.*, 2002).

Cytochrome b_{558}, also called the flavocytochrome, contains flavin adenine dinucleotide (FAD) and two hemes, all situated on $gp91^{PHOX}$ (Babior, 2004; Nisimoto *et al.*, 1995). This heme-binding component of the oxidase has a typical absorbance peak at 558 nm, thus the term cytochrome b_{558} (Dagher *et al.*, 1995). Enzyme-bound FAD is responsible for electron transfer in the active enzyme. This was first suggested after reports that a corner of the benzene ring of the electron-carrying isoalloxazine group on FAD is always exposed to the solvent in cell-free assays (Nisimoto *et al.*, 1995). When FAD is removed from the enzyme, oxidase activity is lost; however, this activity ensues upon refurbishment of the lost FAD. Purified cytochrome b_{558} is able to catalyze superoxide production in the absence of the cytosolic complex, supporting the concept that FAD is the electron carrier for the oxidase (Koshkin and Pick, 1994).

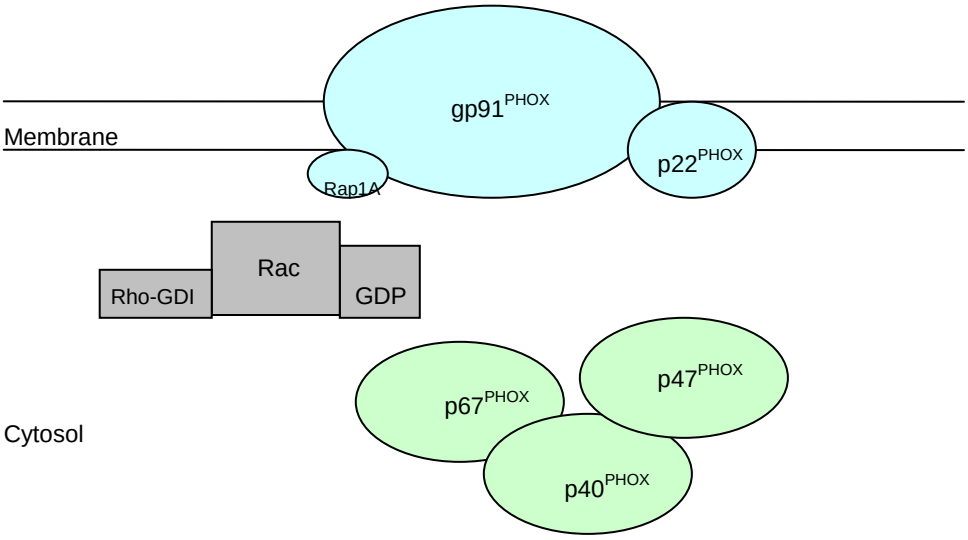

Figure 1.1: Simplified diagram of the inactive NADPH oxidase complex. Cytochrome b_{558} ($pg91^{PHOX}$ and $p22^{PHOX}$) is situated on the membrane along with Rap1A which dissociates upon activation of the enzyme. Rac is located in the cytosol and is complexed with Rho-GDI and GDP. The cytosolic complex of the enzyme is also found in the cytosol and it consists of $p40^{PHOX}$, $p47^{PHOX}$ and $p67^{PHOX}$.

1.2.2.1 Stimulation of NADPH Oxidase

Neutrophils have surface receptors onto which various targets can bind and result in the up- or down-regulation of functional activities of the phagocytic cell. These activities are modulated by second messengers associated with enzyme systems and regulatory proteins, e.g., G-proteins, and they comprise increased adhesion, chemotaxis, phagocytosis, degranulation and production of an oxidative burst (Kogut *et al.*, 2001).

Before the onset of the oxidative burst, cells must be activated by a stimulus such as arachidonic acid, opsonized particles, immunocomplex or phorbol myristate acetate (PMA) leading to a cascade of events (Dusi *et al.*, 1995). These events may be summarized as (1) phosphorylation of oxidase subunits, (2) migration of the cytosolic complex to the membrane, (3) assembly of the active enzyme and (4) resultant production of O_2^- through oxidation of NADPH (Ximenes *et al.*, 2007).

During stimulation, subunits of the enzyme become hyperphosphorylated ensuing in conformational rearrangements of the domains and motifs of each subunit. These conformational changes serve as a switch that triggers assembly of the active enzyme (Fig 1.2). Phosphorylation of $p47^{PHOX}$ is chiefly responsible for translocation of the cytosolic complex to the flavocytochrome and results in 2-20% translocation when cells are adequately activated/stimulated (Forbes *et al.*, 1999).

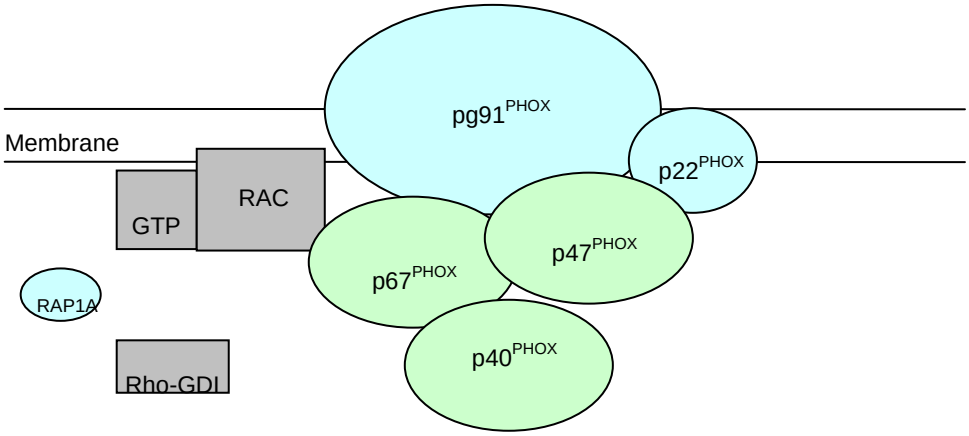

Figure 1.2: Simplified diagram of activated NADPH oxidase complex. Phosphorylation of oxidase subunits allows translocation of the cytosolic complex the membrane and subsequent binding to cytochrome b_{558} follows. Rap1A and Rho-GDI dissociate from the flavocytochrome and Rac, respectively, upon activation on the oxidase.

1.2.2.2 Oxidase Activity

The redox core of the NADPH oxidase appears to be $gp91^{PHOX}$ as all the elements required for electron transfer are bound to this protein. The protein has binding sites for flavin, NADPH and two heme molecules (Bokoch, 1995). An NADPH-binding domain has also been located on $p67^{PHOX}$ (Smith *et al.*, 1996). It has been established that $p47^{PHOX}$ interacts with $p22^{PHOX}$ upon activation of the oxidase, leaving $p67^{PHOX}$ free to bind to $gp91^{PHOX}$. NADPH is thus presumed to bind to $p67^{PHOX}$ and then transferred to $gp91^{PHOX}$. Electrons are then conveyed from NADPH to FAD and the two hemes and finally O_2, the ultimate electron acceptor, to produce O_2^- (Leusen *et al.*, 1996).

1.2.2.3 ROS Production

When a phagocyte receives a stimulus, usually attachment of a microorganism to the cell surface, a remarkable increase in activity of the hexose monophosphate shunt occurs. This results in a vigorous burst of NADPH production which is used to reduce O_2 to an array of radicals (ROS) that act as potent microbicidal agents (Mims *et al.*, 1993).

The most proximally produced ROS by NADPH oxidase is the superoxide anion (O_2^-) (Conner and Grisham, 1996; Rinaldi *et al.*, 2007).

$$2O_2 + NADPH \rightarrow \mathbf{2O_2^-} + NADP^+ + H^+$$

It is formed as the simplest acceptor of electrons rather than as an exclusive free radical product (Segal, 2008). Superoxide is unreactive towards most biological substrates but it spontaneously (sometimes enzymatically) dismutases and serves as a pioneer for formation of other ROS including hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^\bullet$) (Conner and Grisham, 1996).

$$O_2^- + e^- + 2H^+ \rightarrow \mathbf{H_2O_2}$$

$$H_2O_2 + e^- + H^+ \rightarrow H_2O + \mathbf{OH^\bullet}$$

Alternatively, O_2^- may react with endothelium-derived nitric oxide (NO) to form peroxynitrite ($ONOO^-$), which nitrates proteins and mediates lipid peroxidation (Selemidis *et al.*, 2008).

$$O_2^- + NO \rightarrow \mathbf{ONOO^-}$$

These in turn react with other molecules to form highly toxic substances, e.g., singlet oxygen ($^1O_2$) which attacks double bonds and hypochlorous acid (HOCl) (Hampton *et al.*, 1998).

$$H_2O_2 + Cl^- \rightarrow \mathbf{HOCl} + OH^\bullet$$

All ROS produced are responsible for the microbicidal activity of PMNs.

1.2.2.4 Chronic Granulomatous Disease

Phagocytes are sometimes not able to eradicate invading microorganisms due to decreased or complete lack of oxidase activity. This may be due to (1) absence or abnormality of a component(s) of the oxidase complex, (2) decreased affinity of the oxidase for NADPH, or (3) impaired generation of NADPH (Umeki, 1990).

Defective oxidase activity is observed in patients with chronic granulomatous disease (CGD). In these individuals, phagocytes fail to activate the superoxide-generating NADPH oxidase, which is necessary for production of ROS. Their cells can only produce between 10 and 20% of the normal amount of oxidase activity, increasing susceptibility of the patients to microbial infections (Banerjee *et al.*, 2000). Recurrent infections begin from early childhood and are usually fatal because they are very difficult to treat. These infections can usually be controlled with extensive antibiotic therapy, but eradicating the pathogenic microorganisms is often a slow and difficult process (Segal, 2008).

In CGD phagocytes, microorganisms are engulfed into phagosomes but are, however, not destroyed, leading to a chronic inflammatory state with granuloma formation (Heyworth *et al.*, 2003). The most common microorganisms that persist within phagosomal vacuoles are catalase-positive, e.g. Gram-negative enteric bacilli, *Staphylococcus aureus* and *Aspergillus* species, a problematic fungus in immunocompromised humans (Edwards and Watson, 1995; Leusen *et al.*, 1996). Catalase converts H_2O_2 to a nonradical form and functions as a natural antioxidant, preventing phagocytes from using H_2O_2 to kill microbes (Leusen *et al.*, 1996; Pourmorad *et al.*, 2006). Patients may therefore develop chronic inflammation that destroys organs, resulting in death.

1.2.2.5 Inhibition of NADPH Oxidase

Natural and synthetic inhibitors of NADPH oxidase have been used over the years to increase our understanding of the enzyme, how it functions and the molecular aspects of CGD. A number of NADPH oxidase inhibitors have been identified and they may act in one of four ways:

a. Prevention of the assembly of the oxidase

Naturally occurring sulfhydryl blockers, e.g. 4-hydroxynonenal, impede activity of the leukocyte oxidase by preventing phosphorylation of various subunits of the enzyme (Siems *et al.*, 1997). Phosphorylation occurs chiefly on serine residues on the oxidase subunits, therefore, any agent with the ability to mask

sulfhydryl groups on these amino acids should inhibit phosphorylation, and consequently, translocation of the cytosolic complex to the membrane and resultant production of O_2^- (Babior, 1999).

S-nitrosoglutathione (GSNO) impairs the interaction between cytochrome b_{558} and the cytosolic complex by transferring its nitroso group to the sulfhydryl groups in the membrane proteins, but has no effect on O_2^- production once the oxidase has been fully assembled (Park, 1996). This suggests that GSNO inhibits NADPH oxidase activity by altering the conformation of cytochrome b_{558} which is necessary for $p47^{PHOX}$ -binding, thus hampering binding of the entire cytosolic complex and subsequent O_2^- release.

Apocynin inhibits NADPH oxidase activity by preventing $p47^{PHOX}$ translocation (Barbieri *et al.*, 2004). Apocynin is a compound that can oxidize into a dimeric glutathione radical and this radical is proposed to conjugate with thiol residues on $p47^{PHOX}$, preventing formation of the cytosolic complex (Ximenes *et al.*, 2007). Once the oxidase is fully assembled, however, compounds that inhibit translocation or phosphorylation will not have an inhibitory effect on O_2^- production (Fujii *et al.*, 1997).

b. Decrease gene expression of one or more subunits of the enzyme

Oxidase activity may be suppressed by down-regulating the expression of a crucial subunit of the enzyme complex, $gp91^{PHOX}$. Infection of cells with human granulocytic ehrlichiosis (HGE)-causing bacteria has been shown to cause down-regulation of the $gp91^{PHOX}$ protein (Banerjee *et al.*, 2000). A decrease in $gp91^{PHOX}$ also affects $p22^{PHOX}$ as the two proteins cannot exist without each other within the membrane. This diminished level of the membrane complex subunits implies that only limited translocation of the cytosolic complex will occur; thus decreasing or totally preventing O_2^- production. The HGE agent consequently induces a state in which sensitivity of the host to secondary infections is enhanced due to lack of the respiratory burst.

c. Alteration of the electron transport mechanism at the membrane

Production of O_2^- can also be inhibited by blocking the electron transport chain occurring at cytochrome b_{558}. Diphenylene iodonium (DPI) and iodonium diphenyl (IDP), like other iodonium compounds, react with reduced flavin on cytochrome b_{558}, abstracting an electron from FAD to form phenylated products. They form DPI- and IDP-FAD adducts (O'Donnell *et al*., 1993), therefore non-competitively inhibiting NADPH from binding at the flavocytochrome (Jones *et al.*, 2000).

d. Modulation of oxidase activity by modifying upstream activators

Inhibitors of phosphodiesterases (PDEs) are being developed clinically as potential anti-inflammatory agents (Dastidar *et al.*, 2007). PDEs regulate cellular concentrations of cyclic AMP, an important intracellular second messenger (Jacob *et al.*, 2002). Inhibition of PDEs elevates cAMP levels and this elevation inhibits neutrophil activity, lowering O_2^- production (Hwang *et al.*, 2009; Selemidis *et al.*, 2008).

1.2.3 Nitric Oxide Synthases

In addition to NADPH oxidase, cells contain other enzymes that are involved in the production of free-radicals; nitric oxide synthase being a classical example. Nitric oxide synthases (NOS) are a family of enzymes that produce nitric oxide (NO) through the two-step oxidation of L-arginine to L-citrulline (Stuehr *et al.*, 1991). Three isoforms of NOS are known, two of which are constitutively expressed by cells and the third, an inducible enzyme. The constitutively expressed isoforms, NOS-1/nNOS (neuronal) and NOS-3/eNOS (endothelial), are involved in cell communication and vasodilation, respectively. The third isoform, NOS-2/iNOS, must be induced by certain stimuli and is involved in the immune and cardiovascular systems (Knowles, 1996). Expression of iNOS is triggered when cells are stimulated by cytokines, lipopolysaccharide (LPS) – an endotoxin, hypoxia and phorbol esters, among others (Ferreiro *et al.*, 2001; Nathan, 1992; Yoon *et al.*, 1994). iNOS is activated by these stimuli in various cell types, particularly macrophages, neutrophils, chondrocytes, Kupffer cells, hepatocytes, epithelial cells and in vasculature (Lechner *et al.*, 2005).

1.2.3.1 NOS Structure

All three NOS isoforms are homodimers associated with flavin adenine dinucleotide (FAD) and flavine mononucleotide (FMN) (Knowles and Moncada, 1994). The FAD and FMN are clustered together with calmodulin at the carboxy terminus of each monomer of the protein (the reductase moiety) while the amino terminus contains heme, tetrahydrobiopterine (BH_4) and an L-arginine binding-site (the oxygenase moiety) (Eissa *et al.*, 1998). During NO synthesis L-arginine is hydroxylated by O_2 and NADPH to form N-ω-hydroxy-L-arginine, which is further oxidized to L-citrulline with concomitant production of H_2 and NO (Stuehr *et al.*, 1991).

1.2.3.2 Reactive Nitrogen and Oxygen Species Production

Nitric oxide is a small, short-lived free radical that easily diffuses from its production site within a cell to a different site of action (Aktan, 2004). Upon its release by NOS, NO can react with an assortment of other molecules to yield numerous reactive nitrogen and oxygen species (RNOS) that result in the indirect effects attributed to NO (Lechner *et al.*, 2005). Superoxide reacts with NO to generate peroxynitrite ($ONOO^-$) which is then protonated to peroxynitrous acid. Peroxynitrous acid decomposes into nitrate and finally nitrogen dioxide and $OH^\bullet$ (Beckman *et al*., 1992). Under normal conditions, NO is produced only by eNOS and nNOS for physiological function including vasodilation and neurotransmission. Activated cells produce NO at concentrations that are much higher than the concentrations observed under normal cellular conditions due to stimulation of iNOS. This overproduction of NO assists cells in the eradication of invading pathogenic microorganisms through formation of RNOS that are highly microbicidal (Bogdan, 2001).

1.2.3.3 Beneficial Effects of NO

There is increasing evidence that RNOS produced at sites of chronic inflammation have deleterious effects (Ohshima and Bartsch, 1994). However, the role of NO as a cytoprotective molecule is also appreciated, as not only is NO responsible for carcinogenesis, but it also has anti-tumour activity in some cell lines. NO causes up-regulation of p53, a pro-apoptotic tumour suppressor protein (Forrester *et al.*, 1996). This is believed to be a reaction to DNA

damage mediated by NO as increased cell proliferation was noted in tumour cells with mutant p53 after NO exposure (Ambs *et al.*, 1998). However, the apoptotic effects of NO on cells have to be interpreted with caution as they are often multifaceted. These effects depend on:

a. Concentration of NO in affected cells

Prolonged exposure of macrophages to high levels of NO induces apoptosis while lower levels increase the expression of anti-apoptotic proteins (Genaro *et al.*, 1995). However, if apoptosis is impaired by NO, cells that would normally die or stop dividing are able to proliferate and develop into a tumour(s).

b. Cell type

NO triggers apoptosis in macrophages, thymocytes and neurons by up-regulating pro-apoptotic genes but prevents it in B-cells, splenocytes and hepatocytes through up-regulation of anti-apoptotic genes (Chung *et al.*, 2001; Mannick *et al.*, 1994; Tzeng *et al.*, 1998).

c. Availability of transition metals

Observations that NO is able to bind to Fe^{2+} and SH groups on proteins led to the conclusion that the redox state and transition metal complexes within a cell affect the sensitivity of the cell to NO-mediated apoptosis. Indeed, it was reported that preloading cells with Fe^{2+} protects them from apoptosis caused by iNOS activity (Chung *et al.*, 2001).

d. Interaction of NO with free radicals

All three NOS isoforms are capable of O_2- synthesis, with iNOS being the least potent producer of the radical (Alderton *et al.*, 2001). NO reacts with O_2^- to form $ONOO^-$, and with various oxygen related reactive species to yield an array of RNOS (Lechner, 2005). The combined activity of these radicals promotes chronic inflammatory disorders and carcinogenesis.

1.2.4 Cyclooxygenases

Another key enzyme that plays a role during inflammation is cyclooxygenase (COX). COX plays a vital role in the biosynthesis of prostaglandins – lipid mediators that coordinate various physiological processes by binding to membrane receptors on cell surfaces. Prostaglandins are synthesized by cleavage of phospholipids to arachidonic acid by phospholipase A_2 which is then used to synthesize prostaglandins through a reaction mediated by COX (Patrignani *et al.*, 2005).

1.2.4.1 Variations Between COX-1 and COX-2

Two Cox isoenzymes are responsible for prostaglandin synthesis, COX-1 and COX-2. COX-1 is constitutively expressed in tissues, while COX-2 expression has to be induced by specific stimuli (Needleman and Isakson, 1997). Prostaglandins synthesized by constitutively-expressed COX-1 regulate electrolyte balance in kidneys, protect the mucosal lining of the gastrointestinal tract and maintain normal haemostasis, among other functions (Simon, 1999). However, inducible COX-2 is predominantly involved in synthesis of prostaglandins associated with the inflammatory response, i.e. those that enhance vascular permeability, vasodilation and sensitization of pain fibres (Schepelmann *et al.*, 1992). The two COX isoforms have an overall homology of only 60%, but exhibit 90% homology at the active site. At the active site, a distinct difference lies in the substitution of an isoleucine in COX-1 with a valine residue in COX-2 (Gierse *et al.*, 1996). This small variation in amino acid residues allows selective inhibition of COX-2, leaving COX-1 free to perform its homeostatic functions under normal cellular conditions. Synthetic COX inhibitors are designed in such a way that they should fit snugly into the large active site on COX-2, but be restricted from accessing the smaller COX-1 active site.

The COX-2 gene, like many other early response genes, contains regions that allow rapid up-regulation in response to the correct stimuli. Its mRNA also has regions that allow the message to be degraded with resultant down-regulation of the gene in the absence of stimulation. The gene is also down-regulated by anti-inflammatory agents. The COX-1 gene is, however, maximally expressed

throughout all tissues and does not exhibit the control noted with COX-2, e.g. is not down-regulated by anti-inflammatory mediators (Crofford, 1997).

1.2.4.2 Non-Steroidal Anti-Inflammatory Drugs

The most widely used treatments for inflammation, pain and fever are non-steroidal anti-inflammatory drugs (NSAIDs), accounting for a large percentage of both prescribed and over-the-counter medications (Green, 2002). These drugs exert their anti-inflammatory effect chiefly by hampering conversion of arachidonic acid to prostaglandins by COX enzymes. Inhibition of prostaglandin synthesis causes reduction in inflammation as prostaglandins mediate vasodilation, vascular permeability and sensitization of pain fibres (Schepelmann *et al.*, 1992).

There are, unfortunately, adverse effects that manifest as a result of NSAID activity. Traditional NSAIDs inhibit both COX-1 and COX-2-mediated prostaglandin synthesis. Prostaglandins synthesized by COX-1 are involved in maintaining kidney electrolyte balance and protecting the gastric mucosa from acid damage (Simon, 1999). Impeding COX-1 activity consequently prevents protective effects of prostaglandins on the gastrointestinal tract, leading to bleeding and ulcers with long term use of NSAIDs. Prolonged use of NSAIDs has also been reported to cause renal failure, liver diseases, asthma and increased blood pressure; the latter observed in patients taking other drugs in combination with NSAIDs (Green, 2002).

These adverse clinical effects necessitated the search for NSAIDs with fewer harmful effects. Since many side-effects of traditional non-specific NSAIDs are evoked by COX-1 inhibition, COX-2 specific inhibitors were sought after. The first specific inhibitors of COX-2 (Coxibs) were released and introduced into clinical practice in the US in 1999 (Crofford *et al.*, 2000). Coxibs inhibit COX-2 without significantly affecting COX-1, reducing the harsh side-effects observed when both enzymes are targeted. This property of Coxibs is extremely attractive for anti-inflammatory drugs, as inflammation-linked COX-2 can be inhibited without altering constitutively-expressed COX-1.

1.2.5 Antioxidants

The formation and clearance of free radicals in organisms is a controlled process; however, when there is an increase in free radical production; the introduction of exogenous free radical scavengers (antioxidants) becomes vital (Korotkova *et al.*, 2003).

Several naturally occurring antioxidant compounds have been identified and these antioxidants protect living tissues from ROS that are continuously produced during normal metabolism, and consist of both enzymatic and non-enzymatic antioxidants (Conner and Grisham, 1996). Fruits and vegetables are well-appreciated sources of natural antioxidants and their intake is associated with reduced risk of cancer and many other diseases attributed to overproduction of free radicals. Synthetic antioxidants that mimic actions performed by natural antioxidants have also been developed as therapeutic agents against various diseases (Barlow, 1990; Branen, 1975).

Table 1: Common examples of antioxidant compounds

Antioxidant	**Class**	**Type**	**Function**
Albumin	Natural	Non-enzymatic	Binds copper
Carotenoids	Natural	Non-enzymatic	Free radical and $^1O_2$ scavengers
Catalase	Natural	Enzyme	Converts H_2O_2 to H_2O
Glucose	Natural	Non-enzymatic	$OH^{\bullet}$ scavenger
Nitroxides	Synthetic	Non-enzymatic	Mimic superoxide dismutase
Plasmalogens	Synthetic	Non-enzymatic	Free radical and $^1O_2$ scavengers
Pyruvate	Natural	Non-enzymatic	H_2O_2 scavenger
Sulfhydryl groups	Natural	Non-enzymatic	Free radical and HOCl scavengers
Superoxide dismutases	Natural	Enzyme	Convert O_2^- to H_2O_2
Vitamin C	Natural	Non-enzymatic	Free radical scavenger

Adapted from Conner and Grisham (1996).

1.2.6 The Liaison Between Free Radicals and Cancer

When produced at low levels, ROS formed from O_2^- activate many receptor-mediated signaling pathways responsible for regulating cell physiology; from

gene expression to cell proliferation and even apoptosis (Bokoch and Knaus, 2003). On the other hand, along with their regulatory role and eradication of bacteria, extracellular release of ROS may culminate in host tissue injury resulting from elevated levels of these radicals in tissues.

The pathogenesis of many inflammatory diseases is a direct result of dysregulated release of ROS from phagocytes (Rinaldi *et al.*, 2007). Furthermore, oxidative stress also results in mutagenic processes that have been implicated in ageing and clinical conditions, such as cancer (Dröge, 2002). Inflammatory agents increase the level of oxidative DNA damage, transforming inflammation into cancer (Tak *et al.*, 2000). Moreover, H_2O_2 and O_2^- promote mutagenesis by increasing the expression of growth-related genes. Indeed, several human cancers have been associated with multiple chronic inflammatory diseases including inflammatory bowel disease, hepatitis and *Helicobacter pylori* infections (Chen *et al.*, 2007).

When produced in high concentrations, RNOS interact with cellular macromolecules and result in oxidative damage to tissues (Bloodsworth *et al.*, 2000). RNOS are also implicated in deleterious modifications of DNA through oxidation, nitration and nitrosation reactions (Beckman, 1991; Wink *et al.*, 1991). RNOS also inhibit the activity of numerous enzymes by reacting with key sulfhydrate groups or with iron-protein complexes within their active centres (Brown, 2001; Stamler *et al*., 1992). Enzymes containing sulfhydrate groups are modified through *S*-nitrosylation, where cysteine thiols involved in zinc (Zn^{2+}) complexation in zinc finger motifs are the targets (Kroncke, 2001). Zinc finger domains are necessary for interactions between proteins and nucleic acids and are thus present on many enzymes involved in replication, transcription and translation of DNA to functional proteins. The alteration and resultant inhibition of these enzymes (many of them responsible for DNA repair) means RNOS play a critical role in carcinogenesis through (i) DNA damage, (ii) direct inhibition of enzymes, as well as (iii) by rendering cells vulnerable to various other carcinogens.

1.2.7 COX-2 and Cancer

Although expression of COX-2 is associated with inflammation, the protein has also been detected in various tumour cells (Prescott, 2000). The presence of COX-2 in tumour cells implicates the enzyme in carcinogenesis and/or inhibition of apoptosis. Accordingly, COX-2 synthesizes prostaglandins from arachidonic acid, lowering its concentration in the specific cells. Arachidonic acid is a pro-apoptotic molecule and therefore its depletion enhances cell survival, including survival of those cells targeted for programmed cell death. Furthermore, prostaglandin E_2 elevates expression of Bcl-2, an anti-apoptotic protein (Sheng *et al.*, 1998). Prostaglandin synthesis also enhances angiogenesis by modulation of integrin expression, promoting tumour growth by increasing nutrient supply to the growing mass of cells through formation of new blood vessels (Tsujii *et al.*, 1998).

1.2.8 Apoptosis

The term apoptosis (a form of programmed cell death) originates from an ancient Greek root describing the shedding of leaves in autumn by trees (Kerr *et al.*, 1972). Apoptosis occurs alongside cell proliferation to keep cell numbers at a homeostatic level. It is an organism's natural way of balancing cell numbers during development. Furthermore, it is also triggered when there is irreparable DNA damage within a cell. The cell is forced to stop the cell cycle and "commit suicide" if it cannot repair the faulty DNA (Tak *et al.*, 2000). In contrast with necrosis, apoptotic cell death does not initiate an inflammatory response as the dead cells do not release their contents into the cytosol. Necrosis, on the other hand, is caused by damage to cells, e.g. trauma or ischaemia, causing the cells to swell and burst, releasing biologically active cellular contents into the surrounding cytoplasm, consequently initiating an inflammatory response (Sepiashvili *et al.*, 2001).

Apoptosis occurs in multiple stages noted as (i) cytoplasmic shrinkage, (ii) condensation of chromatin, (iii) segregation of the nuclear membrane, (iv) DNA fragmentation, and (v) production of membrane-enclosed "apoptotic bodies" containing intracellular components (Fig 1.3) (Remillard and Yuan, 2004; Wyllie *et al.*, 1980). Two modes of apoptosis are known: the extrinsic or

receptor-mediated pathway, and the intrinsic or mitochondria-mediated pathway (Zhang *et al.*, 2004).

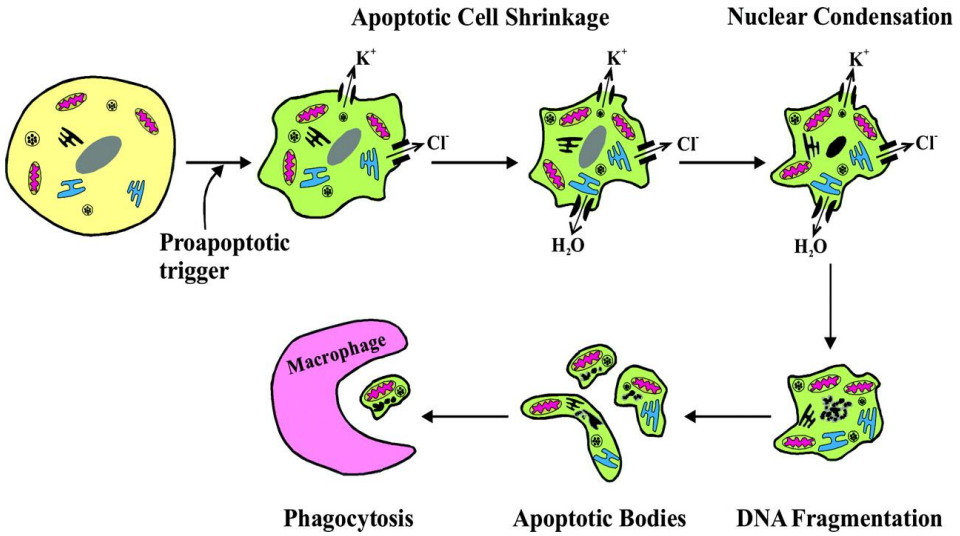

Figure 1.3: Diagram showing the chronological order of morphological and biochemical changes during apoptotis (Remillard and Yuan, 2004).

1.2.8.1 The Extrinsic Pathway

The extrinsic signaling pathway is initiated when cells receive a "death signal" through binding of appropriate ligands on death receptors situated on the cell membrane. These receptors are of the tumour necrosis factor 1 (TNF1) and Fas protein families (Tanaka *et al.*, 1996). Once the ligand-receptor interaction has taken place, Fas-associated death domain (FADD) is activated which in turn activates procaspase-8 to caspase-8 (Liu *et al*., 1996).

1.2.8.2 The Intrinsic Pathway

The intrinsic apoptotic pathway involves permeabilization of the mitochondrial membrane brought about by quantitative imbalance between Bax (a pro-apoptotic protein) and Bcl-2 (an anti-apoptotic protein) on the outer membrane of mitochondria (Korsmeyer, 1996). Intracellular stimuli, e.g. irreparable DNA damage, cause up-regulation of the tumour suppressor gene, *p53*, which itself up-regulates Bax. Under normal cellular conditions Bcl-2 homodimers and Bcl-2:Bax heterodimers exist on the mitochondrial membrane. However, p53

up-regulation culminates in formation of Bax homodimers which result in pore formation and resultant leakage of cytochrome c into the cytoplasm. Cytoplasmic cytochrome c binds to apoptosis protease-activating factor-1 (Apaf-1), enabling it to bind to and activate procaspase-9 to caspase-9 (Liu *et al.*, 1996).

1.2.8.3 Caspases

Cysteine-dependent **aspa**rtate-specific protea**ses** (caspases) are a family of enzymes that have intrinsic proteolytic activity (Zhang *et al.*, 2004). They are synthesized as inactive zymogens and require a minimum of two cleavages for activation into mature, active enzymes. Both the extrinsic and intrinsic apoptotic pathways lead to caspase activation; specifically caspase-8 in the former and caspase-9 in the latter pathway. These caspases activate executioner caspases-3, 6 and 7, which cleave various cellular proteins, ensuing in the hallmark features observed in apoptotic cells (Stennicke and Salvasen, 1999).

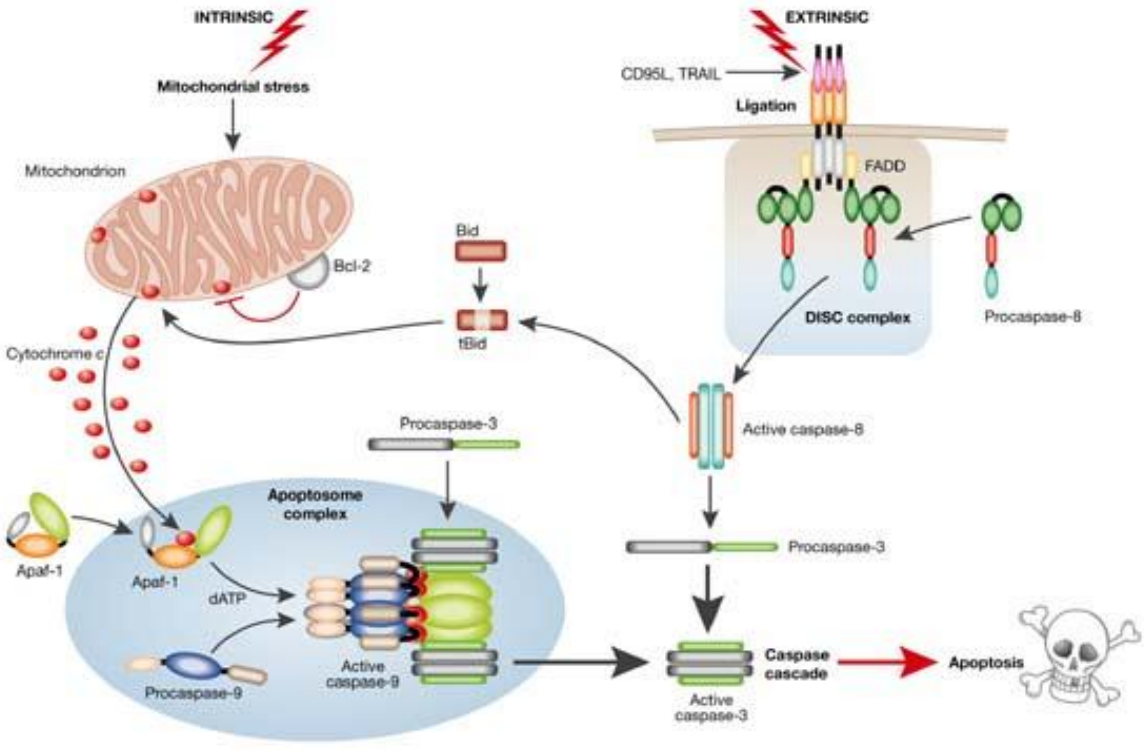

Figure 1.4: A schematic representation of the intrinsic and extrinsic apoptotic pathways (MacFarlane and Williams, 2004).

1.2.9 Plants as Sources of Drugs

Plants have been used over the years to treat a wide variety of ailments, from microbial infections to various forms of cancer. The isolation and characterization of novel compounds from medicinal plants has now become an area of much interest worldwide (Taylor *et al.*, 2001). Medicinal plants hold the best promise in the never-ending search for therapeutic compounds that will be used for many of the infections that mankind is battling to eradicate. There are a number of ways in which traditional medicinal plants have proven to be quite valuable in modern medicine, i.e. (1) they may be used as sources for direct therapeutic agents, (2) they may be used as starting points for the modification of semi-synthetic compounds, (3) they serve as sources of model chemical compounds for novel synthetic compounds, and (4) the taxonomy of some plants aid in the discovery of new compounds from other related plants (Gurib-Fakim, 2006).

Medicinal plant drugs are divided into two classes: those used as pure compounds and those included in complex mixtures. Pure compounds are used when their activity is strong and highly specific. When the activity is weak and less specific, the entire extract is used as a drug (Taylor *et al.*, 2001). The compounds in a plant may work individually or synergistically as treatments. This poses a challenge during the purification of active compounds from a bioactive plant extract because there may be a general loss of activity during the purification procedure if the activity of the extract is due to a synergistic or additive interaction of various compounds (Etken, 1986).

Bioactive phytocompounds isolated from plants include phenolic acids, tannins, flavonoids, etc (Korotkova *et al.*, 2003) and these polyphenolic compounds possess known properties which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes, as well as anti-proliferative and anti-inflammatory actions (Frankel, 1995). Many of these compounds have already been isolated and incorporated into the pharmacology industry for generation of chemotherapeutic drugs. Flavopiridol is a totally synthetic anti-cancer agent, but the basis for its structure was a natural product, rohitukine,

a phytocompound responsible for anti-inflammatory and immunomodulatory activity isolated from *Dysoxylum binectariferum* (Cragg and Newman, 2005). It is from plants such as this that compound(s) with the ability to modulate activity of oxidative enzymes can be obtained.

1.2.10 Rationale and Aim of the Study

Dicerocaryum senecioides is a crawling herb that grows in the southern and south-eastern parts of Africa. Its small, hairy leaves are used as a folk remedy for the treatment of wounds as well as removal of retained placenta in both humans and animals (Barone *et al.*, 1995; Luseba *et al.*, 2007).

Figure 1.5: Image of *Dicerocaryum senecioides* in bloom (http://www.metafro.be./prelude/prelude_pic/Dicerocaryum_senecioides2.jpg)

There is scanty documented information on *D. senecioides*, however in a previous study by Luseba *et al.* (2007), the methanol extract of *D. eriocarpum* demonstrated anti-inflammatory activity which is attributed to its soapy nature that is speculated to a have a soothing effect on inflamed skin and mucosa lesions. It has also previously been demonstrated in our laboratory that the dichloromethane (D2) fraction of *D. senecioides* possesses anti-proliferative, anti-oxidative (Madiga, 2007) and anti-inflammatory properties (Madiga *et al.*, 2009) when tested in various cellular systems.

In order to establish and confirm the anti-proliferative, anti-oxidative, and anti-inflammatory properties of the dichloromethane fraction of *D. senecioides,* the D2 fraction was semi-purified into sub-fractions using HPLC for further analysis. Therefore the specific objectives of the study were to:

i. Evaluate the cytotoxic effect of the D2 fraction on the RAW 267.4 cell line using the MTT assay and real time cell analysis.
ii. Evaluate the effect of the D2 fraction on ROS production using the DCFH-DA assay.
iii. Assess the effect of the D2 fraction on iNOS activity.
iv. Sub-fractionate the D2 fraction using semi-preparative high performance liquid chromatography (HPLC).
v. Use thin layer chromatography to obtain a phytochemical fingerprint of the HPLC sub-fractions and to screen the sub-fractions for antioxidant compounds.
vi. Quantify the antioxidant activity of the D2 HPLC sub-fractions by using the DPPH and FRAP assays.

CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipment

- Waring Commercial Blendor (**Model 32BL79, Dynamics Corporation, New Hartfort, Connecticut, USA**)
- Büchi Rotary Evaporator R-205 (**Büchi Labortechik AG, Switzerland**)
- Microtiter-Plate Multimode Detector (**Model 550 and Model DTX 800, Bio-Rad Laboratories, California, USA)**
- CO_2 incubator (**NAPCO model, Instrulab cc, Johannesburg, SA**)
- Centrifuge (**Model GS-15R, Beckman Coulter, Germany**)
- Light microscope (**Zeiss, Germany**)
- Inverted fluorescence microscope (**Nikon, Japan**)
- HPLC Semi-preparative Chemstation (**1200 Series, Agilent Technologies, Germany**)
- RTCA DP Analyzer (**ACEA Biosciences, Inc., California, USA**)

2.1.2 Chemicals, Cells and Culture Media

- Organic solvents, Potassium ferricyanide, Ferric chloride, Trichloroacetic acid (**Rochelle Chemicals, SA**)
- RAW 264.7 cell culture (**ATCC, Rockville, USA**)
- Fetal bovine serum (FBS) (**Hyclone, Cramlington, UK**)
- RPMI-1640 medium, PSN (penicillin, streptomycin and neomycin cocktail) (**Gibco, Auckland, New Zealand**)
- Dimethylsulfoxide (DMSO), Sodium nitrite (**Merck Chemicals, (PTY) LTD, Darmstadt, Germany**)
- Tris(hydroxymethyl)aminothane (**Melford Laboratories Ltd, Chelsworth, UK**)
- 4′-6-Diamidino-2-phenylindole, Apocynin, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 2′,7′-Dichlorofluorescin diacetate, 2,2-Diphenyl-1-picryl-hydrazyl, Phorbol 12-myristate 13-acetate, Griess reagent, Lipopolysaccharide, Interferon gamma (**Sigma-Aldrich, Saint Louis, Missouri, USA**)

2.2 Methods

2.2.1 Plant Collection and Extraction

The leaves of *Dicerocaryum senecioides* were collected from University of Limpopo (Turfloop campus) grounds in January (summer). The leaves were dried in open air at room temperature for 2 days and placed for a further 2 days in an oven at 40°C. The dried leaves were ground into a fine powder with a Waring Commercial Blendor and extracted exhaustively with absolute methanol (10 g sample/100 ml solvent). The samples were shaken vigorously for 18 hours at room temperature and filtered with Whatman No.1 filter paper. The methanol was evaporated under a stream of air and the dried crude residue was then resuspended in ethanol/water (3:1, v/v) and fractionated using solvent/solvent extraction into 3 fractions: *n*-hexane (D1 fraction), dichloromethane (D2 fraction) and water (D3 fraction). The D1 and D3 fractions were discarded as they had displayed none of the sought-after activities in a previous study (Madiga, 2007). The D2 fraction was then concentrated using a rotary evaporator at 40°C and left to dry under a stream of air at room temperature. A schematic representation of the fractionation procedure is illustrated in Figure 2.1.

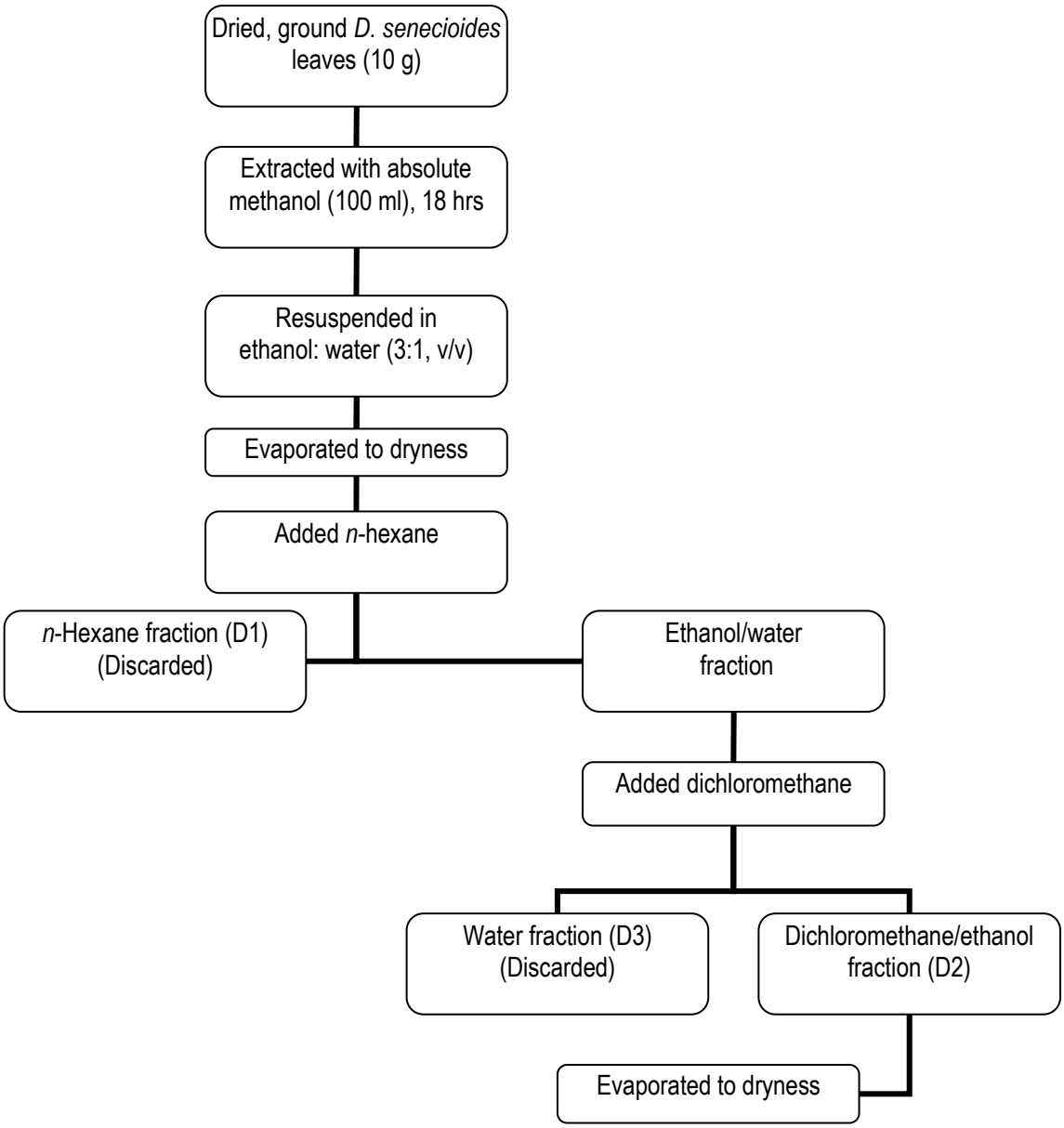

Figure 2.1: Fractionation scheme of ground *D. senecioides* leaves to obtain D2 fraction.

2.2.2 Cell Culture

A murine macrophage cell line, RAW 264.7, was cultured in RPMI-1640 growth medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) PSN (penicillin, streptomycin, neomycin) antibiotic cocktail at 37°C in a humidified atmosphere of 5% CO_2 – 95% air.

2.2.3 MTT Cytotoxicity Assay

Cell viability of D2-treated cells was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (van der Loosdrecht *et al.* 1994). Cells were seeded at 2 x 10^5 cells/ml into 96-well cell culture plates (Nunc™, Roskilde, Denmark) and incubated at 37°C overnight to allow the cells to attach. The cells were exposed to various concentrations (50 µg/ml to 250 µg/ml) of the D2 fraction redissolved in dimethylsulfoxide (DMSO). A mock sample (cells, RPMI-1640 and 0.005% DMSO) served as a negative control. The plates were incubated at 37°C for up to 72 hours after which 40 µl of 5 mg/ml MTT was added to each well followed by incubation at 37°C for 3 hours. The medium was aspirated and the remaining cells were washed once with pre-warmed PBS, pH 7.4. The cells were redissolved in 50 µl DMSO and the absorbance was measured at 595 nm using a microtiter-plate multimode detector. The percentage of viable cells was calculated as follows:

$$\% \text{ viability} = \frac{[A_{595} \text{ of control} - A_{595} \text{ of sample}]}{A_{595} \text{ of control}} \times 100$$

2.2.4 Real Time Cell Analysis

The effect of the D2 fraction on cell proliferation was also assessed by real time cell analysis where RAW 264.7 macrophages were cultured for 24 hours at 37°C in a 16-well plate (E-plate 16) docked in a real time cell analysis dual plate (RTCA DP) analyzer. The cells were then treated with the D2 fraction as above (Section 2.2.3) and cell densities of the treated cells were quantitatively monitored for 48 hours at 37°C and reflected by cell-index values.

2.2.5 Morphological Evaluation of Apoptosis

The D2 fraction was evaluated for pro-apoptotic potential by microscopic analysis of the DNA of extract-treated cells. Apoptotic nuclei are identified by condensed chromatin gathering at the periphery of the nuclear membrane or totally fragmented apoptotic bodies. Briefly, cells were exposed to various concentrations of D2 fraction (50 µg/ml to 250 µg/ml) for 24 hours and washed with PBS. The cells were then stained with 4′-6-diamidino-2-phenylindole

(DAPI), which forms fluorescent complexes with double-stranded DNA by binding in the minor groove of the nucleic acid backbone (www.celldeath.de/apometh/dapi.html). Cells were viewed under a fluorescence microscope.

2.2.6 DCFH-DA Oxidation Assay

The effect of the D2 fraction on ROS production in RAW 264.7 cells was analysed by a fluorescence assay using 2′,7′-dichlorofluorescin diacetate (DCFH-DA). DCFH-DA diffuses through the cell membrane and is hydrolyzed by esterases to non-fluorescent 2′,7′-dichlorofluorescin (DCFH) which is then rapidly oxidized by intracellular ROS to a highly fluorescent 2′,7′-dichlorofluorescein (DCF) (LaBel *et al.*, 1992). The fluorescence intensity of DCF is directly proportional to ROS produced by cells. For experimental setup, RAW 264.7 cells were grown to 90% confluency on microscope-slide cover-slips and treated with various concentrations of D2 fraction for a maximum of 24 hours. A mock sample (cells, RPMI-1640 and 0.005% DMSO) served as a negative control while cells treated with 30 µg/ml apocynin served as a positive control. Cells were then stimulated with 5 nM phorbol 12-myristate 13-acetate (PMA) and 10 µM DCFH-DA was added as a fluorescent probe for detection of available reactive oxygen species (ROS) released by the cells. Following 10 minutes of incubation at room temperature, cells were viewed under an inverted fluorescence microscope.

2.2.7 Fluorimetric DCFH-DA Oxidation Assay

A fluorimetric assay was used to quantify the amount of DCFH-DA oxidized by macrophage-generated ROS. For this assay, RAW 264.7 cells were seeded at 2.5 x 10^5 cells/ml into 96-well black plates (Nunc™, Roskilde, Denmark) and allowed to attach overnight at 37°C. After treatment with 50 µg/ml to 250 µg/ml of the D2 fraction for 1 hour, the cells were loaded with 10 µM DCFH-DA and stimulated with 5 nM PMA for 10 minutes. Fluorescence intensity was measured using a microtiter-plate multimode detector with excitation and emission wavelengths of 483 nm and 535 nm, respectively. A mock sample (cells, DMSO and RPMI-1640) served as a negative control while cells treated

with 30 µg/ml apocynin served as a positive control. ROS production was calculated as a percentage of the fluorescence of resting cells.

2.2.8 iNOS Activity Assay

To determine the effect of the D2 fraction on iNOS activity in RAW 264.7 cells, nitrite formation was measured as an indicator of NO synthesis. Cells were seeded at 2.5 x 10^5 cells/ml into 96-well plates in phenol-free RPMI-1640 and allowed to attach overnight at 37°C. The attached cells were then treated with various concentrations of the D2 fraction (50 µg/ml to 250 µg/ml) for 1 hour followed by activation with 500 ng/ml lipopolysaccharide (LPS). After incubation at 37°C for 18 hours the amount of nitrite was measured by adding supernatants to an equal volume of Griess reagent (1% sulfanilamide and 0.1% ethylene diaminedihydrochloride in 5% H_3PO_4) and reading the absorbance at 550 nm after 30 minutes. The concentration of nitrite synthesized was intrapolated from a standard curve generated using sodium nitrite ($NaNO_2$) (Jin *et al.*, 2010).

2.2.9 Semi-Preparative High Performance Liquid Chromatography

The D2 fraction was further fractionated into 96 sub-fractions using reverse-phase, semi-preparative high performance liquid chromatography (HPLC) using a chemstation equipped with a quaternary pump, diode array detector, autosampler, column thermostat and automatic fraction collector. To obtain the profile of the extract, the following gradient system was used: 25% A with 0% B and 75% C at 0 minutes; gradient to 25% A with 75% B and 0% C at 20 minutes, and finally 25% A with 0% B and 75% C at 30 minutes (Solvent A: methanol, Solvent B: acetonitrile, Solvent C: water). The fraction was sub-fractionated using 25% A, 0% B and 75% C. The column was thermostated at 60°C and the pressure kept at 400 bar. An injection volume of 400 µl was used with a flow rate of 5 ml/min. Analyte detection was performed between 260 and 360 nm.

2.2.10 Phytochemical Analysis by Thin Layer Chromatography

Phytochemical compounds of the D2 HPLC sub-fractions were analyzed by spotting each of the 96 samples (redissolved to 10 mg/ml in DMSO) onto

aluminium-backed, silica gel thin layer chromatography (TLC) plates (Macherey-Nagel, Düren, Germany) and further development of the plates in EMW (ethylacetate: methanol: water, 10:1.35:1, v/v/v). The chromatograms were visualised under ultraviolet light to detect UV-active compounds. The plates were then sprayed with vanillin (0.1 g vanillin, 28 ml methanol, 1 ml sulphuric acid) and heated at 110^{o}C for 3 minutes in an oven to develop colour.

2.2.11 TLC-DPPH Antioxidant Screening

The HPLC sub-fractions were assayed for antioxidant activity by chromatographic separation of the sub-fractions as above (Section 2.2.9) and spraying the plates with 0.2% 2,2-diphenyl-1-picryl-hydrazyl (DPPH) in absolute methanol to visualise the antioxidant compounds present in the fractions. DPPH is reduced from violet to a yellow diphenylpicryl hydrazine in the presence of an antioxidant compound (Pourmorad *et al.*, 2006).

2.2.12 DPPH Radical-Scavenging Activity Assay

The antioxidant activity of each of the 96 HPLC sub-fractions was quantitatively evaluated by a spectrophotometric assay where the percentage of DPPH reduced to hydrazine was measured. This was achieved by adding 0.2% DPPH to 200 µg/ml of each fraction in a 96-well plate and incubating the plate at room temperature for 30 minutes before measuring the absorbance at 490 nm using a microtiter-plate multimode detector. DMSO was used as a blank. The scavenging activity was calculated as follows:

$$\% \text{ inhibition} = \frac{[A_{490} \text{ of blank} - A_{490} \text{ of sample}]}{A_{490} \text{ of blank}} \times 100$$

2.2.13 Ferric Reduction Antioxidant Potential Assay

Reducing potential of D2 fraction, and the HPLC sub-fractions, was determined by means of the ferric reduction antioxidant potential (FRAP) assay: a simple and inexpensive test that measures reduction of ferric ions (Fe^{3+}) to the ferrous form (Fe^{2+}). Various concentrations of D2 fraction (50, 100 and 200 µg/ml) and 200 µg/ml of the 96 HPLC sub-fractions were

prepared in 100 µl of dH_2O. Vitamin C served as a positive control. A volume of 250 µl of phosphate buffer (0.2 M, pH 6.6) was added to each sample followed by 250 µl potassium ferricyanide [$K_3Fe(CN)_6$] (1% w/v). The samples were incubated at 50°C for 20 minutes before addition of 250 µl trichloroacetic acid (TCA) (10% w/v). The samples were then centrifuged at 3000 rpm for 10 minutes and 250 µl of the supernatant from each test sample was aspirated into a clean tube. An equal volume of dH_2O was added to the samples followed by 50 µl ferric chloride ($FeCl_3$) (0.1% w/v). Two hundred microlitres (200 µl) of this mixture was transferred to a 96-well microtiter plate and absorbance was measured at 595 nm using a microtiter-plate multimode detector.

2.2.14 Statistical Analysis

The results of each series of experiments are expressed as mean values ± standard error of the mean (SEM). Levels of statistical significance were calculated using the paired student t-test when comparing two groups, or by analysis of variance (ANOVA) with subsequent Tukey-Kramer multiple comparisons test for multiple groups. *P* values of ≤ 0.05 were considered significant.

CHAPTER 3
RESULTS

3.1 Anti-Proliferative Effect of D2 Fraction on RAW 264.7 Cells

The effect of the D2 fraction on proliferation of RAW 264.7 cells was analyzed by determining viability of treated cells using the MTT assay and real time cell analysis. The D2 fraction decreased cell viability in a time- and dose-dependent mode (Figs. 3.1 and 3.2). Treated cells were shown to die through apoptosis when stained with DAPI nucleic acid stain (Fig. 3.3). The DAPI stain is sensitive to DNA conformation and the state of chromatin in cells, and is thus used to grade nuclear damage. Cells dying through apoptosis displayed nuclei that stained bright blue as their chromatin was condensed, while those that had reached advanced stages of apoptosis showed totally fragmented morphology of nuclear (apoptotic) bodies that also stained bright blue (Fig 3.3).

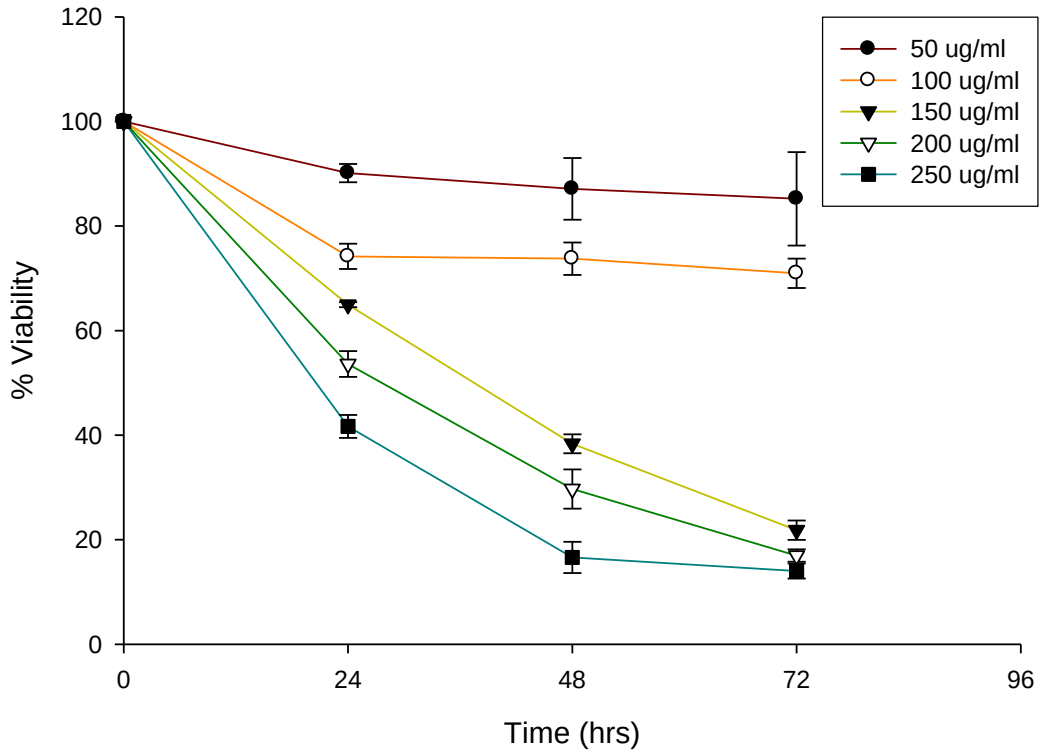

Figure 3.1: The effect of D2 fraction on the viability of RAW 264.7 cells. Cells were treated with various concentrations of the D2 fraction for 72 hours followed by viability determination by the MTT assay. Viability was calculated as percentage of untreated control cells (0.005% dimethylsulfoxide control). The results represent the mean of two independent experiments, each done in duplicate.

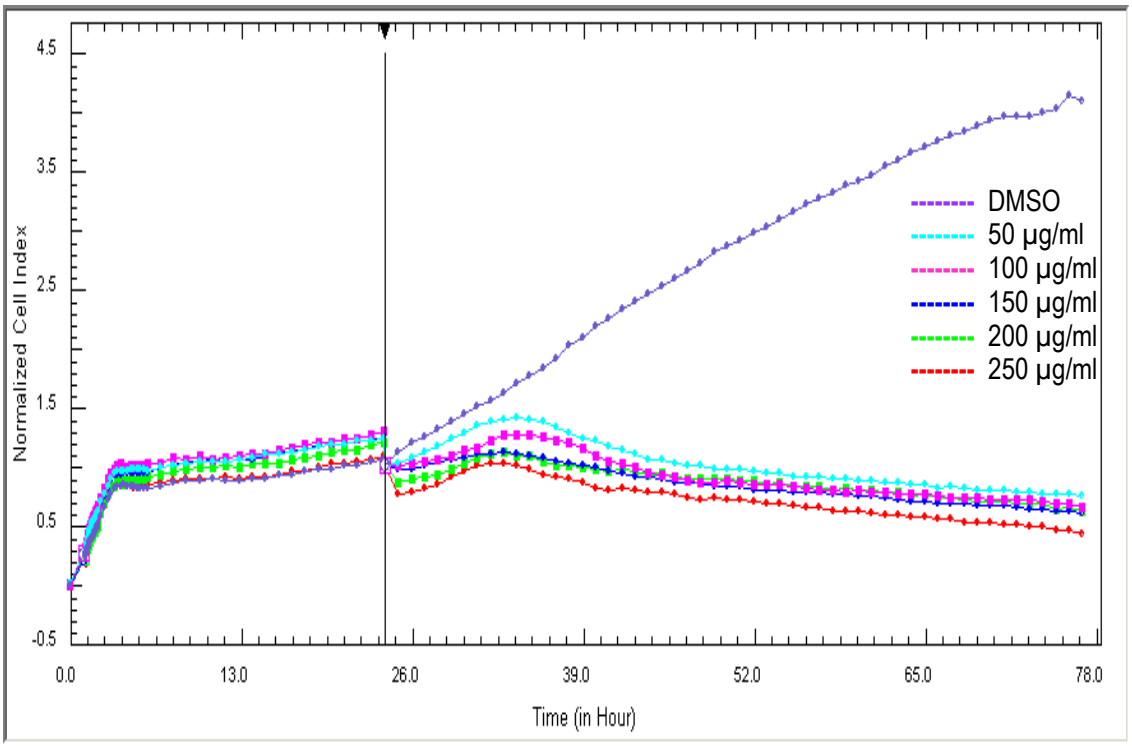

Figure 3.2: Real time cell analysis plot of RAW 246.7 cells treated with the D2 fraction. Cells were cultured for 24 hours followed by treatment with various concentrations of the D2 fraction for 48 hours. Proliferation was measured by continuously monitoring cell indices using an RTCA DP analyzer. Cell indices were normalized at 24 hours. DMSO: 0.005% dimethylsulfoxide.

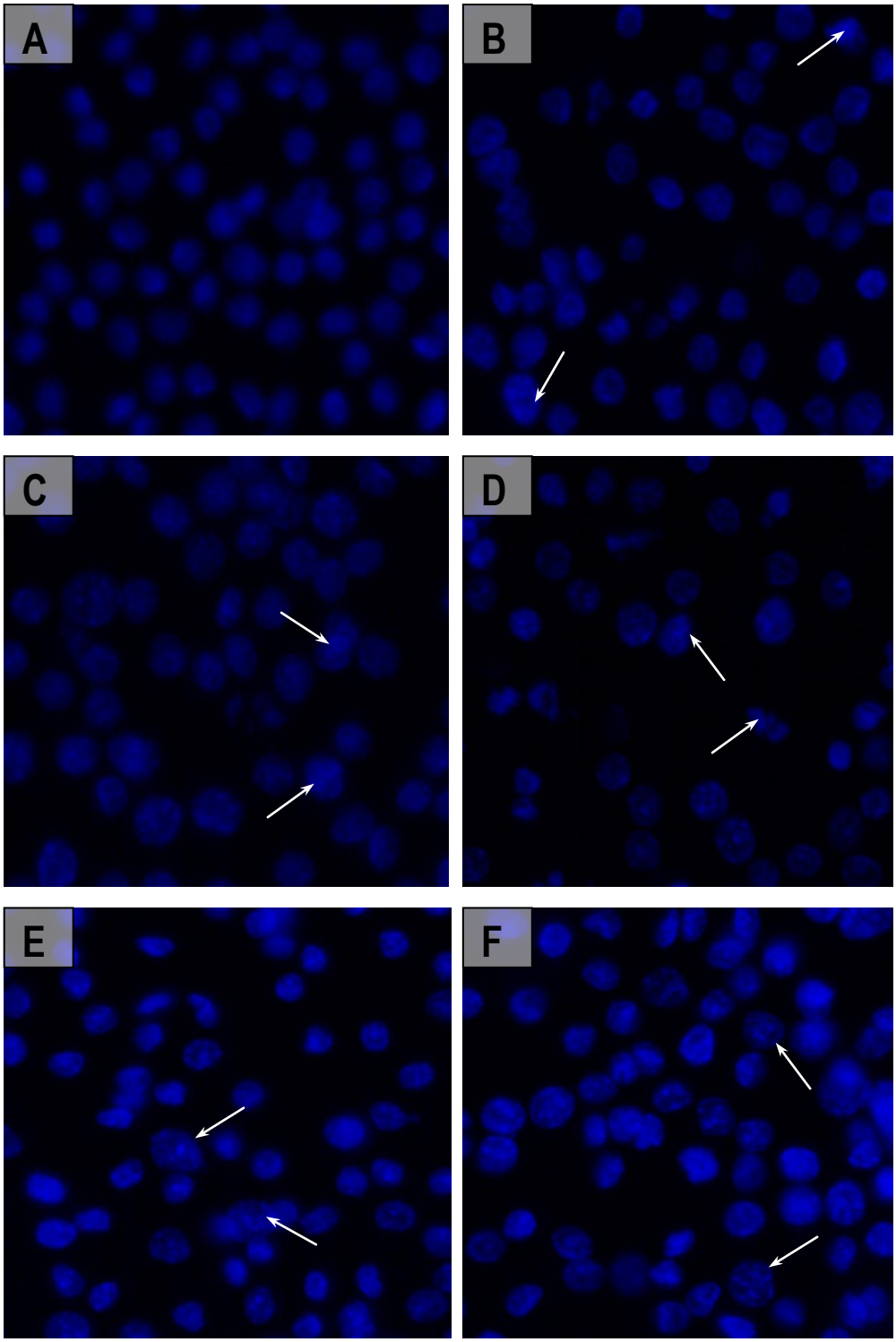

Figure 3.3: DAPI nucleic acid staining showing apoptotic morphology in RAW 264.7 cells exposed to a range of concentrations of *D. senecioides* D2 fraction for 24 hours. Cells were photographed under an inverted fluorescence microscope at 40X magnification. A = DMSO control, B = 50 µg/ml, C = 100 µg/ml, D = 150 µg/ml, E = 200 µg/ml and F = 250 µg/ml. Arrows indicate nuclear shrinkage and chromatin condensation.

3.2 Effect of D2 Fraction on ROS and NO Production in RAW 264.7 Cells

The effect of the D2 fraction on ROS production by PMA-stimulated RAW 246.7 cells was assessed by a fluorescent DCFH-DA assay. The intensity of the fluorescence is proportional to ROS produced within the cells. Cells treated with various concentrations of the D2 fraction exhibited lower fluorescence intensity compared to untreated, stimulated cells (Fig. 3.4). Fluorescence of the D2-treated cells was similar in strength to that of unstimulated cells, as well as that of cells treated with apocynin (a positive control).

ROS produced by RAW 264.7 cells was quantified by a fluorimetric DCFH-DA assay where the fluorescence of D2-treated, stimulated cells was measured with a micro-titer plate reader and compared to fluorescence of resting cells. Fluorescence of resting cells was taken as 100%. The D2 fraction impeded ROS production in all treated cells as fluorescence of treated cells remained at values close to 100%, while that of untreated cells increased by 100% to 206% (Fig. 3.5). All experimental concentrations of the D2 fraction had the same effect on the production of ROS, which was comparable to the effect of apocynin on the cells.

The D2 fraction inhibited nitrite formation in treated RAW 264.7 cells, signifying inhibition of NO production by iNOS. The inhibitory effect of the D2 fraction increased with an increase in concentration; with concentrations above, and including, 100 µg/ml resulting in NO concentrations that were lower than that observed in resting cells (Fig. 3.6).

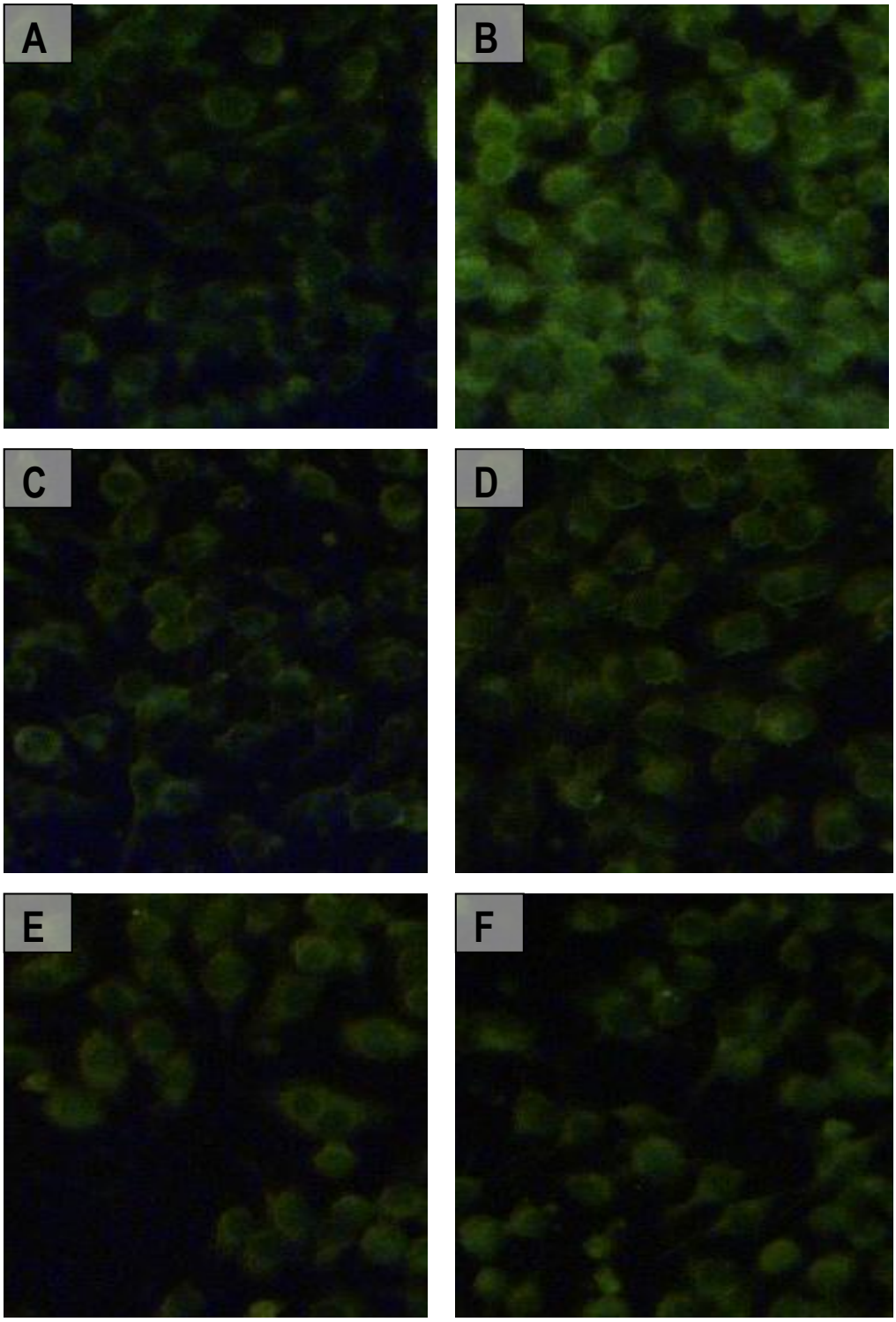

Figure 3.4: DCFH-DA fluorescence of RAW 264.7 cells treated with various concentrations of D2 fraction. Cells were treated with the D2 fraction for 1 hour followed by addition of DCFH-DA and stimulation with PMA for 10 minutes. Cells were viewed under a fluorescence microscope at 40X magnification. A = resting, B = DMSO, C = 50 µg/ml, D = 100 µg/ml, E = 200 µg/ml and F = 30 µg/ml apocynin.

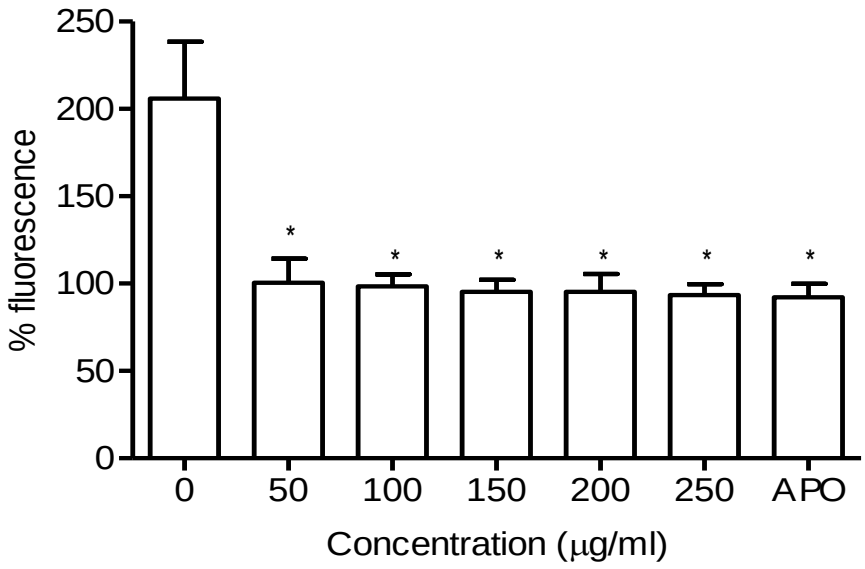

Figure 3.5: D2-induced inhibition on ROS production in RAW 264.7 cells. Cells were treated with the D2 fraction for 1 hour followed by addition of DCFH-DA and stimulation with PMA for 10 minutes. Fluorescence of resting cells was taken as 100% (not shown) and fluorescence of treated cells was compared to the resting fluorescence. APO = 30 μg/ml apocynin (positive control). * Statistically significant ($P < 0.05$)

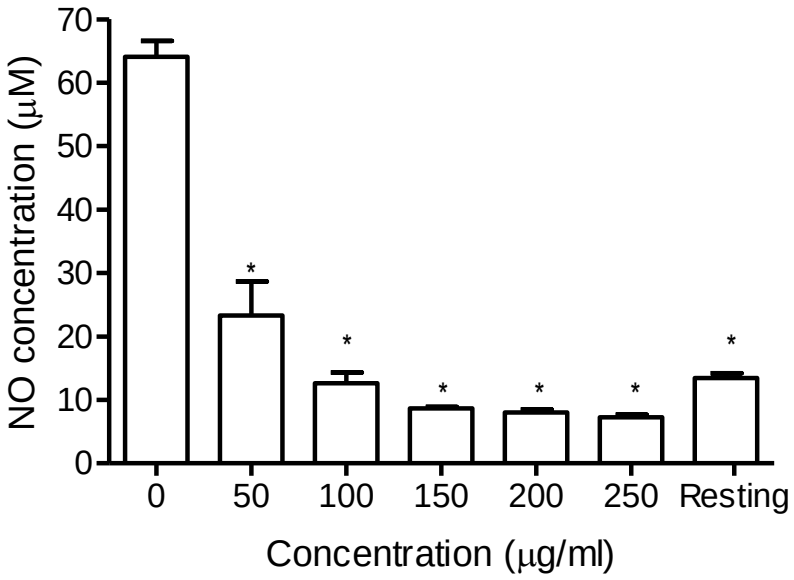

Figure 3.6: D2-induced inhibition of NO production in RAW 264.7 cells. Cells were treated with the D2 fraction for 1 hour followed by stimulation with 500 ng/ml LPS for 18 hours. Griess reagent was added to supernatants before reading the absorbance at 550 nm. Resting cells were not challenged with LPS.

* Statistically significant ($P < 0.05$)

3.3 Semi-Preparative HPLC of D2 Fraction

Fractionation of the D2 fraction into 96 semi-purified sub-fractions was achieved using methanol-water 25:75 (v/v) at a flow rate of 5 ml/min.

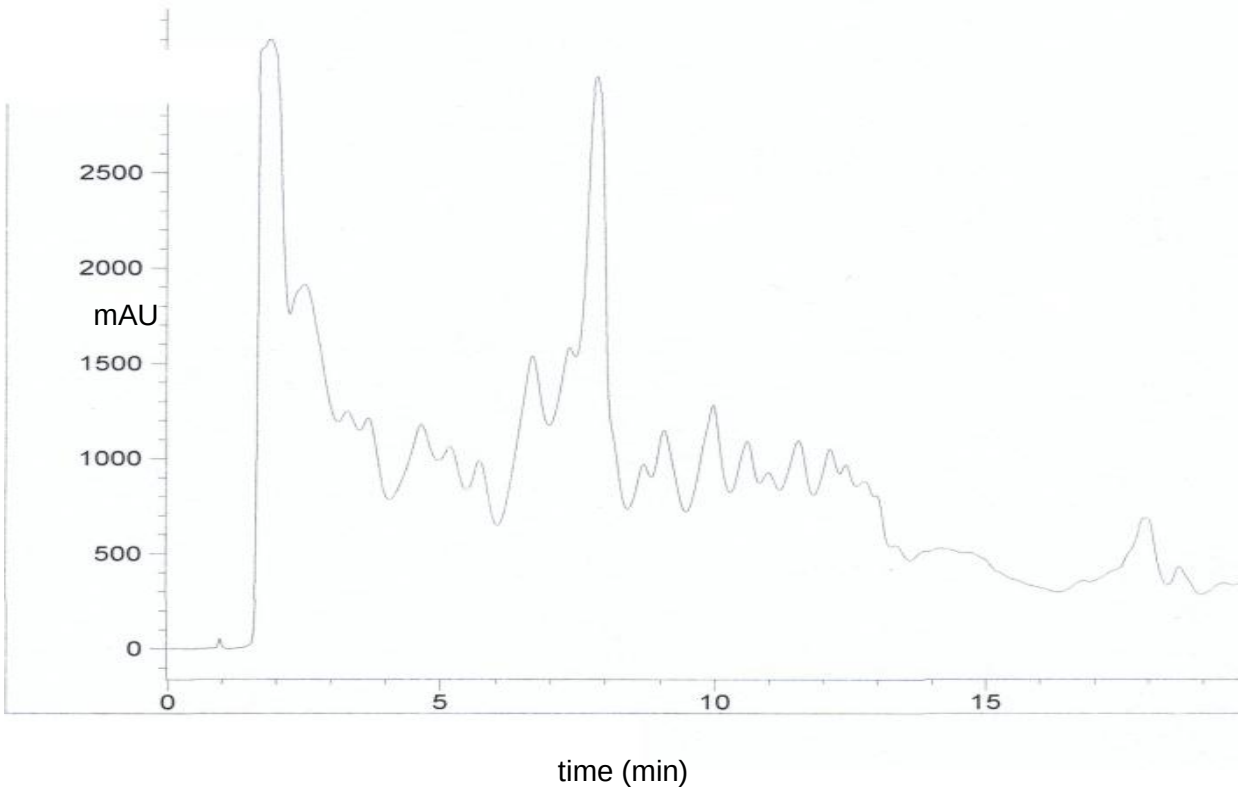

Figure 3.7: Semi-preparative HPLC fingerprint of D2 fraction fractionated with methanol-water 25:75 (v/v) at a flow rate of 5 ml/min. Analyte detection was performed between 260 and 360 nm using a diode array detector.

3.4 TLC-DPPH Antioxidant Screening of D2 HPLC Sub-fractions

Thin layer chromatographs developed in EMW 10:1.35:1 (v/v/v) and sprayed with DPPH were used to screen HPLC sub-fractions for compounds with anti-oxidative activity. An antioxidant compound was present in HPLC sub-fractions 1-43 but strongly pronounced in fractions 1-7 and 33-39, as shown by the high intensity of the yellow colour of the compound in these sub-fractions (Fig. 3.8F-I). The R_f value of the compound in all the fractions was either 0.27 or 0.28, indicating that the same compound is responsible for the scavenging of DPPH in all the fractions.

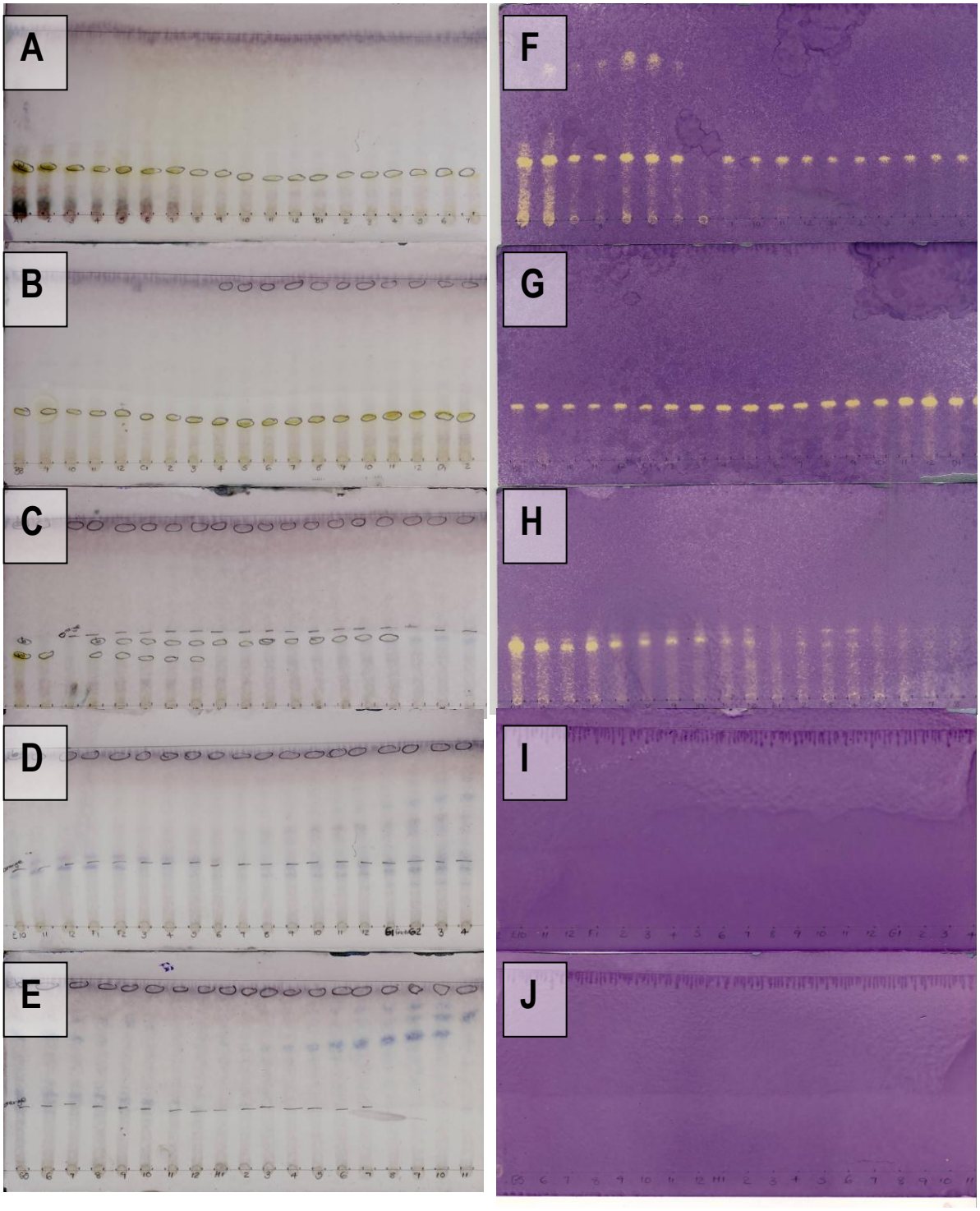

Figure 3.8: Thin layer chromatography profiles of HPLC sub-fractions of the D2 fraction of *Dicerocaryum senecioides* developed in EMW (10:1.35:1, v/v/v). A-E: Plates were sprayed with vanillin-sulphuric acid and heated at 110°C. UV-fluorescent compounds are circled. F-J: Plates sprayed with 0.2% DPPH. Yellow spots are indicative of free-radical-scavenging compounds. A & F = sub-fractions 1-19, B & G = sub-fractions 20-38, C & H = sub-fractions 39-57, D & I = sub-fractions 58-79, E & J = sub-fractions 80-96.

3.5 DPPH Radical Scavenging Assay of D2 HPLC Sub-fractions

A quantitative DPPH-scavenging assay was carried out for quantification of the DPPH scavenged by D2 fraction and its HPLC sub-fractions. Sub-fractions 1-7 and 33-39 quenched more than 50% of the DPPH; moreover, these sub-fractions showed better scavenging activity than crude D2 fraction at the same concentrations (Figs. 3.9–3.11).

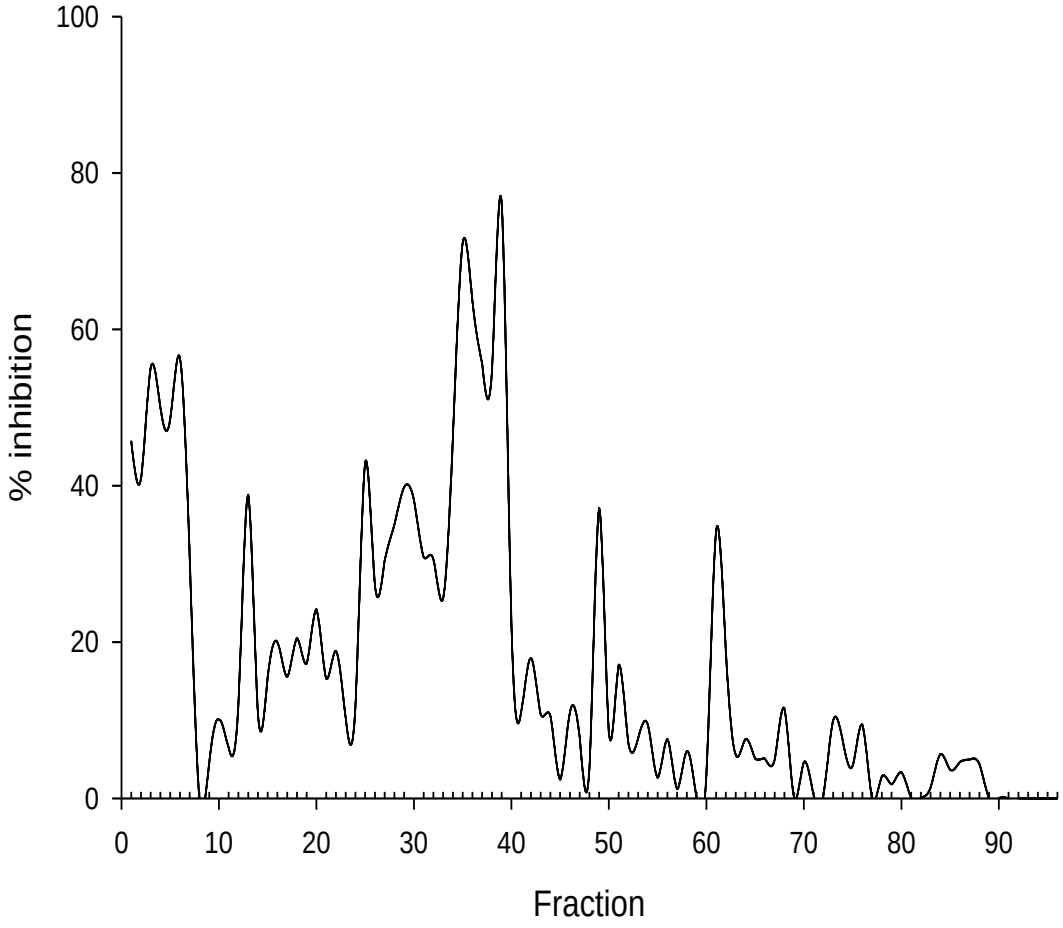

Figure 3.9: DPPH radical scavenging activity profile of 96 HPLC sub-fractions of crude D2 fraction of *D. senecioides.* A solution of 0.2% DPPH in methanol (w/v) was added to 200 µg/ml of each sample and absorbance was read at 490 nm after 30 minutes.

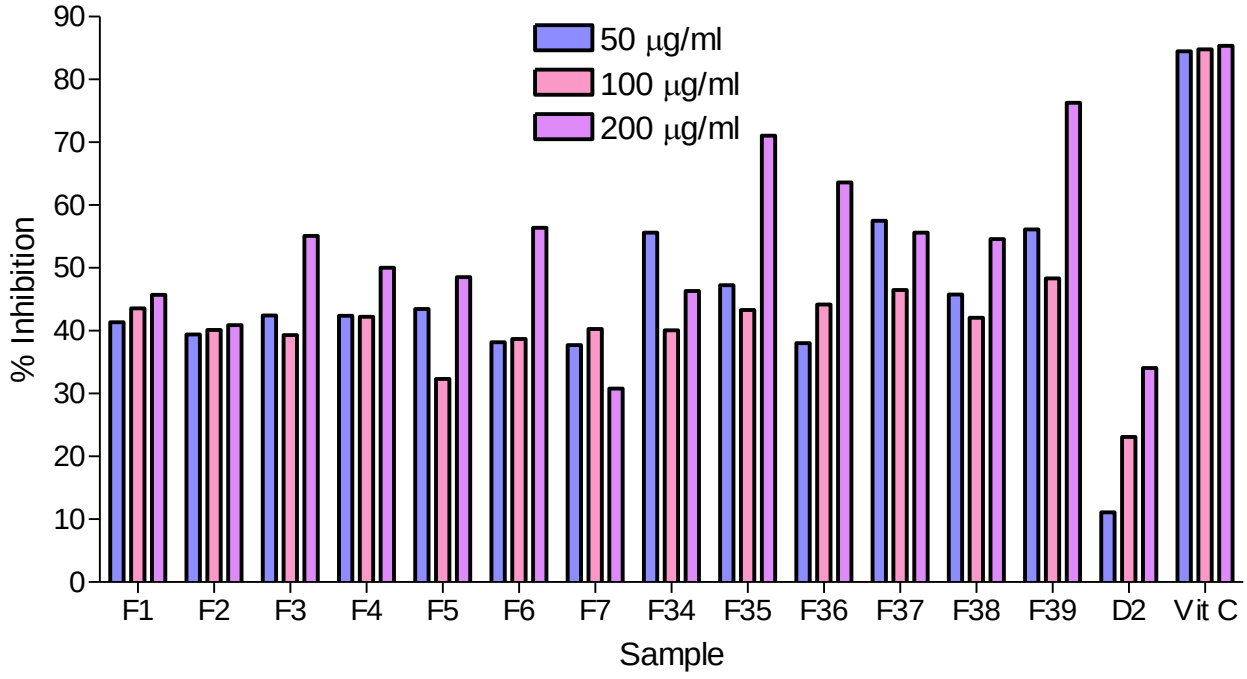

Figure 3.10: DPPH radical scavenging activity of D2 HPLC sub-fractions 1-7 and 34-39 of *D. senecioides.* Samples were diluted to 50, 100 and 200 µg/ml with DMSO followed by addition of 0.2% DPPH in methanol (w/v). Absorbance was read at 490 nm after 30 minutes. Vitamin C was used as comparator.

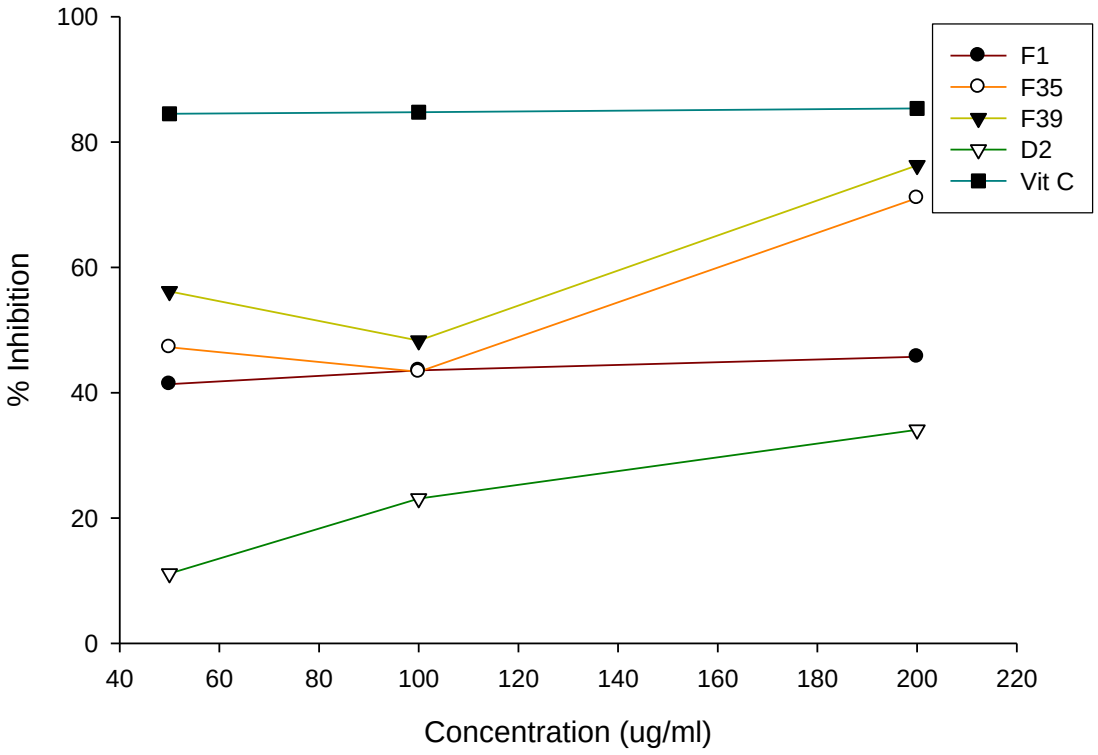

Figure 3.11: DPPH radical scavenging activity of D2 fraction and HPLC sub-fractions 1, 35 and 39 of *D. senecioides.* DPPH in methanol (0.2%, w/v) was added to each sample followed by an absorbance measurement at 490 nm after 30 minutes. Vitamin C was used as comparator.

3.6 FRAP Assay of D2 HPLC Sub-fractions

The reducing potential of the D2 fraction and its HPLC sub-fractions was evaluated by assessing their ability to reduce Fe^{3+} to Fe^{2+} (Fig 3.12). Sub-fractions 1-7 and 34-39 elicited higher reducing power than the crude D2 fraction at all concentrations used. Moreover, some of the sub-fractions showed a higher reducing power than vitamin C (Figs. 3.13-3.14).

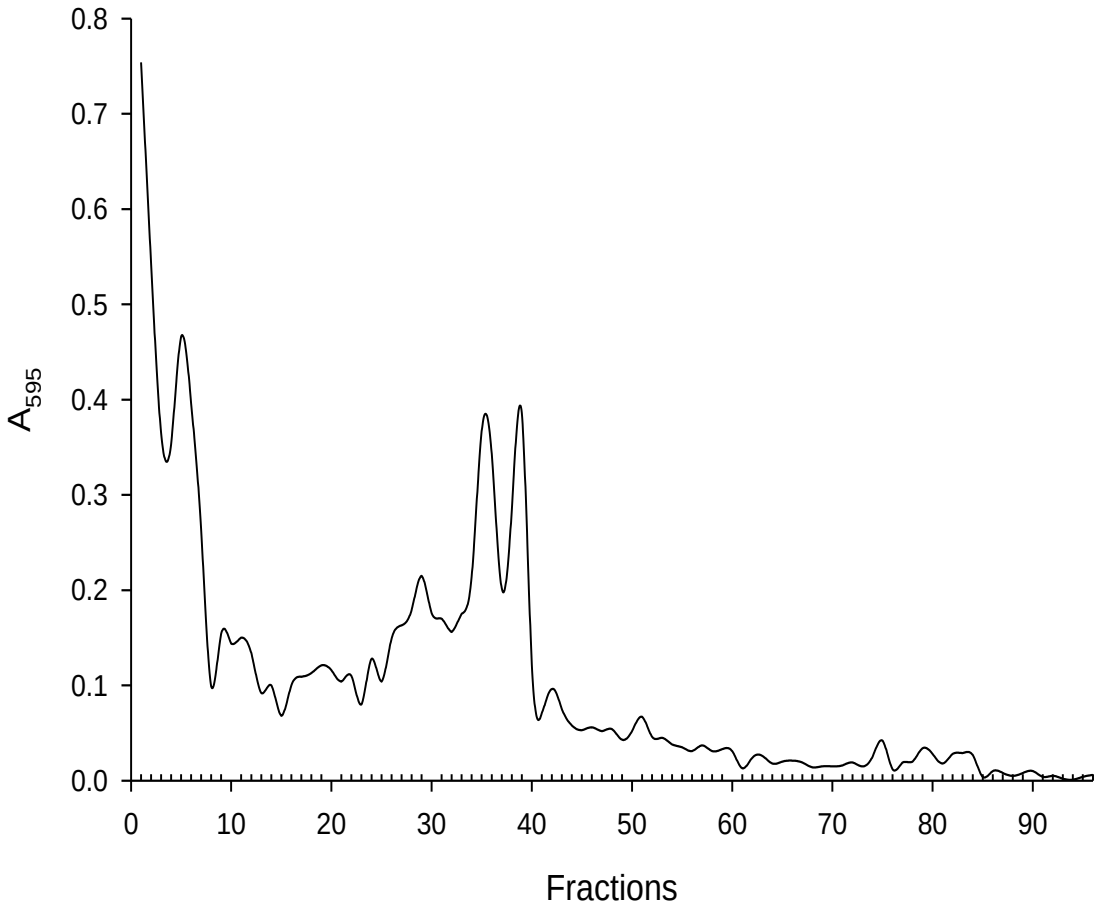

Figure 3.12: Ferric ion reducing power profile of 96 HPLC sub-fractions of crude D2 fraction of *D. senecioides.* Samples were diluted to 200 µg/ml with DMSO followed by addition of $K_3Fe(CN)_6$, TCA and $FeCl_3$. Absorbance was measured at 595 nm.

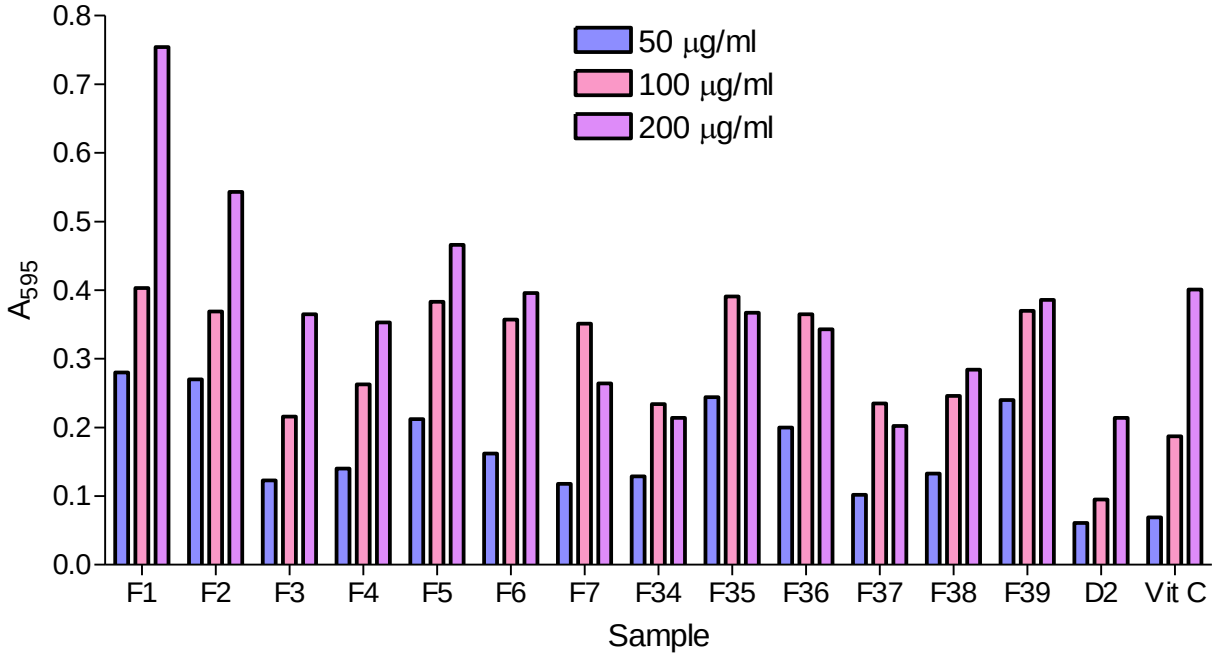

Figure 3.13: Ferric ion reducing power profile of HPLC sub-fractions 1-7 and 34-39 of crude D2 fraction of *D. senecioides.* Samples were diluted to 50, 100 and 200 µg/ml with DMSO followed by addition of $K_3Fe(CN)_6$, TCA and $FeCl_3$. Absorbance was read at 595 nm and vitamin C was used as a comparator.

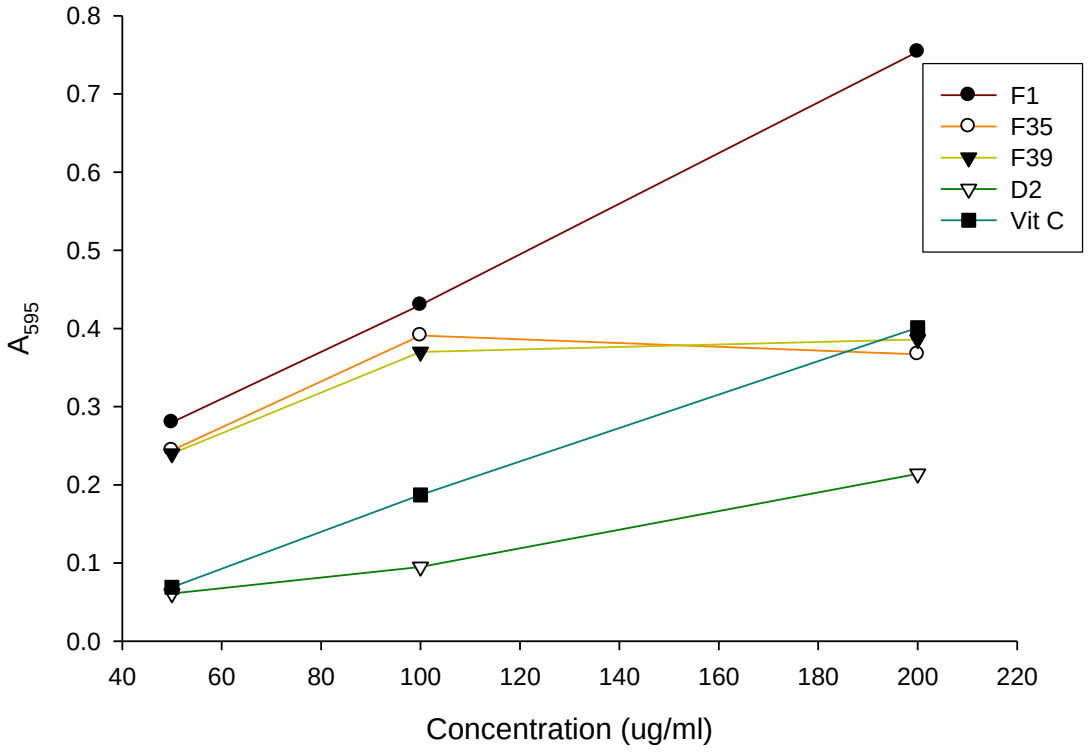

Figure 3.14: Ferric ion reducing power profile of D2 fraction and HPLC sub-fractions 1, 35 and 39 of *D. senecioides.* Samples were diluted to 50, 100 and 200 µg/ml with DMSO followed by addition of $K_3Fe(CN)_6$, TCA and $FeCl_3$. Absorbance was read at 595 nm and vitamin C was used as a comparator.

CHAPTER 4
DISCUSSION AND CONCLUSION

There is indisputable evidence that dysregulated inflammation is the underlying cause of many cancers, arthritis, as well as some neurodegenerative diseases (Halliwell *et al.*, 1992). It is therefore vital to identify and introduce anti-inflammatory therapies that will assist the body in controlling chronic inflammation. Numerous traditional medicinal plants have been documented to possess anti-inflammatory compounds and some of these compounds have already been isolated and incorporated into the pharmacology industry for synthesis of conventional drugs (Cragg and Newman, 2005). Nevertheless, many other plants have yet to be studied in order to validate their use for health purposes. In a previous study, the dichloromethane fraction of *Dicerocaryum senecioides* leaves (D2 fraction) was preliminarily found to exhibit anti-proliferative, anti-inflammatory and antioxidative properties. Further work was, therefore, essential to verify and substantiate the properties reported from this initial study.

The D2 fraction of *D. senecioides* was evaluated for anti-proliferative activity by analysis of its effect on the growth of RAW 264.7 cells, a murine macrophage cell line. The D2 fraction induced a decrease in cell viability in a concentration- and time-dependent manner (Figs 3.1 and 3.2). Figure 3.2 showed that the fraction results in a decrease in the cell index; a unitless, quantitative measure of the number of cells present in a given E-plate 16 well. The measurement of cell proliferation using an RTCA DP analyser allowed constant monitoring of cell death in real-time without interfering with cell behaviour. Cells were shown to die through apoptosis by staining the treated cells with DAPI nucleic acid stain. Cells undergoing apoptosis are characterised by cytoplasmic shrinkage, chromatin condensation, blebbing of the cell membrane and formation of apoptotic bodies (Wyllie *et al.*, 1980). These features were observed in cells treated with the D2 fraction for 24 hours (Fig. 3.3B-F). The chromatin of untreated cells remained intact and unaffected after 24 hours, as indicated by the even spread of the nucleic acid stain in Figure 3.3A.

The anti-inflammatory activity of the D2 fraction was assessed by determining its effect on RAW 264.7 murine macrophages. Macrophages are cells involved in the immune response and their function is to clear up invading microorganisms through phagocytosis, and the production of ROS that is used as a microbicidal agent. Under normal physiological conditions, macrophages produce ROS at basal levels, and this ROS is used primarily for cell signaling. During the immune response, however, macrophages receive signals that induce increased ROS production to obliterate assaulting microorganisms, after which ROS production discontinues. Nonetheless, there are occasions when there is prolonged generation of ROS, resulting in chronic inflammation; and in these rare cases an external agent is required to stop the macrophages from producing ROS. This may be accomplished by inhibiting enzymes known to generate the majority of ROS during inflammation, most notably NADPH oxidase.

The effect of the D2 fraction on ROS production in treated RAW 264.7 cells was evaluated by the DCFH-DA assay using a fluorescence microscope, where fluorescence of the DCF in the treated cells was compared to that of untreated and resting cells. The D2 fraction showed inhibition on the generation of ROS, as fluorescence intensity of the D2-treated cells was lower than that detected in the untreated cells (Fig. 3.4). Resting cells were not stimulated with PMA (a stimulant of many ROS-generating enzymes), consequently resulting in low fluorescence. Fluorescence of the treated cells was similar to that observed in the resting cells, as well as that of stimulated, apocynin-treated cells. Apocynin is a known inhibitor of NADPH oxidase, an enzyme chiefly responsible for superoxide production. Since the assay did not quantify the amount of ROS produced, a quantitative assessment of ROS production was thus conducted to substantiate these findings. While ROS-production in D2-treated cells remained almost unchanged after stimulation for 10 minutes, it increased two-fold in the untreated, stimulated cells (Fig. 3.5). The D2 fraction hindered the production of ROS, in a manner comparable to that of apocynin, thus indicating excellent anti-inflammatory potential. The ability of the D2 fraction to suppress oxidation of DCFH-DA to

DCF illustrates that the fraction may have successfully inhibited NADPH oxidase from generating O_2^- and all the other ROS resulting from it. These findings, however, do not specify the mode of inhibition of the oxidase by the D2 fraction; and further studies will have to be conducted to elucidate the mechanism of action of the D2 fraction on the inhibition of ROS production by NADPH oxidase.

During the inflammatory response, NO production by iNOS in macrophages increases, and the produced NO reacts with other molecules to yield various RNOS that assist cells in eradicating invading pathogenic microorganisms. Unfortunately, the overproduction of these RNOS is also implicated in inflammatory disorders and mutagenesis (Lechner *et al.*, 2005). iNOS activity thus needs to be regulated in order to prevent excess production of NO during inflammation. The effect of the D2 fraction on iNOS activity was indirectly assessed by measuring nitrite accumulation in LPS-activated cells using the Griess reagent. Treatment of RAW 264.7 cells with the D2 fraction significantly reduced nitrite formation in a dose-dependent manner, showing inhibition of NO synthesis by iNOS. It is, however, unknown whether the decrease in NO synthesis is due to inhibition of the expression of iNOS or direct inhibition of its activity. The effect of the D2 fraction on the induction of iNOS expression will need to be further evaluated using Western blot and classical enzyme activity analyses.
Since free-radicals are implicated in tumour formation and progression, the ability of the D2 fraction to hinder reactive nitrogen and oxygen species (RNOS) production illustrates chemoprotective and/or chemotherapeutic activity. The fraction inhibits formation of RNOS that, if produced in excess amounts, could otherwise result in oxidative DNA damage as well as effecting inhibition of the activity of key enzymes involved in DNA repair.

During inflammation, excess NO produced by iNOS reacts with O_2^- to produce $ONOO^-$, a highly reactive radical that causes remarkable DNA damage. In addition, this molecule also plays a role in activating both COX isoforms (Salvemini *et al.*, 1993). It is also interesting to note that some of the transcription factors that regulate iNOS also regulate the inducible COX

isoform, COX-2 (Posadas *et al.*, 2000). Overexpression of both iNOS and COX-2 has been observed in numerous cancer cells, perhaps as a result of this co-regulation between the two enzymes. The observed reduction in NO synthesis by the D2 fraction in RAW 264.7 cells could be occurring concurrently with COX-2 inhibition. However, further experiments need to be conducted to assess the effect of the D2 fraction on COX-2 activity. If both these enzymes are inhibited by the D2 fraction, it could be due to the action of the fraction on the expression or activities of upstream transcription factors that co-regulate both enzymes, and not necessarily through separate modulation of the individual enzymes. It is well-appreciated that phytochemicals often exert multiple effects that involve more than one signaling pathway, and these effects may be antagonistic, synergistic or additive. Indeed, the D2 fraction elicited activities in various cellular pathways by inducing apoptosis and inhibiting both NADPH oxidase and iNOS activities in murine macrophages.

Bioactive compounds within plant extracts are blended with other ineffectual compounds, and the activity of the compounds of interest is often dampened or masked by these unwanted compounds. Plant extracts therefore require purification in order to isolate the active compounds in their pure form, and/or to trim down the redundant, interfering components. The D2 fraction was found to possess antioxidative properties in a previous preliminary study from this laboratory. It was therefore essential to further fractionate the D2 fraction and to evaluate the antioxidative potential of the resultant sub-fractions alongside the crude D2 fraction.

Antioxidant compounds exert their effect either by scavenging free-radicals or by stabilizing the free-radicals through donation of an electron. The scavenging activity of the D2 fraction and its HPLC sub-fractions was assessed by screening each of the fractions for DPPH-quenching activity, using a TLC-DPPH screening method. Compounds with the ability to scavenge DPPH reduce it from a purple hydrazyl to a yellow hydrazine; the intensity of the yellow colour produced depends on the nature and quantity of the compound. Figure 3.7 illustrates the presence of a DPPH-scavenging

compound in HPLC sub-fractions 1-43; however, this particular antioxidant compound was intensely pronounced in sub-fractions 1-7 and 33-39. The R_f (reference to the solvent front) value of this antioxidant compound was either 0.27 or 0.28 in all the analysed sub-fractions, alluding to a similar compound. The difference in band intensities of the compound is therefore attributed to its varying quantities in the different sub-fractions.

The TLC-DPPH assay is a qualitative assay and does not provide the quantity of the DPPH reduced by an antioxidant compound. To obtain a quantitative measure of the reduced DPPH, a spectrophotometric assay was performed. Once again, fractions 1-7 and 33-39 elicited profound DPPH scavenging activity by quenching more than 50% of the DPPH solution. Interestingly, the semi-purified HPLC sub-fractions showed better activity than the crude D2 fraction, scavenging higher percentages of the free-radical than the crude fraction (Figs. 3.8-3.10), thus signifying an improvement in the scavenging ability of the D2 fraction following purification. It should be highlighted that the crude D2 fraction is a concoction of a multitude of compounds, and some of them may undeniably impede the free-radical scavenging activity of this fraction. Upon further fractionation, however, some of these contaminants were removed, thus enhancing the scavenging activity demonstrated by the HPLC sub-fractions.

Antioxidants may also act by donation of an electron to a free-radical (reduction), converting it to a more stable molecule. To assess the reducing potential of D2 HPLC sub-fractions, their ability to reduce the ferric ion (Fe^{3+}) to the ferrous (Fe^{2+}) form was evaluated. The FRAP assay differs from other antioxidant activity assays because no free radicals or oxidants are applied in the system (Cao and Prior, 1998). However, the ability of a compound to reduce Fe^{3+} indicates great reducing potential of that particular compound and it serves as a significant indicator of its antioxidant and anti-inflammatory activity (Meir *et al.*, 1995). Sub-fractions 1-7 and 34-39 demonstrated greater reducing potential than that of the crude D2 fraction. Furthermore, some of these sub-fractions showed reducing power that superseded that of vitamin C at the same concentrations used (Figs. 3.11-3.13), thus suggesting that these

sub-fractions could contain a potent antioxidant compound(s). Nonetheless, additional assays need to be done to further explore the remarkable reducing power elicited by these sub-fractions. From Figure 3.7, one can deduce that sub-fractions 1-7 contain the same compounds, with the quantity of these compounds decreasing from sub-fraction 1 down to sub-fraction 7. The same can be concluded about sub-fractions 33-39.

In conclusion, the D2 fraction of *D. senecioides* displayed properties of an ideal anti-inflammatory agent. Along with the inhibition of ROS and NO production in macrophages, the fraction also showed impressive antioxidant activity both as a free-radical scavenger and as a reducing agent. Moreover, the induction of apoptosis in macrophages suggests that the fraction has the ability to combat chronic inflammation at three stages: i.e., inhibiting initial free radical production, mopping up excess free radicals and eradicating macrophages responsible for the over-production of ROS. The pro-apoptotic activity of the D2 fraction also illustrates a commendable chemotherapeutic potential, as the fraction has the ability to induce cancer-cell death without eliciting the inflammatory response observed in necrotic cell death. Further studies should be aimed at evaluating the active HPLC sub-fractions of the D2 fraction for anti-inflammatory and anti-proliferative properties. The compounds in these fractions will then need to be isolated and identified.

REFERENCES

Aktan, F (2004). iNOS-mediated nitric oxide production and its regulation. *Life Sciences* **75**:639-653.

Alderton, W.K, Cooper, C.E and Knowles, R.G (2001). Nitric oxide synthases: structure, function and inhibition. *Biochemical Journal* **357(3)**:593-615.

Ambs, S, Merriam, W.G, Ogunfusika, M.O, Bennet, W.P, Ishibe, N, Hussain, S.P, Tzeng, E.E, Geller, D.A, Billiar, T.R and Harris, C.C (1998). p53 and vascular endothelial growth factor regulate tumour growth of NOS2-expressing human carcinoma cells. *Nature Medicine* **4(12)**:1371-1376.

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

Babior, B.M (2004). NADPH oxidase. *Current Opinion in Immunology* **16**:42-47.

Babior, B.M, Lambeth, J.D and Nauseef, W (2002). The neutrophil NADPH oxidase. *Archives of Biochemistry and Biophysics* **397(2)**:342-344.

Banerjee, R, Anguita, J, Roos, D and Fikrig, E (2000). Cutting edge: Infection by the agent of human granulocytic ehrlichriosis prevents the respiratory burst by down-regulating $gp91^{PHOX1}$. *The Journal of Immunology* **164**:3946-3949.

Barbeiri, S.S, Cavaica, V, Eligini, S, Brambilla, M, Caiani, A, Tremoli, E and Colli, S (2004). Apocynin prevents cyclooxygenase-2 expression in human monocytes through NADPH oxidase and glutathione redox-dependent mechanisms. *Free Radical Biology and Medicine* **37(2)**:156-165.

Barlow, S.M (1990). Toxicological aspects of antioxidants used as food additives. In *Food Antioxidants*. Hudson BJF (ed.) Elsevier, London. pp 253-307.

Barone, G, Corsaro, M.M, Giannattasio, R.L, Moscariello, M and Parrilli, M (1995). Structural investigation of the polysaccharide fraction from the mucilage of *Dicerocaryum zanguebaricum* Merr. *Carbohydrate Research* **280**:111-119.

Beckman, J.S (1991). The double-edged role of nitric oxide in brain function and superoxide-mediated injury. *The Journal of Developmental Physiology* **15(1)**:53-59.

Beckman, J.S, Ischiropoulos, H, Zhu, I, van der Woerd, M, Smith, C, Chen, J, Harrison, J, Martin, J.C and Tsai, M (1992). Kinetics of superoxide dismutases and iron-catalyzed nitration of phenolics by peroxynitrite. *Archives of Biochemistry and Biophysics* **298(2)**:438-445.

Bloodsworth, A, O'Donnell, V.B and Freeman, B.A (2000). Nitric oxide regulation of free radical- and enzyme-mediated lipid and lipoprotein oxidation. *Arteriosclerosis, Thrombosis and Vascular Biology* **20(7)**:1707-1715.

Bopana, N and Saxena, S (2007). *Asparagus racemosus* – ethnopharmacological evaluation and conservation needs. *The Journal of Ethnopharmacology* **110**:1-15.

Bogdan, C (2001). Nitric oxide and the immune response. *Nature Immunology* **2**:907-916.

Bokoch, G.M (1995). Regulation of the phagocyte respiratory burst by small GTP-binding proteins. *Trends in Cell Biology* **5**:109-113.

Bokoch, G.M and Knaus, U.G (2003). NADPH oxidases: not just for leukocytes anymore. *Trends in Biochemical Sciences* **28(9)**:502-508.

Branen, A.L (1975). Toxicology and biochemistry of butylated hydroxyanisol and butylated hydroxytoluene. *Journal of American Oil Chemists Society* **5**:59-63.

Brown, G.C (2001). Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome c oxidase. *Biochimica et Biophysica Acta* **1504(1)**:45-57.

Burg, N.D and Pillinger, M.H (2001). The neutrophil: function and regulation in innate and humoral immunity. *Clinical Immunology* **99**:7-17.

Cao, G and Prior, R.L (1998). Comparison of different analytical methods for assessing total antioxidant capacity of human serum. *Clinical Chemistry* **44**:1309-1315.

Celsus, A.C (1935). In *De medicina*. Spencer, W.G (ed. and trans.) Heinemann, London.

Chen, K, Huang, J, Gong, W, Iribarren, P, Dunlop, N.M and Wang, J.M (2007). Toll-like receptors in inflammation, infection and cancer. *International Immunopharmacology* **7**:1271-1285.

Chung, H.T, Pae, H.O, Choi, B.M, Billiar, T.R and Kim, Y.M (2001). Nitric oxide as a bioregulator of apoptosis. *Biochemical and Biophysical Research Communications* **282**:1075-1079.

Conner, E.M and Grisham, M.B (1996). Inflammation, free radicals, and antioxidants. *Nutrition* **124**:274-277.

Cragg, G.M and Newman, D.J (2005). Plants as a source of anti-cancer agents. *Journal of Ethnopharmacology* **100**:72-79.

Crofford, L.J (1997). COX-1 and COX-2 tissue expression: implications and predictions. *The Journal of Rheumatology* **24(49)**:15-19.

Crofford, L.J, Lipsky, P.E, Brooks, P, Abramson, S.B, Simon, L.S and van de Putte, L.B (2000). Basic biology and clinical applications of specific COX-2 inhibitors. *Arthritis and Rheumatism* **43**:4-13.

Dagher, M-C, Fuchs, A, Bourmeyster, N, Jouan, A and Vignais, P.V (1995). Small G proteins and the neutrophil NADPH oxidase. *Biochimie* **77**:651-660.

Dastidar, S.G, Rajagopal, D and Ray, A (2007). Therapeutic benefit of PDE4 inhibitors in inflammatory diseases. *Current Opinion in Investigational Drugs.* **8**:364-372.

Dröge, W (2002). Free radicals in the physiological control of cell function. *Physiological Reviews* **82**:47-95.

Dusi, S, Donini, M and Rossi, F (1995). Mechanisms of NADPH oxidase activation in human neutrophils: $p67^{PHOX}$ is required for the translocation of Rac1 but not of Rac2 from cytosol to the membranes. *Biochemical Journal* **308**:991-994.

Edwards, S.W and Watson, F (1995). The cell biology of phagocytes. *Immunology Today* **16(11)**:508-510.

Eissa, N.T, Yuan, J.W, Haggerty, C.M, Choo, E.K, Palmer, C.D and Moss, J (1998). Cloning and characterization of human inducible nitric oxide synthase splice variants: a domain, encoded by exons 8 and 9, is critical for dimerization. *Proceedings of the National Academy of Sciences* **95(13)**:7625-7630.

Etken, N.L (1986). Multi-disciplinary perspectives in the interpretation of plants used in indigenous medicine and diet. Redgrave Publishing Company. New York. pp 2–29.

Fawole, O.A, Amoo, S.O, Ndhlala, A.R, Light, M.E, Finnie, J.F and van Staden, J (2010). Anti-inflammatory, anticholinesterase, antioxidant and phytochemical properties of medicinal plants used for pain-related ailments in South Africa. *The Journal of Ethnopharmacology* **127**:235-241.

Ferreiro, C.R, Chagas, A.C.P, Carvalho, M.H.C, Dantas, A.P, Jantene, M.B, de Souza, L.C.B and da Luz, P.L, (2001). Influence of hypoxia on nitric oxide synthase activity and gene expression in children with congenital heart disease: a novel pathophysiological adaptive mechanism. *Circulation* **103(18)**:2272-2276.

Ferrero-Miliani, L, Nielsen, O.H, Andersen, P.S and Girardin, S.E (2006). Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1β generation. *Clinical and Experimental Immunology* **147**:227-235.

Fialkow, L, Wang, Y and Downey, G.P (2007). Reactive oxygen and nitrogen species as signaling molecules regulating neutrophil function. *Free Radical Biology and Medicine* **42**:153-164.

Forbes, L.V, Moss, S.J and Segal, A.W (1999). Phosphorylation of $p67^{PHOX}$ in the neutrophil occurs in the cytosol and is independent of $p47^{PHOX}$. *FEBS Letters* **449**:225-229.

Forrester, K, Ambs, S, Lupold, S.E, Kapust, R.B, Spillare, E.A, Weingerg, W.C, Felley-Bosco, E, Wnag, X.W, Geller, D.A, Tzeng, E, Billiar, T.R and Harris, C.C (1996). Nitric oxide-induced p53 accumulation and regulation of inducible nitric oxide synthase expression by wild-type p53. *Proceedings of the National Academy of Sciences* **93(6)**:2442-2447.

Frankel, E (1995). Nutritional benefits of flavonoids. International conference on food factors: *Chemistry and Cancer Prevention*, Hamamatsu, Japan. Abstracts, C6-2.

Fujii, H, Ichimori, K, Hoshia, K and Nakazawa, H (1997). Nitric oxide inactivates NADPH oxidase in pig neutrophils by inhibiting its assembling process. *The Journal of Biological Chemistry* **272(52)**:32773-32778.

Genaro, A.M, Hortelano, S, Alvarez, A, Martinez, C and Bosca, L (1995). Splenic B-lymphocyte programmed cell death is prevented by nitric oxide release through mechanisms involving sustained Bcl-2 levels. *The Journal of Clinical Investigation* **95**:1884-1890.

Gierse, J.K, McDonald, J.J, Hauser, S.D, Rangwala, S.H, Koboldt, C.M and Seibert, K (1996). A single amino acid difference between cyclooxygenase-1 (COX-1) and -2 (COX-2) reverses the selectivity of Cox-2 specific inhibitors. *The Journal of Biological Chemistry* **271(26)**:15810-15814.

Green, G.A (2002). Understanding NSAIDs: from aspirin to COX-2. *Sports Medicine* **3(5)**:50-59.

Gurib-Fakim, A (2006). Medicinal plants: Traditions of yesterday and drugs of tomorrow. *Molecular Aspects of Medicine* **27**:1-93.

Halliwell, B, Gutteridge, J.M.C and Cross, C. E (1992). Free radicals, antioxidants and human disease: Where are we now? *The Journal of Laboratory and Clinical Medicine* **119**:598-620.

Hampton, M.B, Kettle, A.J and Winterbourn, C.C (1998). Inside the neutrophil phagosome: oxidants, myeloperoxidase and bacterial killing. *Blood* **92(9)**:3007-3017.

Heyworth, P.G, Cross, A.R and Curnutte, J.T (2003). Chronic granulomatous disease. *Current Opinion in Immunology* **15**:578-584.

Hwang, T-L, Li, G-L, Lan, Y-H, Chia, Y-C, Hsieh, P-W, Wu, Y-H, Wu, Y-C (2009). Potent inhibition of superoxide anion production in activated human neutrophils by isopedicin, a bioactive component of the Chinese medicinal herb *Fissistigma oldhamii*. *Free Radical Biology and Medicine* **46**:520-528.

Jacob, C, Leport, M, Szilagyi, C, Allen, J.M, Bertrand, C and Lagente, V (2002). DMSO-treated HL-60 cells: a model of neutrophil-like cells mainly expressing PDE4B subtype. *International Immunopharmacology* **2**:1647-1656.
Jin, M, Suh, S-J, Yang, J.H, Lu, Y, Kim, S.J, Kwon, S, Jo, T.H, Kim, J.W, Park, Y.I, Ahn, G.W, Lee, C-K, Kim, C-H, Son, J-K, Son, K.H and Chang, H.W (2010). Anti-inflammatory activity of bark of *Dioscorea batatas* DECNE through the inhibition of iNOS and COX-2 expressions in RAW 264.7 cells via NF-κB and ERK1/2 inactivation. *Food and Chemical Toxicology* **48(11)**: 3037-3079.

Jones, R.D, Hancock, J.T and Morice, A.H (2000). NADPH oxidase: A universal oxygen sensor? *Free Radical Biology and Medicine* **29(5)**:416-424.

Kerr, J.F, Wyllie, A.H and Currie, A.R (1972). Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *The British Journal of Cancer* **26**:239-257.

Knowles, R.G and Moncada, S (1994). Nitric oxide synthases in mammals. *Biochemical Journal* **298(2)**:249-258.

Knowles, R.G (1996). Nitric oxide synthases. *Biochemical Society Transactions* **24(3)**:875-878.

Kogut, M.H, Genovese, K.J and Lowry, V.K (2001). Differential activation of signal transduction pathways mediation phagocytosis, oxidative burst, and

degranulation by chicken heterophils in response to stimulation with opsonized *Salmonella enteritidis*. *Inflammation* **25(1)**:7-15.

Korotkova, E.I, Avramchik, O.A, Yusubov, M.S, Belousov, M.V and Andreeva, T.I (2003). Determination of the antioxidant activity of plant extracts by means of cathode voltammetry. *Pharmaceutical Chemistry Journal* **3(9)**: 511-512.

Korsmeyer, S (1996). Regulators of cell death. *Trends in Genetics* **11**:101-105.

Koshkin, V and Pick, E (1994). Superoxide production by cytochrome b_{559}. Mechanism of cytosol-independent activation. *FEBS Letters* **338**:285.

Kroncke, K.D (2001). Cysteine-Zn^{2+} complexes: unique molecular switches for inducible nitric oxide synthase-derived NO. *The FASEB Journal* **15(13)**:2503-2507.

Kwong, C.H, Adams, A.G and Leto, T.L (1995). Chracterization of the effector-specifying domain of Rac involved in NADPH oxidase activation. *The Journal of Biological Chemistry* **270**:19868-19872.

LaBel, C.P, Ishiopoulos, H and Bondy, S.C (1992). Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. *Chemical Research in Toxicology* **5**:227-231.

Lechner, M, Lirk, P and Rieder, J (2005). Inducible nitric oxide synthase (iNOS) in tumour biology: The two sides of the same coin. *Seminars in Cancer Biology* **15**:277-289.

Leusen, J.H.W, Verhoeven, A.J and Roos, D (1996). Interactions between the components of the human NADPH oxidase: Intrigues in the *phox* family. *Journal of Laboratory and Clinical Medicine* **128(5)**:461-476.

Liu, X, Kim, C.N, Yang, J, Jemmerson, R and Wang, X (1996). Induction of apoptotic program in cell-free extracts: requirement of dATP and cytochrome c. *Cell* **86**:147-157.

van der Loosdrecht, A.A, Beelen, R.H.J, Ossenkoppele, G.J, Broekhoven, M.G and Langenhuijsen (1994). A tetrazolium-based colorimetric MTT assay to quantitate human monocyte mediated cytotoxicity against leukemic cells from cell lines and patients with acute myeloid leukemia. *Journal of Immunological Methods* **174**: 311-320.

Luseba, D, Elgorashe, E.E, Ntloedibe, D.T and van Staden, J (2007). Antibacterial, anti-inflammatory and mutagenic effects of some medicinal plants used in South Africa for the treatment of wounds and retained placenta in livestock. *South African Journal of Botany* **73**:378-383.

MacFarlane, M and Williams, A.C (2004). Apoptosis and disease: a life or death decision. *EMBO Reports* **5**:674-678.

Madiga, M.C (2007). Antioxidative, anti-inflammatory and antineoplastic potential of *Dicerocaryum* species. MSc Dissertation, University of Limpopo.

Madiga, M.C, Cockeran, R, Mokgotho, M.P, Anderson, R and Mampuru, L.J (2009). Dichloromethane extract of *Dicerocaryum senecioides* leaves exhibits remarkable anti-inflammatory activity in human T-lymphocytes. *Natural Product Research* **23(11)**:996-1006.

Mander, M and Le Breton, G (2006). Overview of the medicinal plants industry in Southern Africa. In *Commercialising Medicinal Plants – A Southern African Guide*. Diederichs, N (Ed.). Sun Press, Stellenbosch, pp. 1-8.

Mannick, J.B, Asano, K, Izumi, K, Kieff, E and Stamler, J.S (1994). Nitric oxide produced by human B-lymphocytes inhibits apoptosis and Epstein-Barr virus reactivation. *Cell* **79(7)**:1137-1146.

Meir, S, Kanner, J, Akiri, B and Hadas, S.P (1995). Determination and involvement of aqueous reducing compounds in oxidative defense systems of various senescing leaves. *Journal of Agriculture and Food Chemistry* **43**:1813-1817.

Mims, C.A, Playfair, J.H.L, Roitt, I.M, Wakelin, D and Williams, R (1993). In *Medical Microbiology*. 1st ed. Times Mirror International Publishers, Ltd. England, pp. 4.5.

Nathan, C (1992). Nitric oxide as a secretory product of mammalian cells. *The FASEB Journal* **6(12)**:3051-3064.

Needleman, P and Isakson, P.C (1997). The discovery and function of COX-2. *The Journal of Rheumatology* **24(49)**:6-8.

Nisimoto, Y, Otsuka-Murakami, H and Lambeth, D.J (1995). Reconstitution of flavin-depleted neutrophil flavocytochrome b_{558} with 8-mercapto-FAD and characterization of the flavin-reconstituted enzyme. *The Journal of Biological Chemistry* **270**:16428-16434.

O'Donnell, B, Tew, D.G, Jones, O.T.G and England, P.J (1993). Studies on the inhibitory mechanism of iodonium compounds with special reference to neutrophil NADPH oxidase. *Biochemical Journal* **290**:41-49.

Ohshima, H and Bartsch, H (1994). Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. *Mutation Research* **305**:253-264.

Park, J-W (1996). Attenuation of $p47^{PHOX}$ and $p67^{PHOX}$ membrane translocation as the inhibitory mechanism of S-nitrosothiol on the respiratory burst oxidase in human neutrophils. *Biochemical and Biophysical Research Communications* **220**:31-35.

Patrignani, P. Tacconelli, S, Sciulli, M.G and Capone, M.L (2005). New insights into COX-2 biology and inhibition. *Brain Research Reviews* **48**:352-359.

Posadas, I, Terencio, M.C, Guillen, I, Ferrandiz, M.L, Coloma, J, Paya, M and Alcaraz, M.J (2000). Co-regulation between cyclooxygenase-2 and inducible nitric oxide synthase expression in the time-course of murine inflammation. *Naunyn Schmiedebergs Archives of Pharmacology* **361**:98-106.

Pourmorad, F, Hosseinimehr, S.J and Shahabimajd, N (2006). Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. *African Journal of Biotechnology* **5(11)**:1142-1145.

Prescott, S.M (2000). Is cyclooxygenase-2 the alpha and the omega in cancer? *The Journal of Clinical Investigations* **105**:1511-1513.

Remillard, C.V and Yuan, J.X.-J (2004). Activation of K^+ channels: an essential pathway in programmed cell death. American Journal of Physiology **286(1)**:49-67.

Renwick, J, Reeves, E.P, Wientjes, F.B and Kavanagh, K (2007). Translocation of proteins homologous to human neutrophil $p47^{PHOX}$ and $p67^{PHOX}$ to the cell membrane in activated hemocytes of *Galleria mellonella. Developmental and Comparative Immunology* **31**:347-359.

Rinaldi, M, Moroni, P, Paape, M.J and Bannerman, D.D (2007). Evaluation of assays for the measurement of bovine neutrophil reactive oxygen species. *Veterinary Immunology and Immunopathology* **115**:107-125.

Salvemini, D, Misko, T.P, Masferrer, J.L, Seibert, K, Currie, M.G and Needleman, P (1993). Nitric oxide activates cyclooxygenase enzymes. *Proceedings of the National Academy of Sciences, USA* **90**:7240-7244.

Schepelmann, K, Messlinger, K, Schaible, H.G and Schmidt, R.F (1992). Inflammatory mediators and nociception in the joint: excitation and sensitization of slowly conducting afferent fibers of cat's knee by prostaglandin I_2. *Neuroscience* **50**:237-247.

Segal, A.W (2008). The function of the NADPH oxidase of phagocytes and its relationship to other NOXes in plants, invertebrates and mammals. *The International Journal of Biochemistry and Cell Biology* **40**:604-618.

Selemidis, S, Sobey, C.G and Wingler, K (2008). NADPH oxidases in the vasculature: Molecular features, roles in disease and pharmacological inhibition. *Pharmacology and Therapeutics* **120**:254-291.

Sepiashvili, R.I, Shubich, M.G, Kolesnikova, N.V, Slavyanskaya, T.A and Lomtatidze, L.V (2001). Apoptosis in immunologic processes. *Clinical and Applied Immunology Reviews* **1**:163-172.

Sheng, H, Shao, J, Morrow, J.D, Beauchamp, R.D and DuBois, R.N (1998). Modulation of apoptosis and Bcl-2 expression by prostaglandin E_2 in human colon cancer cells. *Cancer Research* **58**:362-366.

Siems, W.G, Capuozzo, E, Verginelli, D, Salemo, C, Crifo, C and Grune, T (1997). Inhibtion of NADPH oxidase-mediated superoxide radical formation in PMA-stimulated human neutrophils by 4-hydroxynonenal-binding to –SH and $–NH_2$ groups. *Free Radical Research* **27**:353-358.

Simon, L..S (1999). Role and regulation of cyclooxygenase-2 during inflammation. *The American Journal of Medicine* **106(5B)**:37-42.

Smith, R.M, Connor, J.A, Chen, L..M and Babior, B.M (1996). The cytosolic subunit of $p67^{PHOX}$ contains an NADPH-binding site that participates in catalysis by the leukocyte NADPH oxidase. *The Journal of Clinical Investigation* **98**:977-983.

Stamler, J.S, Jaraki, O, Osborne, J, Simon, D.I, Keaney, J, Vita, J, Singel, D, Valeri, C.R and Loscalzo, J (1992). Nitric oxide circulates in mammalian plasma primarily as an *S*-nitroso adduct of serum albumin. *Proceedings of the National Academy of Sciences* **89(16)**:7674-7677.

Stennicke H.R and Salvesen, G.S (1999). Catalytic properties of the caspases. *Cell Death and Differentiation* **6**:1054-1059.

Stuehr, DJ, Kwon, NS, Nathan, CF, Griffith, OW, Feldman, L and Wiseman, J (1991). N-ω-hydroxy-L-arginine is an intermediate in the biosynthesis of nitric oxide from L-arginine. *Journal of Biological Chemistry* **266(10)**:6256-6263.

Tak, P.P, Zvaifler, N.J, Green, D.R and Firestein, G.S (2000). Rheumatoid arthritis and p53: how oxidative stress might alter the course of inflammatory disease. *Immunology Today* **21(2)**:78-82.

Tanaka, M, Suda, T, Haze,K, Nakamura, N, Sato, K, Kimura, F, Motoyoshi, K, Mizuki, M, Tagawa, S, Ohga, S, Hatke, K, Drummond, A.H and Nagata, S (1996). Fas ligand in human serum. *Nature Medicine* **2**:317-322.

Taylor, J.L.S, Rabe, T, McGaw, L.J, Jäger, A.K and van Staden, J (2001). Towards the scientific validation of traditional medicinal plants. *Plant Growth Regulation* **34**:23-37.

Tsujii, M, Kawano, S, Tsuji, S, Sawaoka, H, Hori, M and DuBois, R.N (1998). Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell* **93**:705-716.

Tzeng, E, Billiar, T.R, Williams, D.L, Li, J, Lizonova, A and Kovesdi, I (1998). Adenovirus-mediated inducible nitric oxide synthase gene transfer inhibits hepatocyte apoptosis. *Surgery* **124(2)**:278-283.

Umeki, S (1990). Human neutrophil cytosolic activation factor of the NADPH oxidase. *The Journal of Biological Chemistry* **265(9)**:5049-5054.

Wink, D.A, Kasprzak, K.S, Maragos, C.M, Elespuru, R.K, Misra, M, Dunams, T.M, Cebula, T.A, Koch, W.H, Andrews, A.W, Allen, J.S and Keefer, L.K (1991). DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. *Science* **254(5034)**:1001-1003.

Wyllie, A.H, Kerr, J.F and Currie, A.R (1980). The significance of apoptosis. *International Reviews on Cytology* **68**:251-306.

Ximenes, V.F, Kanegae, M.P.P, Rissato, S.R and Galhiane, M.S (2007). The oxidation of apocynin catalyzed by myeloperoxidase: Proposal for NADPH oxidase inhibition. *Archives of Biochemistry and Biophysics* **457**:134-141.

Yoon, H.J, Jun, C.D, Kim, J.M, Rim, G.N, Kim, H.M and Chang, H.T (1994). Phorbol ester synergistically increases interferon-gamma-induced nitric oxide synthesis in murine microglial cells. *Neurotransmodulation* **1(6)**:377-382.

Zhang, A, Wu, Y, Lai, H.W.L and Yew, D.T (2004). Apoptosis – A brief review. *Neuroembryology* **3**:47-59.

http://www.metafro.be./ prelude/prelude_pic/Dicerocaryum_senecioides2.jpg – retrieved March 2011

http://www.celldeath.de/apometh/dapi.html – retrieved September 2011