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In vitro 5-lipoxygenase and anti-oxidant activities of South African medicinal plants commonly used topically for skin diseases

Yakov Frum

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Master of Science in Medicine.

February, 2006

DECLARATION

I, Yakov Frum, declare that this dissertation is my own work. It is being submitted in fulfillment for the degree, Master of Science in Medicine, University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

_____

_____ day of _____, 2006.

I dedicate this dissertation to my loving parents.

Thank you for your support, encouragement and belief.

This work would not have been possible without you.

Thank you for shaping me into the person I am today

and for always encouraging me to be a better person.

ABSTRACT

Thirty plant species traditionally used to treat skin pathologies were chosen from the readily available ethnobotanical literature. Four plants (aqueous or methanol extracts) displayed promising 5-lipoxygenase inhibitory activity with IC_{50} values below 61 ppm. These included *Aloe greatheadii, Melianthus comosus, Pentanisia prunelloides* and *Warburgia solutaris*. Essential oils generally displayed superior 5-lipoxygenase inhibitory activity with IC_{50} values between 22 and 75 ppm. These included the essential oils of *Ballota africana, Helichrysum odoratissimum, Heteropyxis natalensis* and *Lippia javanica*. A large proportion of the plants exhibited dose-dependent DPPH anti-oxidant activity with IC_{50} values between 5 and 94 ppm for the most active. These included *Halleria lucida, Croton sylvaticus, Melianthus comosus, Lippia javanica* and *Pentanisia prunelloides*. Aqueous extracts of *Melianthus comosus* exhibited the most potent anti-inflammatory and anti-oxidant activity.

The methanol extract of the leaves of *Halleria lucida* was subjected to activity guided fractionation and two anti-oxidant molecules were isolated, namely luteolin-5-*O*-glucoside and verbascoside (acteoside). Isobologram construction resulted in a concentration-dependent additive and antagonistic interaction being recognised between the two isolated compounds.

Warburgia salutaris displayed promising 5-lipoxygenase inhibitory activity. Two isolated compounds, mukadiaal and warburganal were found to partially contribute to the anti-inflammatory activity of the plant. The essential oils of *Helichrysum odoratissimum, Heteropyxis natalensis* and *Lippia javanica* were subjected to gas

chromatography and major compounds contributing to possible anti-inflammatory effects identified. These included β-caryophyllene, 1,8-cineole, limonene and α-humulene. Enantiomers and racemic mixtures of limonene displayed significantly different 5-lipoxygenase inhibitory activity suggesting stereoselectivity of the enzyme-catalysed reaction. The monoterpene 1,8-cineole appeared to cause partial potentiation of the anti-inflammatory activity displayed by limonene.

These results provide some *in vitro* scientific rationale for their traditional use as dermatological agents.

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Chapter 1: General Introduction

Medicinal plants have been used for skin pathologies for a number of centuries (Hutchings *et al*., 1996; van Wyk *et al*., 1997; Watt and Breyer-Brandwijk, 1962). In rural areas in developing countries wounds and skin ailments constitute one of the five most common reasons for people to seek medical care (Ryan, 1992). It has been reported that approximately one-third of medicinal plants are used for skin disorders (Mantle *et al*., 2001). A common theme underlying many skin pathologies is inflammation. Delayed healing of wounds and chronic inflammation is a feature of diseases such as leprosy, syphilis, AIDS, filariasis, and leishmaniasis. Furthermore, the production of free radicals is inextricably linked to the inflammatory process (Allen, 2003; Grimble, 1994). Therefore, it is not an unreasonable assumption that medicinal plants currently used as dermatological agents are anti-inflammatory and anti-oxidant in their mechanism of action.

Approximately 80% of the world's population rely on medicinal plants for their health and wellbeing (Mantle *et al*., 2001). Medicinal plants share a history of being used by virtually all cultures at one time or another. Furthermore, the use of curative herbal remedies form the basis of sophisticated plant-based traditional medicine systems that have been in existence for thousands of years in countries such as China and India and have been extensively used in the traditional African health care setting.

The plant kingdom historically became the driving force for the development of novel drugs. Phytochemicals have played an important part in the development of

chemotherapeutic agents throughout the twentieth century. Of 520 new drugs approved worldwide in the period 1983 to 1994, 30 drugs are derived directly from natural sources and 173 are semisynthetic derivatives modelled on parent phytochemicals (De Smet, 1997). Natural products are widely viewed as templates for structure optimization programs with the goal of creating new drugs. Few researchers doubt that plants are superior sources of molecular diversity especially in the areas where good synthetic leads do not exist (Bolton and De Gregorio, 2002). Furthermore, the phytochemical evolutionary engine for the production and selection of diverse bioactive molecules has been in existence for much longer than any pharmaceutical company.

Many of our present medicines are derived directly or indirectly from higher plants. The strong historic bond between plants and human health began in 1897, when Friedrich Bayer and Co. introduced aspirin to the world (Raskin *et al.*, 2002) which is a safer synthetic analogue of salicylic acid obtained from willow bark. Many other phytochemicals have lent themselves to drug development and these include pharmacologically diverse chemicals such as caffeine, alkaloids such as colchicine and galanthamine, digoxin and morphine (De Smet, 1997). Furthermore, anticancer (taxoids and camptothecins) and antimalarial (quinine and artemisinin) drugs have altered our perception of fatal illnesses to diseases which can be managed and cured. One of the misconceptions of the twentieth century was that complex diseases could be treated with a 'single golden molecular bullet'. However, the shortcomings of this approach manifested itself in the emergence of resistance to antimicrobial and anticancer drugs. For plants to rely on a single antibiotic in their biochemical

warfare with pathogens would be equivalent to evolutionary suicide because resistance would develop. As a result, plants have developed various metabolic mechanisms for the production of structurally and functionally diverse compounds. For example, *Berberis fremontii* has been reported to produce both antimicrobial berberine alkaloids and inhibitors of a bacterial multidrug-resistant pump (Hsiang and Lewis, 2000). With the technological strides taking place in the biotechnology arena in recent years, the mechanisms of complex diseases such as diabetes, heart disease, cancer and psychiatric disorders are beginning to be elucidated. The complexity of these illnesses necessitates the development of a multitude of different molecules and the innate diversity of phytochemicals make them ideal candidates for this task.

1.1 Overview of Inflammation

The inflammatory process is an extremely complicated cascade. A full description of the physiological processes which take place is beyond the scope of this work. A brief overview follows.

Inflammation literally means 'to set afire'. It is a collective term used to describe the network of physiological responses of the body to injury (Fig. 1.1.1). Typical clinical signs of inflammation include redness, heat and swelling which are due to vascular alterations in the area of injury. When an area of the body undergoes trauma, blood vessels in proximity dilate and this is termed vasodilation (Ryan and Majno, 1977). This results in an accumulation of blood in the vasodilated blood

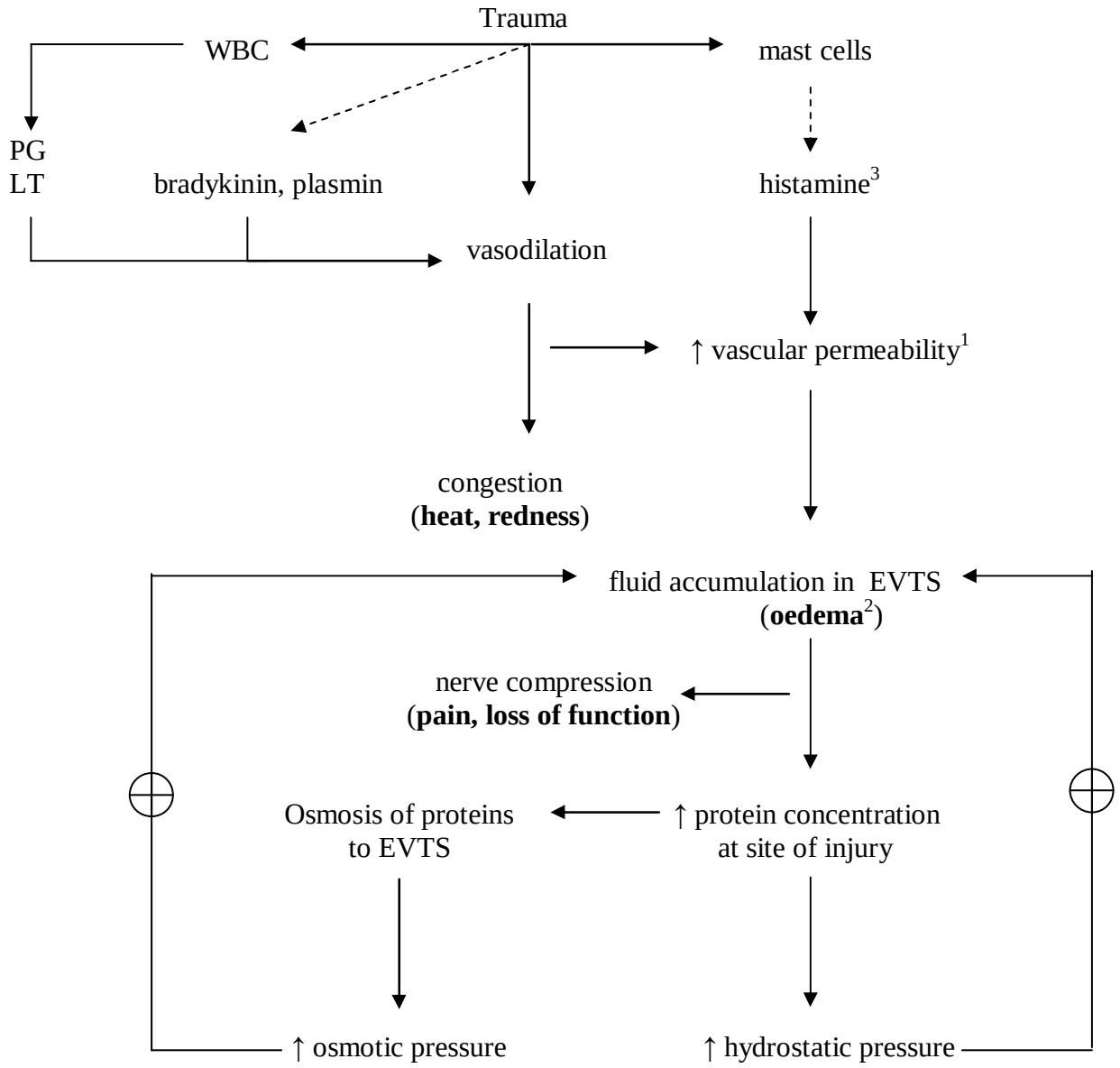
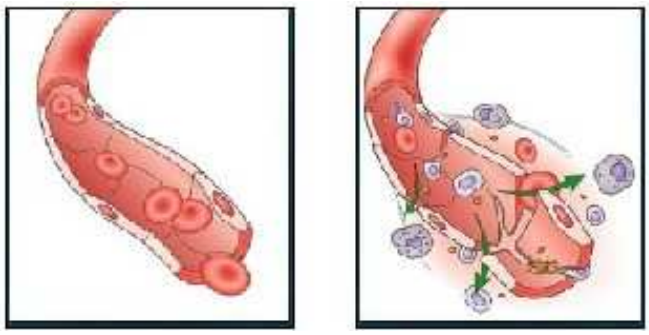


Fig. 1.1.1: Selected physiological pathways of the inflammatory response. Vasodilation and congestion are collectively termed hyperemia. Broken arrows indicate release of pro-inflammatory chemical mediators. Typical inflammatory symptoms are indicated in bold.
EVTS, extravascular tissue space; WBC, white blood cells; PG, prostaglandins; LT, leukotrienes, [1]inhibited by Witch hazel (*Hamamelis virginiana*) and Oak bark (*Quercus alba*), [2]inhibited by *Loasa speciosa* and *Azadirachta idica*, [3]inhibited by chamomile (*Matricaria recutita*).

vessels and this effect is called congestion. Vasodilation and congestion are collectively called hyperemia. Hyperemia results in an increase of vascular permeability and the escape of fluid from blood vessels to extravascular tissue space (Fig. 1.1.1, Fig. 1.1.2). Preparations such as witch hazel (*Hamamelis virginiana*) have shown to reduce vascular permeability, and therefore inflammation, due to a relatively high content of tannins, primarily gallotannins, which are reported to possess astringent properties (Brown and Dattner, 1998). Oak bark (*Quercus alba*) has also been reported to exert the same physiological effects as *H. virginiana* due to a mixture of tannins including catechins, oligomeric proanthrocyanidins and ellagitannins (Graf, 2000).

The loss of fluid from blood vessels results in an increase in concentration of proteins at the site of injury. As a result an increase in hydrostatic pressure takes place and more fluid leaves from the intravascular space to the extravascular tissue space. Plasma proteins also move in the same direction and the accumulation of proteins in the extravascular tissue space manifests in an increased osmotic pressure in the tissue area. This in turn results in more fluid leaving the intravascular space to the extravascular tissue space and again the process is thus exacerbated. The overall accumulation of fluid in the tissue area is termed oedema. Many curative herbal medications such as the leaves of *Loasa speciosa* and *Azadirachta indica* are reported to reduce inflammatory oedema (Badilla *et al.*, 2003; Chattopadhyay *et al.*, 1993).



Normal blood vessel Inflammed blood vessel

Fig. 1.1.2: Vasodilation, congestion, hyperemia and diapedesis. Medicinal plants such as *Loasa speciosa* have been reported to inhibit leukocyte migration and diapedesis (Atherogenics Inc., 2000)

Terms which are often used to describe the clinical signs of inflammation are heat, redness, swelling, pain and loss of function. Heat and redness can be attributed to hyperemia whereas swelling can be attributed to oedema (Fig. 1.1.1). Swelling in turn results in compression of surrounding sensory nerves which can cause pain and loss of function.

The above physiological processes are partly brought about by various chemical mediators. Mast cells are usually present in the tissues. When a tissue is subjected to a traumatic event such as mechanical trauma, radiation or bacterial toxins, mast cells release a chemical mediator known as histamine. Histamine attaches to H_1 receptors of the vascular endothelium which results in increased vascular permeability. Herbal preparations such as chamomile (*Matricaria recutita*) have been shown to inhibit histamine release from mast cells (Norman and Nelson, 2000)

due to the presence of flavonoids such as apigenin and quercetin (Middleton and Drzewiecki, 1982).

Trauma to a blood vessel wall can also sometimes result in the underlying basement membrane to be exposed. This often proves to be a trigger factor for the production of bradykinin and plasmin which result in further vasodilation and vascular permeability.

Some of the most well known chemical mediators of inflammation are leukotrienes and prostaglandins. Both are the end products of arachidonic acid metabolism mainly in white blood cells (Foegh *et al*., 1998). Cell injury results in the liberation of phospholipids from the surrounding plasma membrane (Fig. 1.1.3). Phospholipids undergo conversion to arachidonic acid under the action of phospholipase A_2. Arachidonic acid can be converted further to prostaglandins (PG) and leukotrienes (LT) and to a number of other eicosanoids via oxygenation reactions under the action of cyclooxygenases and lipoxygenases, respectively. Flavonoids such as quercetin have been shown to inhibit both phospholipase A_2 and lipoxygenase enzymes which results in the inhibition of pro-inflammatory prostaglandins and leukotrienes (Graf, 2000). Various subtypes of prostaglandins exist such as PGE_2, PGD_2 and $PGF_{2\alpha}$. Prostaglandins are responsible for various physiological functions such as smooth muscle relaxation, vasodilation, platelet aggregation, cytoprotection, pain and temperature control. Medicinal plants such as *Curcuma longa*, *Salix alba* and *Aloe barbadensis* have been shown to inhibit the physiological actions of prostaglandins (Bedi and Shenefelt, 2002; Graf, 2000; Srivastava and Srimal, 1985).

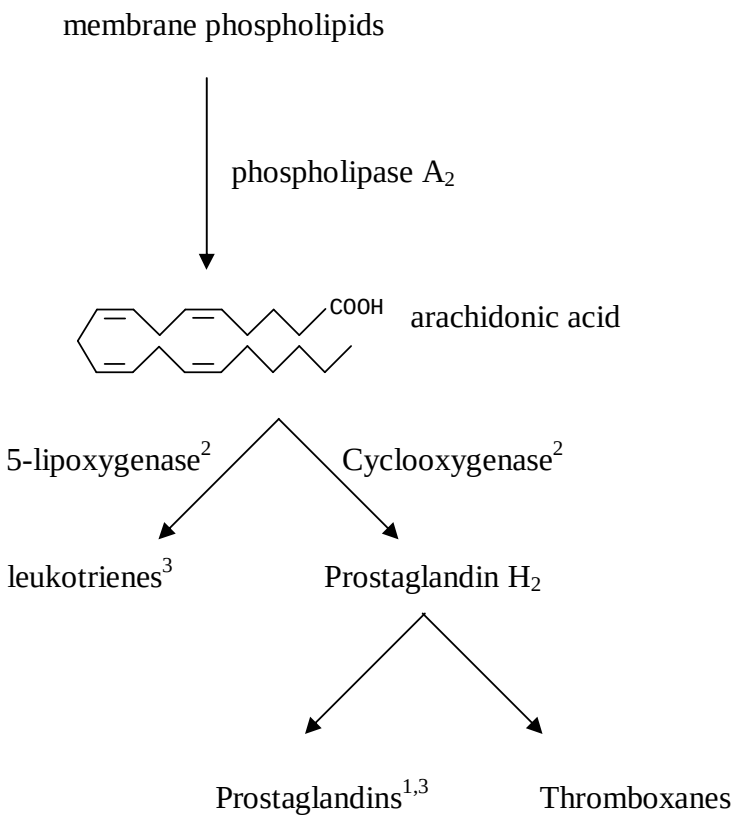


Fig. 1.1.3: Formation of leukotriene and prostaglandin pro-inflammatory mediators. Taken and modified from Foegh *et al*, 1998.

[1] inhibited by *Curcuma longa*, *Salix alba* and *Aloe barbadensis,*[2] inhibited by chamomile (*Matricaria recutita*),[3] inhibited by Turmeric (*Curcuma longa*).

Arachidonic acid undergoes conversion to various chemical mediators under the action of 5-lipoxygenase, 12-lipoxygenase and 15-lipoxygenase. These include hydroperoxyeicosatetraenoic acids (HPETE), hydroxy derivatives (HETE) and leukotrienes. Chamomile preparations have been reported to inhibit both lipoxygenase and cyclooxygenase *in vitro* (Norman and Nelson, 2000). It is thought that the anti-inflammatory properties of chamomile are attributed to α-bisabolol, α-

bisabolol oxides A and B, and matricin which is converted to chamazulene during extraction of the aromatic constituents (Czygan *et al*., 2002). The most actively investigated leukotrienes are those produced by the 5-lipoxygenase present in inflammatory cells such as polymorphonuclear neutrophils (PMN), basophils, mast cells, eosinophils and macrophages. Arachidonate undergoes conversion to the unstable epoxide leukotriene A_4 (LTA_4). Leukotriene A_4 undergoes conversion to dihydroxy leukotriene B_4 (LTB_4) or conjugates with glutathione to yield leukotriene C_4 (LTC_4) which in turn can undergo sequential degradation of the glutathione moiety by peptidases to yield LTD_4 and LTE_4. Leukotriene B_4 has been reported to be responsible for the attachment of white blood cells to the endothelium of damaged blood vessels and also acts as a chemoattractant for phagocytes. Leukotriene C_4 and LTD_4 are potent bronchoconstrictors and have been reported to be secreted in hyperallergenic conditions. Leukotriene C_4, LTD_4 and LTE_4 collectively form the slow-reacting substance of anaphylaxis (SRS-A) which is secreted in hyperallergenic conditions such as asthma and anaphylaxis. Many medicinal plants have been reported to inhibit the formation of leukotrienes such as Turmeric (*Curcuma longa*) which additionally inhibits prostaglandins and stimulates the adrenal glands to secrete cortisone (Srivastava, 1989; Srivastava and Srimal, 1985).

Currently, there are four separate approaches to anti-leukotriene drug development: 5-lipoxygenase enzyme inhibitors, leukotriene receptor antagonists, inhibitors of a membrane-bound 5-lipoxygenase activating protein (FLAP), and phospholipase A_2 inhibitors (Miele, 2003; Peters-Golden, 2003; Samaria, 2004).

Various other chemical mediators exist which also play important roles in the inflammatory process. These include platelet activating factor (PAF) secreted by macrophages, natural killer (NK) and lymphocytic cells; Interleukin (IK) 8 secreted by macrophages; complement cascade proteins, immunoglobulins and cytokines.

Various constituents of herbal preparations such as Zemaphyte® have displayed a reduction of immunoglobulin E (IgE) and low affinity IgE receptors such as CD23 (Banerjee *et al.*, 1998; Latchman *et al.*, 1994; Xu *et al.*, 1997). A variety of medicinal plants such as *Uncaria tomentosa* have been shown to inhibit tumour necrotic factor α (TNF- α).

As discussed above, increased vascular permeability plays an important role in the inflammatory cascade. It has been reported that vascular permeability following a traumatic event follows a bimodal pattern over time. Initial vascular permeability is brought about by the action of histamine whereas more prolonged permeability is caused by a combination of prostaglandins and leukotrienes. Increased vascular permeability is accompanied by WBC migration to the extravascular tissue space. The migration of WBC is termed diapedesis and is accompanied by the release of enzymes and free radicals from the WBC for purposes of destroying foreign matter. Medicinal plants such as *Loasa speciosa* have been shown to reduce leukocyte migration and diapedesis (Badilla *et al.*, 2003).

A number of nuclear transcription factors such as NFκB are responsible for many of the regulatory functions of the inflammatory response such as production of cytokines (IL-1, IL-2, IL-6, IL-8) and tumour necrosis factor α (TNFα). It has been shown that medicinal plants such as arnica (*Arnica montana*) inhibit NFκB (Bedi

and Shenefelt, 2002). It is thought that phytochemicals such as sesquiterpene lactones, isoprenoids, kaurene diterpenoids and polyphenolics are responsible for the inhibitory action on this important transcription factor (Bremner and Heinrich, 2002).

The inflammatory response is beneficial when the destruction of foreign matter is an end in itself. However, often normal inflammatory physiological responses are exaggerated and hypersensitivity reactions follow. Four types of hypersensitivity reactions can take place. Type I are caused by primed mast cells which secrete large amounts of histamine with resultant hyper-vasodilation and is typical of exposure to grasses, pollens, weeds and dust. Medicinal plants such as *Tephrosia purpurea* have been shown to modulate both cell mediated and humoral components of the immune system (Damre *et al*., 2003).

Type II is an immune cytotoxic reaction caused by the destruction of normal self tissue by degradation enzymes secreted by WBC. Type III is an immune complex hypersensitivity reaction characterized by circulating antigens bound to antibodies and is typical of diseases such as rheumatoid arthritis. Type IV is a delayed type hypersensitivity reaction that develops relatively slowly and is typical of contact dermatitis.

The inflammatory process is extremely complex with numerous 'checks and balances'. Abundant targets exist for potential chemotherapeutic agents. The structural and functional diversity of phytochemicals present unique opportunities for their development as chemotherapeutic agents which exert their potential at one or many possible inflammatory targets.

1.2 Overview of Free Radicals and Anti-oxidants

1.2.1 Definition of a Free Radical

A free radical is any species that contains one or more unpaired electrons as part of its molecular structure and is capable of independent existence (Halliwell and Gutteridge, 1999). An unpaired electron is an electron that occupies an atomic or molecular orbital by itself. Free radicals can be formed by the gain or loss of a single electron from a non-radical. In addition, free radicals can be formed by homolytic fission whereby the breakage of a covalent bond between two atoms takes place (Halliwell and Gutteridge, 1999). As a result, one electron from each of the shared pair remains with each individual atom:

$$A{:}B \rightarrow A^{\bullet} + B_{\bullet}$$

$A^{\bullet}$ is an A-radical and $B_{\bullet}$ is a B-radical. Similarly, homolytic fission of one covalent bond in the water molecule will yield a hydrogen radical ($H^{\bullet}$) and a hydroxyl radical ($^{\bullet}OH$).

Alternatively, free radicals can be formed by heterolytic fission in which one atom receives both electrons when a covalent bond is broken (Halliwell and Gutteridge, 1999):

$$A{:}B \rightarrow A{:}^{-} + B_{\bullet}^{+}$$

The extra electron gives A a negative charge and B**.** is a free radical with a positive charge.

1.2.2 Common Free Radicals and their Formation

Aerobic life utilizes oxygen for the oxidation and combustion of hydrogen- and carbon-rich substrates. This process facilitates the production of chemical energy which is essential for life. It also results in the reduction of the oxygen molecule to various free radicals (Gutteridge, 1993). Stepwise reduction of oxygen takes place as follows:

$$O_2 + e + H^+ \rightarrow HO_2^{\cdot} \text{ hydroperoxyl (radical)}$$

$$HO_2^{\cdot} \rightarrow H^+ + O^{2-} \text{ superoxide anion (radical)}$$

$$O^{2-} + 2H^+ + e \rightarrow H_2O_2 \text{ hydrogen peroxide (non-radical)}$$

$$H_2O_2 + e \rightarrow OH^- + {}^{\cdot}OH \text{ hydroxyl (radical)}$$

$${}^{\cdot}OH + e + H^+ \rightarrow H_2O$$

Stepwise reduction of the oxygen molecule results in a number of different radicals as shown above. In addition, the oxygen molecule in the ground state, as it occurs

naturally, qualifies as a free radical (Gutteridge, 1993; Halliwell, 1995; Halliwell and Gutteridge, 1999). Furthermore, the oxygen molecule undergoes dismutation reactions resulting in the formation of hydrogen peroxide which plays a critical role in the production of highly reactive free radical species (Gutteridge, 1993; Halliwell and Gutteridge, 1999). These concepts are examined in more detail in forthcoming discussion sections.

Nitric oxide ($NO^{\bullet}$) is another free-radical found in various biological systems. It is

produced by the vascular endothelium and other cells (Moncada and Higgs, 1991). It has been reported to react with the superoxide anion to produce a peroxynitrite intermediate ($ONOO^{-}$) which is capable of damaging many biological molecules (Beckman *et al*., 1990; Saran *et al*., 1990).

Transition metals in the first row of the d-block of the Periodic Table contain unpaired electrons. As a result, they fall within the broad definition of free-radicals. Furthermore, they display variable valencies which makes them potentially reactive.

A number of other free radicals exist in biological systems such as thioyl, peroxyl and alkoxyl free radicals (Halliwell, 1995).

1.2.3 Pauli's Principle

A potential anti-oxidant will scavenge various free radicals such as the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical by donation of an electron. Reduction of the DPPH free radical by an anti-oxidant is schematically represented in Fig. 3.2. Once an anti-oxidant donates an electron it will itself be transformed into a free radical.

Such electron donation or reduction is dependent upon the premise that the free radical formed is more energetically stable than the free radical reduced (Halliwell and Gutteridge, 1999; and references therein). Free radical stability is inversely related to activity. Free radical activity is determined by Pauli's principle which states that no two electrons can have the same quantum numbers (Halliwell and Gutteridge, 1999; and references therein). Spin quantum numbers have only two possible values (± ½). As a result, electrons sharing a molecular orbital will possess opposite spins. This implies a restriction on electron transfer to a free radical. For example, the oxygen molecule in the ground state, as it occurs naturally, qualifies as a free-radical because it has two unpaired electrons located in different π^* (π antibonding) orbitals (Halliwell and Gutteridge, 1999; and references therein). These two electrons have the same spin quantum numbers or have parallel spins. As a result, transfer of electrons to the oxygen molecule has to take place one at a time. This property contributes to the fact that oxygen reacts sluggishly with many non-radicals. Complex organic compounds of the human body should immediately combust in oxygen of the air but the above spin restriction fortunately slows this process down. On the other hand, a common free radical such as singlet oxygen (Gupta *et al.*, 2003) is more reactive than the oxygen molecule in the ground state because the spin restriction is removed and the oxidizing ability of this free radical is correspondingly increased (Gutteridge, 1993; Halliwell, 1995; Halliwell and Gutteridge, 1999).

1.2.4 Diseases Caused by Free Radicals

Free radicals play an important role in the pathogenesis of many diseases. The production of free radicals *in vivo* takes place randomly and for specific metabolic purposes. An imbalance between free radical generation and body defense mechanisms results in oxidative damage to various cell structures such as DNA, proteins, lipids, etc. It has been reported that various pathological conditions and illnesses such as cancer, atherosclerosis, cardiovascular disease, diabetes, immune system impairment, Parkinson's disease, Alzheimer's disease, arthritis and premature body aging can be linked to the production of very reactive free radicals (Benavente-Garcia *et al*., 1997, Vaughan, 1997). Furthermore, it has been proposed that free-radicals play an important role in the pathogenesis of ischaemic injury (Cotelle *et al*., 1996).

1.2.5 Definition of Anti-oxidants

An anti-oxidant is any substance that when present at low concentrations, compared to those of the oxidizable substrate, significantly delays, or inhibits, oxidation of that substrate (Halliwell and Gutteridge, 1999). Anti-oxidants can act by a number of mechanisms including removal of oxygen or decreasing local O_2 concentrations; removal of catalytic metal ions; removal of reactive oxygen species (ROS) such as superoxide anions and hydrogen peroxide; scavenging of initiating free radicals such as hydroxyl free radicals; scavenging of singlet oxygen; and chain-breakage of an oxidation sequence (Gutteridge, 1993).

Superoxide dismutase (SOD), catalase and glutathione peroxidase are examples of enzymatic innate biological anti-oxidants inherent to the human body. A number of nonenzymatic anti-oxidants also exist such as albumin, ascorbic acid, α-tocopherol, β-carotene, uric acid and bilirubin (Prior and Cao, 1999).

1.2.6 Medicinal Plants as Anti-oxidants

Various medicinal plants have been reported to possess anti-oxidant properties. These include, amongst others, thyme, marjoram (Banias *et al*., 1992), sage (Schwarz and Ternes, 1992; Stephen *et al*., 1977; Svoboda and Deans, 1992) and catnip (Hirosue *et al*., 1988). In addition, a number of phytochemicals which display anti-oxidant activities have been isolated from medicinal plants. These include polyphenols such as tannins and flavonoids (Yokozawa *et al.*, 1998), tocopherols and catechines (Dapkevicius *et al.*, 1998). Organic acids, carotenoids and protein hydrolysates have also displayed anti-oxidant effects or synergistic effects with other anti-oxidants.

1.2.7 Limitations of Current Anti-oxidants

The food industry has traditionally provided the impetus and momentum for the discovery of novel anti-oxidant molecules. Phenolic compounds provided the stepping stone for the production of synthetic anti-oxidants during the 20th century (Sherwin, 1990). Some of the well known anti-oxidants used today include synthetic compounds such as gallates, 2,6-di-tert-butyl-4-methylphenol (BHT), tert-butyl hydroxyanisole (BHA) and tert-butyl hydroquinone (TBHQ). However, in

recent years consumer preference has turned the wheel full-circle in the search for natural products. Tocopherol and ascorbic acid display lower anti-oxidant potentials than synthetic anti-oxidants (Nishina *et al*., 1991) and the safety and toxicity of synthetic anti-oxidants has often been a point of contention and concern with synthetic anti-oxidants reportedly being implicated in causing liver damage and carcinogenesis (Grice, 1986; Wichi, 1988). Furthermore, some of the physicochemical properties of the available synthetic anti-oxidants such as instability and volatility are deemed to be unfavourable. Isolation of novel anti-oxidant phytochemicals and structure-activity analysis could result in the development of an anti-oxidant compound which is superior in terms of physicochemical and pharmacological profiles. This could ultimately yield anti-oxidant compounds of greater therapeutic potential than currently available.

1.3 Inflammation, Free Radical Production and Anti-oxidants

The production of free radicals is inextricably linked to the inflammatory process. The immune system produces a large number of potent chemicals which include free radicals. Free radicals prime the immune response, recruit inflammatory cells and are innately bactericidal (Allen, 2003; Grimble, 1994). Free radicals and oxidants activate NFκB, a nuclear transcription factor, resulting in an upregulation of interleukin-1, interleukin-8 and tumour necrosis factor (TNF). This in turn further stimulates the immune response, increases oxidant production and can lead to further tissue damage. The paragraphs which follow provide a ‘snap-shot’ of an

extremely complicated process describing the interrelated concepts of inflammation and free radical production. As a result, they offer a simplified picture for the sake of brevity and understanding.

1.3.1 Free Radicals and the 'Respiratory Burst'

When human tissue is subjected to injury, an acute inflammatory response often develops as described in Section 1.1. Foreign matter in the inflamed area is engulfed by neutrophils and macrophages reaching the site and this physiological response is called phagocytosis. At the onset of phagocytosis, both neutrophils and macrophages demonstrate an increase in oxygen uptake and this is termed the 'respiratory burst'. This process ultimately results in the formation of toxic free radicals such as superoxide anion and the production of hydrogen peroxide which plays a critical role in the assembly of highly reactive free radical species.

The formation of superoxide anions and hydroxyl free radicals by the respiratory burst has been observed not only in neutrophils and macrophages but also in brain microglial cells, liver Kupffer cell, monocytes, basophils, mast cells and eosinophils. It is also of interest to note that the immunological substance histamine described in section 1.1 has been shown to stimulate basophils, eosinophils and mast cells to produce more superoxide anions.

1.3.2 Free Radicals and Inflammatory Cell Recruitment

As described in section 1.3.1, superoxide anions are produced by phagocytic cells such as neutrophils and macrophages. It has been reported that superoxide anions

react with a component of normal plasma and in so doing are responsible for forming a chemotactic factor for neutrophils. Furthermore, it has been shown that perfusion of tissue with superoxide anions caused an increase in vascular permeability and neutrophil margination and pavementing (Del Maestro *et al*., 1982). In addition, prostaglandins regulate some inflammatory functions (Tanaka *et al*., 2004) and effective anti-oxidant treatment has been found to inhibit prostaglandin synthesis (Rekka *et al.*, 1996). Many other examples are available which demonstrate the close relationship between free radical production and inflammatory cell recruitement (Ayyagarri *et al*., 2003; Grimble, 1994; Safayhi *et al*., 1994).

1.3.3 Free Radicals, Anti-oxidants and the 5-lipoxygenase Enzyme

Molecular structure elucidation of the 5-lipoxygenase enzyme has yielded valuable information about the mechanism of action of this pro-inflammatory enzyme. For maximum activity to take place, it is necessary that this enzyme be converted from an inactive reduced state to an active oxidized state (Young, 1999). The active 5-lipoxygenase enzyme undergoes recycling via formation of a pentadienyl free radical, hydropyroxy radical and oxygen atom insertions at different places of the carbon chain of arachidonic acid (Young, 1999). Anti-oxidants and free radical scavengers could therefore potentially reduce all three radicals mentioned and thus terminate synthesis of leukotrienes. In addition, leukotrienes have been documented to enhance the 'respiratory burst' and thus increase free radical production

(Halliwell and Gutteridge, 1999; Young 1999). Therefore, inhibition of the 5-lipoxygenase enzyme can also indirectly reduce free radical production.

1.3.4 Practical Examples of Free Radicals and Inflammation

A large number of examples are available which are able to depict the close relationship that exists between free radical production and the inflammatory process in a clinical setting (Halliwell and Gutteridge, 1999). For example, patients suffering from psoriasis have been shown to have a serum factor which increases oxygen-radical production by phagocytes which are known to accumulate in epidermal lesions. Macrophages are present in the lesions of myelinated nerves in multiple sclerosis and damage may be exacerbated by the production of free radicals. Lungs of animals which are damaged by elevated oxygen concentrations provoke an inflammatory response characterized by an influx of neutrophils. These in turn release superoxide anions and hydrogen peroxide and thus cause more tissue damage. Inhalation of silica particles and asbestos is known to cause a chronic inflammatory condition accompanied by macrophage destruction and subsequent release of free radicals into the extracellular surrounding medium. Furthermore, some asbestos fibres contain iron which can stimulate lipid peroxidation and hydroxyl-radical formation. Free radical production by asbestos and silica can lead to further tissue destruction and the development of cancer (Halliwell and Gutteridge, 1999).

1.4 Medicinal Plants, Skin Pathologies and Relevance to Inflammation / Free Radicals

Only 1-3% of modern drugs are used for ailments of the skin (Mantle *et al*., 2001). Medicinal plants have been used by traditional people for many different types of skin disorders. (Hutchings *et al*.; 1996; van Wyk *et al*., 1997; Watt and Breyer-Brandwijk, 1962). Currently available ethnobotanical literature often proves to be limited in information when skin pathologies are described. Furthermore, the mechanism of action of medicinal plants used for skin ailments is rarely reported. Without such information it is difficult to justify the rational usage of medicinal plants as effective dermatological chemotherapeutic agents.

Pharmacological and pharmacognostic investigations have resulted in the development of many conventional anti-inflammatory and anti-oxidant agents. These include anti-inflammatory agents such as salicylic acid (Evans, 1996). Likewise, semi-synthetic steroidal drugs are synthesized using plant steroids as intermediates such as diosgenin and hecogenin and steroidal alkaloids of the *Solanaceae* family. Anti-oxidants derived from natural sources have gained popularity in recent years and these include phytochemicals such as catechins and procyanidins (Dragsted, 2003). Therefore, ethnobotanicals commonly used in traditional medicine for skin ailments may possess anti-inflammatory and anti-oxidant phytochemicals which may explain their mechanism of action. These phytochemicals may be used as a foundation and driving force for the development of conventional chemotherapeutic agents. Furthermore, pharmacognostic

investigations of these phytochemicals could yield valuable conclusions about the rationale for using medicinal plants as dermatological agents.

1.5 Rationale for the Usage of the 5-Lipoxygenase and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) Anti-oxidant Assays

Numerous biological assays are in existence for the determination of anti-inflammatory and anti-oxidant activities of therapeutic compounds. Established anti-inflammatory assays include, amongst others, cyclooxygenase-1 (COX-1) anti-inflammatory assay, cyclooxygenase-2 (COX-2) anti-inflammatory assay, interleukin-1 (IL-1) and interleukin-6 (IL-6) anti-inflammatory assays, NFκB anti-inflammatory assay and the 5-lipoxygenase assay (Kankuri *et al*., 2003; Tanaka *et al*., 1992; Wu *et al*., 2004). Anti-oxidant assays have also been in existence for many years and include the oxygen radical absorbance capacity (ORAC), ferric reducing anti-oxidant power (FRAP), total oxidant scavenging capacity (TOSC), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Aruoma, 2003). However, the DPPH anti-oxidant assay involves the use of a stable free radical which is relatively easier to handle than conventional free-radicals used in established *in vitro* anti-oxidant assays. Furthermore, the stability of the DPPH free radical implies that a potential anti-oxidant will react with other well known free radicals which are more unstable and therefore more reactive. Moreover, the DPPH assay is relatively inexpensive compared to other *in vitro* anti-oxidant assays. Although numerous anti-inflammatory assays also exist, the 5-lipoxygenase inhibitory assay was

specifically chosen because the 5-lipoxygenase enzyme is theoretically very sensitive to anti-oxidants because of its non-heme iron atom in the active site of the enzyme which undergoes redox recycling for activation (Schneider and Bucar, 2005; Young, 1999). For maximum activity to take place, it is necessary that the transitional iron atom of the enzyme be converted from an inactive reduced state to an active oxidized state (Young, 1999). Many 5-lipoxygenase inhibitors act as non-selective antioxidants by reducing the active-site ferric iron. Further investigations could potentially reveal the dichotomy or correlation between 5-lipoxygenase inhibitory activity and anti-oxidant activity of selected medicinal plants.

1.6 Study Objectives

The following objectives were proposed for the fulfilment of this academic work:

● Implementation of the 5-lipoxygenase anti-inflammatory assay.

● Screening of selected ethnobotanicals for anti-inflammatory activity using the 5-lipoxygenase enzyme assay. The concentration of selected ethnobotanicals to inhibit 50% of 5-lipoxygenase activity (IC_{50}) will be determined.

● Implementation of the DPPH anti-oxidant assay.

● Screening of selected ethnobotanicals for anti-oxidant activity using the DPPH anti-oxidant assay. The concentration of selected ethnobotanicals to reduce 50% of DPPH radicals (IC_{50}) will be determined.

● Isolation of possible anti-inflammatory compounds from lead crude plant extracts using classical chromatographic techniques.

● Isolation of possible anti-oxidant compounds from lead crude plant extracts using column chromatography and thin layer chromatography.

● Evaluation of IC_{50} values of lead pure compounds with regard to anti-inflammatory and anti-oxidant activity.

● Determination of interaction between isolated anti-inflammatory and anti-oxidant compounds using isobologram construction.

Chapter 2: Collection and Extraction of Plant Material

2.1 Introduction

A thorough literature survey undertaken revealed that medicinal plants have been used for various skin disorders for a number of centuries. However, the precise mechanisms of action and active constituents of these entities have not been fully elucidated. As a result, viable plants were identified, collected and subjected to extraction procedures. It was hoped that crude extracts thus obtained could in future be subjected to biological testing.

2.2 Materials and Methods

2.2.1 Collection of Plant Material

South African indigenous plants were chosen according to their reported use as dermatological agents and availability from various localities (Fig. 2.1). Only plant parts which were reported in literature (Hutchings *et al*., 1996; van Wyk *et al*., 2000; Watt and Breyer-Brandwijk, 1962), to be used for skin ailments were harvested. Thirty plants were collected from various localities or were obtained from the Walter Sisulu Botanical Garden (WSBG) and National Botanical Institute (NBI). Table 2.1 depicts medicinal plants chosen which may possibly display anti-inflammatory / anti-oxidant activity correlating with their traditional use. Plant family divisions allow insight into anti-inflammatory and / or anti-oxidant actions across genus and species in a particular family. Voucher specimens are housed in the Department of Pharmacy and Pharmacology, University of the Witwatersrand,



(a)



(b)



(c)



(d)

Fig. 2.1: Collection of various medicinal plants used for dermatological pathologies. (a) collection at Walter Sisulu Botanical Garden (WSBG); (b) *Croton sylvaticus*; (c) *Bulbine* spp.; (d) *Pentanisia prunelloides.*

Table 2.1: South African medicinal plants chosen based on documented evidence as dermatological agents with corresponding topical uses and chemical compounds previously isolated.

Species[a]	**Family**	**Common topical uses**	**Chemical compounds and pharmacological action**
Aloe ferox[1,2]	*Asphodelaceae*	Arthritis, eczema, venereal sores, skin lesions, pimples, blisters, ringworm, boils. Contains wound-healing, hydrating, insulating and protective properties.	Glycoproteins have wound healing effects.
Aloe greatheadi[1,2]			
Aloe maculata[1,2]			
Artemisia afra[1,2,3]	*Compositae*	Neuralgia, swellings in mumps, throat inflammation, eye lotion, haemorrhoid lotion, measles rash, earache, relieves pain of gumboils, relieves toothache.	The volatile oil contains 1,8-cineole, α-thujone, β-thujone, camphor and borneol. Also contains terpenoids of the eudesmadien- and germacratien types, as well as coumarins and acetylenes.
Ballota africana[3]	*Lamiaceae*	Lotion for sores on the head, thrush.	Diterpenoid lactones of the labdane type.
Bulbine spp. [1,2,3]	*Asphodelaceae*	Rheumatism, sciatica, rashes, itches, ringworms, cracked lips, mouth ulcers, rheumatism.	Glycoproteins in the leaf gel, such as aloctin A and aloctin B. Also contains chrysophanol.
Carpobrotus edulis[1,2]	*Aizoaceae*	Antiseptic, severe weeping infantile eczema, wounds, burns, ringworm, blue-bottle stings.	Tannins, malic acid, citric acid have antiseptic, vasoconstricting and tissue regenerating properties.
Clausena anisata[1,2,3]	*Rutaceae*	Wounds which are usually infested.	Various terpenes.
Clerodendrum glabrum[1,2,3]	*Verbenaceae*	Decoction of the leaf used to prevent the development of Maggots, blowflies and other parasites in wounds on animals.	Chemistry poorly studied.
Cotyledon orbiculata[1,2,3]	*Crassulaceae*	Earache, toothache, boils, inflammation	Contains cardiac glycosides of the bufadienolide type but there is no clear link to reported analgesic effects.
Crinum bulbispermum[1,2,3]	*Amaryllidaceae*	Aching joints, swollen joints, rheumatism, varicose veins, backache, septic sores, abscesses, earache.	Various alkaloids have been shown to have analgesic activity.
Croton gratissimus[1,2]	*Euphorbiaceae*	Inflammation, bleeding gums, wounds, boils.	Saponins, monoterpenoids, diterpenoids.
Croton sylvaticus[1,2]			
Datura stramonium[1,2,3]	*Solanaceae*	Wounds, sores, swellings, tumours, rheumatic pain, gout, boils, abscesses, bruises, inflammation, pus.	Alkaloids.
Gloriosa superba[1,2,3]	*Colchicaceae*	Skin eruptions, bruises, haemorrhoids, sprains, pimples, wound disinfectant, toothache, rheumatism and gout.	Colchicine, phenolic alkaloid, steroids, salicylic acid.

Species	Family	Common topical uses	Chemical compounds and pharmacological action
Halleria lucida[1]	*Scrophulariaceae*	Earache, skin ailments.	Chemistry poorly studied. Compounds appear to be mildly astringent.
Harpagophytum procumbens[2]	*Pedaliaceae*	Rheumatism, arthritis, sores, ulcers,boils.	Triterpenoids and flavonoids.
Helichrysum odoratissimum[1,2,3]	*Compositae*	Popular ingredient for wound dressings, wound infections, acute dermatoses.	Flavonoids, sesquiterpenoids and acylated phloroglucinols. The antimicrobial flavonoid (chalcone) helichrysetin has been isolated. The essential oil contains α-pinene and α-humulene.
Heteropyxis natalensis[1,2,3]	*Heteropyxidaceae*	Steaming of the face, nose and mouth in nose bleeding, bleeding gums.	The essential oil contains monoterpenoids such as β-ocimene, 1,8-cineole and limonene
Kigelia africana[1,2,3]	*Bignoniaceae*	Ulcers, sores, syphilis, rheumatism, acne and toothache.	Dihydroisocoumarins and their glycosides.
Leonotis leonurus[1,2]	*Lamiaceae*	Boils, eczema, skin ailments, itching, fever, arthritis, swollen glands, mouth ulcers.	Diterpenoids.
Lippia javanica[1,2]	*Verbenaceae*	Scabies, lice, sprained joints, rashes, urticaria.	Volatile oil contains monoterpenoids such as Myrcene, caryophyllene, linalool, ρ-cymene and ipsdienone. Contains various organic acids, alcohols, iridoid glycosides and triterpenoids.
Melianthus comosus[1,2]	*Melianthaceae*	Sores, rheumatism, painful feet, bruises.	Triterpenoids, possibly heart glycosides.
Pentanisia prunelloides[1,2]	*Rubiaceae*	Burns, swellings, sore joints, rheumatism.	Naphthoquinones. Chemistry poorly studied.
Rauvolfia caffra[1,2,3]	*Apocynaceae*	Analgesic properties, rashes including rashes caused by measles, urticaria.	None of the compounds isolated thus far have been shown to have dermatological effects.
Rothmannia capensis[1,2,3]	*Rubiaceae*	General rashes, rash caused by measles.	Indole alkaloids such as reserpine and ajmalicine (raubasine).
Scilla natalensis[1,2,3]	*Hyancinthaceae*	Various skin ailments including sprains, fractures, boils and sores.	Saponins and flavonoids.
Trichilia emetica[1,2]	*Meliaceae*	Eczema, skin ailments, ulcers, rheumatism, gout.	Limonoids.
Warburgia salutaris[1,2]	*Canellaceae*	Penial irritation, urethral inflammation, sores, skin irritation.	Drimane sesquiterpenoids.
Ziziphus mucronata[1,2,3]	*Rhamnaceae*	Boils, sores, swellings, pain relief, promotion of healing, glandular swellings, carbuncles.	Various alkaloids.

[a] Referencing indicated by number. [1] Hutchings *et al*., 1996; [2] van Wyk *et al*., 2000; [3] Watt and Breyer-Brandwijk, 1962.

Johannesburg, South Africa. Seasonal and regional variations were not taken into consideration and plants were collected between June 2003 and June 2004.

2.2.2 Preparation of Crude Plant Extracts

Plants were air dried at room temperature for three days and macerated. Indigenous people often utilize aqueous solvents for extraction procedures in a rural setting. However, phytochemists have in the past been reported to use various volatile solvents for extraction procedures because of easy manipulation and handling in a laboratory setting. This invariably confounds the data available in various literature and as a result both methanol and aqueous solvents were used for extraction procedures. Furthermore, the polarity index of methanol is relatively similar to that of water with both solvents reportedly classed as polar. In addition, the mode of application of medicinal plants in rural settings can sometimes be direct without further extraction procedures but this is not feasible for *in-vitro* biological tests. Correlation in biological activity between the two types of extracts was subsequently investigated. Extraction with aqueous and methanol solvents was not performed successively. Rather, approximately 5 g of separate macerated plant material was subjected to one of the two extraction solvents. Cold extraction took place for eight hours and the samples were re-extracted with the same solvent for an additional eight hours. Methanol extracts were placed in a fume chamber to dry or alternatively subjected to rotary vacuum evaporation and aqueous extracts were lyophilized using a freeze drier (Virtis) for 48 hours. All plant extracts were stored at room temperature.

2.2.3 Extraction of Essential Oils

Seven of the selected plants (*Artemesia afra, Ballota africana, Clerodendrum glabrum, Heteropyxis natalensis, Helichrysum odoratissimum, Leonotis leonurus, Lippia javanica*) are, according to literature, aromatic and the essential oils were obtained according to the method of Yayli *et al.* (2005) with slight modification. Briefly, crude plant material was hydro-distilled in 500 ml of water for three hours using a Clevenger-type apparatus. The temperature of the apparatus was kept constant at 104 °C. Essential oil and water was collected in the collection arm of the apparatus and separation was due to density differences. The essential oils were stored in amber vials which offered protection against light-initiated degradation. The amber vials were then stored at 4 °C to prevent essential oil resinification.

2.3 Results and Discussion

2.3.1 Non-volatile Compound Yield

Yield of aqueous and methanol extracts obtained is expressed as a percentage of fresh plant material weighed before extraction procedures were initiated. All species investigated, locality and percentage yield are given in Table 2.2.

Resource limitation prevented the collection of all plants identified from literature which are used for various dermatological pathologies. An effort was made to obtain aqueous or methanol extracts of plants which were limited by distribution. Alternatively, if a plant was aromatic and methanol or aqueous extracts could not be obtained, essential oils were obtained by hydro-distillation.

Table 2.2: Plant name, locality, plant part and percentage yield of South African medicinal plants commonly used topically for skin diseases.

Plant	**Voucher / locality**	**Plant part**	**Methanol / Aqueous / Essential oil**	**% Yield[a]**
Aloe ferox	WSBG[b]	Leaves	Aqueous	20.48
			Methanol	59.52
		Exudate	-	nd[e]
Aloe greatheidii	WSBG[b]	Roots	Aqueous	NPM[f]
			Methanol	5.18
Aloe maculata	WSBG[b]	Leaves	Aqueous	NPM[f]
			Methanol	15.90
		Roots	Aqueous	NPM[f]
			Methanol	11.11
Artemisia afra	Klipriversberg (Gauteng)	Leaves	Aqueous	NPM[f]
			Methanol	NPM[f]
		Aerial parts	Essential oil	0.35
Ballota africana	Vergelegen (Southern Cape)	Aerial parts	Aqueous	20.60
			Methanol	20.60
			Essential oil	0.012
Bulbine spp.	WSBG[b]	Leaves	Aqueous	28.75
			Methanol	23.75
Carpobrotus edulis	WSGB[b]	Leaves	Aqueous	16.89
			Methanol	45.85
Clausena anisata	WSBG[b]	Leaves	Aqueous	23.21
			Methanol	21.27

Plant	**Voucher / locality**	**Plant part**	**Methanol / Aqueous / Essential oil**	**% Yield[a]**
Clerodendrum glabrum	North-west of Warmbad (Northern Province)	Leaves	Aqueous	18.35
			Methanol	9.63
		Aerial parts	Essential oil	NPM[f]
Cotyledon orbiculata	WSBG[b]	Leaves	Aqueous	29.00
			Methanol	43.16
Crinum bulbispermum	WSBG[b]	Bulb	Aqueous	NPM[f]
			Methanol	2.84
	UW[d]	Flowers	Aqueous	40.42
			Methanol	26.39
Croton gratissimus	WSBG[b]	Bark	Aqueous	4.49
			Methanol	8.97
Croton sylvaticus	NBI[c]	Roots	Aqueous	15.20
			Methanol	64.80
Datura stramonium	South of Warmbad	Fruit	Aqueous	18.95
			Methanol	5.44
		Leaves	Aqueous	26.14
			Methanol	13.71
Gloriosa superba	WSBG[b]	Corms	Aqueous	26.20
			Methanol	2.49
Halleria lucida	WSBG[b]	Leaves	Aqueous	15.64
			Methanol	19.52
		Roots	Aqueous	11.96
			Methanol	4.91
Harpagophytum procumbens	Commercial product	Roots	Aqueous	17.40
			Methanol	52.80

Plant	Voucher / locality	Plant part	Methanol / Aqueous / Essential oil	% Yield[a]
Helichrysum odoratissimum	Amatola Mountains (Eastern Cape)	Leaves	Aqueous	NPM[f]
			Methanol	NPM[f]
		Aerial parts	Essential oil (AV 587)	0.064
			Essential oil (AV 590)	0.14
Heteropyxis natalensis	Cullinan (Gauteng)	Whole plant	Aqueous	NPM[f]
			Methanol	NPM[f]
		Aerial parts	Essential oil	nd[e]
Kigelia africana	Northern Province (commercial product)	Fruit	Aqueous	34.00
			Methanol	22.71
Leonotis leonurus	WSBG[b]	Leaves	Aqueous	8.81
			Methanol	20.59
		Aerial parts	Essential oil	nd[e]
Lippia javanica	Melville Koppies (Gauteng)	Leaves and stems	Aqueous	12.40
			Methanol	12.80
		Aerial parts	Essential oil	0.054
Melianthus comosus	WSBG[b]	Leaves	Aqueous	27.46
			Methanol	27.71
Pentanisia prunelloides	NBI[c]	Roots	Aqueous	6.00
			Methanol	52.00
Rauvolfia caffra	WSBG[b]	Bark	Aqueous	11.39
			Methanol	2.17
		Stem	Aqueous	14.92
			Methanol	7.69

Plant	Voucher / locality	Plant part	Methanol / Aqueous / Essential oil	% Yield[a]
Rothmania capensis	WSBG[b]	Fruit	Aqueous	36.32
			Methanol	37.49
		Roots	Aqueous	15.01
			Methanol	5.80
Scilla natalensis	WSBG[b]	Bulb	Aqueous	14.88
			Methanol	35.98
Trichilia emetica	Swaziland	Leaves	Aqueous	20.67
			Methanol	12.00
Warburgia solutaris	WSBG[b]	Leaves	Aqueous	26.32
			Methanol	23.30
Ziziphus mucronata	Melville Koppies	Leaves	Aqueous	14.81
			Methanol	12.88

[a] percentage yield expressed for aqueous and methanol extracts per dry weight and for essential oil per wet weight of plant material weighed.
[b] Walter Sisulu Botanical Garden, Johannesburg, South Africa.
[c] National Botanical Institute, Pretoria, South Africa.
[d] University of the Witwatersrand Medical School, Johannesburg, South Africa.
[e] not determined.
[f] no plant material.

2.3.2 Essential Oil Yield

Seven of the selected plants (*Artemesia afra, Ballota africana, Clerodendrum glabrum, Heteropyxis natalensis, Helichrysum odoratissimum, Leonotis leonurus, Lippia javanica*) are aromatic and the essential oil yield expressed as a percentage of fresh plant material is represented in Table 2.2.

Chapter 3: 5-Lipoxygenase and Anti-oxidant Activities of Medicinal Plant Extracts

3.1 Introduction

The mechanism of action of medicinal plants used for various skin disorders remains an unanswered question. Without such information (albeit *in vitro* data) it is difficult to justify the rational usage of medicinal plants as effective therapeutic agents. The intricate interplay between inflammation and oxidation prompted the investigation of anti-inflammatory and anti-oxidant activities of medicinal plants collected. The 5-lipoxygenase anti-inflammatory and the DPPH anti-oxidant assays were chosen as yardsticks to represent such biological activity for reasons explained in Section 1.5. Preliminary screening offered a benchmark for subjecting specific medicinal plants to further biological tests and classical chromatographic techniques.

3.2 Materials and Methods

3.2.1 Materials

Tween® 20 was obtained from Merck (S.A); dimethyl sulfoxide (DMSO) was from Saarchem (S.A); potassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), linoleic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ascorbic acid were from Fluka (S.A); potato 5-lipoxygenase enzyme and nordihydroguaiaretic acid (NDGA) were from Cayman (U.S.A); methanol (HPLC

grade) was from Ultrafine Limited (S.A). The materials were used without further purification. Double distilled de-ionised water was used for reconstitution.

3.2.2 Biological Testing

3.2.2.1 5-lipoxygenase Assay

Possible inhibition of 5-lipoxygenase activity was determined by the method of Sircar *et al*. (1983) and modified by Evans (1987). This assay was implemented by myself in collaboration with Ms. S. Baylac (Robertet, France) in October 2003. All concentrations refer to final concentrations in 3 ml cuvettes maintained at 25 °C in a thermostated bath. Stock solutions were made up in accordance to Appendix 1. The standard assay mixture contained 10 μL of plant extract dissolved in a 3 % w/w solution of Tween® 20 in DMSO, 0.1 M potassium phosphate buffer (pH 6.3, 2.95ml) prepared with analytical grade reagents and linoleic acid (100 μM, 50 μL). The initial concentration of plant extract was 100 ppm with at least a threefold stepwise serial dilution used for determination of 5-lipoxygenase inhibitory activity. An extract showing no or weak (< 50 % inhibitory activity) at 100 ppm was not subjected to further testing and stepwise serial dilution. At least three readings were taken for each extract. The final concentration of the Tween® 20 in DMSO solution in the reaction mixture was 0.3 % v/v. Linoleic acid, rather than arachidonic acid, was used due to its ease of handling and stronger affinity for the 5-lipoxygenase enzyme resulting in greater UV absorbance readings (Baylac and Racine, 2003; Shimizu *et al.*, 1990). The reaction was initiated with the addition of 100 U (12 μL) isolated 5-lipoxygenase diluted with equal volume potassium phosphate buffer

maintained at 4 °C. The stock concentration of the enzyme was 8333 U ml^{-1}. Potato 5-lipoxygenase was used rather than human equivalent due to greater accessibility, cost and greater purity with concomittant reduction in side-reactions. The increase in absorbance at 234 nm was recorded for 10 minutes with a single beam spectrophotometer (Analytikjena Specord 40) linked to a PC by the Winaspect® software (Fig. 3.1 (a)-(e)). Decreasing amounts of extract were added and initial reaction rate was determined from the slope of the straight line portion of the curve.

The percentage enzyme activity was calculated by comparison with the negative control (DMSO and Tween® 20) according to the following equation:

$$\% \text{ activity} = S_{PE} / S_{NC} \times 100 \quad (1)$$

where S_{PE} and S_{NC} are the slope of graph due to plant extract and slope of graph due to negative control, respectively. Nordihydroguaiaretic acid represented the positive control and its activity was assessed with an initial concentration of 20 ppm with at least a threefold reduction in concentration using serial dilutions (Baylac and Racine, 2003). Percentage enzyme activity was plotted against concentration of plant extract. The concentration of plant extract that caused 50% enzyme inhibition (IC_{50}) was determined using Enzfitter® version 1.05 software.



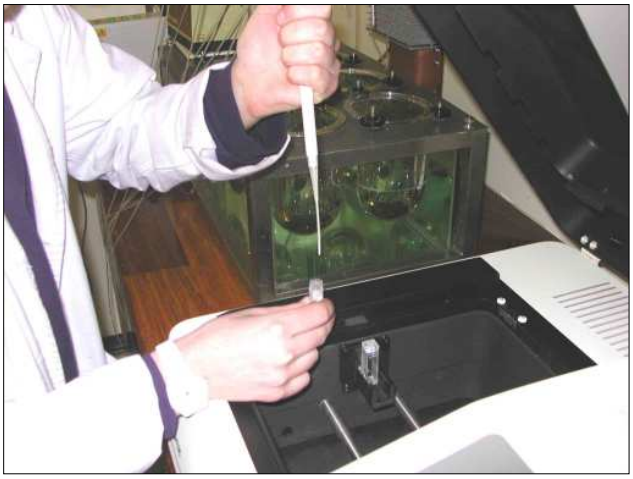
(a)



(b)



(c)



(d)



(e)

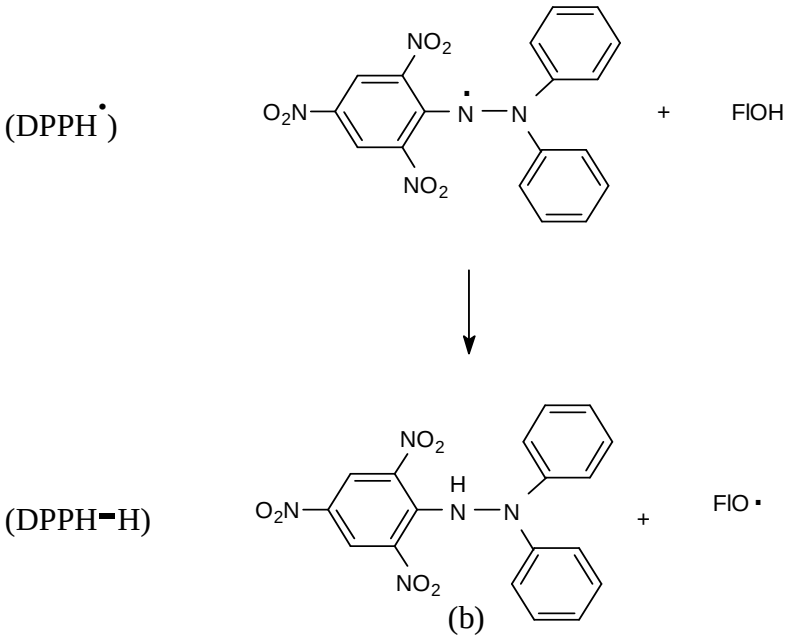
Fig. 3.1: The 5-lipoxygenase assay; (a) weighing of the plant extract; (b) addition of plant extract dissolved in DMSO to give a final concentration of 100 ppm in cuvette; (c) addition of linoleic acid (substrate) to cuvette; (d) addition of 5-lipoxygenase (enzyme); (e) monitoring of reaction at 254 nm over a period of 10 min.

3.2.2.2 DPPH Anti-oxidant Assay

The anti-oxidant activity of each extract was determined based on the colorimetric method described by Shimada *et al*. (1992) to test for DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity. This assay was implemented by myself in collaboration with Ms. M. Muranova (CSIR, Pretoria, South Africa) in January 2004. A 96 well microtitre plate was used to generate a quantitative measure of radical scavenging activities of extracts. All concentrations refer to final concentrations in each well. Stock solutions were made up in accordance to Appendix 2. The reaction mixture contained 50 µL extract dissolved in pure DMSO and 200 µL DPPH (0.077 $mmol.L^{-1}$) dissolved in HPLC grade methanol. The final concentration of DMSO in the reaction mixture was 20 % v/v. Reaction mixtures for negative controls contained extracts and methanol without DPPH. Each extract was tested at an initial concentration of 100 ppm with at least a threefold reduction in concentration using serial dilutions to determine final anti-oxidant activity. The plate was shaken for 2 minutes and stored in the dark for an additional 30 minutes. Percentage decolourisation was obtained spectrophotometrically at 550 nm using a Labsystems Multiskan RC microtitre plate reader (Fig. 3.2). Each assay was performed in triplicate. Extracts which displayed promising activitiy ($\geq$ 50% decolourisation at 100 ppm) were retested at lower concentrations using serial dilutions. Analytical grade ascorbic acid and NDGA constituted the positive controls (Dupont *et al*., 2001; Song *et al*., 2005). The activities of the positive controls were assessed with an initial concentration of 20 ppm with at least a threefold reduction in concentration using serial dilutions. The concentration of the



(a)



(b)

Fig. 3.2: Principles of the DPPH anti-oxidant assay; (a) a 96 well microtitre plate containing an active medicinal plant extract. Yellow wells indicate strong free radical scavenging activity while purple wells indicate weak free radical scavenging activity. Colourless wells are used to subtract colour interference due to plant extracts. Top and bottom rows constitute negative controls; (b) Chemical reaction representing the reduction of DPPH by a flavonoid (FlOH) compound. DPPH in its unreduced form is purple but undergoes a colour change to yellow once reduced.

extract that caused 50% decolourisation (IC_{50}) was determined using Enzfitter® version 1.05 software.

3.2.2.3 Statistical Analysis

Student's *t*-test was used to determine statistical differences in biological activity with a significance level of $P \leq 0.05$. All analysis were performed with GraphPad Prism® version 4.03 software.

3.3 Results and Discussion

3.3.1 Anti-inflammatory Activity

The vast majority of medicinal plants did not exhibit any significant anti-inflammatory activity in the 5-lipoxygenase assay at an arbitrarily chosen concentration of 100 ppm. This concentration value was chosen because of the inherent limitations associated with localised and transdermal drug delivery (TDD). Optimistic expectations of TDD were often thwarted by many pitfalls inherent to this science. Most notably, the outermost nonviable layer of the epidermis, the stratum corneum, has proved particularly resistant to TDD due to its lipid bilayer architectural design (Elias, 1983; Meidan and Michniak, 2004). As a result, this places a major limitation on the physicochemical profile displayed by compounds which qualify as candidates for TDD. Such physicochemical limitations include, amongst others, molecular weights less than 500 daltons, log P (octanol-water) between one and three and Pka values between six and nine (Barry, 2001; Meidan

and Michniak, 2004). As a result, a maximum concentration value of 100 ppm chosen is extremely conservative because the limitations described mean that only a small percentage of applied drug eventually reaches the target site. A larger value would entail a scaling up of the concentration of the applied formulation to the skin which could decrease patient compliance and increase toxicity. Statistical analysis for this set of results was, for the greater part, deemed unnecessary as this formed a preliminary screening exercise. However, statistical tests were included sporadically and in ensuing investigations. Furthermore, the *in vitro* activity of medicinal plant extracts were compared to NDGA and Vit C and an arbitrary scale of relative *in vitro* activity was defined which allowed the identification of the maximum IC_{50} regarded as indicating worthwhile anti-inflammatory activity as follows (Baylac and Racine, 2003):

++++	$IC_{50} < 10$ ppm	(very strong)
+++	10 ppm $\leq IC_{50} \leq$ 30 ppm	(strong)
++	31 ppm $< IC_{50} \leq$ 50 ppm	(moderate)
+	51 ppm $< IC_{50} \leq$ 100 ppm	(weak)

Inactive above 100 ppm

Colour interference prevented obtaining credible 5-lipoxygenase inhibitory activity for *Croton sylvaticus*. This phenomenan has been reported to exist for compounds such as citral (Baylac and Racine, 2003).

Large discrepancies ($P < 0.05$) in 5-lipoxygenase anti-oxidant activities between aqueous and methanol extracts of some plants indicates that a realistic approach in a

laboratory setting would be to use extraction solvents identical to those used in clinical settings. Such discrepancies include the aqueous and methanol extracts of *Melianthus comosus, Pentanisia prunelloides* and *Warburgia salutaris*.

Only four plants (aqueous and methanol extracts) displayed 5-lipoxygenase inhibitory activity representing 13.33 % of medicinal plants selected. NDGA represented the positive control with an IC_{50} value of 5 ± 0.50 ppm (Abad *et al*., 1995; Hope *et al*., 1983; Safayi *et al*., 1992). In addition, NDGA represented one of the positive controls in the DPPH anti-oxidant assay with an IC_{50} value of 2.73 ± 0.50 ppm. The dual contribution to anti-inflammatory and anti-oxidant activities of NDGA represented an innate and inherent correlation between anti-inflammatory and anti-oxidant activities and chemistries.

The aqueous extract of *Melianthus comosus* displayed the strongest 5-lipoxygenase inhibitory activity with an IC_{50} value of 13.84 ± 1.18 ppm (Table 3.1). However, it should be noted that no significant difference between all medicinal plants (methanol and aqueous extracts) displaying 5-lipoxygenase inhibitory activity was observed ($p > 0.05$). However, a statistically signicant difference in 5-lipoxygenase inhibitory activity was observed between the methanol and aqueous extracts of *M.comosus* ($p < 0.05$). This again indicated the importance of using extraction solvents similar to those used in clinical settings. The wound-healing properties of *M. comosus* are poorly understood. However, it has been reported that the leaves and roots of *M. comosus* contain triterpenoid molecules (van Wyk *et al*., 1997) which have been shown to possess anti-inflammatory activity (Liu, 1995). It would be of interest to determine the active principles through a reductionist approach.

Table 3.1: Plant name, plant part, 5-lipoxygenase inhibitory and DPPH anti-oxidant activities of South African medicinal plants commonly used topically for skin diseases.

Species	**Plant part**	**Methanol / Aqueous / Essential oil**	**IC_{50} (ppm)[a]**	**IC_{50} (ppm)[b]**
Aloe ferox	Leaves	Aqueous	> 100	77.93 ± 1.55
		Methanol	> 100	19.11 ± 0.10
	Exudate	-	> 100	17.72 ± 0.35
Aloe greatheadii	Roots	Aqueous	*	*
		Methanol	30.33 (1.59)[c]	> 100
Aloe maculate	Leaves	Aqueous	*	*
		Methanol	> 100	> 100
	Roots	Aqueous	*	*
		Methanol	> 100	> 100
Artemisia afra	Leaves	Aqueous	*	*
		Methanol	*	*
	Aerial parts	Essential oil	> 100	> 100
Ballota africana	Aerial parts	Aqueous	> 100	> 100
		Methanol	> 100	> 100
		Essential oil	29.99 (2.14)[c]	> 100
Bulbine spp.	Leaves	Aqueous	> 100	> 100
		Methanol	> 100	> 100
Carpobrotus edulis	Leaves	Aqueous	> 100	> 100
		Methanol	> 100	13.35 ± 0.13
Clausena anisata	Leaves	Aqueous	> 100	20.45 ± 0.76
		Methanol	> 100	20.91 ± 0.23

Species	**Plant part**	**Methanol / Aqueous / Essential oil**	**IC_{50} (ppm)[a]**	**IC_{50} (ppm)[b]**
Clerondendrum glabrum	Leaves	Aqueous	> 100	> 100
		Methanol	> 100	> 100
	Aerial parts	Essential oil	*	*
Cotyledon orbiculata	Leaves	Aqueous	> 100	> 100
		Methanol	> 100	93.38 ± 6.33
Crinum bulbispermum	Bulb	Aqueous	*	*
		Methanol	> 100	> 100
	Flowers	Aqueous	> 100	> 100
		Methanol	> 100	42.17 ± 0.12
Croton gratissimus	Bark	Aqueous	> 100	> 100
		Methanol	> 100	> 100
Croton sylvaticus	Roots	Aqueous	nd[e]	11.28 ± 0.23
		Methanol	nd[e]	5.82 ± 0.10
Datura stramonium	Fruit	Aqueous	> 100	> 100
		Methanol	> 100	> 100
	Leaves	Aqueous	> 100	> 100
		Methanol	> 100	> 100
Gloriosa superba	Corms	Aqueous	> 100	> 100
		Methanol	> 100	> 100
Halleria lucida	Leaves	Aqueous	> 100	80.58 ± 0.79
		Methanol	> 100	8.49 ± 0.12
	Roots	Aqueous	> 100	83.36 ± 1.25
		Methanol	> 100	14.93 ± 0.24
Harpagophytum procumbens	Roots	Aqueous	> 100	71.42 ± 1.40
		Methanol	> 100	19.84 ± 0.13

Species	**Plant part**	**Methanol / Aqueous / Essential oil**	**IC_{50} (ppm)[a]**	**IC_{50} (ppm)[b]**
Helichrysum odoratissimum	Leaves	Aqueous	*	*
		Methanol	*	*
	Aerial parts	Essential oil –AV 587	22.46 (1.70)[c]	> 100
		–AV 590	35.90 (6.10)[c]	> 100
Heteropyxis natalensis	Whole plant	Aqueous	*	*
		Methanol	*	*
	Aerial parts	Essential oil	46.63 (1.09)[c]	> 100
Kigelia africana	Fruit	Aqueous	> 100	> 100
		Methanol	> 100	> 100
Leonotis leonurus	Leaves	Aqueous	> 100	34.21 ± 0.73
		Methanol	> 100	> 100
	Aerial parts	Essential oil	> 100	> 100
Lippia javanica	Leaves and stems	Aqueous	> 100	50.07 ± 1.85
		Methanol	> 100	11.42 ± 0.08
	Aerial parts	Essential oil	74.26 (1.54)[c]	> 100
Melianthus comosus	Leaves	Aqueous	13.84 (1.18)[c]	5.27 ± 0.15
		Methanol	55.05 (0.00)[c]	5.60 ± 0.12
Pentanisia prunelloides	Roots	Aqueous	> 100	38.02 ± 0.49
		Methanol	32.71 (9.06)[c]	11.79 ± 0.14
Rauvolfia caffra	Bark	Aqueous	> 100	> 100
		Methanol	> 100	> 100
	Stem	Aqueous	> 100	> 100
		Methanol	> 100	63.91 ± 1.46
Rothmania capensis	Fruit	Aqueous	> 100	> 100
		Methanol	> 100	> 100
	Roots	Aqueous	> 100	> 100
		Methanol	> 100	> 100

Species	**Plant part**	**Methanol / Aqueous / Essential oil**	**IC_{50} (ppm)[a]**	**IC_{50} (ppm)[b]**
Scilla natalensis	Bulb	Aqueous	> 100	> 100
		Methanol	> 100	> 100
Trichilia emetica	Leaves	Aqueous	> 100	43.89 ± 0.91
		Methanol	> 100	17.90 ± 0.30
Warburgia salutaris	Leaves	Aqueous	> 100	34.43 ± 0.74
		Methanol	32.11 (11.95) [c]	15.38 ± 0.20
Ziziphus mucronata	Leaves	Aqueous	> 100	32.75 ± 1.17
		Methanol	> 100	21.51 ± 0.32
NDGA	-	-	5 ± 0.50[d]	2.73 ± 0.06
Vit C	-	-	> 100	2.46 ± 0.01

[a] 5-lipoxygenase inhibitory activity.
[b] DPPH anti-oxidant activity, values are mean ± S.D (n=3).
[c] Value in brackets reflects standard error of data points fitted to obtain a sigmoidal curve.
[d] Value is mean ± S.D (n=3).
[e] Not determined due to colour interference.
* Not determined due to insufficient plant material.

The mode of application of medicinal plants might selectively release molecules to increase their specificity. For example, it has been reported that traditional healers use *Heteropyxis natalensis* for steaming the face, nose and mouth in nose bleeding (Van Wyk *et al*., 1997; Watt and Breyer-Brandwijk, 1962). It could be assumed that such application results in the release of volatile compounds which could be responsible for the pharmacological and therapeutic effects of the plant.

Another example is *Leonotis leonurus* which is sometimes applied as an ointment containing powdered leaves for pain above the eye. This method of application could possibly selectively diffuse lipophilic active molecules to the necessary dermatological site.

The methanol extract of the roots of *Pentanisia prunelloides* displayed moderate 5-lipoxygenase inhibitory activity with an IC_{50} value of 32.71 ± 9.06 ppm. Little appears to be known about the chemistry of *P. prunelloides* but it has been reported to possess anti-inflammatory activity by inhibiting the cyclooxygenase-1 enzyme (Yff *et al*., 2002). It is interesting to note that *M. comosus* and *P. prunelloides* are all traditionally used for rheumatism which is characterized by exaggerated immunological responses such as increased prostaglandin and autoantibody production resulting in pathological manifestations of the disease such as pain, bone destruction and deformation (Cha *et al*., 2004; Westman *et al*., 2004). Furthermore, the traditional Zulu name for *P. prunelloides* is 'Icishamlilo' which literally means 'the extinguisher of the fire' referring to the fact that in traditional health-care settings it is used for pain control (Ngwenya *et al*., 2003). Pain is often a manifestation of immunological chemical mediators such as prostaglandins.

The methanol extract of the leaves of *Warburgia solutaris* also displayed moderate 5-lipoxygenase inhibitory activity (IC_{50} = 32.11 ± 11.95 ppm). The relatively large coefficient of variation implies poor fit to a sigmoidal curve suggesting that this particular plant does not conform to the standard kinetics of enzyme inhibition and this needs to be investigated further. Furthermore, the frequency distribution of 5-lipoxygenase inhibitory activity could be skewed which could explain the large coefficient of variation. Limited resources prevented further verification of this and this warrants further investigation. The leaves of *W. solutaris* are used for skin diseases but in practice the bark is also used. The leaves and bark of *W. solutaris* have been shown to display equipotent activity against the cyclooxygenase-1 enzyme and possess similar antibacterial properties (Zschocke *et al*., 2000). In addition, Zschocke *et al*. (2000) reported that the TLC-fingerprints of the leaf and bark extracts of *W. salutaris* were very similar. These results may indicate similar molecular profiles in the leaves and bark of *W. solutaris*. Furthermore, Drewes *et al*. (2001) suggested possible substitution of leaves in place of bark of *W. solutaris* due to the plant's over-exploitation in the context of ethnomedicine and the present threat of extinction in the wild. In the same publication it was noted that drimane sesquiterpenoids, warburganal and polygodial, were isolated from both the bark and the leaves of *W. solutaris*. Another drimane sesquiterpenoid, muzigadial, has been isolated from the bark of *W. solutaris* and therefore there is a possibility that it is present in the leaves of the same plant (Rabe and van Staden, 2000). Drimane sesquiterpenoids have been reported to possess a wide spectrum of biological activities (Jansen and de Groot, 1991) while polygodial has already been shown to

display anti-inflammatory activity (da Cunha *et al*., 2001). In addition sesquiterpenoids such as β-caryophyllene and germacrene D have in the past been reported to display anti-inflammatory activity and this could explain the anti-inflammatory action of the leaves of *W. solutaris* (Baylac and Racine, 2003). The common name of *W. solutaris* is fever tree and a close association between fever and inflammation exists. Due to the interesting anti-inflammatory pharmacological profile displayed by *W. solutaris* and the interesting chemistry presented by the drimane sesquiterpenoids, it was decided to test two drimane sesquiterpenoid molecules from this plant in the 5-lipoxygenase anti-inflammatory assay (Chapter 5).

The methanol extract of the roots of *Aloe greatheadii* was one of the few plants which displayed any activity in the 5-lipoxygenase assay. Furthermore, neither *Aloe ferox* nor *Aloe maculata* displayed any anti-inflammatory effects. *Aloe barbadensis* has been shown to decrease thromoxane A_2, thromboxane B_2, and prostaglandin 2α (Bedi and Shenefelt, 2002; and references therein) and prevent the oxidation of arachidonic acid (Reynolds and Dweck, 1999). In addition, anti-inflammatory compounds such as salicylic acid have been shown to be present in *Aloe barbadensis*. Relief of inflammation is also thought to be due to the presence of gel polysaccharides, especially the acetylated mannans (Reynolds and Dweck, 1999). Anti-inflammatory compounds such as C-glucosyl chromones have been isolated from *Aloe barbadensis* and have been reported to display topical anti-inflammatory activity (Hutter *et al*., 1996). It would be of interest to subject such compounds to the 5-lipoxygenase assay. Moreover, depending on the biological assay employed

different molecules from *A. barbadensis* such as anthraquinones can be shown to act as anti-inflammatory agents (Davis *et al*., 1989).

A number of essential oil-containing plants displayed strong to weak 5-lipoxygenase inhibitory activity with IC_{50} values ranging from 22.46 ± 1.70 ppm to 74.26 ± 1.54 ppm. The essential oil of *Helichrysum odoratissimum* displayed the most potent 5-lipoxygenase inhibitory activity which was significantly greater than the essential oils of *Ballota africana*, *Heteropyxis natalensis* and *Lippia javanica* ($p < 0.05$). However, no significant difference in 5-lipoxygenase inhibitory activity was observed between the two essential oil of *H. odoratissimum* ($p > 0.05$). The essential oils of *Leonotis leonurus* and *Artemisia afra* displayed no activity at a concentration of 100 ppm. Essential oils have been reported to display anti-inflammatory activity while essential oil constituents such as terpenic hydrocarbons, sesquiterpenic hydrocarbons and sesquiterpenic alcohols have been reported to display 5-lipoxygenase inhibitory activity (Alexander, 2001; Baylac and Racine, 2003). Essential oil activity could be related to types of chemicals present, amounts of chemicals present (concentration effect), synergism, antagonism, additiveness and potentiation. As a result, it was decided to subject a selection of essential oils to gas chromatography (GC) and GC coupled with mass spectroscopy (MS) for identification of constituents and for further tests designed to elucidate any synergism, antagonism and additive interactions between essential oil constituents (Chapter 6). The inherent chemical nature of essential oil constituents means that they are lipid soluble, low molecular weight chemicals which can diffuse through

the skin barrier and reach the underlying tissues. These properties make essential oils ideal candidates for future development.

3.3.2 Anti-oxidant Activity

The maximum concentration of 100 ppm was chosen for the same reasons as those described in Section 3.3.1. Likewise, comparison with the positive controls (NDGA and Vit C) allowed the definition of an arbitrary scale of relative *in vitro* anti-oxidant activity. This allowed the identification of the maximum IC_{50} regarded as indicating worthwhile anti-oxidant activity. In essence, the same criteria were used as those defined in Section 3.3.1 for the 5-lipoxygenase assay. Furthermore, a thorough investigation of various literature indicated that the range chosen for maximum IC_{50} value correlated well with that indicated in literature (Ahmad *et al*., 2005; Juma and Majinda, 2004; Luo *et al*., 2002; Salehi *et al*., 2005; Valentova *et al*., 2005; Zou *et al*., 2004). In addition, statistical analysis for this set of results was deemed unnecessary as this formed a preliminary screening exercise. However, statistical tests were included sporadically and in ensuing investigations.

The vast proportion of medicinal plants tested displayed dose-dependent DPPH anti-oxidant activity. All essential oils tested displayed no activity at a concentration of 100 ppm which could be due to their low polyphenolic content. Polyphenolics are able to act as free radical scavengers by electron donation due to their inherent molecular structure (Prior *et al*., 2000). Donation of an electron could result in a stable polyphenolic molecule because electron delocalisation in the aromatic rings ensures stability (Gillespie *et al*., 1986). The doughnut-shaped electron clouds

above and below aromatic rings could possibly ensure stable free radical formation which may perhaps explain the biological activity observed.

The methanol extract of the leaves of *Aloe ferox* and the leaf exudates displayed statistically different anti-oxidant activities ($p < 0.05$). Aloesin derivatives such as isorabaichromone, feruloyaloesin and p-coumaroylaloesin have been isolated from *A. ferox* and shown to be potent DPPH radical and superoxide anion scavengers (Yagi *et al*., 2002). Further investigation is warranted to determine the chemical constituents which result in significantly different anti-oxidant activity between the methanol extract and leaf exudate of *A. ferox*. Little appears to be known about the chemical profile of *Carpobrotus edulis*. However, antibacterial activity has been related to the presence of flavonoids isolated from the same plant (van der Watt and Pretorius, 2001). Flavonoids have in the past been shown to attribute to anti-oxidant activity and this could explain the strong anti-oxidant activity displayed by *C. edulis* (Cotelle *et al*., 1996).

The flowers of *Crinum bulbispermum* exhibited moderate DPPH anti-oxidant activity as opposed to the bulbs which displayed no activity at 100 ppm. This difference between aerial and subterranean plant parts could be due to harnessing of anti-oxidant molecules for protection against ultra-violet radiation. Likewise, the methanol extract of the leaves and roots of *Halleria lucida* displayed significantly different anti-oxidant activity ($p < 0.05$). It would be interesting to determine the difference, if any, in anti-oxidant activity between aerial and subterranean parts in other plants. It is also interesting to note that the methanol extract of the leaves of *H. lucida* displayed more potent anti-oxidant activity than the methanol extract of

the roots of *Harpagopytum procumbens* ($p < 0.05$) but *H. procumbens* displayed superior efficacy compared to *H. lucida* (Fig. 3.3).

Another interesting result was that *Croton gratissimum* and *Croton sylvaticus* displayed completely different anti-oxidant activity ($p < 0.05$). Given that biological activity is simply a manifestation of chemical profiles this warrants further investigation.

Methanol extracts were generally more active ($P < 0.05$) than aqueous extracts as free radical scavengers which again indicates that a wise approach to high throughput screening would be to utilize extraction solvents in laboratory settings identical to solvents used in clinical settings. Examples of this include, but are not limited to, methanol and aqueous extracts of *Aloe ferox, Halleria lucida* and *Rauvolfia caffra*. The only two exceptions to this were the aqueous extract of the leaves of *Leonotis leonurus* which displayed greater anti-oxidant activity than the corresponding methanol extract ($P < 0.05$) and the aqueous extracts of the leaves of *Melianthus comosus* which displayed greater anti-oxidant activity than the corresponding methanol extract ($P < 0.05$). Little is known about the anti-oxidant activities of *Clausena anisata, Lippia javanica, Rauvolfia caffra, Trichilia emetica and Ziziphus mucronata* and this warrants further investigation.

Medicinal plants which displayed the most potent anti-oxidant activities included *C. sylvaticus* (methanol extract), *H. lucida* (methanol extract) and *M. comosus* (aqueous and methanol extract) relative to the arbitrary scale of relative *in vitro* anti-oxidant

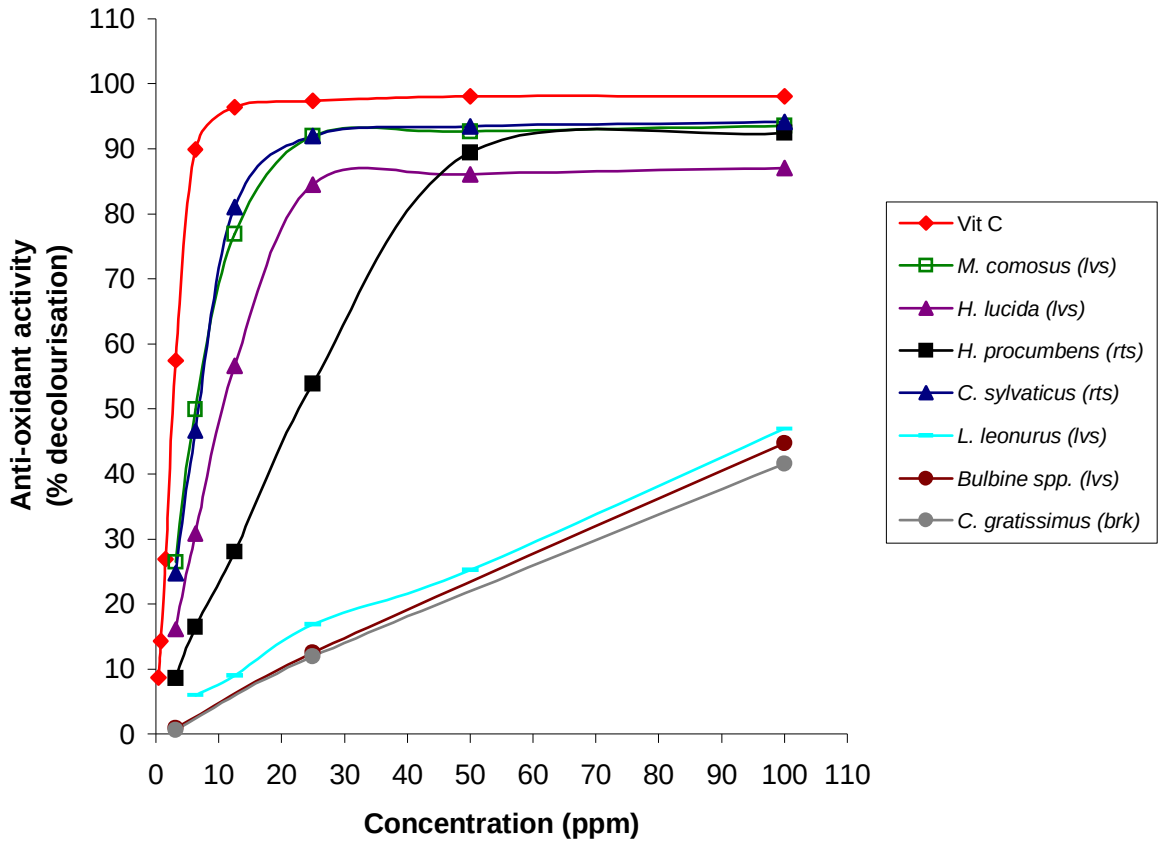


Fig. 3.3: Anti-oxidant / DPPH free radical scavenging activity of selected methanol extracts of South African medicinal plants commonly used topically for skin diseases. Percentage decolourisation reflects the amount of DPPH free radical reduced by a potential anti-oxidant. Potency of anti-oxidant activity is extrapolated from the greatest % decolourisation achieved for an extract.

activity defined previously. All three medicinal plants mentioned displayed anti-oxidant activity statistically different from the positive controls of Vit C and NDGA. The vast proportion of medicinal plants chosen displayed anti-inflammatory and / or anti-oxidant activity. The inflammatory and oxidation cascades are extremely complex with a number of potential pharmacological targets. A medicinal plant showing little or no activity in the 5-lipoxygenase assay or DPPH anti-oxidant assay might display biological activities in a selection of different assays. Furthermore, results obtained by experimentation are simply a manifestation of the method used. It is not an unreasonable assumption that medicinal plants tested might exert potent activities in anti-inflammatory and anti-oxidant assays such as the iron chelation assay, hydrogen peroxide assay, cyclooxygenase-1 assay etc. Correlation between *in vitro* and *in vivo* tests also needs to be verified for a complete assessment of anti-oxidant and anti-inflammatory activity. In addition, further work is required to assess the toxicity profile of medicinal plants which are used topically for skin disease.

Due to the promising activities presented by *Halleria lucida*, *Warburgia solutaris* and essential oils (*Helichrysum odoratissimum*, *Heteropyxis natalensis*, *Lippia javanica*) and their availability from natural localities, these medicinal plants were subjected to further biological tests and classical chromatographic techniques documented in the following sections.

The anti-oxidant potential of various medicinal plants displayed in Table 3.1 may be due to concentration differences in anti-oxidant molecules. Alternatively, it may be due to the stability of the free-radical formed by the anti-oxidant molecules in a

medicinal plant relative to the stability of the DPPH free-radical (Pauli's principle). Such stability differences need to be analysed further using electron spin resonance (ESR) in the pursuit of novel anti-oxidant molecules. This warrants further investigation.

Chapter 4: *Halleria lucida*: Isolation and Further Biological Studies

4.1 Introduction

Preliminary screening of the anti-oxidant activity of *Halleria lucida* yielded promising results with an IC_{50} value of 8.49 ± 0.12 ppm for the methanol extract of the leaves. Given this result and the interesting ethnopharmacologic uses of this medicinal plant, a decision was taken to subject the methanol extract of the leaves of *Halleria lucida* to activity-guided fractionation. It was hoped that such activities would result in the isolation of anti-oxidant compounds. Isolated compounds were subsequently characterised by utilizing HPLC and NMR and retested in biological assays. Further, their potential for interaction was investigated.

4.1.1 Botanical Description

A small to medium-sized tree. It can reach 20 metres in height. The leaves are drooping, ovate, thinly leathery, shiny bright green and hairless. The apex tapers to attenuate and the base very broadly tapers to square. The margin of the leaves is finely toothed or scalloped. The flowers are cream, brick-red or orange in colour, tubular and often grow in clusters. The fruit is ovoid to almost round, fleshy and black. The bark is pale greyish brown in colour, longitudinally fissured and flaky (Coates-Palgrave, 2003; Palmer, 1981).



Fig. 4.1: *Halleria lucida* in flower.

4.1.2 Distribution

Olifants River Mountains in the Western Cape but not confined to this area (Coates-Palgrave, 2003). Occuring in forests, forested ravines and grasslands often along streams or in rocky places (van Wyk *et al.*, 2000).

4.1.3 Dermatological Uses

Used for a variety of skin complaints. The Zulu use the dried leaf moistened with water and the juice is squeezed into the ear for the relief of earache. (Coates-Palgrave, 2003; Hutchings *et al.*, 1996; Watt and Breyer-Brandwijk, 1962).

4.2 Materials and Methods

4.2.1 Thin Layer Chromatography (TLC)

Preliminary screening of the methanol extract of the leaves of *Halleria lucida* indicated promising anti-oxidant / free radical scavenging activity. The methanol extract of the leaves of *H. lucida* was subjected to activity-guided fractionation using TLC. This technique is simple, rapid and cost effective and allows for easy zoning and separation of biologically active molecules. The methanol extract of the leaves of *H. lucida* was diluted (1:10) with methanol (Rochelle Chemicals) and approximately 2 µl was applied to a silica gel (Alugram® Sil G/UV_{254}, Macherey-Nagel) TLC plate using a calibrated glass capillary tube (Hirschmann Laborgerate). The TLC plate was developed in a mobile phase comprising methanol:water:ethyl acetate (16.5:13.5:100). The TLC plate was then removed, air-dried for 1-2 min., placed under UV light of wavelength 254 and 356 nm and viewed (Camag). Positive free radical scavenging / anti-oxidant activity was confirmed by spraying the plate with DPPH solution (0.04**%** in HPLC grade methanol from Ultrafine Limited) using an atomizer. Colour development occurred spontaneously and active molecules appeared as yellow zones against a purple background (Fig. 4.2).

4.2.2 Silica Gel Column Chromatography (SGCC)

A glass column resistant to solvents and able to withstand pressure differentials was used. The column was clamped in an upright position. Wet column packing was used in which silica gel (size 0.063-2 mm, Macherey-Nagel) was mixed with the appropriate mobile phase and poured into the column as a compact even suspension

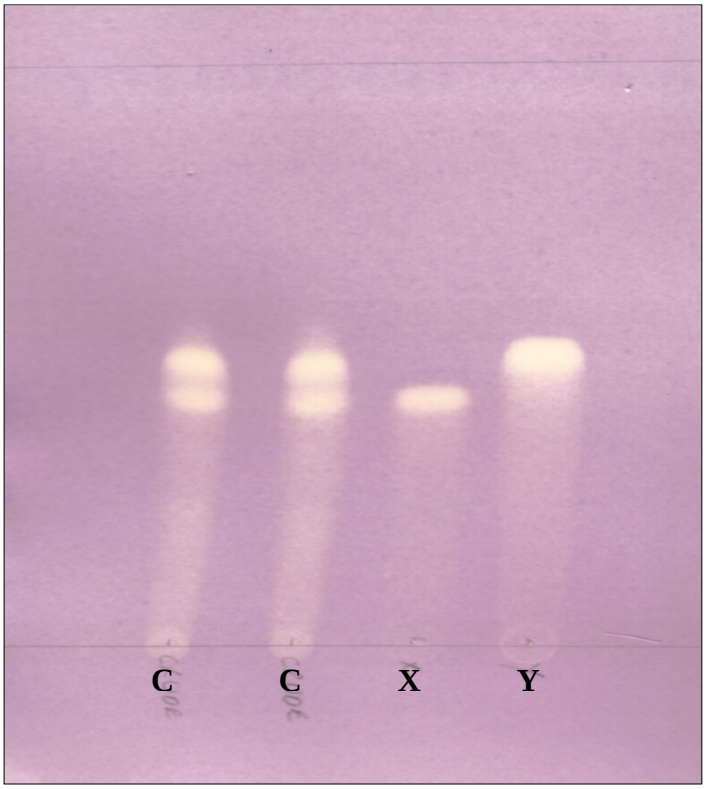


Fig. 4.2: Final isolation of two anti-oxidant compounds using PTLC. Active molecules appear as yellow zones against a purple background following atomization of a TLC plate with DPPH. C, methanol extract of leaves of *Halleria lucida*; X, compound X; Y, compound Y.

to form the stationery phase. Particular attention was paid to ensure a small volume of mobile phase supernatant. Approximately 1 g extract per 100 g silica was weighed (1200 mg) and dissolved in approximately 2.5 ml of the mobile phase. The extract solution was then mixed with approximately 2.5 % stationary phase and allowed to dry in a fume-cupboard. The dry mass mixture of plant extract and silica gel was then introduced to the supernatant mobile phase in the column. Flow rates of the column were adjusted appropriately. Gradient elution of increasing polarity

was initiated consisting of successive elutions of hexane and dichloromethane (9:1); dichloromethane and methanol (6:1); and methanol. The fractions were collected from the column (F1-F6) and free radical scavenging / anti-oxidant activity of each fraction was monitored and confirmed by TLC under UV light and atomisation as described in Section 4.2.1. Fractions displaying free radical scavenging / anti-oxidant activity (F3-F5) were dried using a rotavapour (Büchi R-114). Fractions with similar R_f values for potential anti-oxidant compounds were considered as fractions with similar components. Fraction 5 was chosen for further fractionation procedures as it appeared to contain fewer biologically inactive compounds. This was used as rationale for choosing F5 over F3 and F4 as isolation of active compounds could be achieved more efficiently. SGCC was performed on F5 using a mobile phase system comprising methanol:water:ethyl acetate (16.5:13.5:100). Active fractions displaying similar R_f values for potential anti-oxidant compounds were recombined and dried yielding a final mass of 900 mg. In all cases fractions were generally stored in cool, dark places between procedures to prevent UV initiated degredation.

4.2.3 Size Exclusion Column Chromatography (SECC)

SECC, also known as gel filtration or gel permeation chromatography, was used subsequent to SGCC. In this method molecular size differences account for separation of biologically active molecules. The stationary phase consisted of a porous three-dimensional polymeric matrix (Sephadex LH-20, Pharmacia Biotech) with a fractionation range < 1.5 daltons. The polymeric matrix was initially

immersed in methanol for several hours to facilitate swelling to occur prior to use. Thereafter, the matrix was introduced into a glass column and the biologically-active fraction (F5, 300mg) was applied to the supernatant as described in Section 4.2.2. Methanol was used as mobile phase. Fourty fractions were eluted and collected and biological activities and fraction composition (R_f values of active compounds) monitored using TLC and DPPH atomisation. Fractions displaying similar biological activities and chemical composition (similar R_f values) were combined: F9-F40 combined while F1-F8 were discarded. Combined fractions were then subjected to drying using a rotavapour and further fractionation. In all cases fractions were generally stored in cool, dark places between procedures to prevent UV initiated degredation.

4.2.4 Flash Column Chromatography (FCC)

FCC comprised flash silica (size 0.04-0.063 mm, Macherey-Nagel) as the stationary phase and methanol, water and ethyl acetate (33:13.5:150) as the mobile phase. The glass column was packed by the same method described in section 4.2.2 with F9-F40 subjected to further fractionation. The eluate was collected and parallel biological and chemical profiling (R_f values) of fractions was performed by TLC and DPPH atomisation. Fractions displaying free radical scavenging / anti-oxidant activity and similar chemical composition (similar R_f values) were combined and dried using a rotavapour. In all cases fractions were generally stored in cool, dark places between procedures to prevent UV initiated degredation.

4.2.5 Crystallisation

Further purification of compounds was attempted by crystallisation from methanol following FCC. The dry active fraction (following combination) was dissolved in methanol and placed at room temperature in the dark upon which visual inspection of crystallization took place. Crystals were removed and washed with acetone over filter paper (55 mm, Whatman). Purity and chemical composition (R_f values) of crystals were assessed by TLC and DPPH atomisation.

4.2.6 Preparative / Analytical TLC (PTLC)

FCC and crystallization appeared to have caused derivatisation of one of the active anti-oxidant / free radical scavenging compounds of *H. lucida*. As a result, F5 was discarded and further isolation procedures reverted to F4 discussed in Section 4.2.2. A total of 79.6 mg of F4 was dissolved in methanol and applied as a band to two TLC plates (size 20 x 20 cm, thickness 0.5 mm, SIL G-50 UV $_{254}$ from Macherey-Nagel). The plates were then developed in a mobile phase system comprising methanol:water:ethyl acetate (16.5:13.5:100) for approximately 120 min in the dark. The plate was then air-dried for 5-10 minutes and visualized under UV light for detection of compounds. Positive identification of two bioactive molecules was made by spraying with a DPPH atomizer along a narrow strip at the edge of each plate. Correlation with previously determined R_f values for the two bioactive compounds aided positive identification. The colorimetric reaction which took place was used to estimate the position of the bands that had been protected from the spray and subsequently marked. The marked band was scraped off the plate,

mixed with an excess volume of methanol, filtered and dried in a fume-chamber. Purity of compounds obtained by PTLC was initially assessed using TLC and DPPH atomization (Fig. 4.2). Correlation with previously determined R_f values for the two isolated compounds was also evident. PTLC yielded approximately 7.8 mg and 9 mg of two anti-oxidant / free radical scavenging bioactive molecules, respectively, which were subsequently stored in the dark. Both compounds isolated were subsequently subjected to the DPPH anti-oxidant assay and the 5-lipoxygenase assay.

4.2.7 High Performance Liquid Chromatography (HPLC)

HPLC / UV-MS was performed in collaboration with Mr. P. Steenkamp (Forensic Toxicology Research Unit, Forensic Chemical Laboratory, Johannesburg, South Africa). Identification and detection was performed using a Waters 2690 HPLC System (Phenomenex Aqua C18 column, 250 x 2.1 mm) equipped with a 996 photodiode array (PDA) detector and a Thermabeam mass selective detector (TMD). The TMD detector was operated in electron impact mode with the ioniser at 70 eV with a gain of 10 and scanning mass range of 50-550 amu. The Z spray mass selective detection was operated in positive mode with no flow splitting. The flow rate of the HPLC was 0.2 ml/min and the gas flow through the nebuliser 30 l/h. The nebuliser, expansion region and source temperatures were maintained at 80 °C, 90 °C and 225 °C, respectively. Isolated compounds described in section 4.2.6 were injected separately and compared to the HPLC fingerprint obtained by injecting the methanol extract of the leaves of *H. lucida*. Final analysis and identification was

confirmed by convolution of retention times and UV profiles using Empower® software.

4.2.8 Nuclear Magnetic Resonance (NMR)

Final chemical characterization of two isolated anti-oxidant molecules was confirmed by NMR in collaboration with Prof. F. van Heerden (RAU, Johannesburg, South Africa). NMR spectroscopy experiments on the compounds were performed on a Varian Inova 2000 300 MHz spectrophotometer. All spectra were recorded at 25 °C in deuterated chloroform. Chemical shifts were recorded in ppm referenced to tetramethyl silane (TMS) as internal standard. Chemical shifts, coupling constants and signal multiplicity were reported with the following designations: δ = chemical shift in ppm; J = coupling constant; *d* = doublet; *dd* = double doublet; *m* = multiplet; *s* = singlet; *t* = triplet. Unfortunately, Mass Spectrometry and Infrared Spectrometry could not be performed in tandem to NMR due to insufficient isolated compound material.

4.2.9 Biological Testing

The two isolated anti-oxidant molecules were subsequently subjected to the 5-lipoxygenase assay and the DPPH anti-oxidant assay as described in Section 3.2.2.1 and Section 3.2.2.2

4.2.10 Isobologram Construction for Anti-oxidant Compounds

The synergistic, antagonistic or additive interaction between the two isolated molecules was determined. Different concentrations of one isolated molecule were prepared using serial dilutions and these were combined with serial dilutions of the second isolated molecule. The DPPH assay (Section 3.2.2.2) was used and IC_{50} values for each combination determined. Isobolograms were constructed by plotting fractional IC_{50} values for one compound against fractional IC_{50} values for the second compound. The different fractional IC_{50} values for each combination were calculated and plotted. Points lying above the straight diagonal line (convex in shape) and those lying below the straight diagonal line (concave in shape) indicated an antagonistic or synergistic interaction, respectively (Fig. 4.3) (Berenbaum, 1978). Points lying on the straight line indicated that the combination of two compounds in question have an additive interaction.

4.2.11 Statistical Analysis

Student's *t*-test was used to determine statistical differences in biological activity with a significance level of $P \leq 0.05$. All analysis were performed with GraphPad Prism® version 4.03 software.

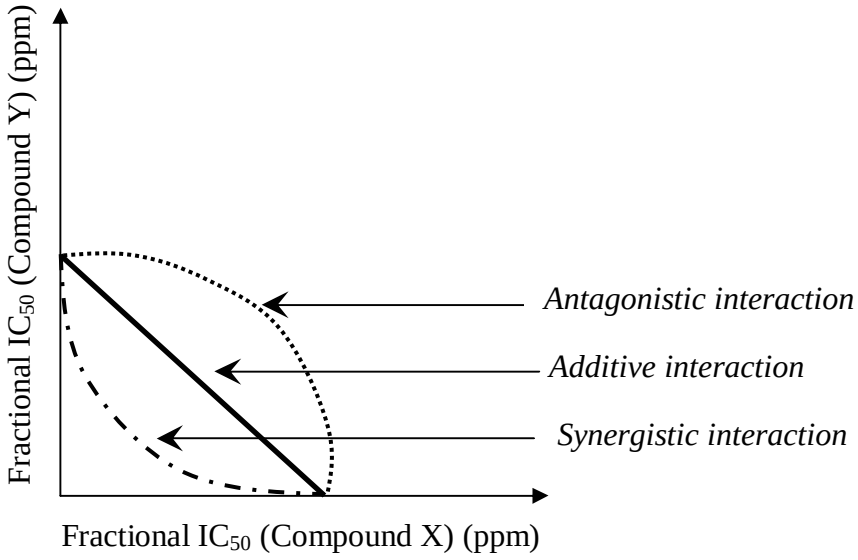
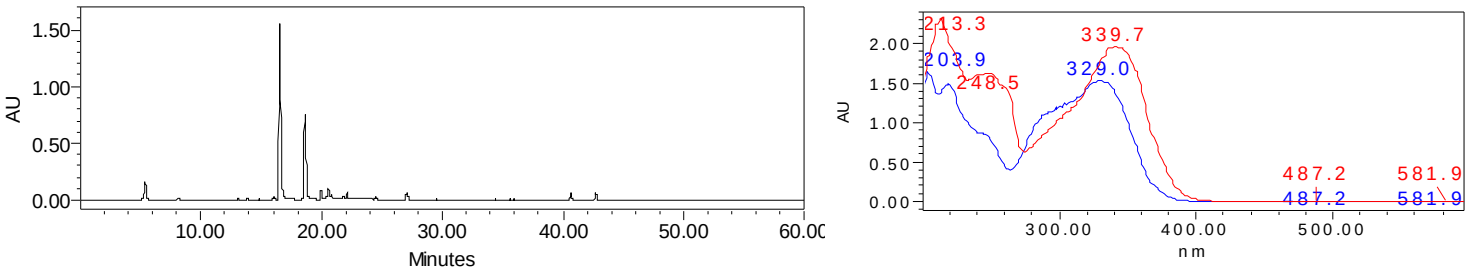


Fig 4.3: Graphical shapes produced by synergistic, antagonistic and additive isobolograms for a combination of two compounds (Berenbaum, 1978). Taken and modified.

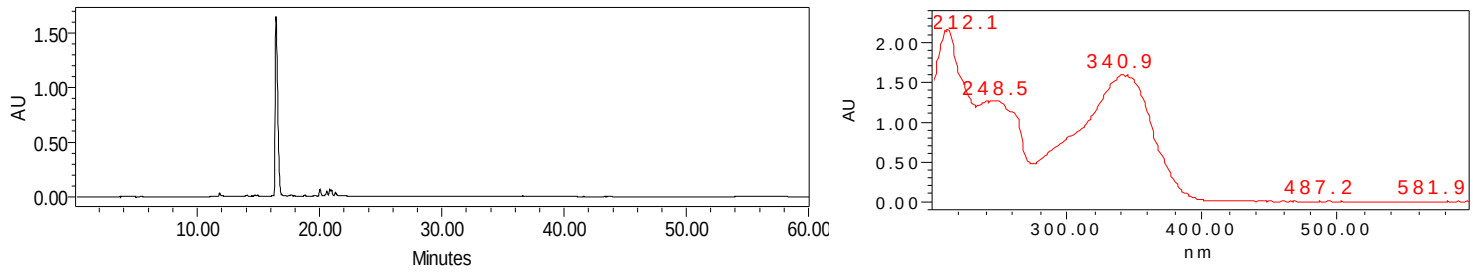
4.3 Results and Discussion

4.3.1 HPLC

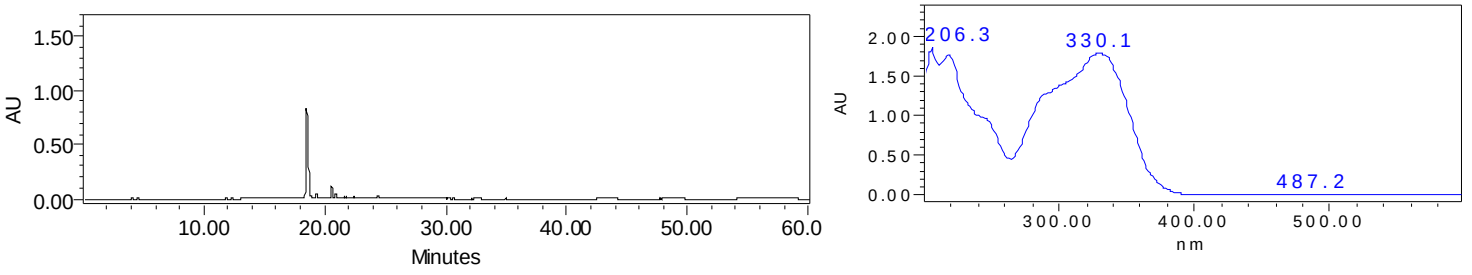
HPLC analysis was undertaken to determine purity of compounds prior to chemical characterization using NMR. Furthermore, the UV spectra generated by the PDA detector offered valuable information regarding the type of anti-oxidant molecules isolated. This provided a platform to attempt final chemical elucidation using NMR. For convenience, the two compounds were labelled compound X and compound Y, respectively (Fig. 4.4).



(a)



(b)



(c)

Fig. 4.4: HPLC and UV profiles of *Halleria lucida* and associated anti-oxidant molecules isolated from the same plant determined at 254 nm; (a) methanol extract of the leaves of *Halleria lucida*; (b) compound X; (c) compound Y.

Compound X had a retention time of 16.6 min. The λ_{max} values observed are 248.5 nm and 340.9 nm. The UV spectrum extracted provisionally identified compound X as a flavone-type flavonoid. Typical flavone UV spectra exhibit two major absorption peaks in the region 240-400 nm (Mabry *et al*., 1970). These two peaks are commonly referred to as Band I (300-380 nm) and Band II (240-280 nm) and are due to absorption caused by the B-ring cinnamoyl system and the A-ring benzoyl system, respectively. The λ_{max} values for compound X are 248.5 nm and 340.9 nm representing peak wavelength values for Band II and Band I, respectively (Fig. 4.4 (b)) The UV spectrum extracted for compound X was similar to the profile reported for luteolin-7-*O*-glucoside but the λ_{max} value for the latter was reported to be at a longer wavelength. This could possibly be due to a 5-hydroxyl group substitution which could in theory have caused a hypsochromic shift. As a result it was suggested that glycosidic linkage is confined to carbon-5 resulting in the luteolin-5-*O*-glycoside isomer.

Purity of compound X was assessed prior to chemical characterization by determination of peak purity using the Empower® software. Peak purity is used to evaluate whether a chromatographic peak is spectrally homogenous. Spectral heterogeneity can indicate the presence of coelution. An integration algorithm confirmed purity angle to be less than purity threshold and was a final indicator of spectral homogeneity and subsequent purity. A decision was subsequently made to subject compound X to NMR analysis.

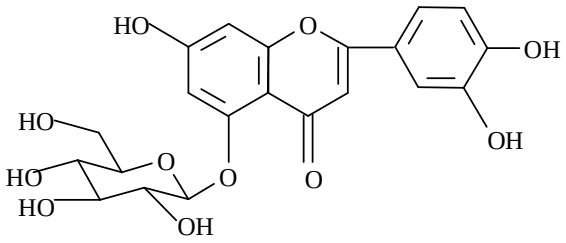
Compound Y had a retention time of 18.5 min. The λ_{max} value of 330.1 nm (Fig. 4.4 (c)) offered little in the way of final chemical characterisation. The area of the peak

for compound Y is less than that observed for compound X. This could possibly be due to compound Y absorbing less strongly than compound X at 254 nm. Differences in peak areas could be indicative of a greater concentration of compound X compared to compound Y in the dry leaf. Alternatively, the method of isolation of both compounds, namely PTLC, could be more efficient for compound X than compound Y. Spectral homogeneity and peak purity were similarly determined for compound Y as for compound X. A decision was subsequently taken to subject compound Y to NMR analysis.

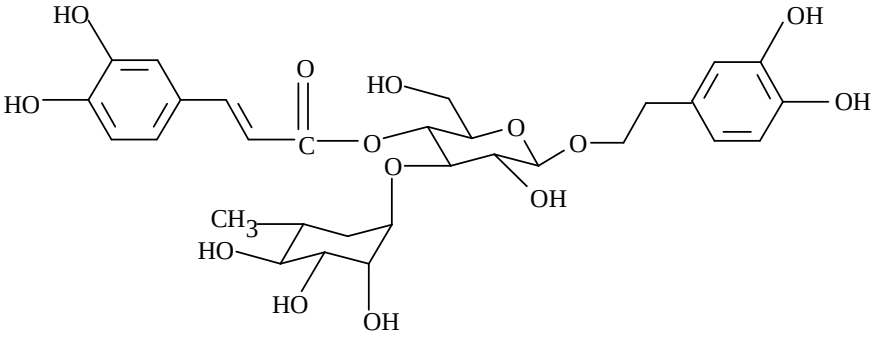
4.3.2 Chemical Structure Elucidation (NMR)

4.3.2.1 Luteolin-5-*O*-*β*-D-glucoside

The more polar compound (7.8 mg, R_f 0.51) was eventually isolated by PTLC as discussed in section 4.2.6 and identified as luteolin-5-*O*-*β*-D-glucoside (Fig. 4.5 (a)). NMR spectra are presented in Appendix 3. ^{1}H-NMR δ 7.39~7.43 (2H, *m*, H-2', 6'), 6.93 (1H, *d*, J = 8.9 Hz, H-5'), 6.85 (1H, *d*, J = 2.4 Hz, H-8), 6.72 (1H, *d*, J = 2.2 Hz, H-6), 6.57 (1H, *s*, H-3), 4.87 (1H, *d*, J = 7.7, H-1''). ^{13}C-NMR δ 180.4 (C-4), 165.8 (C-2), 164.3 (C-7), 160.7 (C-5), 160.1 (C-9), 150.8 (C-4'), 147.0 (C-3'), 123.5 (C-1'), 120.0 (C-6'), 116.7 (C-5'), 113.9 (C-2'), 108.9 (C-10), 106.4 (C-3), 105.1 (C-1''), 105.5 (C-6), 99.4 (C-8), 78.5 (C-5''), 77.3 (C-3''), 74.7 (C-2''), 71.2 (C-4''), 62.5 (C-6''). The NMR data correlated to that given in literature (Lin *et al*., 2001). Hydrogen shift correlations excluded luteolin-7-O-*β*-D-glucoside isomer and glucoside substitution on the B-ring cinnamoyl system (El-Negoumy *et al*., 1986; Feeny *et al*., 1988; Lewis *et al*., 1998).



(a)



(b)

Fig. 4.5: Chemical structures of isolated compounds from the methanol extract of the leaves of *Halleria lucida*. (a) luteolin-5-*O*-glucoside; (b) verbascoside (acteoside).

4.3.2.2 Verbascoside

The less polar compound (9.0 mg, R_f 0.57) was eventually isolated by PTLC as discussed in section 4.2.6 and identified as verbascoside or acteoside (Fig. 4.5 (b)). NMR spectra are presented in Appendix 3. ^{1}H-NMR δ 7.57 (1H, *d*, J = 15.9 Hz, H-7), 7.05 (1H, *d*, J = 2.1 Hz, H-2), 6.94 (1H, *dd*, J = 1.8 Hz and 8.4 Hz, H-6), 6.77 (1H, *d*, J = 7.8 Hz, H-5), 6.69 (1H, *d*, J = 2.1 Hz, H-2'), 6.66 (1H, *d*, J = 8.7 Hz, H-

5'), 6.55 (1H, *dd*, J = 1.8 Hz and 8.1 Hz, H-6'), 6.25 (1H, *d*, J = 15.9 Hz, H-8), 5.18 (1H, *d*, J= 1.8 Hz, H-1 Rham), 4.37 (1H, *d*, J = 7.8 Hz, H-1 Gluc), 4.10–3.20 (Carbohydrate-H), 2.80 (1H, *t*, J = 7.2 Hz, H-7'), 1.08 (3H, *d*, J = 6.3 Hz, H-6 Rham). ^{13}C-NMR δ 168.3 (C-9), 149.8 (C-3), 148.0 (C-7), 146.9 (C-4), 146.1 (C-4'), 144.7 (C-3'), 131.4 (C-1'), 127.6 (C-1), 123.2 (C-6), 121.2 (C-6'), 117.1 (C-5'), 116.5 (C-2'), 116.3 (C-5), 115.2 (C-8), 114.6 (C-2), 104.2 (C-1, Gluc), 103.1 (C-1, Rham), 81.7 (C-3, Gluc), 76.2 (C-5, Gluc), 76.0 (C-2, Gluc), 73.8 (C-4, Rham), 72.3 (C-8'), 72.3 (C-2, Rham), 72.0 (C-3, Rham), 70.5 (C-4, Gluc), 70.4 (C-5, Rham), 62.3 (C-6, Gluc), 36.6 (C-7'), 18.5 (C-6, Rham). The NMR data correlated to that given in literature (Andary *et al*., 1982).

Luteolin-5-*O*-*β*-D-glucoside and verbascoside (acteoside) have both been identified and characterized in the leaves of *H. lucida* (Abdullahi *et al*., 1986).

4.3.3 Documented Anti-inflammatory and Anti-oxidant Activities of Luteolin-5-*O*-glucoside and Verbascoside

Verbascoside, a phenylpropanoid glycoside, has been reported to be present in a number of plants (Franzyk *et al*., 2004; Lin *et al*., 2004; Pinar *et al*., 2004). The anti-oxidant activity of phenylpropanoid glycosides has been investigated and it has been reported that they display radical scavenging activity against DPPH, hydroxyl and superoxide anion (Gao *et al*., 1999; Kyriakopoulou *et al*., 2001). In addition, verbascoside has been shown to inhibit lipid peroxidation (Lin *et al*., 2004).

Furthermore, verbascoside has been reported to inhibit leukocyte migration in rat glomeruli (Akbay *et al*., 2002; Hayashi *et al*., 1994).

Information regarding the biological activities of luteolin-5-*O*-glucoside is limited. However, luteolin has been reported to display DPPH radical scavenging properties (Edenharder and Grünhage, 2003). In addition, luteolin and corresponding glycosides have also been shown to inhibit lipid peroxidation (Lee *et al*., 2002) while luteolin-7-glucoside has been used as a reference material for inhibition of lipid peroxidation in scientific studies (Fejes *et al*., 2000). Furthermore, the anti-inflammatory activities of luteolin and derivatives thereof have been studied and documented and include inhibition of IL-5, degranulation, cyclooxygenase and 5-lipoxygenase activity (Cheong *et al*., 1998; Park *et al*., 1999; Williams *et al*., 1999).

4.3.4 DPPH and 5-lipoxygenase Activities of Luteolin-5-*O*-glucoside and Verbascoside

Isolated compounds were again subjected to further biological tests and the results were as depicted in Table 4.1. Both luteolin-5-*O*-glucoside and verbascoside did not display any 5-lipoxygenase inhibitory activity which was consistent with the activity displayed by the methanol extract of the leaves of *Halleria lucida* from which they were isolated. The anti-oxidant activities were significantly greater for the isolated compounds than for the crude extract ($P < 0.05$). This result was expected as the crude extract has a lower yield of the two anti-oxidant compounds than pure compounds in the DPPH anti-oxidant assay. As a result one would expect a far

Table 4.1: Comparison of the DPPH anti-oxidant and 5-lipoxygenase activities of *Halleria lucida*, luteolin-5-*O*-glucoside and verbascoside.

Plant/Compound	Plant part	Methanol/Aqueous extract	IC_{50} (ppm)[a]	IC_{50} (ppm)[b]
Halleria lucida	Leaves	Methanol	> 100	8.49 ± 0.12
luteolin-5-*O*-glucoside	-	-	> 100	6.12 ± 0.40
verbascoside	-	-	> 100	7.18 ± 0.08
NDGA			5 ± 0.50[c]	2.73 ± 0.06
Vit C			> 100	2.46 ± 0.01

[a] 5-lipoxygenase inhibitory activity.
[b] DPPH anti-oxidant activity, values are mean ± S.D (n=3).
[c] Value is mean ± S.D (n=3).

greater IC_{50} value for the crude extract compared to the IC_{50} values of the two isolated compounds. However, possible antagonism between luteolin-5-*O*-glucoside and verbascoside could also explain this behaviour and was therefore investigated. As a result isobologram construction was undertaken.

4.3.5 Isobologram Construction

4.3.5.1 *H. lucida* and Vitamin C (Ascorbic Acid)

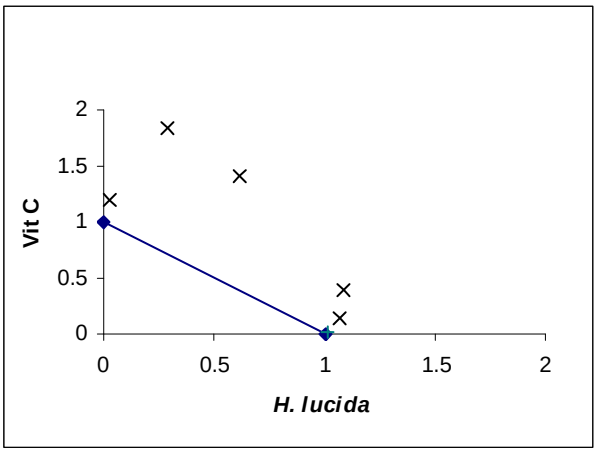
Isobologram construction was undertaken as described in Section 4.2.10 and was used as a tool for predicting the synergistic, antagonistic or additive anti-oxidant interaction between luteolin-5-*O*-glucoside and verbascoside. It was necessary to subject the two isolated compounds for further biological testing following

isobologram construction. Therefore, it was essential to dissolve the isolated compounds in a volatile solvent which allowed subsequent drying and reutilization of compounds in further biological tests. A decision was made to compare and contrast DMSO and methanol as solvents for the methanol extract of the leaves of *Halleria lucida* and Vit C.

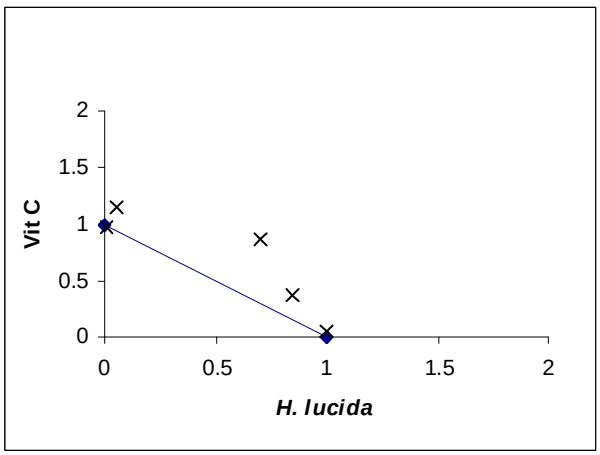
Isobologram construction using DMSO and methanol produced similar profiles (Fig. 4.6 (a) and (b)). This allowed us to proceed with further tests relating to isobologram construction. In addition, these preliminary results offered valuable insight into the usage of *H. lucida* with conventional anti-oxidants. It appears that a pattern of antagonism exists. The potential for interaction between medicinal plants and conventional medicines has been reported (Miller *et al*., 2004).

4.3.5.2 Luteolin-5-*O*-glucoside and Verbascoside

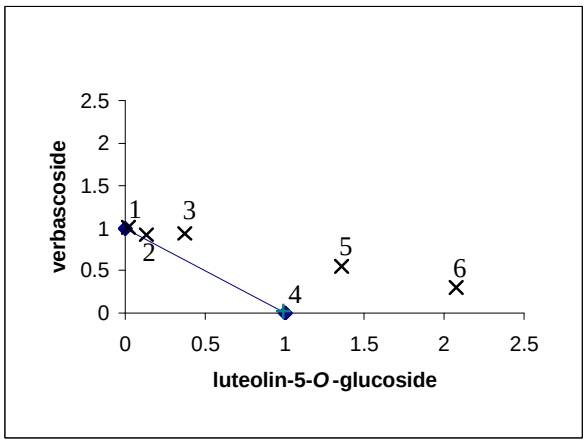
Investigation of the interaction between luteolin-5-*O*-glucoside and verbascoside resulted in an isobologram displaying marked antagonism (Fig. 4.6 (c)). Isobolograms are useful not only in displaying synergism, antagonism and additivity but allows one to correlate such activity over a range of concentrations for two compounds. Raw data generated for the isobologram constructed in Fig. 4.6 (c) is displayed in Table 4.2.



(a)



(b)



(c)

Fig. 4.6: Isobologram constructions. (a) methanol extract of the leaves of *H. lucida* and Vit C dissolved in DMSO; (b) methanol extract of the leaves of *H. lucida* and Vit C dissolved in methanol; (c) luteolin-5-*O*-glucoside and verbascoside. Ratio values for (a), (b) and (c) denote the quotient of fractional IC_{50} values and are therefore unitless. Indices denote individual points.

Table 4.2: Raw data generated for construction of isobologram to display interaction between luteolin-5-*O*-glucoside and verbascoside.

Plate	Concentrations (ppm)		IC_{50} Values (ppm)				Ratio Values[d]	
	X[a]	Y[b]	X	s.d.[c]	Y	s.d.[c]	X	Y
1	50	0	9.75	0.24	0.00	0.00	1.00	0.00
	50	1	9.65	0.37	0.19	-	0.99	0.02
2	35	5	20.24	0.27	2.89	-	2.08	0.30
	25	10	13.23	0.39	5.29	-	1.36	0.55
3	10	25	3.62	-	9.05	0.26	0.37	0.94
	5	35	1.28	-	8.94	0.19	0.13	0.93
4	1	50	0.20	-	9.75	0.24	0.02	1.01
	0	50	0.00	0.00	9.65	0.37	0.00	1.00

[a] luteolin-5-*O*-glucoside.
[b] verbascoside.
[c] standard deviation (n=3),
[d] ratio values denote the quotient of fractional IC_{50} values and are therefore unitless.

Raw data presented in Table 4.2 reveals the existence of two patterns of interaction. Luteolin-5-*O*-glucoside (X) and verbascoside (Y) appear to interact in an additive manner when one of the two compounds are present in low concentrations. Such low concentrations of either compound could presumably negate any antagonism. An indication of the statistical significance of each point (Fig. 4.6) relative to the additive line on the same graph was determined. Statistical significance was determined by geometric iteration of the shortest linear distance of individual points, represented by indices 1-6 (Fig. 4.6), to the additive line represented by the equation $y = -x + 1$ where x and y represent the x- and y-axis, respectively. Points 1 and 4 displayed additive activity in relation to ideal additive biological behaviour ($p > 0.05$) while points 2,3,5 and 6 displayed marked antagonism ($p < 0.05$). Furthermore, points 2,3,5, and 6 were compared and statistical significance between

all four points was observed ($p < 0.05$). Additivity is apparent for low concentrations of luteolin-5-*O*-glucoside and verbascoside i.e point 1 and 4 are both additive in relation to the ideal additive line ($p > 0.05$). The greatest antagonism was observed for the combinations in plate 2 i.e. 35 ppm of luteolin-5-*O*-glucoside with 5 ppm of verbascoside and 25 ppm of luteolin-5-*O*- glucoside with 10 ppm of verbascoside ($p < 0.05$). Antagonism was therefore found to be concentration dependent. A number of explanations can be proposed to explain the antagonistic phenomena observed. It is possible that luteolin-5-*O*-glucoside and verbascoside interact chemically to form a complex resulting in lower anti-oxidant potential. NMR analysis could verify this. Furthermore, the pKa values could be similar which would indicate an equal likelihood, and therefore competition, for scavenging the DPPH free radical. In addition, the concentrations of luteolin-5-*O*-glucoside and verbascoside might be reaching saturation level with regards to DPPH free radical scavenging activity. However, such a scenario is unlikely as raw data generated by the DPPH anti-oxidant assay for the combination tests did not indicate saturation (all combinations $\leq$ 90% DPPH free radical scavenging / decolourisation) at the highest concentration tested. Other compounds in the crude methanol extract of *H. lucida* could also contribute to the observed anti-oxidant activity. This could involve the prevention of decomposition or autoxidation of luteolin-5-*O*-glucoside or verbascoside (Palozza and Krinsky, 1992) or alternatively by a mechanism involving recycling of luteolin-5-*O*-glucoside and verbascoside free radicals which could perhaps increase antagonistic behaviour via scavenging competition.

Furthermore, it is important to remember that the isolation procedures for luteolin-5-*O*-glucoside and verbascoside involve "naked-eye" visualisation of TLC plates atomised with DPPH. Although this invariably results in activity-guided isolation of major anti-oxidant compounds, it remains a subjective effort in the sense that an individual's visual cortex has its own limit of detectibility of colour change. It could possibly prove more accurate to utilize a camera which demarcates a colorimetric reaction with its own standardised limits of detectibility. This would standardise activity-guided fractionation and zoning of all viable anti-oxidant compounds regardless of the level of their activity. The potential for interaction, and therefore competition, between all viable anti-oxidant compounds is enormous and could further explain the higher IC_{50} value of the crude methanol extract of *H. lucida* in relation to the isolated compounds.

In addition, given the predominance of luteolin-5-*O*-glucoside and verbascoside in the HPLC profile of the methanol extract of the leaves of *Halleria lucida* (Fig. 4.4) it is a possibility that other compounds which contribute to the anti-oxidant activity and possibly interaction and competition are non-UV active. If this is indeed the case visualisation of such compounds under UV light at wavelength 254 and 356 nm would prove unsuccessful and other techniques of isolation would be warranted.

In conclusion it would appear that isolation of active anti-oxidant constituents of *H. lucida* via a reductionist approach yielded two viable anti-oxidant molecules. It appears that the two molecules react in an antagonistic manner when in combination. This could explain the higher IC_{50} value of the crude methanol extract in relation to the isolated compounds. Isolated compounds in low concentrations resulted in an

additive phenomenon being observed. These low concentrations probably mimic the concentrations of the two isolated compounds in the methanol extract of the *H. lucida* and therefore additivity is probably observed in the same extract. As a result, the high IC_{50} values of the crude extract in relation to the isolated compounds is probably due to lower yield. Usage of both compounds appear to cause marked antagonism when combined according to various concentration ratios. Therefore, isobologram construction in tandem to reductionist approaches is recommended for the isolation and identification of viable anti-oxidant molecules in various medicinal plants and for their possible subsequent clinical use in combination.

Chapter 5: *Warburgia salutaris*: Isolated Compounds and Further Biological Studies

5.1 Introduction

Preliminary screening of various South African medicinal plants used topically for skin diseases was described in Chapter 3. The methanol extract of the leaves of *Warburgia salutaris* was shown to display moderate but promising 5-lipoxygenase inhibitory activity with an IC_{50} of approximately 32.11 ppm. Various drimane sesquiterpenoids such as warburganal and polygodial have been isolated from *W. salutaris* (Mashimbye *et al.*, 1999). It is possible that these compounds are not present in the aqueous or methanol extracts of *W. salutaris* given the polarity of the extraction solvents. However, various sesquiterpenoids have in the past been extracted and identified with various aqueous methanolic solutions (Jang *et al.*, 2004; Kitajima *et al.*, 2002; Zidorn *et al.*, 2000). Furthermore, present ethnobotanical literature indicates that ointments are made from pounded leaves and stalks of *W. salutaris*, mixed with bark and any kind of fat, and used in cases involving inflammation, sores and skin irritation (Hutchings *et al.*, 1996). This would allow selective diffusion of drimane sesquiterpenoid compounds to areas of local skin pathology. Given the interesting chemistry and wide spectrum of biological activities intrinsic to drimane sesquiterpenoids and the interesting ethnopharmacological profile reported for *W. salutaris*, a decision was taken to subject these compounds to further biological tests. Furthermore, the over-

exploitation of *W. salutaris* and consequent threat of extinction means that there is an urgent need for the accumulation of more scientific knowledge about this medicinal plant.

5.1.1 Botanical Description

Slender in stature, usually 5-10 m in height but can occasionally reach a height of 20 m. Leaves are elliptic to lanceolate, glossy dark green above, paler green below, hairless, covered with clear gland dots and slightly aromatic. The apex and base taper. The flowers are white or greenish in colour and axillary. The fruit is a spherical berry with leathery skin, green to black in colour and covered with gland dots. The bark is brown in colour, rough in texture and the branchlets are long and lax (Coates-Palgrave, 2003; van Wyk *et al*., 1997).



Fig. 5.1: *Warburgia salutaris.* Taken from van Wyk *et al*. (1997).

5.1.2 Distribution

Restricted to a few localities in the north-eastern parts of South Africa where it has been heavily exploited for its bark. Usually found along the eastern seaboard of the province of KwaZulu-Natal and extending northwards through Mpumalanga and the Northern Provinces. Occurs in evergreen forest, wooded ravines and bushveld in rocky or sandy places (Coates-Palgrave, 2003; van Wyk *et al*., 1997).

5.1.3 Dermatological Uses

W. salutaris has been used since early times, as indicated by its specific name which means 'salutary' or 'health giving' (Coates-Palgrave, 2003). It has been used medicinally for penial irritation, urethral inflammation and sores (Hutchings *et al*., 1996; van Wyk *et al*., 2000). The bark is often powdered and applied to sores and inflammation (Rabe and van Staden, 2000). In addition, ointments made from pounded leaves and stalks, mixed with bark and any kind of fat, are used in cases of inflammation, sores and skin irritations (Hutchings *et al*., 1996).

5.2 Materials and Methods

5.2.1 Reference Compounds

Preliminary screening of the methanol extract of the leaves of *Warburgia salutaris* yielded promising 5-lipoxygenase inhibitory activity. Due to the expensive nature of the assay it was unpractical to conduct biologically-guided fractionation for zoning out viable anti-inflammatory compounds. A literature review revealed that traditional healers often use the bark, leaves and stalks of *W. solutaris* as ointments

for penial irritation, lotions for inflammation of the urethra and generalized sores (Hutchings *et al.*, 1996; van Wyk *et al.*, 1997). Furthermore, a compound by the name of warburganal has been identified and isolated from both bark and leaves of *W. salutaris* as a major compound and subsequently the leaves represented a good alternative source of major active compounds found in the bark (Mashimbye *et al.*, 1999). Drimane sesquiterpenoid standards, warburganal and mukaadial, were made available by Prof. S.E. Drewes (University of Natal, Durban, South Africa) utilizing SGCC and crystallization as previously published (Mashimbye *et al.*, 1999) while polygodial had undergone polymerisation. Drimane sesquiterpenoids have been reported to possess a wide spectrum of biological activities such as antibacterial, antifungal, anticomplemental, antifeedant, plant-growth regulatory, cytotoxic, phytotoxic, piscicidal, and molluscicidal. As a result, a decision was made to subject warburganal and mukaadial to further biological tests.

5.2.2 Biological Testing

The two isolated molecules were subjected to the 5-lipoxygenase assay and the DPPH anti-oxidant assay as described in Section 3.2.2.1 and Section 3.2.2.2, respectively.

5.2.3 Statistical Analysis

Student's *t*-test was used to determine statistical differences in biological activity with a significance level of $P \leq 0.05$. All analysis were performed with GraphPad Prism® version 4.03 software.

5.3 Results and Discussion

5.3.1 DPPH and 5-lipoxygenase Activities of *W. salutaris*, Mukaadial and Warburganal

The DPPH anti-oxidant and 5-lipoxygenase inhibitory activites of the methanol extract of the leaves of *W. salutaris* were promising (see Table 3.1). Previous studies reported the existence of a number of drimane sesquiterpenoid compounds to be present in the leaves and bark of *W.salutaris* as major compounds (Drewes *et al*, 2001; Mashimbye *et al*., 1999; Rabe and van Staden, 2000). Furthermore, the biological activities of drimane sesquiterpenoids are wide and varied (Jansen and de Groot, 1991). This was used as a rationale for subjecting two drimane sesquiterpenoids, namely warburganal and mukaadial, to further biological tests. The results are displayed in Table 5.1. It is apparent that warburganal displays moderate 5-lipoxygenase inhibitory activity with an IC_{50} of 61.86 ppm. Mukaadial displayed an IC_{50} value > 100 ppm in the 5-lipxoygenase assay. However, this translated into 46.10 % enzyme inhibition at 100 ppm. From the results it is apparent that both mukaadial and warburganal are contributing to the 5-lipoxygenase inhibitory activity of *W. salutaris*. However, the difference in activity between the two isolated compounds and *W. salutaris* is significant ($p < 0.05$) and can possibly be due to other compounds being present in the methanol extract of *W. salutaris*. Limited isolated compound material prohibited further biological testing.

Table 5.1: Comparison of the DPPH anti-oxidant and 5-lipoxygenase activities of *Warburgia salutaris*, mukaadial and warburganal.

Plant/Compound	Plant part	Methanol/Aqueous extract	IC_{50} (ppm)[a]	IC_{50} (ppm)[b]
Warburgia salutaris	leaves	methanol	32.11 (11.95)[c]	15.38 ± 0.20
mukaadial	-	-	> 100[d]	> 100
warburganal	-	-	61.86 (0.078)[c]	> 100
NDGA			5 ± 0.50[e]	2.73 ± 0.06
Vit C			> 100	2.46 ± 0.01

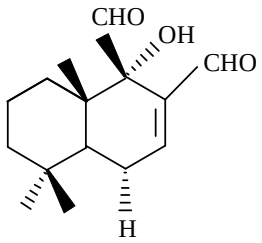
[a] 5-lipoxygenase inhibitory activity.
[b] DPPH anti-oxidant activity, values are mean ± S.D (n=3).
[c] Value in brackets reflects standard error of data points fitted to obtain a sigmoidal curve.
[d] 46.10 % inhibition at 100 ppm.
[e] Value is mean ± S.D (n=3).

In addition, *W. salutaris* contains a number of other drimane sesquiterpenoids such as polygodial, isopolygodial (isotadeonal), muzigadial and salutarisolide and ugandensidial (cinnamodial) (Mashimbye *et al*., 1998; Rabe and van Staden, 2000) and it is possible that such compounds are also contributing to the anti-inflammatory activity of the crude extract. This warrants further investigation and the procurement of HPLC profiles of *W. salutaris* in tandem with biological assays would yield valuable information with regard to the presence and activity of these compounds in the crude plant extract.

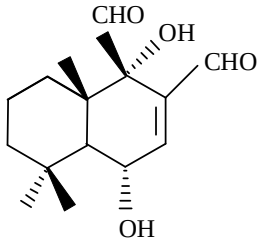
It is apparent from Table 5.1 that maukaadial and warburganal are not contributing to anti-oxidant activity. Anti-oxidant activity of the methanol extract of *W. salutaris* could possibly be due to the presence of flavonoid compounds. Flavonoids such as quercetin and kaempferol have been isolated from *Warburgia stuhlmannii* and these compounds could possibly be present in the leaves of *W. salutaris* ([a]Manguro *et al.*, 2003).

5.3.2 Drimane Sesquiterpenoids as a Class of Bioactive Molecules

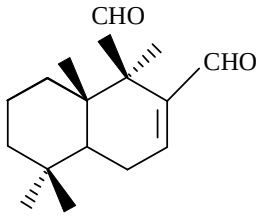
A drimane sesquiterpenoid is a bicyclic sesquiterpene. It contains the classic A,B ring system of many di- and triterpenes. Therefore, it may be considered as the missing biogenetic link between lower and higher terpenes (Jansen and de Groot, 1991; and references therein). *Warburgia salutaris* has been reported to contain a number of drimane sesquiterpenoids (Fig. 5.2). Both warburganal and mukaadial contain two aldehyde groups and differ only in the latter possessing a hydroxyl group at C-6. This hydroxyl group appears to be responsible for a large reduction in 5-lipoxygenase inhibitory activity compared to warburganal. As mentioned in Section 1.3.3, the 5-lipoxygenase enzyme undergoes conversion from an inactive reduced state to an active oxidized state (Young, 1999). It is possible that the hydroxyl group on C-6 of warburganal alters the redox potential of this reaction by nucleophilic attack on the Fe^{2+} of 5-lipoxygenase. Alternatively, the hydroxyl group



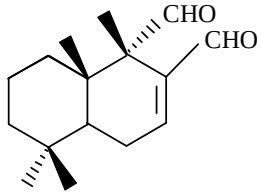
(a)



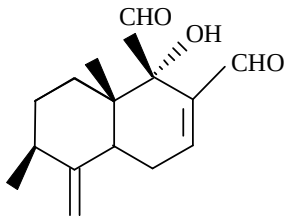
(b)



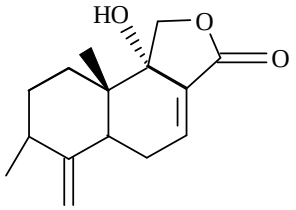
(c)



(d)



(e)



(f)

Fig. 5.2: Drimane sesquiterpenoids reported to be present in *Warburgia salutaris*. (a) warburganal; (b) maukaadial; (c) polygodial; (d) isopolygodial (isotadeonal); (e) muzigadial; (f) salutarisolide (Drewes *et al*., 2001; Mashimbye *et al*., 1999; Rabe and van Staden, 2000).

may interact with the 5-lipoxygenase enzyme via hydrogen bond formation. It has also been reported that biological activity of the enal-aldehydes is primarily related to their ability to form adducts with amino groups and this might explain the 5-lipoxygenase activity observed (D'Ischia *et al.*, 1982; Jonassohn *et al.*, 1997). Furthermore, it has been reported that racemic mixtures of warburganal halved allergenic responses compared to a single enantiomer (Stampf *et al.*, 1982). It is possible that the 5-lipoxygenase reaction is enantiomeric specific and that selection of warburganal enantiomers might provide more promising 5-lipoxygenase inhibitory activity and this warrants further investigation. In addition, the large class of bioactive molecules represented by drimane sesquiterpenoids may provide a 'stepping-stone' for chemical synthesis, structure-activity elucidation and enantiomeric selection in the search for a lead anti-inflammatory agent. It is also of interest to note that both warburganal and polygodial have been implicated in the formation of skin irritation and the development of contact dermatitis (Jansen and de Groot, 1991). This is unusual when one takes into consideration the dermatological uses of *W. salutaris*.

Various anti-inflammatory activities have been attributed to drimane sesquiterpenoids. The bark of *Drimys winteri* has been shown to display anti-asthmatic, anti-allergic, anti-inflammatory and anti-nociceptive effects which were related, at least partially, to the presence of polygodial (Malheiros *et al.*, 2001; and references therein). Furthermore, polygodial has been shown to significantly inhibit mouse paw oedema, exudation and cell influx (da Cunha *et al.*, 2001).

Drimane sesquiterpenoids are known to have antibacterial, antifungal, cytotoxic, molluscicidal and insect-antifeedant activities (Fukuyama *et al*., 1982; Taniguchi and Kubo, 1993). Consequently, the bark of *W. salutaris* is one of the most sought-after medicinal plant commodities in the South African traditional health care sector (Mander, 1997; Williams, 1996). The wide-spectrum of activity displayed by drimane sesquiterpenoids is promising. However, such non-selectivity of action can result in toxicity and subsequent restricted use and further structure-activity relationship elucidation is warranted. Given the large number of drimane sesquiterpenoids identified, synthesis of an ideal candidate as a potential 5-lipoxygenase inhibitor seems feasible and eminent.

5.3.3 Documented Anti-inflammatory and Anti-oxidant Activities of the Genus *Warburgia*

To the best of my knowledge no quantitative information appears to be available regarding the DPPH anti-oxidant and 5-lipoxygenase anti-inflammatory biological activities of the genus *Warburgia*. As a result, evidence-based extrapolation to other species within the genus was attempted as well as to related molecules isolated. As mentioned previously, anti-oxidant activities of *Warburgia salutaris* could be related to flavonoids such as quercetin and kaempferol which have been isolated from *Warburgia stuhlmannii* and these compounds could possibly be present in the leaves of *W. salutaris* ([a]Manguro *et al*., 2003). In addition, several flavonol glycosides have been isolated from the leaves of *Warburgia ugandensis*. ([b]Manguro *et al*., 2003). Drimane sesquiterpenoids with rearrangement of the drimane skeleton has

yielded novel anti-inflammatory molecules such as dysidotronic acid which is reported to inhibit human phospholipase A_2 selectively (Giannini *et al.*, 2000). It is encouraging that such molecular rearrangement can enhance selectivity and potency of drimane sesquiterpenoids (Fig. 5.3).

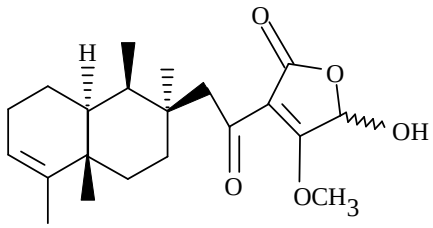


Fig. 5.3: Dysidotronic acid – a sesquiterpenoid with a rearranged drimane skeleton displaying enhanced potency and selectivity towards human phospholipase A_2 (Giannini, 2000).

Sesquiterpenoids have in the past been shown to display anti-inflammatory activity (da Cunha *et al.*, 2001). Furthermore, sesquiterpenic hydrocarbons and sesquiterpenic alcohols have been reported to display 5-lipoxygenase inhibitory activity (Baylac and Racine, 2003).

Further investigations into the 5-lipoxygenase inhibitory activity of a large number of drimane sesquiterpenoids and optimisation of structure-activity relationships could result in a novel anti-inflammatory compound with superior selectivity,

potency and inferior cytotoxicity compared to conventional anti-inflammatory agents.

Chapter 6: Essential Oils: Isolation and Further Biological Studies

6.1 Introduction

The essential oils of *Heteropyxis natalensis, Helichrysum odoratissimum and Lippia javanica* displayed promising 5-lipoxygenase inhibitory activity with IC_{50} values between 22 ppm and 75 ppm (Table 3.1). As a result, GC and GC / MS analysis was undertaken in order to identify compounds possibly responsible for the observed pharmacological effect. Insufficient plant material prevented further analysis of the essential oil of *Ballota africana*.

6.1.1 *Helichrysum odoratissimum*

6.1.1.1 Botanical Description

A perennial aromatic herb or shrublet up to 600 millimetres in height containing densely hairy or woolly leaves, thin greyish white stems and persistent flower heads (Pooley, 2003; van Wyk *et al*., 1997) (Fig. 6.1 (a)).

6.1.1.2 Distribution

Widely spread over large areas of South Africa, extending between the Cape to Zimbabwe. Occuring in large clumps, on damp grass slopes, particularly around rocks and at the foot of cliffs (Pooley, 2003; van Wyk *et al*., 1997)).



(a)



(b)



(c)

Fig. 6.1: Aromatic plants commonly used topically for skin diseases. These were hydrodistilled and the essential oil subjected to further biological testing. (a) *Helichrysum odoratissimum*; (b) *Heteropyxis natalensis*; (c) *Lippia javanica*.

6.1.1.3 Dermatological Uses

Helichrysum odoratissimum is a popular ingredient for wound dressings. Leaves are widely used on wounds to prevent infection. The Zulu use a decoction of the leaf and twig to swab skin in acute dermatoses (van Wyk *et al*., 1997; Watt and Breyer-Brandwijk, 1962).

6.1.2 *Heteropyxis natalensis*

6.1.2.1 Botanical Description

A small tree up to 10 metres in height with a branched trunk. The branches are densely leafy and the foliage is strongly aromatic. The bark has a distinctive mottled appearance due to regular flaking. The leaves are simple, oblong and shiny green but can be tinged red when young. The flowers are yellow and are followed by small dry capsules (Coates-Palgrave, 2003; Palmer, 1981) (Fig. 6.1 (b)).

6.1.2.2 Distribution

Distributed over the north-eastern parts of South Africa (Von Breitenbach, 1986). Occurs in bushveld, forest margins in riverine margins and also on rocky hillsides (Coates-Palgrave, 2003).

6.1.2.3 Dermatological Uses

Used by the Venda for steaming the face, nose and mouth in nose bleeding and bleeding gums (van Wyk *et al*., 1997; Watt and Breyer-Brandwijk, 1962).

6.1.3 *Lippia javanica*

6.1.3.1 Botanical Description

An erect, woody shrub up to two metres in height. The leaves are hairy containing conspicuous veins and are highly aromatic. The flowers are small and yellow-white in colour and produced in dense rounded heads (van Wyk *et al*., 1997) (Fig. 6.1 (c)).

6.1.3.2 Distribution

Occurs over large parts of South Africa. The distribution extends northwards into tropical Africa (van Wyk *et al*., 1997). Usually found in woodland, bushveld and grassland (Coates-Palgrave, 2003).

6.1.3.3 Dermatological Uses

The Zulu use it to treat measles, urticaria, rashes and sprained joints. Applied topically to treat scabies and lice and infusions are used for rashes (Hutchings *et al*., 1996; van Wyk *et al*., 1997).

6.2 Materials and Methods

6.2.1 Gas Chromatography (GC)

Gas chromatography uses temperature differentials to separate essential oil components. Differences in volatility ensures adequate separation. Samples to be injected were diluted 2:8 with hexane and 1 µl was injected into the GC system (Shimadzu 17A gas chromatograph). The following operating conditions were maintained: Column: J&W-DBI (60m x 0.25 mm id., 0.25 µm film thickness), temperatures: injection port 230 °C, column 60 °C for 1 min., 5 °C / min. to 180 °C, 180 °C for 2 min., (total = 25 min.) and flame ionization detector (FID) 260 °C.

6.2.2 Gas Chromatograpy / Mass Spectrometry (GC / MS)

Essential oils were analysed by GC / MS using a Hewlett-Packard G1800A GCD system. An Innowax FSC column (60 m x 0.25 mm diameter, 0.25 µm film thickness) was used. Helium was used as carrier gas at a flow rate of 0.8 ml / min. GC oven temperatures were maintained at 60 °C for 10 min., 4 °C / min. to 220 °C, 220 °C for 10 min., 1 °C / min. to 240 °C (total = 80 min.). Split flow was adjusted at 50 ml / min. Injector and detector temperatures were maintained at 250 °C. Mass spectra were taken at 70 eV. Mass range was from m/z 35 to 425. Base peaks and molecular ion (M^+) were identified. Kovats Indices (KI) for all compounds were determined and relative percentage amounts of separated essential oil constituents were calculated from peak areas of the total ion chromatogram (TIC). Library searches were carried out using the Wiley GC / MS Library and TBAM Library.

Matching both retention indices and mass spectral fragmentation patterns offered positive identification of essential oil constituents.

6.2.3 Combination Tests for Anti-inflammatory Compounds (Essential Oil Standards)

Isobologram construction for the assessment of the anti-inflammatory potential of combinations of essential oil standards proved to be impractical. This was mainly due to the expensive nature of the 5-lipoxygenase assay. As a result, single IC_{50} values for combinations of essential oil standards were determined. Particular attention was paid to the avoidance of enzyme saturation.

6.2.4 Statistical Analysis

Student's *t*-test was used to determine statistical differences in biological activity with a significance level of $P \leq 0.05$. All analysis were performed with GraphPad Prism® version 4.03 software.

6.3 Results and Discussion

6.3.1 *Helichrysum odoratissimum*

6.3.1.1 Essential Oil Composition

Plants from two different populations were collected from the Amatola Mountains which were close to each other within the locality. Voucher numbers were AV 587 and AV 590. Gas chromatographic profiles (Fig. 6.2 and Fig. 6.3) and gas chromatography coupled with mass spectroscopy results (Table 6.1) were obtained.

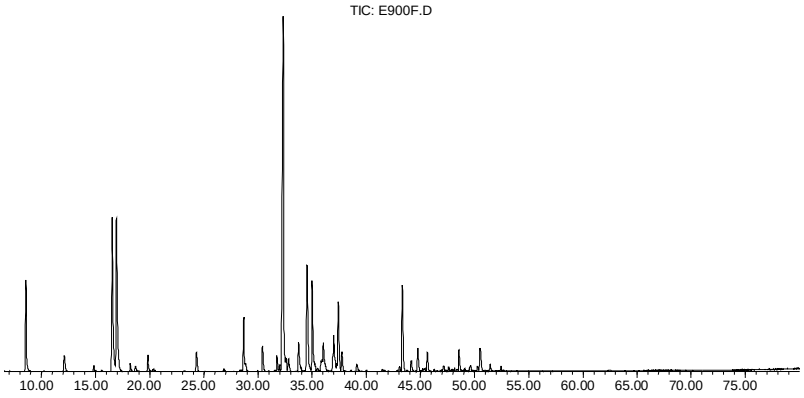


Fig. 6.2: Gas chromatography profile of *Helichrysum odoratissimum* (AV 587)

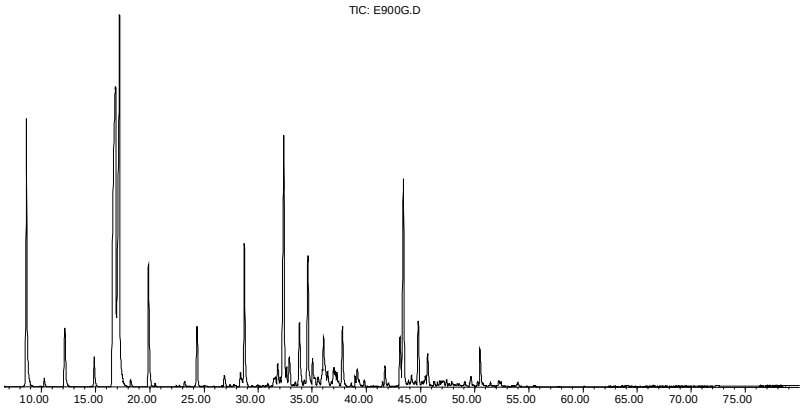


Fig. 6.3: Gas chromatography profile of *Helichrysum odoratissimum* (AV 590)

Table 6.1: GC / MS results of *Helichrysum odoratissimum* from two populations from the Amatola Mountains designated as AV 587 and AV 590.

RRI*	**Area percentage (AV 587)**	**Area percentage (AV 590)**	**Compound**
1032	4.8	**8.3**	**α-pinene**
1076	-	0.3	camphene
1118	1.0	1.9	β-pinene
1174	0.4	1.0	myrcene

RRI*	Area percentage (AV 587)	Area percentage (AV 590)	Compound
1203	**11.6**	**19.6**	**limonene**
1213	**11.2**	**17.1**	**1,8-cineole**
1246	0.5	0.2	(Z)-β-ocimene
1255	0.3	-	γ-terpinene
1280	1.0	3.7	p-cymene
1348	-	0.04	6-methyl-5-hepten-2-one
1386	1.1	1.7	1-octenyl acetate
1391	-	0.05	(Z)-3-hexen-1-ol
1393	-	0.02	3-octanol
1413	-	0.03	rosefuran
1429	-	0.03	Perillen
1452	-	0.03	p-cymenene = α,p-dimethylstyrene
1452	0.1	0.4	1-octen-3-ol
1468/1469	-	0.08	*trans* 1,2-limonene-epoxide
1482	-	0.1	fenchylacetate
1493	-	0.4	α-ylangene
1497	3.2	4.6	α-copaene
1544	-	0.07	α-gurjunene
1553	1.5	0.05	italicene
1568	-	0.04	1-methyl-4-acetyl-cyclohex-1-ene
1597	-	0.2	bornyl acetate
1594	0.8	0.6	*trans*-β-bergamotene
1612	**25.2**	**9.3**	**β-caryophyllene**
1628	0.8	0.4	aromadendrene
1648	-	0.03	myrtenal
1661	1.7	1.9	alloaromadendrene
1678	-	0.1	*cis*-p-mentha-2,8-diene-1-ol
1687	**7.2**	4.0	**α-humulene**
1704	5.4	-	γ-curcumene
1704	-	0.6	γ-muurolene
1726	-	0.1	β-chamigrene
1730	0.4	-	δ-guaiene
1740	1.33	-	*cis*-α-bisabolene
1741	-	0.4	β-bisabolene
1742	-	1.7	β-selinene
1751	-	0.4	carvone
1758	-	0.05	*cis*-piperitol
1771	0.6	0.2	γ-bisabolene
1773	2.0	0.3	δ-cadinene
1776	-	0.2	γ-cadinene
1782	0.09	0.2	*cis*-carvylacetate

RRI*	Area percentage (AV 587)	Area percentage (AV 590)	Compound
1786	3.9	-	ar-curcumene
1796	1.1	1.7	selina-3,7-(11)-diene
1838	-	0.09	2-phenyl ethyl acetate
1845	-	0.2	*trans*-carveol
1853	0.2	0.5	*cis*-calamenene
1864	-	0.2	p-cymen-8-ol
1868	-	0.04	(E)-geranyl acetate
1882	-	0.1	*cis*-carveol
1941	0.1	0.1	α-calacorene
2001	0.1	1.3	iso caryophyllene oxide
2008	4.9	5.9	caryophyllene oxide
2045	0.5	0.2	Humulene epoxide I
2071	1.3	1.7	Humulene epoxide II
2081	-	0.06	Humulene epoxide III
2088	0.08	-	1-epi-cubenol
2104	1.0	0.8	viridiflorol
2144	-	0.095	spathulenol
2170	0.3	-	β-bisabolol
2184	-	0.1	*cis*-p-menth-3-en-1,2-diol
2187	0.2	0.1	T-cadinol
2205	0.1	-	clovenol
2239	0.07	0.03	carvacrol
2256	-	0.07	cadalene
2273	0.2	0.3	porosadienol
2312	0.7	1.1	9-geranyl-p-cymene
2389	0.4	-	caryophyllenol I
Total	**96.67**	**94.21**	

RRI* - relative retention indices calculated against n-alkanes.

Fourty compounds were identified in the essential oil of the aerial parts of ***Helichrysum odoratissimum*** (AV 587). The major compounds accumulated were β-caryophyllene (25.2 %), limonene (11.6 %), 1,8-cineole (11.2 %) and α-humulene (7.2 %).

Sixty-five compounds were identified in the essential oil of the aerial parts of ***Helichrysum odoratissimum*** (AV 590). The major compounds accumulated were

limonene (19.6 %), 1,8-cineole (17.1 %), α-pinene (8.3 %) and β-caryophyllene (9.3 %).

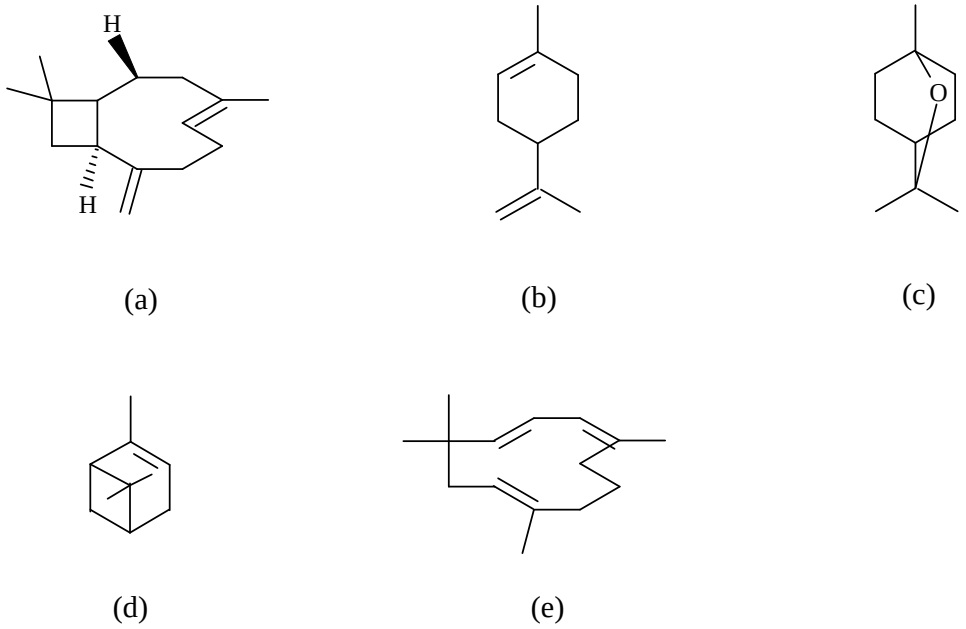


Fig. 6.4: Chemical structures of some of the major compounds accumulated in the essential oil of the aerial parts of *Helichrysum odoratissimum***.** (a) β-caryophyllene; (b) limonene; (c) 1,8-cineole; (d) α-pinene; (e) α-humulene. Taken and modified from Guenther and Althausen (1949).

6.3.2 *Heteropyxis natalensis*

6.3.2.1 Essential Oil Composition

A gas chromatographic profile (Fig. 6.5) and gas chromatography coupled with mass spectroscopy results (Table 6.2) were obtained.

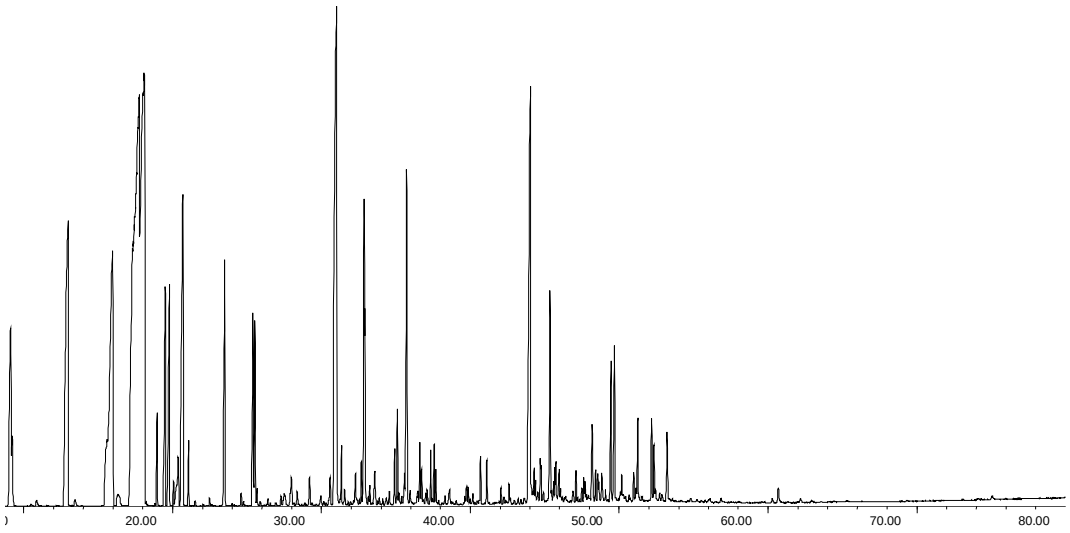


Fig. 6.5: Gas chromatography profile of *Heteropyxis natalensis.*

Table 6.2: GC / MS results of *Heteropyxis natalensis.*

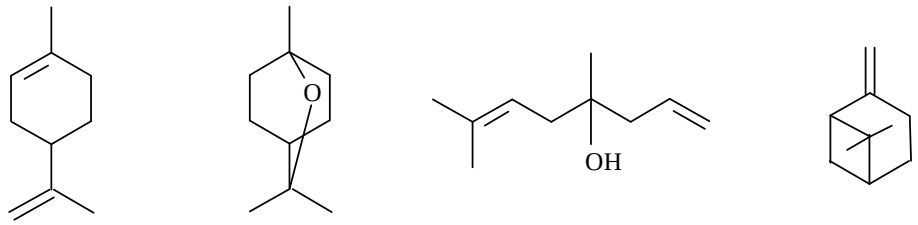
RRI*	Area percentage	Compound
1032	3.7	α-Pinene
1035	tr[a]	α-Thujene
1076	0.1	Camphene
1118	**7.1**	**β-Pinene**
1132	0.1	Sabinene
1174	0.9	Myrcene
1183	6.3	p-Mentha-1,7(8)-diene (=Pseudolimonene)
1188	0.2	α-Terpinene
1203	**24.4**	**Limonene**
1213	**21.2**	**1,8-Cineole**
1246	0.4	(Z)-β-Ocimene
1255	1.6	γ-Terpinene
1266	1.8	(E)-β-Ocimene
1272	0.1	Vinyl benzene (=styrene)
1273	tr[a]	2-heptyl acetate
1275	0.4	2-Methylbutyl butyrate
1280	2.9	p-Cymene
1290	0.3	Terpinolene
1327	tr[a]	(Z)-3-hexenyl acetate
1379	tr[a]	3-Methyl-3-butenyl isovalerate

RRI*	Area percentage	Compound
1398	1.0	2-Nonanone
1452	tr[a]	α,*p*-Dimethylstyrene
1463	0.1	(E)-2-hexenyl butyrate
1482	0.1	Fenchyl acetate
1497	0.1	α-Copaene
1521	0.1	2-Nonanol
1553	**10.0**	**Linalool**
1565	0.1	Linalyl acetate
1571	0.1	*trans-p*-Menth-2-en-1-ol
1597	0.1	Bornyl acetate
1604	0.1	2-Undecanone
1612	tr[a]	β-Caryophyllene
1630	0.1	Terpinen-4-yl acetate (=4-Terpinenyl acetate)
1661	tr[a]	*trans*-Pinocarvyl acetate
1670	tr[a]	*trans*-Pinocarveol
1682	0.2	δ-Terpineol
1687	0.3	α-Humulene
1689	tr[a]	*trans*-Piperitol (=*trans-p-Menth-1-en-3-ol*)
1704	0.1	γ-Muurolene
1706	1.6	α-Terpineol
1719	tr[a]	Borneol
1733	tr	Neryl acetate
1742	0.2	β-Selinene
1744	0.1	α-Selinene
1751	tr[a]	Carvone
1758	tr[a]	*cis*-Piperitol
1758	0.1	(E,E)-α-Farnesene
1765	0.1	Geranyl acetate
1773	0.2	δ-Cadinene
1776	0.1	γ-Cadinene
1799	tr[a]	Cadina-1,4-diene (=Cubenene)
1811	tr[a]	*trans-p*-Mentha-1(7),8-dien-2-ol
1845	0.1	*trans*-Carveol
1853	0.1	*cis*-Calamenene
1857	tr[a]	Geraniol
1871	tr[a]	p-cymen-8-ol
1889	tr[a]	Benzyl butanoate
1901	0.1	Geranyl butyrate
1941	0.1	α-Calacorene
1945	0.1	1,5-Epoxy-salvial(4)14-ene
2000	tr[a]	Eicosane

RRI*	Area percentage	Compound
2008	3.6	Caryophyllene oxide
2029	0.1	Perilla alcohol
2037	tr[a]	Salvial-4(14)-en-1-one
2045	0.1	Humulene epoxide-I
2050	0.1	(E)-Nerolidol
2051	tr[a]	Gleenol
2071	0.8	Humulene epoxide-II
2080	tr[a]	Cubenol
2081	tr[a]	Humulene epoxide-III
2088	0.1	1-epi-Cubenol
2098	0.1	Globulol
2100	tr[a]	Heneicosane
2144	tr[a]	Rosifoliol
2144	0.1	Spathulenol
2153	tr[a]	Neointermedeol
2179	0.1	6-epi-cubenol
2200	0.2	γ-Eudesmol
2204	0.2	T-Cadinol
2208	0.1	Eremoligenol
2209	0.1	T-Muurolol
2223	tr[a]	Hinesol
2232	0.1	Clovenol
2239	tr[a]	Carvacrol
2250	0.4	α-Eudesmol
2255	0.6	α-Cadinol
2271	tr[a]	(2E,6E)-Farnesyl acetate
2273	0.1	Selin-11-en-4α-ol
2316	tr[a]	Caryophylla-2(12),6(13)-dien-5β-ol (=Caryophylladienol I)
2324	0.3	Caryophylla-2(12),6(13)-dien-5α-ol (=Caryophylladienol II)
2384	tr[a]	Hexadecanol
2389	0.3	Caryophylla-2(12),6-dien-5α-ol (=Caryophyllenol I)
2392	0.2	(2E,6E)-Farnesol
2396	tr[a]	Eudesma-4(15),7-dien-1β-ol
2397	0.3	Caryophylla-2(12),6-dien-5β-ol (=Caryophyllenol II)
2622	tr[a]	Phytol
Total	**94.9**	

RRI* - relative retention indices calculated against n-alkanes.
[a] trace constituent.

Ninety-six compounds were identified in the essential oil of *Heteropyxis natalensis*. The major compounds accumulated were limonene (24.4 %), 1,8-cineole (21.2 %), linalool (10.0 %) and β-pinene (7.1%).



(a) (b) (c) (d)

Fig. 6.6: Chemical structures of some of the major compounds accumulated in the essential oil of *Heteropyxis natalensis*. (a) limonene; (b) 1,8-cineole; (c) linalool; (d) β-pinene. Taken and modified from Guenther and Althausen (1949).

6.3.3 *Lippia javanica*

6.3.3.1 Essential Oil Composition

A gas chromatographic profile (Fig. 6.7) and gas chromatography coupled with mass spectroscopy results (Table 6.3) were obtained.

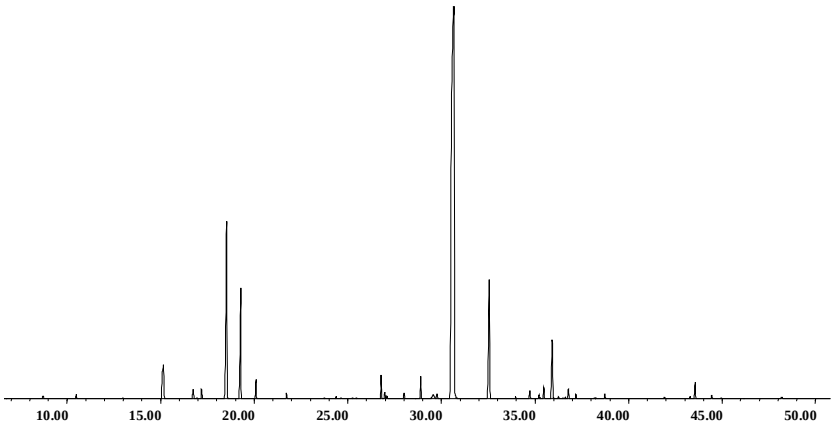


Fig. 6.7: Gas chromatography profile of *Lippia javanica*.

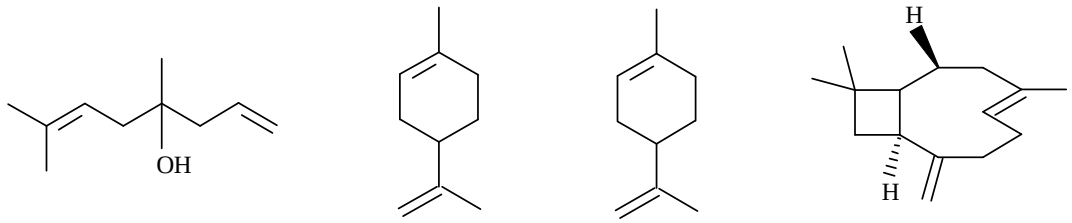
Table 6.3: GC / MS results of ***Lippia javanica***.

RRI*	Area percentage	Compound
1032	0.17	α-pinene
1076	0.3	camphene
1174	2.6	myrcene and α-phellandrene
1203	0.45	limonene
1218	0.46	β-phellandrene
1246	**12.97**	**(*Z*)-β-ocimene**
1266	**6.21**	**(*E*)-β-ocimene**
1280	0.87	p-cymene
1319	0.21	dihydrotagetone
1382	0.18	*cis*-alloocimene
1444	0.79	ipsenone (tentative, Wiley)
1450	0.2	*trans*-linalool oxide (*furanoid*)
1452	0.12	1-octen-3-ol
1478	0.16	*cis*-linalool oxide (*furanoid*)
1497	0.42	α-copaene
1500	0.18	*cis*-tagetone
1522	0.33	*trans*-tagetone
1532	0.18	camphor
1553	**65.19**	**linalool**
1612	**3.58**	**β-caryophylline**
1661	0.07	alloaromadendrene
1687	0.19	α-humulene
1704	0.1	γ-muurolene

RRI*	Area percentage	Compound
1719	0.37	borneol
1726	1.48	germacrene-d
1740	0.09	α-muurolene
1755	0.09	bicyclogermacrene
1758	0.21	(*E*,*E*)-α-farnesene
1773	0.16	δ-cadinene
1830	0.1	2,6-dimethyl-3(*E*),5(*E*),7-octatriene-2-ol
2001	0.07	isocaryophylline oxide
2008	0.42	caryophylline oxide
2050	0.06	(*E*)-nerolidol
2071	0.03	humulene epoxide II
Total	**99.01**	

RRI* - relative retention indices calculated against n-alkanes

Thiry-four compounds were identified in the essential oil of the aerial parts of *Lippia javanica*. The major compounds accumulated were linalool (65.19 %), (Z)-β-ocimene (12.97 %), (*E*)-β-ocimene (6.21 %) and β-caryophylline (3.58 %).



(a) (b) (c) (d)

Fig. 6.8: Chemical structures of some of the major compounds accumulated in the essential oil of the aerial parts of *Lippia javanica*. (a) linalool; (b) (E)-β-ocimene; (c) (Z)-β-ocimene; (d) β-caryophyllene. Taken and modified from Guenther and Althausen (1949).

6.3.4 Correlation between Chemical Composition and Anti-inflammatory Activity

Major compounds previously identified in the essential oil of *Helichrysum odoratissimum* include 1,8-cineole, α-pinene, β-caryophyllene and α-humulene (Gundidza and Zwaving, 1993; Kuiate *et al*., 1999; *Lwande et al*., 1993). To the best of my knowledge limonene-rich essential oils for this plant have not been identified. In the same published reports the monoterpene, limonene, has been reported to be present albeit in lesser or trace quantities. Such discrepancies could be due to seasonal and population variability (Lourens *et al*., 2004). Both *Helichrysum odoratissimum* populations displayed strong to moderate 5-lipoxygenase inhibitory activity with IC_{50} values of 22.46 ppm and 35.90 ppm for AV 587 and AV 590, respectively. The essential oil of *H. italicum* has been reported to display moderate 5-lipoxygenase inhibitory activity (Baylac and Racine, 2003). Both β-caryophyllene and limonene have been shown to display strong 5-lipoxygenase inhibitory activity (Baylac and Racine, 2003). These two compounds might in part explain the strong anti-inflammatory activity displayed by AV 587 and AV 590. Notably, AV 587 contains almost three fold more β-caryophyllene than AV 590 which might explain the lower IC_{50} value of AV 587 compared to AV 590. Another sesquiterpene similar in structure to β-caryophyllene is α-humulene whose 5-lipoxygenase inhibitory activity has not been reported. Given the structural similarities between β-caryophyllene and α-humulene, it remains a possibility that the latter compound contributed to the 5-lipoxygenase inhibitory activity observed.

It would be interesting to subject α-humulene to the 5-lipoxygenase assay as it could reveal how subtle structural modifications could influence 5-lipoxygenase inhibitory activity. One could hypothesise that an increase in double bonds and a consequent reduction in attached hydrogen atoms could result in a reduction of hydrogen bonding and affinity between α-humulene and the 5-lipoxygenase enzyme. This observation might increase our understanding of structure-activity relationships. Limonene, a monoterpene, is present in AV 587 and AV 590 as a major compound. This compound has been shown to display strong 5-lipoxygenase inhibitory activity (Baylac and Racine, 2003). Furthermore, limonene has been shown to inhibit lipoplysaccharide induced inflammation and inflammatory cell migration (Souza *et al*., 2003). Such pharmacological effects might make this compound an attractive target for future development whereby chemical modification might enhance its selectivity.

Aliphatic aldehydes are said to be predominantly anti-inflammatory and compounds such as *trans*-2-decenal, dodecanal and decanal have been reported to display strong 5-lipoxygenase inhibitory activity (Baylac and Racine, 2003). The strong activity displayed by aliphatic aldehydes might be due to strong structural similarities to linoleic acid. Opening closed-ring structures of terpenoids such as β-caryophyllene and limonene in an effort to mimic linoleic acid structurally might potentially increase their activity.

The compound 1,8-cineole is a major compound of both AV 587 and AV 590. The only structural difference between 1,8-cineole and limonene is an oxygen bridge

between positions 1 and 8. It is probable that such a compound would display poor 5-lipoxygenase inhibitory activity due to the electronegative nitrogen atoms of the enzyme which could contribute to electronegative repulsion. This might explain the weak activity displayed by alcohols such as geraniol, linalool and phenylethyl alcohol (Baylac and Racine, 2003). However, it is interesting to note that it was proposed that 1,8-cineole may possibly afford gastroprotection by anti-inflammatory and anti-oxidant mechanisms (Santos and Rao, 2001, Santos *et al*., 2004). In the same publication it was also stated that 1,8-cineole may possess 5-lipoxygenase inhibitory activity.

Major compounds identified in the essential oil of *Heteropyxis natalensis* correlated well with those identified for the same oil in literature (Weyerstahl *et al*., 1992). In the same publication it was noted that limonene, 1,8-cineole and linalool are all major constituents of the essential oil of *Heteropyxis natalensis* while *p*-mentha-1(7),8-diene was acknowledged as being a derivative of β-pinene pyrolysis. The 5-lipoxygenase inhibitory activity displayed by the essential oil of *Heteropyxis natalensis* could be due to the monoterpene limonene which makes up 24.4 % of the essential oil composition and has been shown to display 5-lipoxygenase inhibitory activity (Baylac and Racine, 2003). The lower activity of the essential oil of *H. natalensis* compared to *Helichrysum odoratissimum* might be due to multiple factors such as the absence of β-caryophyllene, the hypothesized weak activity of 1,8-cineole (as discussed) and linalool which has been reported to display weak 5-lipoxygenase inhibitory activity (Baylac and Racine, 2003). The effect of 1,8-cineole and linalool on limonene can potentially be one of diluting an active

principle. However, this needs to be confirmed with further testing. Of interest is that β-pinene is another major constituent accumulated in the essential oil of *H. natalensis*. The 5-lipoxygenase inhibitory activity of β-pinene has not been documented. However, the structural isomer α-pinene has been reported to display moderate 5-lipoxygenase inhibitory activity (Baylac and Racine, 2003). It would be of interest to investigate the difference in activity, if any, between these two isomers. In addition, the influence of one isomer on the other could reveal interesting details about their chemistries and mode of action.

Literature reports indicate that major compounds of the essential oil of *Lippia javanica* include linalool and β-caryophyllene (Ngassapa *et al*., 2003). However, (*Z*)-β-ocimene and (*E*)-β-ocimene, have only been described as being present in minor, trace or absent quantities (Muyima *et al*., 2004; Mwangi *et al*., 1991; Ngassapa *et al*., 2003). This is in agreement with a previous study which indicated high variability in essential oil constituents of *Lippia javanica* both within and between natural plant populations (Viljoen *et al*., 2005). The same report outlines the existence of various chemotypes of *Lippia javanica* including the one described in this report. It would be interesting to subject various natural plant populations of *Lippia javanica* to the 5-lipoxygenase assay and to record the difference, if any, in anti-inflammatory activity. The relatively poor 5-lipoxygenase inhibitory activity of the essential oil of *Lippia javanica* might be due to linalool which makes up 65.19 % of the essential oil composition and has been shown to display no 5-lipoxygenase inhibitory activity at a concentration of 100 ppm (Baylac and Racine, 2003). Furthermore, the relatively strong 5-lipoxygenase inhibitory activity reported for β-

caryophyllene in the same publication explains the weak anti-inflammatory activity of the essential oil given that β-caryophyllene only makes up 3.58 % of the total essential oil composition. Baylac and Racine (2003) reported that the potency of β-caryophyllene as a 5-lipoxygenase inhibitor is greater than that of linalool which was shown to have no activity. The two isomers, (E)-β-ocimene and (Z)-β-ocimene, are also major compounds in the essential oil of *L. javanica*. It would be interesting to determine the level and difference in 5-lipoxygenase inhibitory activity, if any, between these two isomers. Correlation between stereoconfiguration and anti-inflammatory activity has been documented (You *et al*., 2002).

Essential oils are highly complex mixtures of different compounds. A molecule which exists in minor quantities might exert a pharmacological effect in a synergistic, antagonistic, additive or potentiating mechanism. As a result, it remains an 'open-debate' as to which chemicals are directly or indirectly involved in exerting a 5-lipoxygenase inhibitory effect. Analysis of the contribution of each essential oil constituent in a complex essential oil mixture to the anti-inflammatory activity remains a daunting task.

6.3.5 Limonene and 1,8-cineole

The 5-lipoxygenase inhibitory activity, gas chromatographic profile and mass spectroscopic analysis of various essential oils was described in the preceding sections. Two of the most promising essential oils were *Helichrysum odoratissimum* (AV 587) and *Heteropyxis natalensis*. These two essential oils share common chemical compounds as major constituents, namely 1,8-cineole and limonene. The

strong 5-lipoxygenase inhibitory activity of limonene has already been reported (Baylac and Racine, 2003). Limonene contains a chiral centre and as such can exist in two enantiomeric forms. Gas chromatographic seperation of racemic limonene can only be undertaken by utilisation of a chiral column which undergoes diastereomeric interactions with chiral analytes. As a result, a decision was taken to test each enantiomer separately and as a racemic mixture in the 5-lipoxygenase assay. The compound 1,8-cineole does not contain a chiral centre and as such exists in one structural form. A decision was taken to test various combinations of 1,8-cineole and limonene for determination of any synergism, antagonism, additivity or potentiation.

6.3.5.1 Limonene

Limonene can exist in two isomeric forms, namely (R)-(+)-limonene and (S)-(-)-limonene (Fig. 6.9). These enantiomers were tested individually and in a 1:1 racemic mixture. The results are displayed in Table 6.4. The (R)-(+)-limonene enantiomer was approximately three-fold less active than the (S)-(-)-limonene enantiomer ($P < 0.05$). Statistical significance was observed between the (S)-(-)-limonene enantiomer and the racemic mixture ($P < 0.05$). Furthermore, the 1:1 racemic mixture of limonene enantiomers displayed 5-lipoxygenase inhibitory activity intermediate between the values for the two isomers when tested separately. However, statistical significance was only observed at a 10 % level of significance ($p = 0.05059$) when comparing 5-lipoxygenase inhibitory activity of the (R)-(+)-limonene enantiomer and the 1:1 racemic mixture. These results indicate that the

enzymatic reaction for the conversion of linoleic acid to a conjugated diene is stereoselective.



(R)-(+)-limonene (S)-(-)-limonene

Fig. 6.9: Chemical structures of the enantiomeric forms of limonene. Limonene exists as the (R)-(+)-limonene and the (S)-(-)-limonene enantiomeric forms. The racemic mixture of both forms is a major constituent of the essential oil of *Helichrysum odoratissimum* and *Heteropyxis natalensis*. Taken and modified from Guenther and Althausen (1949).

Table 6.4: 5-lipoxygenase inhibitory activity of limonene enantiomers tested separately and in a 1:1 racemic mixture.

Compound	**IC_{50} (ppm)[a]**
(R)-(+)-limonene	36.92 (4.87)[b]
(S)-(-)-limonene	11.50 (4.78)[b]
limonene racemic (1:1)	26.37 (1.80)[b]
NDGA	5 ± 0.50[c]

[a] 5-lipoxygenase inhibitory activity.
[b] Value in brackets reflects standard error of data points fitted to obtain a sigmoidal curve.
[c] Value is mean ± S.D (n=3).

This is in agreement with published data which states that the lipoxygenase enzyme is responsible for the oxygenation of unsaturated fatty acids and that this reaction is stereoselective (Yamamoto, 1990). A number of reports document different anti-inflammatory activity relating to different isomers of the same compound (Patrignani *et al*., 1990; Vàzquez *et al*., 1997). However, such activity for limonene and its isomers appears to be unprecedented. These results hold important implications in the sense that an essential oil's anti-inflammatory activity might be considerably altered depending on the enantiomers present and the proportions of each enantiomer in a racemic mixture. Furthermore, enantiomer selection can greatly effect permeation through the skin (Touitou *et al.,* 1994). This has important implications for essential oils which are used for dermatological applications.

6.3.5.2 1,8-cineole

This compound displayed no significant 5-lipoxygenase inhibitory activity at 100 ppm. Various combinations of 1,8-cineole and limonene (racemic mixture) were prepared. Only the combination of 1,8-cineole at 50 ppm and limonene (racemic mixture) at 50 ppm displayed greater 5-lipoxygenase inhibitory activity than limonene (racemic) tested alone ($p < 0.05$) (Table 6.5). Similar results were obtained for combinations of 1,8-cineole and both enantiomers of limonene ($p < 0.05$).

Although these results are unlikely to change the final IC_{50} values for the racemic mixture of limonene and its individual enantiomers (verified and not statistically significant at $p \leq 0.05$), it does present an interesting result. It would appear that

Table 6.5: Typical 5-lipoxygenase inhibitory values produced by various combinations of limonene with 1,8-cineole.

Compound[a]	**% enzyme inhibition**[c]	**Combination**[b]	**% enzyme inhibiton**[c]
(R)-(+)-limonene	80.65 (1.70)	(R)-(+)-limonene & 1,8-cineole	87.10 (1.65)
(S)-(-)-limonene	89.68 (3.66)	(S)-(-)-limonene & 1,8-cineole	96.13 (2.83)
limonene (1:1 racemic mixture)	87.10 (5.26)	limonene (1:1 racemic mixture) & 1,8-cineole	96.13 (4.70)

[a] compounds tested at a concentration of 50 ppm.
[b] each compound making up a combination constituted a final concentration of 50 ppm in the cuvette.
[c] Value in brackets reflects standard error of data points.

1,8-cineole acts in a potentiating manner. It is possible that 1,8-cineole acts as a more natural solubilising agent than DMSO and Tween® 20 thereby potentially increasing the contact points between the enzyme and limonene. Interestingly, 1,8-cineole and limonene differ structurally only by the addition of a 1-8 oxygen bridge in cineole. This oxygen might, to some extent, play a role in the redox recycling of 5-

lipoxygenase. These results are unprecedented and warrant further investigations to elucidate the biochemical mechanisms of this interaction.

Chapter 7: Conclusions

● The mode of action of medicinal plants which are used as dermatological agents is generally by inhibition of inflammation and / or oxidation. However, the inflammatory cascade and oxidation pathways are extremely complex. As a result, a medicinal plant not displaying activity at one pharmacological site might exert an effect at other potential targets. Furthermore, more sensitive and specific biological assays need to be developed for proper assessment of the mode of action of medicinal plants.

● A dichotomy appears to be present between the anti-inflammatory and anti-oxidant activities of medicinal plants commonly used topically for skin diseases (Table 3.1). Anti-inflammatory agents generally do not display anti-oxidant effects and vice versa. However, the dual activities of the positive control, NDGA, is promising and encouraging.

● Isobologram construction for *Halleria lucida* and Vitamin C serendipitously revealed the potential for interaction between medicinal plants and "conventional" medication (Fig. 4.6). This holds important implications for the concurrent use of traditional and "conventional" medication in a health care setting.

● Isobologram construction for anti-oxidant molecules also confirmed that the combinations of pharmacologically active compounds is inevitably unpredictable and warrants further investigation (Fig 4.6 and Table 4.2).

● A number of drimane sesquiterpenoids appear to be able to inhibit the 5-lipoxygenase enzyme (Table 5.1). Possible modes of action include alterations of redox potential, nucleophilic attack on the Fe^{2+} of 5-lipoxygenase, hydrogen bond formation and, most plausibly, the formation of adducts with amino groups. Deconvolution of the mode of action is warranted. Furthermore, a number of drimane sesquiterpenoids have been shown to be present in *Warburgia salutaris* and the potential for interaction is possible. Further experimental evidence is needed for proper judgement and insight.

● Essential oils generally display no anti-oxidant activity and this may be due to low polyphenolic content (Table 3.1). However, they generally exhibit promising 5-lipoxygenase inhibitory activity. GC / MS profiling and reductionist approaches for isolation of biologically-active essential oil standards is often shortsighted. Essential oils are complex mixtures and the potential for interaction between different constituents is enormous.

● The 5-lipoxygenase enzymic reaction is stereospecific (Table 6.4). This holds enormous implications for essential oil production and aromatherapy. Enhancing the biological effect of an essential oil might depend on selective separation of one isomer. This invariably leads to higher costs of production for pharmaceutical companies.

● The potential of apparently 'inactive' medicinal plant constituents such as 1,8-cineole testifies to the shortcomings of the techniques available for zoning in on

chemical compounds (Table 6.5). Given the results obtained in the preceding sections, it is easy to overlook many novel and promising pharmacological agents.

● Our findings support the rationale for the traditional use of various South African medicinal plants as dermatological agents.

Chapter 8: Further Work

Isolation of active anti-inflammatory and anti-oxidant molecules is only the first step in the long journey of discovering novel anti-oxidant and anti-inflammatory molecules. The next step would be to determine the percutaneous penetration through the epidermis by measuring parameters such as partition coefficients and flux. This would finally determine whether such compounds do indeed reach the required site to exert a pharmacological effect.

Furthermore, essential oils are extremely complex mixtures sometimes comprising hundreds of different compounds. As seen from experiments described, hundreds and thousands of different combinations and permutations are possible. Isobologram construction is also limitless and can extend to two, three or even four molecules. Furthermore, the correlation between *in vitro* and *in vivo* pharmacological effects is often clouded by the latter's inherent biological complexity. In addition, toxicity screening of active medicinal plants and compounds could yield valuable information. Therefore, the elusive search for novel anti-inflammatory and anti-oxidant compounds has only just begun.

Conferences and Presentations

Conferences

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1. Viljoen, A.M., van Vuuren, S., Frum, Y., Hutchings, A. 2004. South African plants used in traditional healing to treat infection and inflammation. 6th International Symposium on Aromatherapy and Medicinal Plants. Grasse, France.

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1. Frum, Y., Viljoen, A.M. 2004. *In vitro* 5-lipoxygenase and anti-oxidant activities of South African medicinal plants commonly used topically for skin diseases. Skin Pharmacol Appl Skin Physiol [submitted for publication and awaiting review].

2. Frum, Y., Viljoen, A.M. 2004. *In vitro* 5-lipoxygenase activity of indigenous South African aromatic plants used in traditional healing and the stereospecific activity of limonene in the 5-lipoxygenase assay. J Essent Oil Res [submitted for publication and awaiting review].

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Appendix 1

Stock solutions of reagents for usage in the 5-lipoxygenase assay (Chaper 3, Section 3.2.2.1) were prepared.

Tween® 20 in DMSO (dimethyl sulfoxide) solution

Tween® 20 (1.5 g) was poured into a beaker. DMSO was added to make a final weight of 55 g. The contents of the beaker were then stirred to give a final 3 % w/w solution of Tween® 20 in DMSO.

Potassium phosphate buffer

A 0.2 M KH_2PO_4 and 0.2 M K_2HPO_4 solutions were prepared. Approximately 77.5 ml of K_2HPO_4 was added to 22.5 ml KH_2PO_4 and adjusted to 200 ml with H_2O to obtain 0.1 M potassium phosphate buffer at pH 6.3.

Linoleic acid

Approximately 0.16 g of linoleic acid was placed in a volumetric flask and dissolved to 100 ml with ethanol. The solution was then thoroughly stirred.

Appendix 2

Stock solutions of reagents for usage in the DPPH (2,2-diphenyl-1-picrylhydrazyl) anti-oxidant assay (Chaper 3, Section 3.2.2.2) were prepared.

DPPH solution

Approximately 0.038 g of DPPH was dissolved in methanol and made up to 1 L with the same solvent. This gave a final concentration of 0.096 mM.

Appendix 3

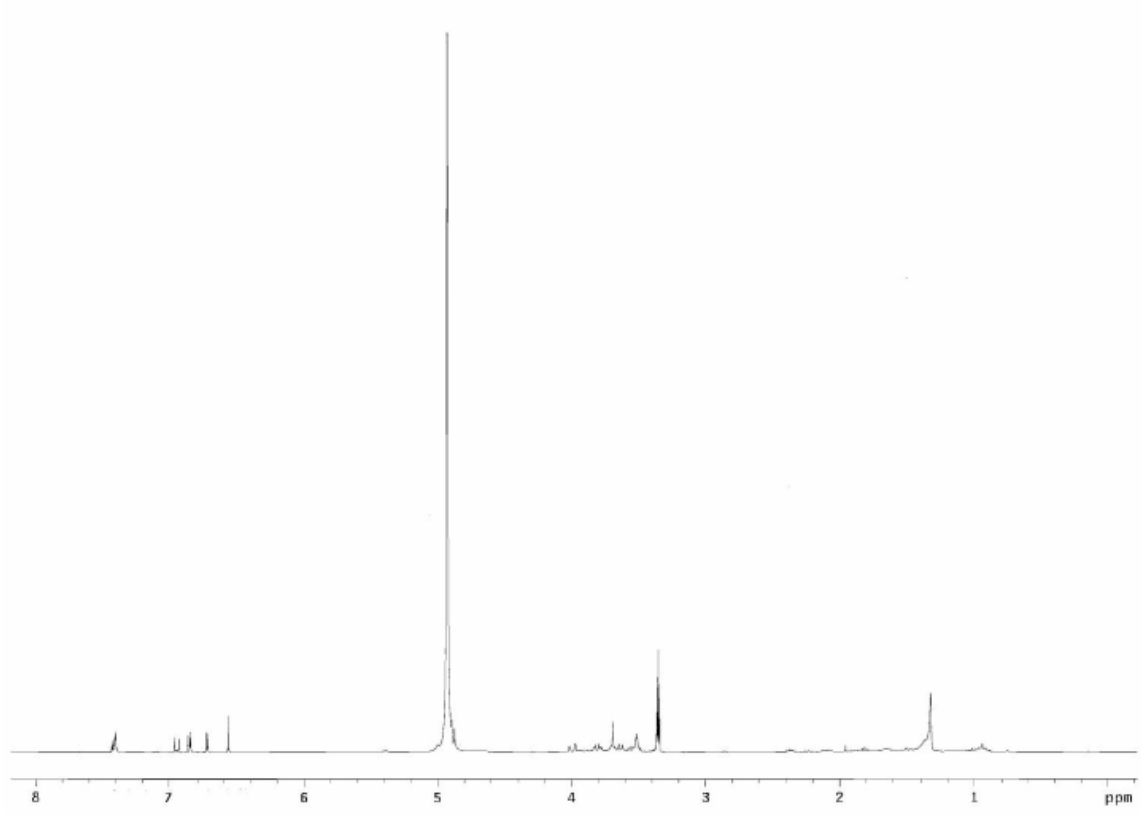


Fig. A1: ^{1}H-NMR spectrum of luteolin-5-*O*-*β*-D-glucoside obtained in deuterated chloroform. Chemical shifts are recorded in ppm referenced to tetramethyl silane as internal standard.

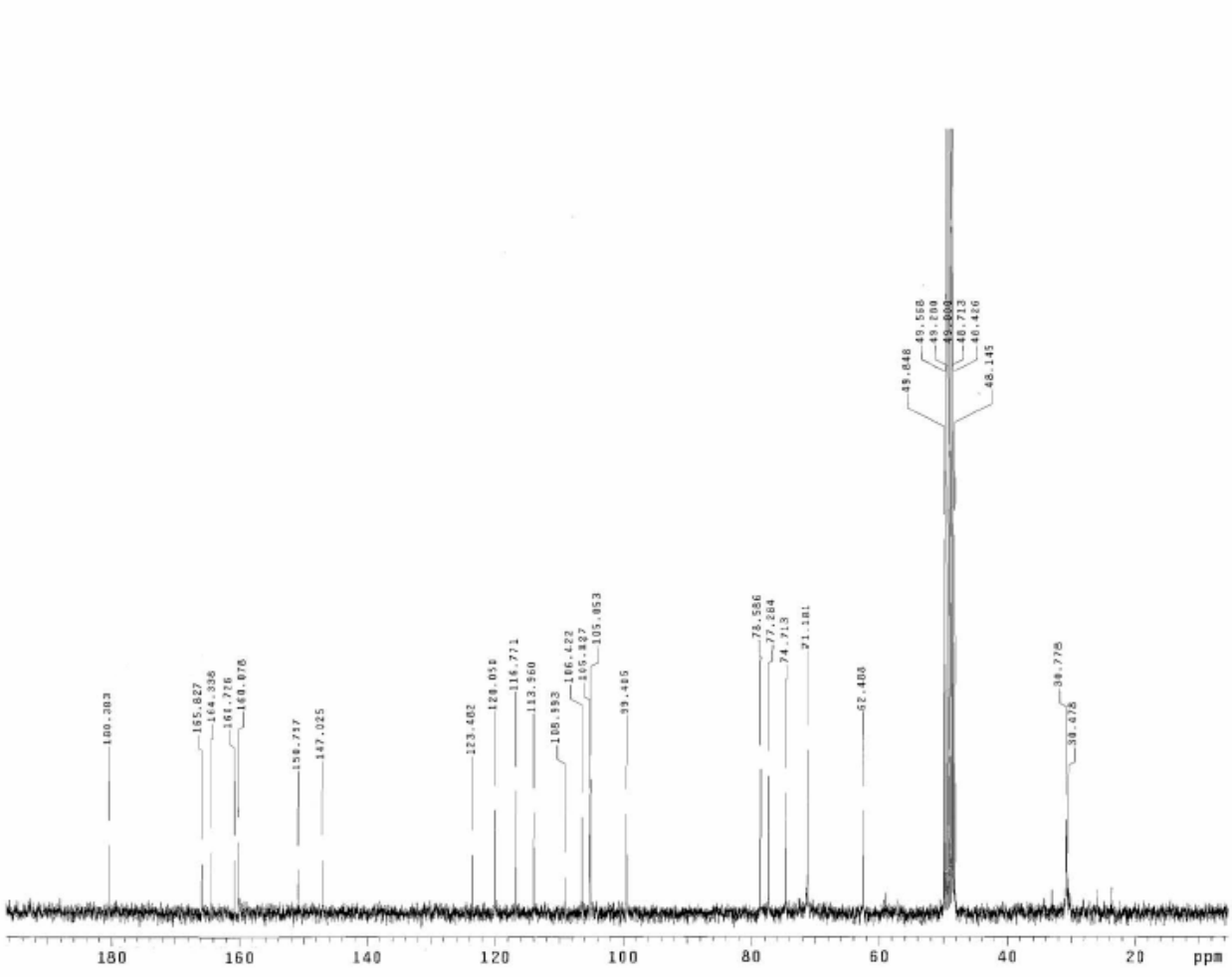


Fig. A2: ^{13}C-NMR spectrum of luteolin-5-*O*-*β*-D-glucoside obtained in deuterated chloroform. Chemical shifts are recorded in ppm referenced to tetramethyl silane as internal standard.

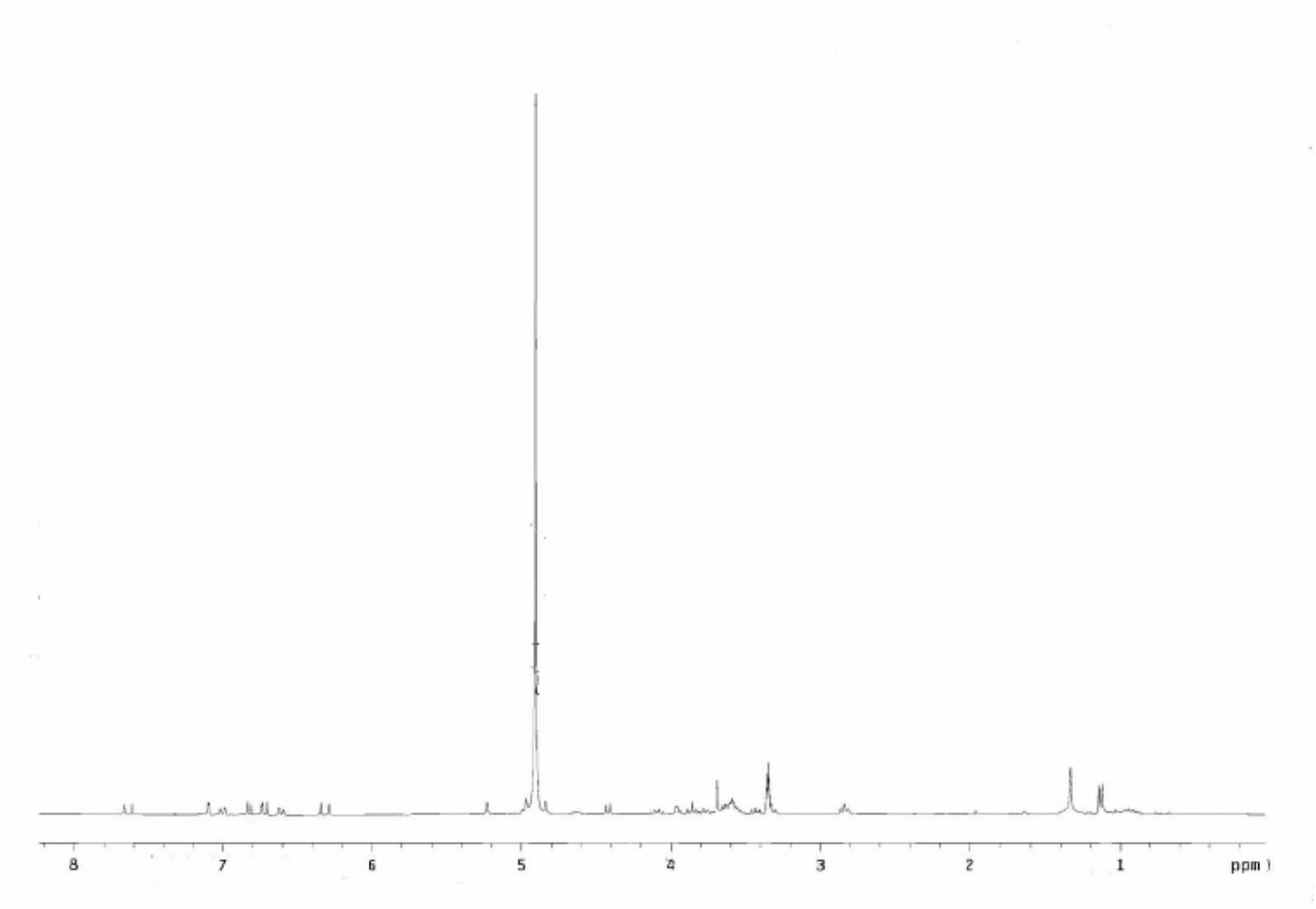


Fig. A3: ^{1}H-NMR spectrum of verbascoside obtained in deuterated chloroform. Chemical shifts are recorded in ppm referenced to tetramethyl silane as internal standard.

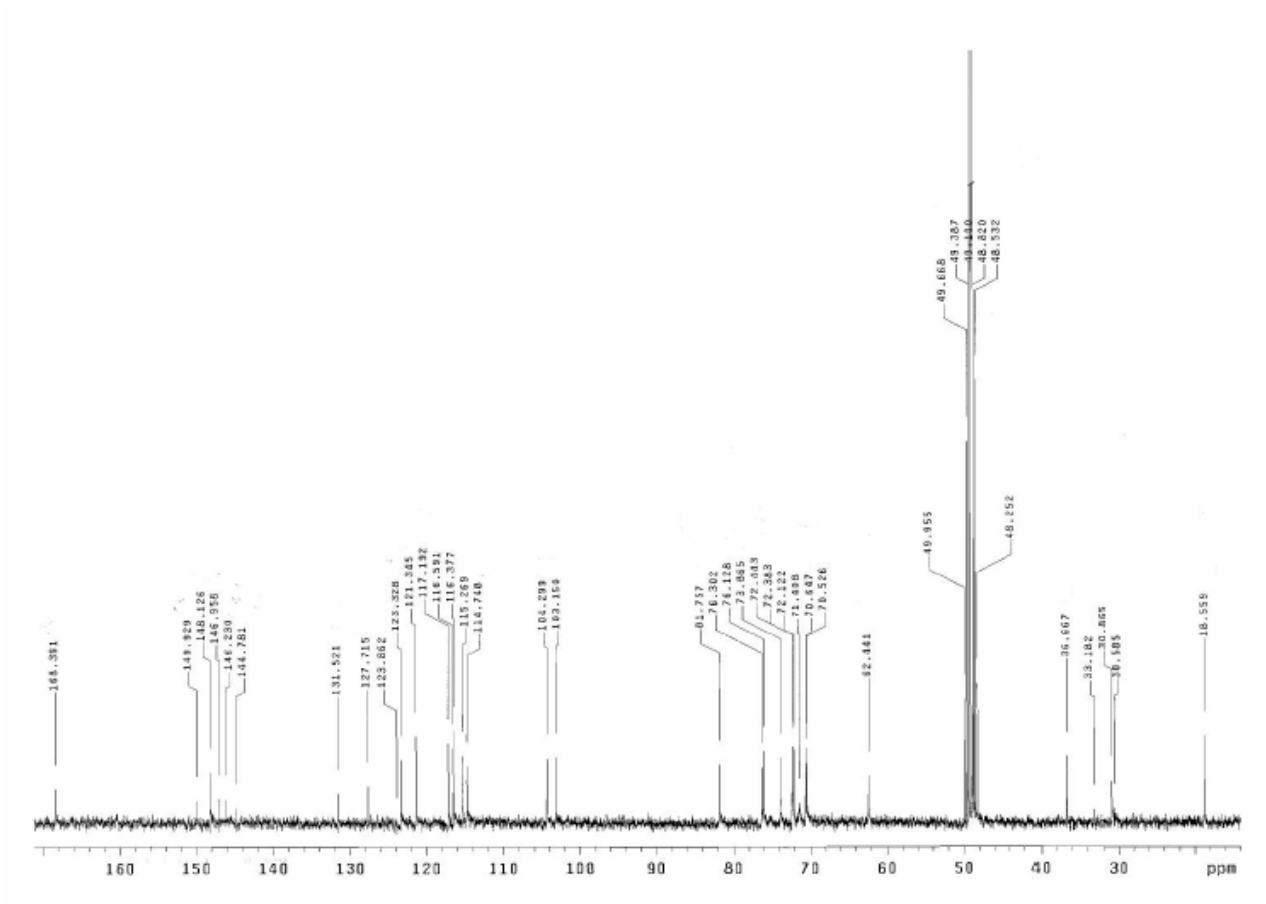


Fig. A4: ^{13}C-NMR spectrum of verbascoside obtained in deuterated chloroform. Chemical shifts are recorded in ppm referenced to tetramethyl silane as internal standard.

Appendix 4

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