



**ANTI ASTHMATIC AND ANTI COUGH ACTIVITIES OF THE
ESSENTIAL OIL OF *EUCALYPTUS GRANDIS* W. HILL EX
MAIDEN**

BY

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Declaration

This is to certify that the work reported in the dissertation entitled “ANTI ASTHMATIC AND ANTI COUGH ACTIVITIES OF THE ESSENTIAL OIL OF *EUCALYPTUS GRANDIS* W. HILL EX MAIDEN” is an original work by me, Mr. SOYINGBE OLUWAGBEMIGA SEWANU, carried out under my supervision and directions. The dissertation has been submitted to fulfill the requirements for the degree (PhD) with the approval of the undersigned.

I, O.S. SOYINGBE, declare that the dissertation has not been previously submitted by me for a degree at this or any other University, that this is my own work in design and in execution, and that all the material contained therein has been duly acknowledged.

Soyingbe Oluwagbemiga Sewanu

Prof. A.R. Opoku

DEDICATION

This work is dedicated to the evergreen memory of my beloved mother

Mrs Modupe Olusola Soyngbe (1942 – 2005)

ACKNOWLEDGEMENTS

I wish to express my profound gratitude to the Almighty God for making this day and this dream a reality.

My sincere and unreserved gratitude goes to my supervisor Professor A.R. Opoku for the time taken and detailed supervision in making this research work come into being.

I am grateful to the following people for also supporting my research: Dr. Singh, Mr. Sibusiso Buthulezi, the University of Zululand for funding, and all the staff and colleagues of the Department of Microbiology and Biochemistry of the University of Zululand.

Am forever grateful to my family for their support, prayers and financial assistance. Ms Langelihle Mahlangu and Master Soyingbe Oluwagbemiga. My Father (Mr. T.A. Soyingbe, brother (Mr. Adeola soyingbe) and sisters (Mrs. Titilayo Agoro. Miss Adedoyin Soyingbe and Mrs Iyabo Salu) are much appreciated. I would also like to thank anyone who through their support and prayer has made this day possible. Thank you all and God bless you all.

ABSTRACT

Asthma is a chronic inflammatory disorder of the airways. It is characterized by an inflammation of the airways causing airway dysfunction. Asthma is associated with widespread airflow obstruction, with an associated increase in airway responsiveness to a variety of stimuli. An asthma attack is accompanied by wheezing, shortness of breath, chest tightness and coughing. This project aims to investigate the essential oil of *Eucalyptus grandis*, a medicinal plant used by Zulu traditional healers for its anti-asthmatic and anti-cough activities in the treatment of respiratory tract infections.

The anti-asthmatic and anti-cough activities of the essential oils and 1, 8-cineole on rats were assessed. These activities were induced and challenged with histamine and acetylcholine using an ultrasonic nebulizer for asthma and exposure to ammonia for coughs. The assessment of the chemical composition of the essential oils hydrodistilled from the fresh and dry leaves of *Eucalyptus grandis* was carried out using a GC and GC-MS analysis. Column chromatography was used to isolate 1,8-cineole and terpinen-4-ol components of the essential oils. Agar well diffusion was used to assess antibacterial (*Klebsiella pneumoniae*, *Staphylococcus aureus* and *Moraxella catarrhalis*) susceptibility to the essential oil. Cytosolic LDH was released and efflux pump inhibition activity was monitored to determine the apparent bactericidal mechanism of the essential oils. Antioxidant activity (free radical scavenging of nitric oxide, hydroxyl radical, superoxide anion, and also the sulfhydryl, NADH as well as the malondialdehyde (MDA)—TBARS contents) was determined.

Anti-inflammatory activities of the essential oils and 1,8-cineole were determined using the cotton pellet granuloma test. Biochemical estimates were carried out on the catalase activity, superoxide dismutase, *in vitro* COX-1 and COX-2 inhibition assay and the acetylcholinesterase inhibitory activity. Muscle contraction studies were carried out using the vascular reactivity on aortic smooth muscle, and cytotoxicity assay done using the MTT assay on human embryonic kidney cells (HEK293) and human hepatocellular carcinoma cells (HepG2). The percentage yield of the essential oils from the fresh and dry leaves was 0.19% and 0.40% respectively. The identified main components of the essential oil of the fresh leaves constituted 99.25% and the major constituents were: α -pinene (29.69%), p-cymene (19.89%), 1,8-cineole (12.80%), α -terpineol (6.48%), borneol (3.48%) and d-limonene (3.14%). The identified main components of the essential oil of the dry leaves was 92.63%, with the major constituents being: 1,8-cineole (47.44%), d-limonene (13.34%), α -pinene (7.49%), (-)-spathulenol (7.13%) and benzene,1-methyl-4-(1-methylethyl)-(5.42%). The oils exhibited concentration dependent anti-asthma and anti-cough activities. Significantly, 1,8-cineole isolated and purified from the essential oil showed a concentration dependent anti-inflammatory, anti-cough and anti-asthma activity. The oils inhibited the growth of the microorganisms studied. The minimum inhibitory concentration (MIC) ranged from 0.3125 mg/ml to 1.25 mg/ml, and the minimum bactericidal concentration (MBC) ranged from 0.625 mg/ml to >5 mg/ml. The LDH release assay (membrane damage) revealed bacterial membrane damage ranging from 1% to 11% in comparison with the standard tritonX-100. Accumulation of rhodamine 6G in bacterial cells, which was used to determine the activity of the essential oils as drug efflux pump inhibitors (EPIs), showed that the

essential oils were effective as EPIs; the essential oils were also seen to be concentration dependent in inhibiting the activity of COX 2, with no significant effect on COX 1. The essential oils showed weak antioxidant activity in scavenging free radicals (IC₅₀ for nitric oxide scavenging of 4.34 µg/ml and 3.65 µg/ml for the fresh and dry respectively, and >5 µg/ml for hydroxyl radical). Sulfhydryl contents were 9.00 µg/g(w/w) and 13.14 µg/g(w/w) for the oils from the fresh and dry leaves respectively. The essential oils showed vasorelaxant activity; cytotoxicity levels of the oils indicated that the oils were not toxic on cell lines, with IC₅₀ of 2291, 2189 on HEK 293 cell, HEPG2 for the essential oils from the fresh leaves and 1875 and 1942 for the essential oils from the dry leaves on HEK293 and HEPG2 respectively. It is concluded that the essential oils have the potential to be used as an anti-asthma and anti-cough therapy. This study also justifies its use by traditional healers in the treatment of asthma and coughs in Zulu folklore medicine.

LIST OF ABBREVIATIONS

AA ascorbic acid

BHT butylated hydroxytoluene

CCM cell culture medium

DMSO dimethyl sulfoxide

EDTA ethylenediaminetetra-acetic acid

GC gas chromatography

GC-MS gas chromatography-mass spectroscopy

IC₅₀ inhibitory concentration with 50%

LC₅₀ lethal concentration with 50% inhibition

LDH lactate dehydrogenase

LPS lipopolysaccharide

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

PBS pbuffer saline

RNS reactive nitrogen species

ROS reactive oxygen species

TBA 2-thiobarbituric acid

UV ultraviolet light

WHO World Health Organisation

MIC minimum inhibitory concentration

MBC minimum bactericidal concentration

TAE tris base, acetic acid and EDTA buffer.

CONTRIBUTION TO KNOWLEDGE

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Chapter 1

INTRODUCTION

Asthma is a common physiological disorder encountered in clinical medicine that affects both adults and children. Asthma is also a serious chronic inflammatory disorder that affects the airways (Masoli *et al.*, 2004). It is generally characterized by inflammation of the airways, which causes airways dysfunction (Patel *et al.*, 2009). There are various other symptoms of asthma characterized by and associated with airflow obstruction, which causes an associated increase in airway responsiveness to a variety of stimuli. An asthma attack is accompanied by wheezing, shortness of breath, chest tightness and coughing (Bousquet *et al.*, 2008). Asthma is currently a worldwide problem, with around 300 million people suffering from it around the globe. About 250 thousand annual world deaths are reported to be caused by asthma (Archana *et al.*, 2008). Asthma cases are increasing at a rate of 50% every decade and according to the WHO (2005), by the year 2020, Asthma will become the third leading cause of death.

Inhaled bronchodilators and anti-inflammatory drugs are available and are quite effective in the management of asthma but, they require long-term use and are associated with various side effects (Bryan *et al.*, 2000; Angsten 2000). To minimize and possibly prevent these side effects alternative and complementary medicine is being sought.

Plants are exploited for their medicinal properties as they are a rich source of phytochemicals. Estimates have shown that there are about 400,000 species of higher or vascular plants (Govaerts, 2001; Thorne, 2002). While some of these species have no medicinal benefits, it has been observed and documented that somewhere between

a quarter and a third of all species have been previously used for their medicinal properties (Small and Catling, 2002). Plants as a whole are, therefore, an important source for drug discovery and modeling of new drugs (Jean-Paul *et al.*, 2005).

Essential oils extracted from plants have a long history in folk medicine and food preservation; essential oils are a known store of natural secondary metabolites with lots of biological activity such as antimicrobial and antioxidant activity with many others (Deans and Svoboda, 1990; Cowan, 1999). However, misuse of essential oils have been established as harmful to humans and cause problems such as skin irritation, headaches and nausea (Aromacaring, 2004). Aromatherapy has become popular and has revived interest in the use of essential oils; it is a form of alternative medicine that makes use of specific essential oil aromas for healing.

An ethnobotanical survey carried out by this researcher (see Appendix F) in the kwaZulu-Natal region of South Africa confirms that numerous plants are used to relieve the devastating effect of asthma. Most of these plants are being under-utilized by traditional healers and the native people due to the lack of scientific screening of these plants (Da Silva *et al.*, 2006). *Eucalyptus grandis* was one plant singled out as being used the most.

Eucalyptus grandis is a medicinal plant indicated by Zulu traditional healers for the treatment of respiratory tract infections, bronchial infections, asthma and coughs. An investigation of the medicinal properties of the essential oil of this plant will help to verify the rationale behind the plants use as a cure for these illnesses.

1.2 DISSERTATION OUTLINE

The dissertation is set out in 6 chapters and appendices, as follows:

- Chapter 1 gives a brief background to and motivation for the study
- Chapter 2 brings together the literature review where it explains the two diseases: asthma and cough, the place of traditional healing, and the bioactivity of essential oils
- Chapter 3 presents the methodology used in the study and outlines the materials used. The methodology explaining the extraction of the oils, the chemical characterization and isolation of active compounds of the essential oils, the antioxidant and antimicrobial activities, biochemical estimations of enzymes, anti-asthma and anti-cough activities as well as the cytotoxicity activities of the essential oils are also presented in this chapter.
- Chapter 4 summarizes the data from the experiments without discussing the implications. The data is organized in the form of tables, figures, graphs and photographs.
- Chapter 5 emphasizes the interpretation of the overall findings and data obtained in the study.
- Chapter 6 gives an overall conclusion drawn from results obtained in the study and provides suggestions for further studies.

Chapter 2

Literature review

2.1. ASTHMA

The National Heart, Lung and Blood Institute of the United States of America defines asthma “as a common chronic disorder of the airways that is complex and characterized by variable and recurring symptoms, airflow obstruction, bronchial hyper-responsiveness (bronchospasm), and an underlying inflammation” (Murray and Nadel, 2005). Asthma is a physiologically partial but reversible obstruction of air flow, with pathological overdevelopment of mucus glands, and thickening as well as shortening of the airways which is caused by scarring and inflammation and bronchoconstriction resulting from surrounding smooth muscle tightening. Bronchial inflammation causes the narrowing of the bronchia as a result of edema and swelling is mostly caused by an immune response to allergens. Asthma causes a recurring period of wheezing, chest tightness, shortness of breath, and coughing. The underlining cough most often occurs early in the morning or during the night.

The actual cause of asthma is not yet fully understood. But, researchers believe that a combination of factors, including family genes and certain environmental factors, combine to cause asthma (Martinez, 2007). Other causative factors include: an inherited tendency to develop allergies, called atopy, that is inherited from parents who have asthma; respiratory infections which might have occurred during childhood; exposure to some airborne allergens and or viral infections in infancy, or during early childhood when the immune system is still developing; exposure to airborne allergens

such as house dust mites, cat or dog dander, and certain irritants such as tobacco smoke cause the airways to be more sensitive to substances in the air that is breathed (Martinez, 2007).

An asthma attack (or bronchospasm) is a sudden worsening of symptoms relating to asthma as a result of tightening of the muscles around the airways. Also, during an asthma attack, the lining of the airways becomes swollen or inflamed and results in the production of an excess in thick mucus. Inflamed airways are often triggered by environmental factors such as smoke, dust or pollen causing the airways to narrow, and the production of excess mucus makes breathing difficult. All in all, asthma is believed to be triggered as a result of an immune response in the bronchial airways (Maddox, and Schwartz, 2002). The airways of asthmatics become hypersensitive to certain triggers and, in response to these triggers, the bronchi contract into spasm. Inflammation follows, leading to a further narrowing of the airways and an excess mucus production, which in turn leads to coughing and other breathing difficulties. (See Figure 2.1)

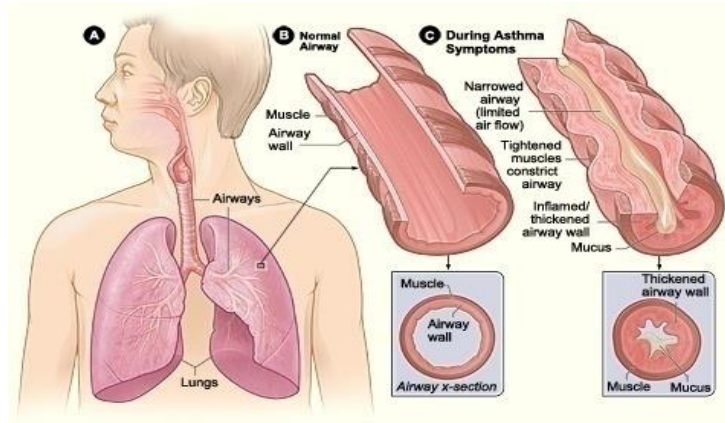


Figure 2.1 Diagram showing a normal airway and airways during an asthma attack. (National Heart Lung and Blood Institute. U.S. Department of Health and Human Services. <http://www.nhlbi.nih.gov/health/health-topics/topics/asthma/> February 2014).

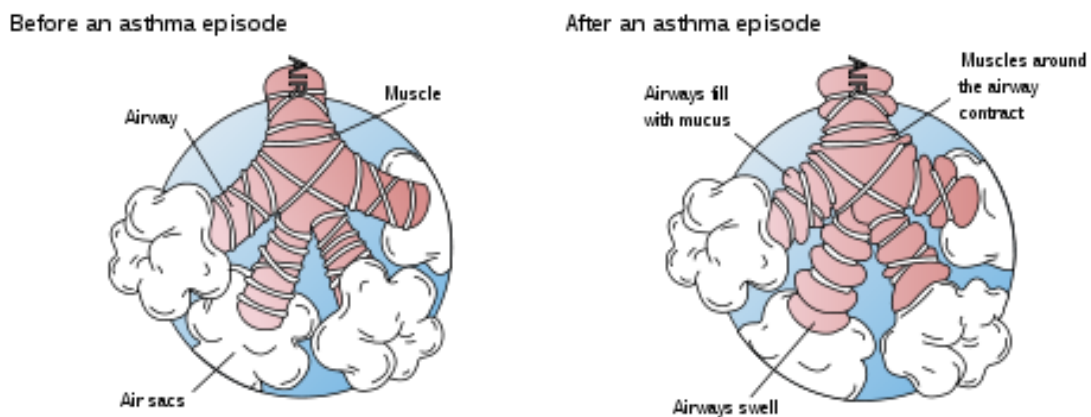


Figure 2.2 Airways and muscles of the lungs before and after an asthma attack. (National Heart Lung and Blood Institute. U.S. Department of Health and Human Services. <http://www.nhlbi.nih.gov/health/health-topics/topics/asthma/> February 2014)

Asthmatics and non-asthmatic individuals inhale allergens every day and these allergens find their way into their airways. The allergens inhaled are ingested by cells known as antigen-presenting cells and immune cells (T_H0 cells). These cells check and usually ignore the allergen molecules. In asthmatics, however, these cells mutate into a different type of cell (T_H2 cells) for reasons not understood. These mutated T_H2 cells activate the immune system, which is the humoral immune system, thereby producing antibodies against the allergen inhaled. When these allergens are inhaled and get into the airways, the antibodies produced attack the airways as well as the bronchial wall, which in turn causes an asthma attack. Inflammation then occurs as a result of a chemical release of inflammatory proteins such as histamine and cytokines from cells damaged by the produced antibodies, thereby causing the wall of the airway to thicken (hyperplasia), in turn causing mucus-producing cells to grow larger (hypertrophy) and produce more and thicker mucus (Kuwano *et al.*, 1993; Knox, 1994).

Inflamed airways are more hyper-reactive and prone to bronchospasm (Tippets and Guilbert, 2009). The release of IL-6 from alveolar macrophages in asthmatic patients after allergen challenge increases (Gosset *et al.*, 1991) with an increased basal release compared with non-asthmatic subjects (Broide *et al.*, 1992). Increased levels of IL-6 can be measured in nasal washings of children following a rhinovirus infection (Zhu *et al.*, 1996). In addition, IL-6 mRNA expression with increased NF κ B-DNA binding activity can be induced by rhinovirus infection of cells *in vitro* (Zhu *et al.*, 1996). The parasympathetic reflex loop consists of different afferent nerve endings that originate under the inner lining of the bronchus. When these afferent nerve endings are

stimulated by asthmatic triggers an impulse is sent to the brain-stem vagal center, and, through vagal efferent pathways, back to the small bronchial airways. Acetylcholine, a neurotransmitter, is released from these efferent nerve endings. The acetylcholine released results in the excessive formation of inositol 1, 4, 5-trisphosphate (IP3) which are agonists that contract airway smooth muscle. Acetylcholine (ACh), histamine and leukotriene D₄ (LTD₄) act on the seven transmembrane domain receptors which are coupled to G proteins of the G_q family. Receptor activation then leads to dissociation of the α-subunit of G_q from the β -subunit and the γ-subunit thereby subsequently activates phospholipase C (PLC). PLC then hydrolyzes phosphatidylinositol 4,5-biphosphate, resulting in inositol 1,4,5-triphosphate (IP3) production. IP3 production causes increased intracellular Ca²⁺ concentration by the release of Ca²⁺ from the sarcoplasmic reticulum. Ca²⁺ then binds with calmodulin, which activates myosin light-chain kinase causing the phosphorylation of the 20 kDa myosin light chain (MLC). In smooth muscle,MLC phosphorylation is required for actin–myosin interactions and muscle contraction. IP3 increases intracellular calcium leading to the activation of myosin light chain kinase, myosin phosphorylation, and the consequent muscle contraction which occurs in the bronchial smooth muscle cells leads to muscle shortening and then bronchoconstriction (Maddox and Schwartz, 2002).(See Figure 2.2)

2.1.1 TREATMENT OF ASTHMA

Symptomatic control of wheezing and the underlining shortness of breath are treated mainly with fast-acting bronchodilators like short-acting, selective beta₂-adrenoceptor agonists, anticholinergic drugs, as well as inhaled glucocorticoids. Long-acting beta₂-

adrenoceptor agonists are used, as well as sustained-release oral albuterol. They are available in pocket-sized, metered-dose inhalers (MDIs). Many steroids, specifically glucocorticoids, reduce inflammation or swelling by binding to cortisol receptors. Non-steroidal anti-inflammatory drugs (NSAIDs) alleviate pain by inhibiting cyclooxygenase (COX) enzyme. COX enzyme are known to produce prostaglandins, creating inflammation. On the whole, the NSAIDs stop the synthesis of prostaglandins thereby reducing or eliminating pain. A nebulizer is used to provide a larger and more continuous dose of bronchodilators. Nebulizers effectively vaporizes doses of medication mixed in saline solution into a steady stream of foggy vapour, which is administered to the patient and which the patient inhales continuously until the full dosage is administered.

2.1.2 CYCLOOXYGENASE (COX-1 AND COX-2)

Cyclooxygenase (COX) is a physiological enzyme that produces important biological mediators called prostanoids, which include prostacyclin, thromboxane and prostaglandins. COX activates the conversion of arachidonic acid to prostaglandins; the produced prostaglandins are responsible for inflammation, pain and fever. Two types of cyclooxygenase enzyme are known, namely COX-1 and COX-2 (see figure 2.3). COX-1, a constitutive enzyme, is required daily for physiological functions; while, on the other hand, COX-2 is an inducible enzyme which is released during inflammation (Kurumbail *et al.*, 1996).

The major significant difference between these two isoenzymes, is located at position 523. COX-1 has isoleucine, while COX-2 consists of valine. Val₅₂₃ residue in COX-2

gives these enzymes their hydrophobic side-pocket property (Kurumbail *et al.*, 1996). NSAIDs which are drugs used to relieve the effect of inflammation are not selective because they inhibit both COX-1 and COX-2. The inhibition of COX-2 by NSAIDs gives them their anti-inflammatory activity, while the unfortunate inhibition of COX-1 leads to side effects such as kidney problems, ulcers and prolonged bleeding (Hawkey 2001 and Schachter, 2003).

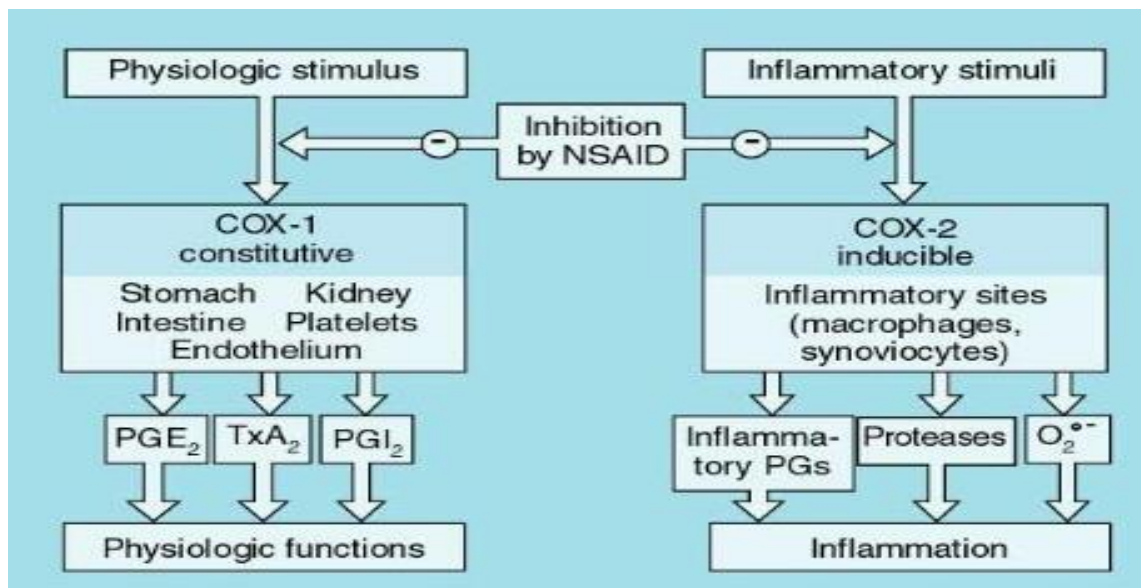


Figure 2.3 Pathway of the production of postagladins by COX-1 and COX-2 (adapted from Herschman 1996, Dubois *et al.*, 1998).

2.1.3 COMMERCIAL ANTI-INFLAMMATORY DRUGS

Drugs well-known for the treatment of chronic inflammation are corticosteroids, as well as non-steroidal anti-inflammatory drugs (NSAIDs), such as ibruprofen and aspirin. NSAIDs only help the patients with symptomatic relief, blocking certain steps of the pathways above, but they do not alter the pathogenesis of inflammation (Ford-Hutchinson *et al.*, 1981). COX enzymes produce prostaglandin which stimulates

inflammation, fever and pain. However, COX-1 mainly produces the prostaglandins needed by platelets and needed for the protection of stomach and intestinal integrity. NSAIDs, however, block the COX enzymes and reduce prostaglandin throughout the body by blocking the action of COX 1 and COX 2. As a result, inflammation, fever and pain are reduced. Since the prostaglandins that support platelets, blood clotting and protect the stomach are inhibited, NSAIDs are a major cause of ulcers in the stomach and support bleeding. They increase blood pressure in hypertensive patients thereby preventing the action of drugs used for hypertension. Also, asthmatic patients are at a higher risk of experiencing serious allergic reactions to NSAIDs.

It has also been observed that patients have and are developing resistance to the drugs currently being used (Bagheri *et al.*, 2000). Celebrex is the first COX-2 selective NSAID that was developed in the late 1990s, after which Vioxx and Bextra were introduced. However, Vioxx and Bextra have since been redrawn from the market as a result of the high risk of heart attacks and strokes caused by their use, making Celebrex the only commercially available COX-2 inhibitor (Sahoo 2005). The ever increasing incidence of inflammatory diseases and inflammatory related diseases, side effects to commercially used drugs, drug resistance and patients not easily tolerating currently used anti-inflammatory drugs, has created a need for research into the development of new anti-inflammatory drugs of plant based origin which are better tolerated, with little or no side effects.

Current research on asthma is aimed at finding safer, better drugs to combat the devastating effects of asthma and relieve the effects of asthma attacks. Since there is

no known cure for asthma, emphasis is being laid on finding drugs to effectively manage the disease (Downs *et al.*, 2001).

Epidemiological evidence have proposed that changes in diet, particularly reduced antioxidant intake, is a contributing factor to increases in developing asthma which indicates that dietary interventions may assist in controlling asthma (Fogarty and Britton, 2000). Lipid peroxidation has a significant effect in asthma. Oxidative stress has been linked to the pathophysiology of asthma (Niki *et al.*, 2007; Barnes, 1990; Doelman and Bast, 1990; Mak and Chang-Yeung, 2006), which could be a common pathway causing tissue damage (Pinto *et al.*, 2012). Substances such as allergens which include, pollutants as well as bacteria and viruses (Levine,1995) lead to an increment and activation of inflammatory cells which then produces oxidants in the airways of asthmatics (Pinto *et al.*, 2012). Activated eosinophils, neutrophils, monocytes, macrophages as well as resident cells, like bronchial epithelial cells, also generate oxidants (Barnes, 1990; Barnes *et al.*,1998; Rahman and MacNee, 2002). Therefore, a good antioxidant could be highly effective as an anti-asthma agent. Experimental evidences have demonstrated that ethanolic extracts of red alga, *L. undulate*, containing large amount of polyphenols inhibit ovalbumin (OVA) induced airways hyper-responsiveness and inflammation in a murine model of asthma (Jung *et al.*,2009).

Some respiratory pathogens which affect the respiratory tracts have been observed to trigger asthma attacks. *Streptococcus pneumonia* a gram positive bacterium is one of such, it is one of the common causes of pneumonia - an acute illness which causes the lung's alveolar air spaces to become filled with fluids and white blood cells (Scott *et al.*,

2008). Another is *Klebsiella pneumonia*, a gram negative bacterium also known to cause pneumonia with chills, fever and the development of mucoid sputum, coughing and chest pain. The sputum also becomes bloody (Nester *et al.*, 2001). *Cryptococcus neoformans*, causes a fungal infection called Cryptococcosis, with symptoms like coughing and chest pains (Nunez *et al.*, 2000; lindell *et al.*, 2005). *Moraxella catarrhalis*, a lower respiratory infection causing bacterium, leads to otitis media that is coupled with sinusitis, shortness of breath, chronic bronchitis and cough which eventually leads to respiratory insufficiency (Nester *et al.*, 2001 Van Wyk and Wink, 2004). *Staphylococcus aureus* is one bacterium responsible for lower respiratory tract infections like pneumonia (Nester *et al.*, 2001). *Mycobacterium tuberculosis* is a bacterium that causes tuberculosis with symptomatic effects of a chronic cough, fever and bloody sputum (Nester *et al.*, 2001).

It is apparent that the search for an effective anti-asthmatic drug for the symptomatic relief of, and possible cure for, asthma be directed towards finding agents that are antioxidant, antimicrobial, anti-inflammatory, anti-allergenic, and immune-boosting in nature.

2.2 COUGH

A cough is a sudden and often repetitive reflex that helps clear the air passages from secretions, irritants and foreign particles, including microbes. The cough reflex occurs when there is a forced and sudden exhalation against the closed glottis which then results in a massive release of air from the lungs, after the opening of the glottis,

accompanied by a distinctive sound (Chung and Pavord, 2008). Coughing can be due to a respiratory tract infection or choking, smoking, air pollution, asthma, chronic bronchitis, medications and a variety of many other factors which include acetylcholinesterase (ACE) inhibitors (Chung and Pavord, 2008).

2.3 MEDICINAL PLANTS

Some of the medicinal and therapeutic properties of medicinal plants have been attributed to the phytochemical constituents of such plants. Phytochemicals are non-nutritive plant chemicals with protective or disease preventive activities. They are nonessential nutrients and as a result they are not needed for the everyday function of the human body. Phytochemicals are also believed to be produced by plants for their protection (against insect attacks and plant diseases). Recent research suggests that they can also be utilized for the protection of humans against diseases (Schultz 2000).

The use of plants for medicine is a basic part of African culture (Hutchings *et al.*, 1996) and one of the oldest and most diverse practices all over the globe (Van Wyk and Wink, 2004). In South Africa, a country also regarded as a developing nation, the use of indigenous African medicine is widely used alongside Western allopathic medicine (Van Wyk *et al.*, 2003). Traditional healing with local herbs is widely practiced in Zululand (Gumede, 1989). The medicinal properties and effects of various plants traditionally used for a wide variety of diseases has been well documented (George *et al.*, 2001; Opoku *et al.*, 2002; Jean- Paul *et al.*, 2005; Iwalewa *et al.*, 2007; Bibhabasu Hazra *et al.*, 2008). Even in our laboratory at the University of Zululand, the effects of various

medicinal plants used by Zulu traditional healers have been well documented (Soyingbe *et al.*, 2013 a, b; Simelane *et al.*, 2013; Mosa *et al.*, 2014 and Machaba *et al.*, 2014). Globally, a number of studies focusing on the scientific validation of plants traditionally used to treat respiratory ailments have been carried out; the testing of such plants specifically against pathogens related to respiratory diseases has been carried out (Van Wyk and Wink, 2004; Van Wyk *et al.*, 2009; Crouch *et al.*, 2006; Tabuti *et al.*, 2010; Mohamad *et al.*, 2011).

Recent reports in literature have indicated that extracts of some medicinal plants exhibit anti-asthmatic (Jung *et al.*, 2009; Sunilson *et al.*, 2010; Pinto *et al.*, 2012), and anti-cough (Batugal *et al.*, 2004; Punjani and Kumar, 2002; Maroyi, 2013; Mulaudzi *et al.*, 2012) activities.

2.4 EUCALYPTUS GRANDIS W. HILL EX MAIDEN

Eucalyptus grandis is a plant which was originally native to the east coast of Australia. It is commonly known as rose gum or flooded gum (a misnomer). It is a premier forest species in Queensland and New South Wales, Australia and can grow to 43 to 55m tall (Figure 2.3) (Hall *et al.*, 1970). Massive planting programs have since been implemented to plant the species in South Africa and Brazil (Jacobs, 1976).



Figure 2.3 *Eucalyptus grandis* W. Hill ex Maiden (www.forestryimages.org October 2014)

The traditional use of *Eucalyptus* by traditional healers to treat a wide range of diseases such as general infections, colds, flu, sore throats, bronchitis, pneumonia, aching, stiffness, and neuralgia (Hutching *et al.*, 1996) and its use as an antibiotic (Hopkins-Broyles 2004) has been documented. Vivik (2008) reported the use of *Eucalyptus* as an antifungal agent for the treatment of some skin infections. Sisay (2010) reported that the essential oils of *Eucalyptus globules* and *Eucalyptus citriodora*, with about 70% of their constituent being 1,8-cineole (Eucalyptol), stimulate respiration, and can be used to relieve the effect of a cough, to expel mucus, and as a vasorelaxant of the respiratory muscles, thereby indicating the usefulness of the oils in the management of bronchitis, asthma, catarrh, sinusitis and throat infections.

Traditional healers interviewed (see Appendix F) on how they treat asthma and coughs using various medicinal plants made reference to steam inhalation as a method of

choice. It is apparent that the volatile compound of the plants is being used, and thus the investigation of the essential oils in this study.

2.5 ESSENTIAL OILS

An essential oil is a concentrated, hydrophobic liquid containing volatile aroma compounds derived from plants. Essential oils are sometimes referred to as volatile or ethereal oils, or as oils of the plant they were extracted from, such as *oil of rose*. Essential oils are referred to as "essential" as they carry a distinctive scent, or essence, of the plant they are extracted from. Essential oils don't necessarily have any specific chemical properties, apart from conveying characteristic fragrances. Essential oils are found in the leaves, flowers, buds, twigs, barks, fruits, and roots of plants while a few are derived from animal sources and micro-organisms. The different odours of the oils can be detected at low concentration as the odours are well-defined. Essential oils consist mainly of aliphatic hydrocarbons, monoterpenoids, diterpenoids and sesquiterpenoids. They can also contain benzyl, phenylpropanoids, and carboxylic acids with other compounds like sulphur and nitrogen (Ahmad *et al.*, 2006).

2.5.1 USES OF ESSENTIAL OILS

Essential oils have been exploited for their medicinal properties throughout history (Buchbauer, 2000). Essential oils have been used for and are believed to treat a numerous diseases, from skin infections to cancer. The use of essential oils has been revived recently with the revitalization of aromatherapy. Aromatherapy is a form of alternative medicine that makes use of specific essential oil aromas through diffusion of

essential oil into the air with a nebulizer, by burning oil over the flame of a candle, or as incense (Lehrner *et al.*, 2005).

Worldwide research is increasing with the screening of plants for active biological activities as well as essential oil activities, ranging from chemical and pharmacological properties to therapeutic properties (Sonbolia *et al.*, 2005; Skaltsa *et al.*, 2003; Tzakou and Skaltsa, 2003; Lawal and Oyedeji, 2009).

The essential oils of various plants have been studied over the years and seen to possess useful biological and pharmacological properties, such as antimicrobial (Kezemi *et al.*, 2011; Vale-Silva *et al.*, 2009; Gulluce *et al.*, 2006; Altanlar *et al.*, 1999; Janssen *et al.*, 1987 and Kurita *et al.*, 1981), antinociceptive (Quintão *et al.*, 2010; Sulaiman *et al.*, 2009), anti-inflammatory (Özbek *et al.*, 2007; Chao *et al.*, 2005), vesorelaxant (Chiara *et al.*, 2010) and antioxidant properties (Kezemi *et al.*, 2011; Kadri *et al.*, 2011; Gulluce *et al.*, 2006).

2.5.2 EXTRACTION OF ESSENTIAL OILS

Essential oils are most often extracted by means of distillation. Some of the other processes used include expression and solvent extraction.

2.5.2.1 HYDRODISTILLATION

This widely used process is also known as water distillation. It is the process most used for the extraction of essential oils. Plant samples are suspended in water and boiled at about 300°C, the vapour condensed and the oils separated using a Clevenger type apparatus (Baser, 1995; Guenther, 1949; Koedam, 1987; Crespo *et al.*, 1991). Essential

oils extracted using this method have a low yield due to the absence of non-volatile compounds (Ernest, 1921).

2.5.2.2 STEAM AND WATER DISTILLATION

This method employs the use of water and steam to extract essential oils from plant materials. This method does not see the plant materials mixed with water. Instead, the steam generated passes through the plant material to obtain the essential oils from the plant. The loss of oxygenated constituents is minimal compared with hydrodistillation (Baser 1995).

2.5.2.3 SOLVENT EXTRACTION

This method makes use of solvents that have low boiling points and are free of odour and impurities to extract essential oils from delicate plant materials. The solvents mostly employed in this method are: purified petroleum ether, hexane and benzene. The solvents used do not react with the oils and can be easily separated from the oils under reduced pressure (Baser 1995; Koedam 1987).

2.5.2.4 SUPERCRITICAL CARBON DIOXIDE EXTRACTION

Supercritical CO₂ extraction uses carbon dioxide under extremely high pressure to extract essential oils. CO₂ is passed through a tank containing plant materials and, under high pressure, the CO₂ turns liquid and acts as a solvent. When the pressure is decreased the CO₂ returns to its gaseous form, leaving the residue essential oils behind. This method uses a lower temperature and gives a higher yield of the essential oils compared with the other methods of extraction (Baser 1995; Temelli *et al.*, 1988).

2.5.3 ANALYSIS OF ESSENTIAL OILS

2.5.3.1 PHYSICOCHEMICAL PROPERTIES

Physicochemical properties determine the overall qualities of essential oils extracted. (Fabiane *et al.*, 2008). Sensory evaluation and the refractive index are two methods of quality evaluation frequently used (Guenther *et al.*, 1971; Ambrose and Ambrose, 1961).

2.5.3.2 REFRACTIVE INDEX

The refractive index or the index of refraction of a substance or medium is regarded as the measure of the speed of light that pass through that medium. The refractive index is expressed as the speed of light in a vacuum relative to that in the considered medium. The refractive index can be used to distinguish other solvents from water and to confirm the purity of essential oils (Young and Freedman, 2008).

2.5.3.3 SENSORY EVALUATION

Analyzing essential oils by smell is a crucial method in determining this physical property. The sensory evaluation is carried out immediately after isolation and it is conducted using a number of panelists to evaluate and recognize the distinctive smell of the essential oils. GC-OA gas chromatograph equipped with a DB-Wax fused silica capillary column, and an FID can also be used (Minh Tu *et al.*, 2003) to determine the characteristic smell of essential oils (Baser, 1995; Sawamura *et al.*, 2001; Gedney *at al.*, 2004).

2.5.3.4 CHEMICAL COMPOSITION OF ESSENTIAL OILS

A variety of plant-derived essential oils have been studied, and their chemical compositions analyzed using GC and GC-MS to chemically characterize the various constituents and compounds in the essential oils, and the results documented.

Gas chromatography (GC) or Gas-Liquid chromatography (GLC) is a chromatography technique used for the identification and quantitative analysis of essential oils and mixtures of complex compounds (Baser 1995; Eiceman 1994; Adams 1991; Vernin 1987). A mobile and a stationary phase are required. The mobile phase describes the use by a carrier gas of an inert gas such as helium, argon, or nitrogen. The stationary phase consists of a packed column (Eiceman *et al.*, 1994).

Gas chromatography–mass spectrometry (GC-MS) is a method that combines gas-liquid chromatography and mass spectrometry in order to identify different substances in a compound. GC-MS is used for identifying the complex properties of essential oils and is also used in drug detection and the identification of unknown samples (Babushok *et al.*, 2007; Shibamoto 1987; Herent 2007). Different retention times are required for molecules to elute from the gas chromatograph. The mass spectrometer breaks molecules into ionized fragments, detecting fragments by using their mass to charge ratio (Eiceman 1994).

Use of the two components together provides better substance identification than either unit used separately. Combining the two processes assists greatly in eliminating the possibility of error, as two different molecules would be unlikely act the same way when using a gas chromatograph and a mass spectrometer (McLafferty *et al.*, 1999).

2.5.4 IDENTIFICATION OF CONSTITUENTS OF ESSENTIAL OILS

2.5.4.1 KOVÁTS RETENTION INDEX

The time taken by the sample after injection and the recording of the maximum peak of the component is the retention time (t_R). In an ideal situation and in operational conditions, a given solute should have the same retention time. With varying temperature and flow rates maintaining a constant condition during analysis, there is a need to express analytical results in a more uniform and reproducible manner; hence, the retention indices (Baser, 1995; Babushok *et al.*, 2007; Shibamoto, 1987; Herent *et al.*, 2007; Scott, 1958). *n*-Paraffin hydro-carbons are employed as the homologous series in the Kováts retention index system *KI*. Kováts retention index system *KI* is measured on a polar and or a non-polar stationary phase (Merfort, 2002; Shibamoto, 1987).

GC and GC-MS have been used by various researchers to establish the chemical composition of the essential oils of flowers, leaves, and stems. For example, twenty-six constituents which represented 86.0 - 99.6% of the total composition of two *Senecio polyanthemoides* Sch. Bip. from South Africa collected from two different localities within the city of uMhlatuze have been reported by Lawal and Oyedeji (2009). The main components were seen to be limonene, ρ -cymene, β -selinene, α -pinene, β -pinene and 1,8-cineole. The essential oil of *Tulbaghia violacea* Harv L.F. contains 2,4-dithiapentane, ρ -xylene, chloromethylmethyl sulfide, σ -xylene, thiodiglycol and ρ -xylol (Soyingbe *et al.*, 2013 b).

2.5.5 CYTOTOXICITY ACTIVITY

The cytotoxicity testing of essential oils is carried out to determine the level of toxicity of the oils in damaging tissues, the cell wall and cell membrane. Essential oils easily pass through the cytoplasmic membrane, which makes it easy to disrupt the structure of the membrane and cause it to permeabilize. Essential oils can coagulate the cytoplasm, thereby damaging lipids and proteins (Burt, 2004). The possibility of using essential oils in anticancer and antitumor treatments can therefore be verified by cytotoxicity testing.

Cell line based bioassays have been seen to provide reliable and valid results (Mire-Sluis *et al.*, 1995). They are also explored as a better replacement for animal based methods such as the brine shrimp lethality test. The cytotoxicity of essential oils is determined by exposing cell cultures to the essential oils. Thereafter, cell death can be determined. Cell lines have also been widely used in research and drug development as models for normal and cancer tissues. Various cytotoxicity analyses have shown that essential oils are generally nontoxic. Lawal and Oyedeji (2009) reported the cytotoxicity of the essential oils of *Cyperus distans*, *C. Papyrus*, *C. rotundus*, *Senecio polyanthemoides* and *S. pterophorus*. Using the brine shrimp lethality assay the results showed a concentration dependent cytotoxicity activities of all the oils which were directly proportional to the different concentrations of 10 µg/ml – 250 µg/ml. Soyingbe *et al.*, (2013b) reported the cytotoxicity of the essential oil of *Tulbaghia violacea* Harv L.F. using the MTT cell proliferation assay on human embryonic kidney cells (HEK293) and human hepatocellular carcinoma cells (HepG2). The oils showed low cytotoxicity IC50 values of 1218 and 1641 µg/ml against HEK293 and HepG2 cells, respectively. Döll-

Boscardin (2012) reported the *in vitro* cytotoxic potential of essential oils of *Eucalyptus benthamii* and its related terpenes on tumor cell lines. The volatile oils from the young and adult leaves of *E. benthamii* showed some degree of cytotoxicity against the studied cells with IC₅₀ values ranging from 108.33 µg/mL at 24 h to 56.51 µg/mL at 72 h, when compared to J77A.1 cells with IC₅₀ values ranging between 287.98 µg/mL at 24 h to 166.87 µg/mL at 72 h. The essential oil obtained from the adult leaves of *E. benthamii* showed similar values for these two cell lines. Studies previously documented suggest that the cytotoxic effect of essential oils with IC₅₀ values between 10– 50 µg/mL suggest a strong cytotoxic activity, while IC₅₀ values ranging between 50–100, 100-200, and 200-300 µg/mL indicate moderate, weak, and very weak cytotoxic properties, respectively (Sylvestre *et al.*, 2006). Compounds with IC₅₀ values higher than 300 µg/mL display no cytotoxic activity (Sylvestre *et al.*, 2006).

2.5.6 ANTIBACTERIAL ACTIVITIES

2.5.6.1 ANTIMICROBIAL RESISTANCE

Antibiotic resistance is a worldwide problem. The Centre for Disease Control and Prevention (CDC) estimates that, in the United States, more than 2 million people are sick with antibiotic resistant infections and about 23,000 die as a result of antibiotic resistance, including fungal and bacterial infections. Antibiotic resistance occurs as a result of bacteria change thereby reducing or eliminates the activity of the drug designed to cure or prevent the infection. The bacteria survive and continue to multiply, causing more harm (Bisht *et al.*, 2009). Antibiotic resistance can happen as a result of the

incorrect use of antibiotics, underdose or overdose (Roger *et al.*, 2003). Antibiotic resistance results in consequences such as: longer periods of hospitalization and high-cost treatments (CDC, 2013; Bisht *et al.*, 2009).

Active efflux is a very important mechanism of bacterial resistance to most classes of antibiotics. This mechanism powered by an efflux pumps system (figure 2.4), is a component associated with active movement by promoting the extrusion of toxic compounds, as well as antibiotics, from the cell (Webber and Piddock, 2003; Li and Nikaido, 2004; Piddock, 2006). These active transporters are present in both specie of bacteria (Wexler, 2012). Efflux pumps confer a multiple drug resistance (MDR) phenotype (Zechini and Versace, 2009). Antimicrobial resistance of efflux mutant is due to an effective efflux pump which makes it more efficient at export; the intracellular antibiotic concentration decreases and the bacterium become less susceptible to the compound used (Li and Nikaido, 2004; Piddock, 2006; Starvi *et al.*, 2007; Levy, 2002; Poole, 2005). Multidrug resistant bacteria that are most problematic include: methicillin resistant *Staphylococcus aureus* (MRSA), Vancomycin resistant enterococci (VRE) and *Mycobacterium tuberculosis* (Kariuki and Hart, 2001; Aqil *et al.*, 2006).

There is a need therefore to develop new antibacterials, either by improving the molecular design of old antibiotics or by developing efflux pump inhibitors (EPIs) (Nikaido, 1996; Zechini and Versace, 2009). EPIs can become active against MDR pumps by binding directly to the pump and blocking it, in a competitive or non-competitive manner (Mahamoud *et al.*, 2007; Lomovskaya and Bostian, 2006).

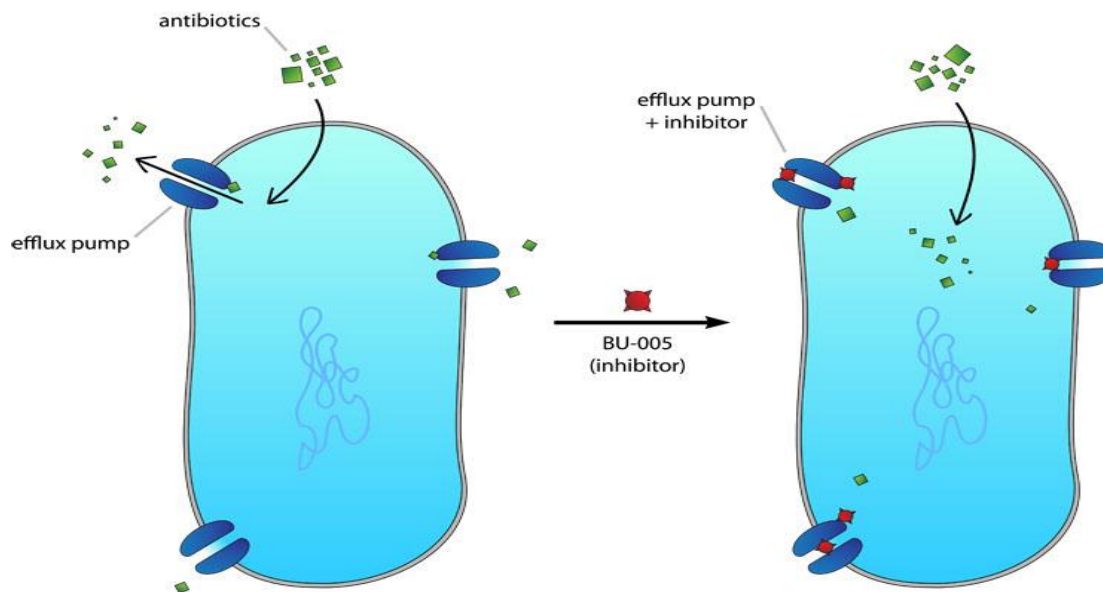


Figure 2.4 Mechanism of efflux pumps system, which is responsible for removing toxic compounds and antibiotics from the cell. (news.brown.edu/2011/bacteria)

Plants are a source of biologically active compounds which have the ability to inhibit the activities of bacteria in the spoilage of food, inhibit the activity of fungi as well as animal pathogens (Zaika, 1989, Dean and Svoboda, 1990; Lin *et al.*, 1999; Penduka *et al.*, 2014). Plant volatiles confer antimicrobial ability to most plants taking into consideration plant maturity, distillation methods, the distilled part of the plant and harvesting periods (Loziene and Venskutonis, 2005; Boira and Blanquer, 1998; Burbott and Loomis, 2000; Panizzi *et al.*, 1993; Lahlou, 2004). Some animal and human viruses have also been reported to be susceptible to essential oil. Research in this field is still ongoing (Dorman and Dean, 2000; Connor and Beuchat, 1984; Deans and Ritchie, 1987; Deans and Svoboda, 1988; Roussis *et al.*, 2002; Kilani *et al.*, 2008).

PrabuSeenivasan *et al.*, (2006) studied the *in vitro* antibacteria activity of six essential oils and reported that cinnamon, clove and lime oils showed varying degrees of

antibacterial activity on both specie of the bacteria tested. The minimum inhibitory concentration and minimum bactericidal concentration values observed for the essential oils of *Eucalyptus globulus* and that of *Thymus algeriensis*, showed that the essential oils of both plants can be used as natural agents in food preservation and as antibiotic compounds (Abdenour *et al.*, 2011). *Blumea megacephala* essential oils have been seen to be a potential source of natural antimicrobial compounds (Liang Zhu *et al.*, 2011). Lalitha *et al.*, (2011) suggests that many essential oils, as well as extracts of the associated plants, have *in vitro* antifungal and antibacterial activity.

Susceptibility testing methods used to determine the activities of essential oils in inhibiting the growth of bacteria include agar well diffusion, agar disk diffusion methods, kill-time analysis and broth dilution analysis (Prabuseenivasan *et al.*, 2006; Cimanga *et al.*, 2002; Skaltsa *et al.*, 2003; Nevas *et al.*, 2004; Dorman and Deans, 2000; Vijoen *et al.*, 2006; van Vuuren and Vijoen, 2006). Other methods employed include the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), membrane damage activity (LDH assay) and the efflux pump inhibitory assay (Dikshit and Husain, 1984)

2.5.7 ANTIOXIDANT ACTIVITIES

The antioxidant activities of essential oils have been widely studied and documented. Ramzi (2011), working with the essential oils of *Nepetade flersiana* found in Yemen, reported that the essential oil reduced DPPH and demonstrated moderate antioxidant activity, although the observed low antioxidant activity was associated with low contents of phenolic compounds found in the essential oil, such as thymol and carvacrol.

The essential oil extracted from guava stem bark showed a weak proton donor in DPPH reaction. However, it was observed to compare favorably with α -tocopherol, a good scavenger of hydroxyl radicals (Fasola *et al.*, 2011). Kadri *et al.*, (2011) reported that the essential oil from the aerial parts of *Artemisia herba-alba* growing in the Tunisian semi-arid region could be a rich source of natural antioxidants in foods as an alternative to synthetic antioxidant, and could assist the pharmaceutical industry with the prevention and treatment of various human diseases. Inès *et al.*, (2011) observed that the essential oil obtained from the flowers of *G. sanguineum L.* possesses antibacterial and antioxidant activities. The antioxidant properties of essential oils invariably make them very good candidates for use as natural antioxidants and also as models for new free radical scavenging drugs.

2.6 AIMS & OBJECTIVES

Despite the many and varying pharmaceutical properties of *Eucalyptus grandis* that are exploited by traditional healers, there has been little or no scientific verification of their therapeutic activities. To the best knowledge of this researcher, there is no mention of the anti-asthmatic and anti-cough activities of the essential oil of this plant in literature. Such knowledge is essential for the complete (medicinal) exploitation of the plant.

2.6.1 HYPOTHESIS

The essential oils of *Eucalyptus grandis* have anti-asthmatic and anti-cough activities that relieve the effects of asthma and a cough.

2.6.2 AIMS

This project aims to extract and investigate the variations in essential oil composition of the dry and fresh leaves of *Eucalyptus grandis*, the anti-asthmatic and anti-cough activities of the essential oil and some of the bioactivities (including the anti-oxidant, antimicrobial, anti-inflammatory activities) of the essential oil hydrodistilled from dry and fresh leaves of *Eucalyptus grandis*.

2.6.3 OBJECTIVES

- I. To collect and identification of *Eucalyptus grandis* (W. Hill ex Maiden);
- II. To extract essential oils from the fresh and dry leaves by hydrodistillation;
Investigation of the variation in chemical composition of the dry and fresh leaves;
- III. To Investigate of the antioxidant and antimicrobial properties of the extracted essential oils;

- IV. To evaluate the cytotoxicity of the extracted essential oils;
- V. To evaluate the anti-asthmatic and anti-cough properties of the essential oils of *Eucalyptus grandis*;
- VI. To isolate the active agent in the essential oil.

Chapter 3

MATERIALS AND METHODS

The materials used in this research are listed below as well as a brief description of the methods used in the study are given; a full and detailed method preparation of the reagents and methodology are presented in Appendix A and B respectively.

3.1. MATERIALS

3.1.1. CHEMICALS AND REAGENTS

(See Appendix A for reagent details)

The potassium persulfate, trichloroacetic acid (TCA), ascorbic acid, trolox, ferric chloride (FeCl_3), iron (ii) chloride tetrahydrate (FeCl_2), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid sodium salt (ferroxine), sodium nitropruside, sulphanic acid, glacial acetic acid, naphthyl ethylenediamine dihydrochloride (naphthylamine), potassium chloride (KCl), sodium chloride, potassium hydroxide, potassium ferricyanide, sodium hydroxide, ferrous ammonium sulfate (FAS), pyridine, sodium carbonate, xanthine, copper chloride (CuCl_2), ferrous sulfate (FeSO_4), hydrogen peroxide (H_2O_2), gallic acid, sodium carbonate, phenol reagent, dimethyl sulfoxide, and p-iodonitotetrazolium violet (INT) used were all obtained from Sigma-Aldrich Co., Germany.

The sulphuric acid (H_2SO_4), ammonium molybdate, potassium ferricyanide, methanol (MeOH), hexane, glacial acetic acid, sulphuric acid (H_2SO_4), hydrochloric acid (HCl), nutrient agar, and nutrient broth used were all obtained from Merck. The ampicillin and neomycin were obtained from Oxiod.

3.1.2. EQUIPMENT

Rotor evaporator (Heidolph—Laborota 4000)

Spectrophotometer (Spekol 1300)

Centrifuge (Eppendorf—5804 R)

pH meter (Hanna Instruments)

Incubator (Labcom)

BiotekEix 808 UI plate reader (Biotek instrument suppliers)

GC-MS (Agilent technologiesGC 7890A equipped with an Agilent mass spectrometry system (5975C VL MSD with triple axis detector)

3.1.3. BACTERIA STRAINS USED IN THE STUDY

The bacteria strains used in this study consisted of the reference strains *klebsiella pneumoniae*, (ATCC31488), *staphylococcus aureus* (ATCC 25925) and clinical isolate *morexella catarrhalis*. *S. aureus* and *K. pneumoniae* were collected from uMhlatuze Water Municipality and *M. catarrhalis* was collected from Nkonjeni Hospital in Nongoma, KZN Province, South Africa. These are pathogens known to be involved in respiratory infections which could trigger an asthma attack. The stock cultures were maintained at 4°C in Müller-Hinton agar (Oxoid, Germany).

3.2 METHODOLOGY

(See appendix B for details)

3.2.1 ETHICAL CLEARANCE

Ethical clearance (UZREC 171110-030 PGD 2013/26) was obtained from the Research Animal Ethics Committee (RAEC) of the University Of Zululand. Sprague-Dawley rats were collected from the animal house in the Department of Biochemistry, University of Zululand. Experimental research was carried out following the guideline for care and supervision of experiments on animals. The animals were housed in standard cages and maintained at room temperature with 12:12-h light: dark cycle. All rats had free access to drinking water and standard rat feed in the experimental environment for 1 week before the experiments were conducted.

3.2.2 COLLECTION AND IDENTIFICATION OF PLANT MATERIAL

Eucalyptus grandis W.Hill ex Maiden leaves were collected from kwaDlangezwa in Empangeni, kwaZulu-Natal Province, SA. The leaf of the plant was taken to the Department of Botany, University of Zululand for identification and voucher specimens (OS.01UZ) were deposited at the University of Herbarium.

3.2.3 EXTRACT PREPARATION

The leaves of *Eucalyptus grandis* were picked from the stalk of the freshly collected plant and a portion of the collected leaves were dried at room temperature. The leaves were subjected to more than three hours of hydrodistillation using a Clevenger-type

apparatus (British pharmacopoeia). The essential oil obtained was collected over water and dried over anhydrous sodium sulfate and then dissolved in methanol which was then stored at -5°C until required.

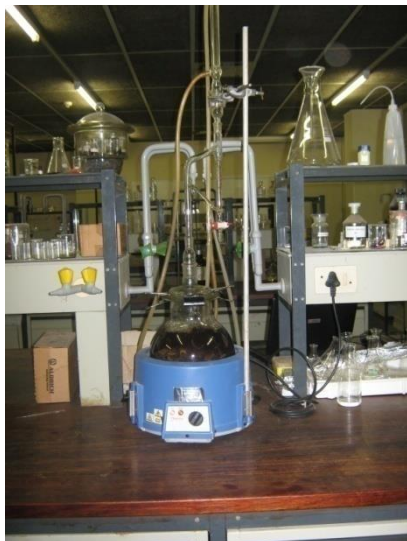


Figure 3.1 Hydrodistillation of essential oil using a Clevenger type apparatus

3.3. PHYSICAL PROPERTIES

The quality assessment of essential oils requires different analyses, including sensory evaluation and refractive index analysis.

The sensory evaluation (color, odour, etc.) of the extracted essential oils was done by a panel of staff and students in the Department of Biochemistry and Microbiology. The refractive index was determined with the Abbey refractometer.

3.4. CHEMICAL COMPOSITION OF ESSENTIAL OILS

The Gas Chromatography/Mass Spectrometer (GC/MS) of the essential oils extracted was carried out using an Agilent Gas Chromatography (7890 A) equipped with an

Agilent 190915 (30m × 250 μm × 0.25 μm calibrated) attached with an Agilent mass spectrometer system (5975C VL MSD with Triple Axis Detector). The oven temperature was set from 45°C – 310 °C. Helium was used as the carrier gas at a flow rate of 5 ml/min with a split ratio of 1: 200. The essential oil (1 μl) was diluted in hexane and 0.5 μl of the solution was manually injected into the GC/MS. The chemical compositions of the essential oil of the leaves of *E. grandis* were determined according to their retention time and spectrometric electronic libraries (WILEY NIST) equations.

3.4.1 ISOLATION OF ACTIVE COMPOUNDS.

The isolation and purification of components from the essential oils of the fresh and dry leaves of *Eucalyptus grandis*, were performed using a column chromatography packed with chromatographic silica gel (0.063-0.200 mm). Firstly 80 g of LC 60A 40-63 silica gel was slurred with 120 mL n-hexane and poured into a 25mm I.D. × 300mm glass column, after which 3.2 grams of essential oil was dissolved in 5mL n-hexane. The mixture was run through the silica gel column and washed with n-hexane and ethanol to give two fractions. Hexane was used to elute (20 ml/min) the terpene hydrocarbon from the LC60A 40-63 silica gel, thereafter, two oxygenated compounds were easily washed off the column with ethanol (20 ml/min). These fractions were then analyzed using thin layer chromatography for purity. After this, a GC-MS analysis was done on the ethanol fraction.

3.5. BIOLOGICAL ACTIVITIES OF ESSENTIAL OILS

3.5.1 ANTIMICROBIAL ACTIVITY OF THE ESSENTIAL OILS.

3.5.1.1 AGAR DISK DIFFUSION METHOD

The antibacterial properties of the essential oils were carried out using the agar disk diffusion method (Van Vuuren and Vijoën, 2006). Bacteria were incubated in 10ml nutrient broth at 37°C overnight. Next, the cultures were diluted to the McFarland no.5 standard (1.0×10^8 CFU/ml). Standard Petri dishes with nutrients agar were inoculated with the bacteria suspension (1.0×10^8 CFU/ml). Sterile paper disks (6 mm) were then placed on the inoculated plates and 10 µl of 10 mg/ml of the essential oils dissolved in 10% DMSO was added to the paper disks. Tests were performed in triplicates; ampicillin and neomycin at 10 µg/ml were used as positive control.

3.5.1.2 MINIMUM INHIBITORY CONCENTRATION

The minimum inhibitory concentrations (MIC) of the essential oils were determined by the Eloff's (1998) method. Nutrients broth (50 µl) was added to all the wells of the microtiter plates; 50 µl of 10 mg/ml of the essential oils dissolved in 10% DMSO was then added to all the wells in the row and then serially diluted down the rows from the first row. Bacteria culture (50 µl) of McFarland standard were added to all the wells and then incubated at 37 °C for 24 hrs. A 20 µl of 0.2 mg/ml p-iodonitotetrazolium violet (INT) solution was then added to each well and incubated at 37 °C for 30 mins. The MIC, which is the lowest concentration at which no visible microbial growth is seen, was recorded. Bacteria treated with ampicillin and neomycin at 10 µg/ml were used as positive controls.

3.5.1.3 MINIMUM BACTERICIDAL CONCENTRATION

The minimum bactericidal concentration (MBC) which is regarded as the lowest concentration of the sample at which inoculated bacterial strains are completely killed, were confirmed by re-inoculating 10µl of each culture medium from the microtiter plates, which was used for MIC, on nutrient agar plates and incubated at 37 °C for 24 hrs (Soyingbe *et al.*, 2013). Bacteria treated with ampicillin and neomycin at 10 µg/ml were used as positive controls.

3.5.1.4 MEMBRANE DAMAGE (CLEAVAGE) LDH ASSAY

The susceptible organisms were grown and incubated with the MBC concentration of the essential oil overnight. The microbial cultures were then centrifuged (5000 g; 5 mins). The supernatant (100 µl) was then mixed with 100 µl of lactic acid dehydrogenase substrate mixture of 54 mM lactic acid, 0.28 mM of phenazine methosulfate, 0.66 mM p-iodonitrotetrazolium violet, and 1.3 mM NAD⁺. The release of the cytosolic lactate dehydrogenase (LDH) reflects the loss of membrane integrity in dying cells, and it was assessed by colorimetric assay. The cytotoxicity in the LDH release test was calculated using the formula: $(E-C)/(T-C) \times 100$, where E is the experimental absorbance of the cell cultures, C is the control absorbance of the cell medium, and T is the absorbance corresponding to the maximal (100%) LDH release of Tri- ton X-100 lysed cells (positive control). (Badovinac *et al.*, 2000).

3.5.1.5 EFFLUX PUMP INHIBITION-RHODAMINE 6G UPTAKE

The activities of the essential oils were tested for their MDR inhibition of Rhodamine 6G (R6G) accumulation using the method of Maesaki *et al.*, (1999) with some modifications. Bacteria were cultured overnight at 37 °C with shaking (110rpm). After 24 hrs cells were centrifuged at 4000xg for 5 min and washed twice with phosphate buffer saline (PBS, pH 7.2). Cells were centrifuged again and re-suspended at 40 mg/ml in PBS containing 10mM sodium azide (NaN₃). R6G was added to a final concentration of 10 µM and cells placed in an incubator for 1hr. Cells were then divided into two aliquots, tube 1 and tube 2. Cells were centrifuged for 5 min at 4000rpm. Cells in tube 1 were re-suspended in PBS containing 1M glucose while the cells in tube 2 were re-suspended in PBS alone. Essential oils were then added to the cells containing glucose to a final concentration of 100 µM. Both tubes were then placed in an incubator with agitation for 30 min at 37 °C. Cells were centrifuged and the supernatant discarded. The remaining pellet was re-suspended in 0.1 m glycine HCl, pH 3 and placed in the shaking incubator overnight. After 24 hr, cells were centrifuged for 10 min at 4000xg and the supernatant collected and the absorbance reading was 527 nm. The accumulation of the R6G was expressed as a percentage accumulation in the cells.

The percentage accumulation of R6G inside cells after exposure to glucose, extract and standards was calculated with the formula:

$$(1-A_t / A_o) \times 100$$

Where A_t is the absorbance of the test compound and A_o is the absorbance of the control in the presence of glucose only, Beberine was used as positive controls.

3.5.2 ANTIOXIDANT ACTIVITY

3.5.2.1 SUPEROXIDE ANION SCAVENGING ACTIVITY

The method used by Nagai *et al.* (2001) for determination of superoxide dismutase was followed, with some modification. The method uses the extract ability to inhibit and reduce nitroblue tetrazolium (NBT) in the riboflavin–light–NBT system (Beauchamp and Fridovich, 1971). A reaction mixture of 0.02 ml each of sodium carbonate buffer (50 mM, pH 10.5), 0.15 % bovine serum albumin, 3 mM Xanthine, 3 mM EDTA, 0.75 mM NBT and 0.02 ml of essential oil (0-5 mg/100 ml) were incubated with 0.02 ml of Xanthine oxidase (6 mU) for 20 min at 25 °C. The production of blue formazan was monitored after the addition of 0.02 ml of 6 mM CuCl₂, at 560 nm.

3.5.2.2 NITRIC OXIDE SCAVENGING ACTIVITY

The nitric oxide radical scavenging activity was determined using the method of Garret (1964). Methanolic solution of the essential oil at different concentration (0.5 ml of 5 mg/ml – 100 mg/ml) were added to a reaction mixture of (2 ml of 10 mM) of sodium nitroprusside saline phosphate buffer (0.5 ml of 0.01 M, pH 7.4), incubated for 150 min at 25 °C. Sulphanilic acid reagent (1 ml of 0.33%) was then added to 0.5 ml of the incubated solution and allowed to stand for 5 min. Naphthyl ethylenediamine dihydrochloride (1 ml of 0.1%) was added and the mixture incubated at 25 °C, after which absorbance was measured at 540nm and the percentage scavenging estimated.

3.5.2.3 HYDROXYL RADICAL (-OH) SCAVENGING ACTIVITY

The assay is based on benzoic acid hydroxylation method as described by Osawa, *et al.*, (1997). In a screw-capped tube, 0.2 ml each of FeSO₄, 7H₂O (10 mM), EDTA (10 mM), different concentrations of the essential oil (0-5 mg/100 ml), DNA (10 mM) and phosphate buffer (pH 7.4; 0.1 mol) were mixed. Then 200 µl of H₂O₂ solution (10 mM) was added. The reaction mixture was incubated at 37 °C for 2 hr. After that, 1.0 ml TCA (2.8%) and 1.0 ml of 1% TBA was added and the mixture boiled and allowed to cool on ice. Absorbance was read at 520nm.

3.5.2.4 SULFHYDRYL (SH) CONTENT

The SH content of the oils was done using the method described by Cohen and Lyle (1966). Methanolic solutions of the essential oils were added to 0.5 ml phosphate buffer (0.1M, pH 8.0) and 0.1 ml of 1% σ--phthalaldehyde thereafter allowed to stand for 20mins at room temperature. The absorbance was read at 420nm. The -SH content of the oils was estimated from the standard graph of glutathione.

3.5.2.5 NADH CONTENT

The of Stern *et al.* (2002) with some modification was used to determine the NADH content in the essential oil. The essential oil (1:5w/v) was mixed with a phosphate buffer containing 20 mM NaHCO₃, 100 mM Na₂CO₃ and 10 mM nicotinamide. The filtrate was heated on a dry heating block at 60 °C for 30 min to destroy NAD⁺/NADP⁺, and quickly chilled to 0 °C. Thereafter, 1 ml of a reaction mixture containing 100 µmol Tris-HCl (pH 8), 2 µmol phenazineethosulfate, 0.5 µmol Tetrazolium bromide, 0.2 mg

alcohol dehydrogenase, and 0.2ml of the essential oil was added then incubated for 5 min at 37 °C. Thereafter 60 µmol ethanol was then added and incubated for 10 min at 37 °C and the absorbance was read at 570 nm.

3.5.2.6 DETERMINATION OF MALONDIALDEHYDE (MDA)—TBARS

Determination of MDA was done using the method of Halliwell and Gutteridge (1990). To 100µl of the reaction mixture, 1.5ml of TCA (10 %) was added and incubated for 10 minutes. Then centrifuged at [3500 rpm for 20 min] the resultant supernatant was mixed with 1.5 ml of 1% TBA. The mixture heated in a bath of boiling water for 30 minutes, allowed to cool, 2ml of n-butanol was then added. The absorbance of the butanol layer was measured at 532nm.

Unless otherwise stated, ascorbic acid, trolox and BHT were used as standards. All assays were repeated three times and the mean ± S.E reported. The inhibitory effects of the extracts on each parameter were calculated as:

$$\% \text{ Inhibition} = \left(1 - \frac{A_t}{A_0}\right) \times 100$$

where A_0 is the absorbance value of the fully oxidized control and A_t is the absorbance of the extract. The inhibitory concentration providing 50% inhibition (IC_{50}) was determined using statistical package Origin 6.1.

3.6. CYTOTOXICITY ASSAY

Human embryonic kidney cells (HEK293) and Human hepatocellular carcinoma cells (HepG2) were the cells used for the assay. Cells were cultured in 25cm² flask to confluency, the cells were then trypsinised and plated into 48 well plates at 2.5×10^4 seeding density per well. Next, the cells were incubated overnight at 37°C. Fresh medium (MEM +Glutmax + antibiotic) was added. The essential oils (50 µg -350 µg) were added in triplicate and incubated for 4 hr. The medium was removed and replaced by complete medium (MEM + Glutmax + antibiotics + 10% Fetal bovine serum). After 48hrs cells were subjected to the MTT assay (Mosman, 1983).

3.7 MUSCLE CONTRACTION STUDIES (VASCULAR REACTIVITY ON AORTIC SMOOTH MUSCLE)

The study was undertaken to evaluate the effect of the essential oils and 1,8-cineole on the isometric contraction of isolated rat aortic rings. Healthy, young adult, male and female Wistar albino rats weighing 250-300 g were used. The animals were kept and maintained under conventional laboratory conditions of controlled temperature, humidity and light, and allowed free access to standard pellet diet and water *ad libitum*. All the animals used were fasted for 18 hr, but still allowed access to water before the commencement of the experiments. The rats were sacrificed by cervical dislocation. The thoracic aorta was quickly removed, freed of connective tissue and placed in a petri-dish containing physiological salt solution (PSS). The aortic lumen was gently flushed with PSS and sectioned into 2 mM ring segments. Each aortic ring was suspended in a 60 ml jacketed tissue bath containing PSS with the following composition (Ebeigbe and Aloamaka, 1987; Obiefuna *et al.*, 1991; Ojeikere *et al.*, 2003; Salahdeen and Murtala,

2012) (mmol/L): NaCl, 118.0; KCl, 4.7; KH₂PO₄, 1.2; MgSO₄, 1.2; NAHCO₃, 15.0; CaCl₂, 1.6 and glucose, 11.5. The temperature of the bath was maintained at 37°C and the solution bubbled with a 95% O₂, 5% CO₂ gas mixture (pH 7.3 - 7.4). An initial tension of 2 g was applied to all arterial rings. An equilibration period of 60-90 min was allowed before the start of experiments, and during this time it was stimulated thrice with 10⁻⁶ M noradrenaline for 5 min at 30 min intervals. At the end of the equilibration period, the vessel was subjected to the following procedures. Cumulative doses of noradrenaline (NA) were added to the PSS to obtain concentrations of 10⁻⁹ to 10⁻⁵ M and the concentration–response curves (CRCs) were determined. Following incubation with essential oil the extract CRCs were determined for NA.

3.8 ACETYLCHOLINESTERASE INHIBITORY ACTIVITY

The method of Ellman *et al.*, (1961) was used to determine acetylcholinesterase inhibitory assay. Acetylcholine iodide was used as a substrate for the assay. The mixture containing 1 ml of acetylcholine iodide, 1 ml of 0.1 mM sodium phosphate buffer (pH 7.4), and the compound (0-5 mg/100 ml), were incubated at 37°C for 5 min, and 0.1 ml of the enzyme (acetylcholinesterase) was added. The mixture was incubated at 37°C for a further 8 min. The reaction was terminated by adding 1 ml of 3 % sodium dodecyl sulphate (SDS), then 0.1 ml of 0.2 % 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) was added to produce the yellow anion of 5-thio-2-nitrobenzoic acid. The colour was measured at 440 nm for 10 min at 2 min intervals. Tacrine was used as a positive control. The enzyme activity was calculated using the formula: $R = 574 \times 10^{-4} \times A/C$.

Where: R = rate in moles of substrate hydrolysed/ min/ mg; A= change in absorbance/ min; C = original concentration of the substrate (mg/ml). Percentage inhibition was calculated using the formula: % Inhibition = $(1 - V_0 \text{ Sample} / V_0 \text{ Blank}) \times 100$. Where $V_0 \text{ Sample}$ and $V_0 \text{ Blank}$ represent the initial velocities of samples and blank velocities.

3.9. INVESTIGATION OF ANTI-INFLAMMATORY EFFECTS.

3.9.1. COTTON PELLETT GRANULOMA TEST

The proliferation phase of inflammation was investigated by the cotton pellet granuloma model (Meier *et al.*, 1950). The essential oils at 50 and 150 mg/ kg and indomethacin as the control drug at 10 mg/kg body weight were dissolved in 10% Tween-20 and were given to the rats orally. After 30 min the animals were anesthetized. Sterile cotton pellets of 20mg each were implanted at an interscapular depth under the skin under sterile conditions. The essential oils and control drug were administered daily for a period of seven days. The rats were sacrificed after anesthesia on the eighth day and the pellets surrounded by granuloma tissues were dissected out, weighed, dried for 24 h at 40°C temperature and weighed again. The increment between dry and wet pellet weights were taken as a measure of granuloma formation and compared with those of the control.

The collected, dried and weighed granulations were separately homogenized, and used to estimate catalase, superoxide dismutase (SOD) and protein content.

3.9.2. CATALASE ACTIVITY

The catalase activity was measured according to method of Shinha *et al.* (1971). A 0.1 ml of the homogenate was mixed with 1.0 ml of 0.01 M phosphate buffer (pH 7.4), and incubated with 0.4 ml of 0.2 M H₂O₂ at 37 °C accurately for 1.0 min and reaction was stopped with 2.0 ml of 5 % potassium dichromate (1:3 with glacial acetic acid). The samples were incubated in a bath of boiling water for 15 min, centrifuged at 5000 rpm for 15 min and supernatant was used to quantify the amount of H₂O₂ to calculate catalase activity at 570nm. One unit represents 1.0 μmole of H₂O₂ consumed/min/mg protein.

3.9.3 SUPEROXIDE DISMUTASE

The method of Hwang *et al.* (1999) was used to determine the SOD content. One gram of fresh tissue was homogenized with 10ml of ice cold 50mM sodium phosphate buffer containing 1mM phenyl methyl sulfonyl fluoride (PMSF).. Superoxide dismutase activity was assayed as described by Beauchamp and Fridovich (1971). The reaction medium was prepared and to 3 ml reaction medium, 1ml of enzyme extract was added. The reaction mixture contained 1.17×10^{-6} M riboflavin, 0.1 M methionine, 2×10^{-5} potassium cyanide and 5.6×10^{-5} M nitroblue tetrasodium salt, dissolved in a 0.05 M sodium phosphate buffer (pH 7.8). The mixture was illuminated in fluorescent glass test tubes. The absorbance was read at 560 nm in the spectrophotometer against the blank. The amount of change in the absorbance by 0.1 per hour per milligram protein under the assay condition was taken as the SOD activity

3.9.4. IN VITRO COX-1 and COX-2 INHIBITION ASSAY

The ability of the essential oils to inhibit COX-1 and COX-2 was determined using an EIA kit as described by Gautam *et al.*, (2010). COX-1 and COX-2 catalyzes the biosynthesis of prostaglandins (PG) H₂ from arachidonic acid, and PGF₂α produced from PGH₂ by reduction with stannous chloride is measured by an acetylcholinesterase competitive EIA kit. The essential oils (50 µg/ml) were dissolved in ethanol and used. The percentage inhibition was calculated by the comparison of compound treated and control incubations.

3.10 ANTI-ASTHMATIC ACTIVITY OF ESSENTIAL OILS

The animal experiments were carried out using Sprague-Dawley rats. Rats obtained from the animal house were allowed to acclimatize for 4 days. The rats were then divided into 5 groups of 5 rats each: group 1 was the control group that received only the carrier solvent (10% Tween-20); group 2 was the positive control, and were treated with Aminophylline (125 mg/kg body weight); groups 3, 4 and 5 received 50 mg/kg, 100 mg/kg and 250 mg/kg in body weight, respectively of the extract dissolved in 10% Tween-20 (Fig 3.2). All the rats were fed with a standard diet (Doghouse, SA) and water *ad libitum*. The rats were administered with their respective drugs through the oral cannula for 3 consecutive days. One hour after the last administration the rats were exposed to irritant agents (histamine and acetylcholine mixture 1:1) for 30seconds using an ultrasonic nebulizer via whole body exposure. The latent periods of asthma were recorded.

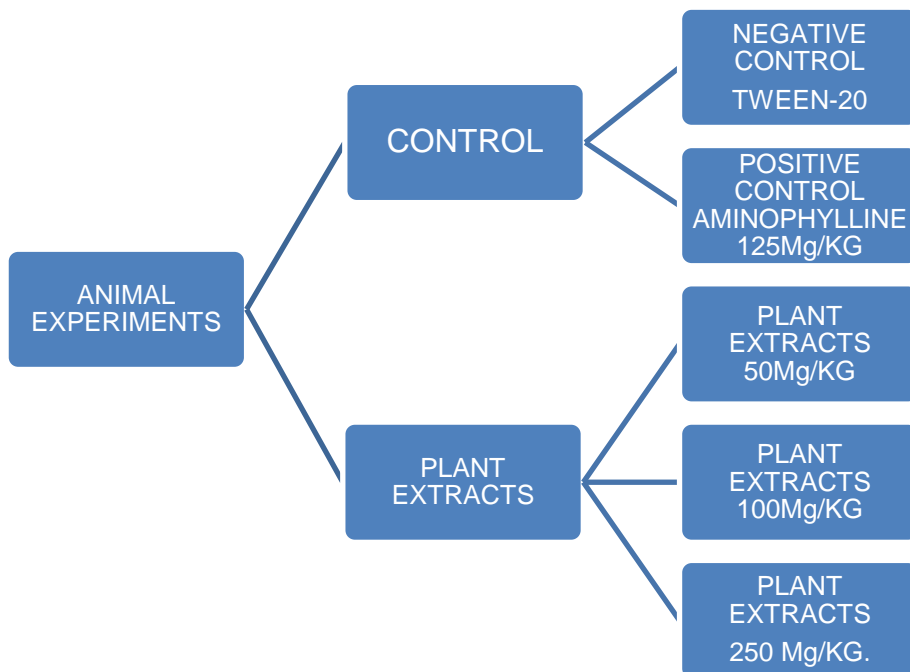


Figure 3.2 Animal experimental set-up.

3.11. ANTI-COUGH ACTIVITY OF ESSENTIAL OILS

The experimental design was similar to that reported for the anti-asthma (see section 3.11). Codeine phosphate (5 mg/kg of body weight) was used as the positive control. Coughing was induced by exposing rats to ammonia for 5 sec, and the latent period of coughing (seconds from the expose to the first cough) and the number of coughs during a 2 minute period recorded.

3.12 A549 LUNGS CELL CULTURES

A549 lungs cells were cultured (37 °C, 5% CO₂) to confluency in 25 cm³ culture flask in complete culture media (CCM). CCM was made up of eagle's minimum essential medium (EMEM), 10% foetal calf serum, 1% L-glutamine and 1% penstrepfungizone. Growth of cells was monitored and confluent flasks were trypsinized using 1ml trypsin. Cell numbers were counted with the aid of trypan blue dye stains. The cytotoxicity effect of the essential oils was determined using (MTT) assay. A549 cells (15,000 cells/ well) were seeded into a 96-well microtiter plate and incubated at (37 °C, 5% CO₂) with a range of essential oil dilutions, in triplicate, for 24 hr. A549 cells incubated with CCM only were used as a positive control. 120 µl of CCM/MTT salt solution at a concentration of 5mg/ml was added to each well and incubated for 4 hr at 37 °C. Following incubation, the supernatant were removed and 100 µl DMSO was added to each well and incubated for 1 hour. The optical density of the formazan produced was measured at 570nm with a reference wavelength of 690nm. The percentage cell viability was then determined (% viability = average OD of treatment/average OD of untreated control X 100).

3.13 INDUCTION OF INTERLEUKIN (IL-6) SECRETION (CYTOKINE ASSAYS)

The levels of cytokines interleukin 6 (IL-6) in A549 cells was determined by enzyme-linked immunosorbent assay. (ELISA) IL-6 secretion was induced by the addition at time zero of 10ul of a stimulatory agent to the test wells. Supernatants were removed from triplicate wells for each stimulus at the four experimental time points. The triplicates were combined into one sample and immediately frozen at -20°C. A control sample obtained from unstimulated cells was treated similarly. Samples from a single experiment were analyzed in the same IL-6 assay.

3.14 HISTOLOGICAL STUDIES

An *in vivo* study using rat models of lipopolysaccharide (LPS)- induced acute lung injury and its effect on the lung inflammation were viewed digitally by histological observation. LPS 50 µl (2 mg/kg) was administered to the rats intranasal. After 48 hrs of the last challenge of LPS, lungs were removed from the rats after they had been sacrificed. Prior to the removal of the lungs, the lungs and trachea were filled intratracheally with a fixative (4% paraformaldehyde) using a ligature around the trachea. Lung tissues were fixed with 10% (v/v) paraformaldehyde. The specimens were dehydrated and embedded in paraffin. Histology studies were carried out at the Vet Diagnostix laboratories (Pietermaritzburg, SA) by a qualified pathologist having no prior knowledge of which group the specimens belonged. The lung tissues were stained with haematoxylin and eosin (H & E). This method allowed for an unbiased description of the histological lesions which were either present or absent in the samples.

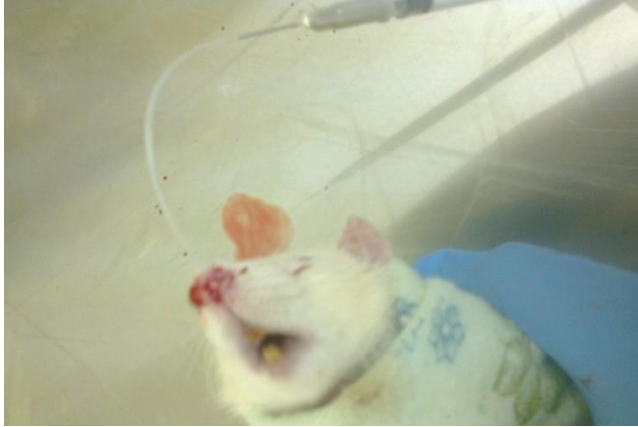


Figure 3.2 intranasal administration of LPS.

3.14 STATISTICAL ANALYSIS

Data was expressed as means \pm S.E.M. Students' t-tests were used to analyze data between the groups and analyses of variance (ANOVA) conducted among groups, followed by Dunnet's t-test for multiple comparisons. The statistical significance will be set at $P < 0.05$.

Chapter 4

RESULTS

The hydrodistilled volatile oils of both the fresh and dry leaves were examined by GC-MS to determine the components of both sets of leaves. The oils were investigated for their anti-asthma and anti-cough properties. Studies of the antioxidant properties as well as their antimicrobial properties were carried out. In addition, studies of the cytotoxicity and the anti-inflammatory properties were carried out. The results obtained are presented in this chapter

4.1 PHYSICOCHEMICAL PROPERTIES OF THE ESSENTIAL OILS.

The physicochemical properties of the essential oils that were isolated from both the fresh and dry leaves of *Eucalyptus grandis* are summarised in Table 4.1 and were assessed using the method described in the British Pharmacopoeia (1998). The results showed a percentage yield of 1.10% for the dry leaves and 0.43% for the fresh leaves; the characteristic color of the oils were green for the fresh leaves and lighter shade of green for the dry leaves. It is apparent that the drying process affected the physical properties of the extracted oil.

Table 4.1 Physicochemical properties of the essential oils of the fresh and dry leaves of *Eucalyptus grandis*.

Properties	Fresh leaves	Dry leaves
% yield	0.43%	1.10%
Refractive index	1.4685	1.4773
Color	Green	light green
pH	3.651 @ 28 ^o	4.065 @ 28 ^o

4.2 CHEMICAL COMPOSITION OF THE ESSENTIAL OILS

The chemical composition of the essential oils (identified by their relative retention indices and percentage composition) are shown in Table 4.2. A total of 33 compounds, representing 99.3%, were identified in the fresh leaves and 14 from the dry leaves, representing 89.2 %. The main components of the oils from the fresh leaves were α -pinene (29.67%), p-cymene (19.89%), 1,8-cineole (12.80%) and α -terpineol (6.48%). The main components of the dry leaves were 1,8-cineole (47.44%), limonene (13.34%), α -pinene (7.49%) and spathulenol (7.13%). (see appendix C for spectra).

Table 4.2 Chemical composition of the essential oils of the fresh and dry leaves of *Eucalyptus grandis*

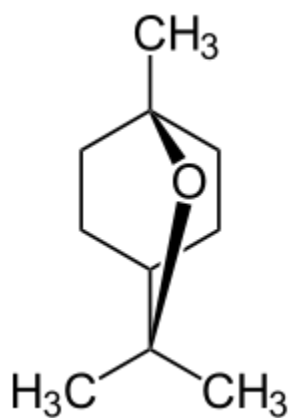
Chemical composition of essential oils of <i>Eucalyptus grandis</i>				
Compound	RT ^a	KI ^b	Percentage composition	
			Fresh	Dry
α - pinene	8.10	936	29.6	7.5
camphene	8.50	950	1.5	
β - pinene	9.31	964	-	0.2
β -myrcene	9.69	993	-	0.3
p-cymene	10.72	1025	19.8	5.4
limonene	10.84	1029	3.1	13.3
1,8-cineole	10.92	1031	12.8	47.4
γ -terpinene	11.71	1060	2.1	1.5
terpinolene	12.60	1089	0.3	1.6
carene	12.90	1148	0.2	-
β -fenchol	13.35	1122	1.2	-
<i>trans</i> -pinocarveol	13.73	1139	0.6	-
camphor	14.11	1146	2.5	-

sabinyl acetate	14.82	1166	0.5	-
borneol	14.90	1169	3.4	-
terpinen-4-ol	15.22	1177	0.8	-
α -terpineol	15.62	1189	6.4	1.2
<i>cis</i> -carveol	16.38	1231	0.4	-
thymol	18.19	1290	0.2	-
carvacrol	18.63	1299	0.3	-
terpinyl acetate	19.97	1349	1.6	-
caryophyllene	21.90	1419	1.8	-
alloaromadendrene	26.12	1441	0.2	-
γ -gurjunene	25.53	1447	0.3	-
viridiflorene	22.83	1497	1.5	-
α -calacorene	24.87	1546	0.4	-
spathulenol	25.76	1578	1.4	7.1
caryophyllene oxide	25.92	1583	1.5	2.7
α -eudesmol	26.35	1632	0.7	0.3
<i>cis</i> -cadin-4-en-7-ol	26.65	1637	1.7	-
epoxy-allo- alloaromadendrene	26.82	1641	1.0	-
cadine-1,4-diene	26.82	1646	0.4	0.5
amiteol	27.13	1660	0.1	-
monoterpene hydrocarbons			56.6	29.9
oxygenated monoterpenes			31.0	48.7
sesquiterpene hydrocarbons			4.9	0.5
oxygenated sesquiterpenes			6.8	10.1
Total identified			99.3	89.2

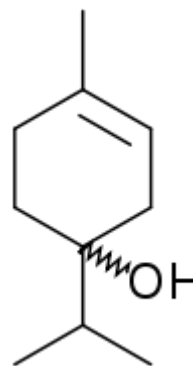
*Kovats index on a DB-5 column in reference to n-alkanes (Adams 1995, 2001). MS, NIST and Wiley libraries spectra and the literature; KI, Kovats index.

4.3 ISOLATION OF COMPOUNDS

Two compounds were isolated from the essential oils: 1,8-cineole (Fig 4.1) from the dry leaves and terpinen-4-ol (fig 4.2) from the fresh leaves. The spectra obtained showed pure compounds. (see Appendix C).



1,8-cineole



terpinen-4-ol

Figure 4.1 1,8-cineole

Figure 4.2 Terpinen-4-o

4.4 CYTOTOXICITY OF THE ESSENTIAL OILS

The cytotoxicity of the essential oils against HEK 293 and HEP G2 cell lines are presented in Table 4.3.

Table 4.3 Percentage inhibition of HEK 293 and HEP G2 by the essential oils

(µg/200µl)	<i>E. grandis</i> (Dry leaves)		<i>E. grandis</i> (Fresh leaves)	
	HEK293	HEPG2	HEK293	HEPG2
100	1.51	10.11	-1.06	-1.32
150	12.03	14.01	-0.21	0.49
200	8.77	13.21	-1.00	1.72
250	13.40	15.80	3.48	4.62
300	17.42	24.45	8.10	8.45
350	25.92	28.68	16.81	13.42
IC 50 µg/ml	1875	1942	2291	2189

0 = Control values expressed as mean ± SEM, (n=3)

The cytotoxicity of the essential oils showed a concentration dependent activity with IC₅₀ values of 1875 µg/ml on HEK293 and 1942 µg/ml on HEP G2 for the fresh leaves, and 2291 µg/ml on HEK293 and 2189 µg/ml on HEP G2 for the dry leaves. Even though the essential oil from the dry leaves appeared to contain more toxic materials than the oil from the fresh leaves, the IC₅₀ values suggest that the essential oils are not cytotoxic.

4.5. ANTIOXIDANT ACTIVITIES

The results of the antioxidant activities of the essential oils of the fresh and dry leaves of *Eucalyptus grandis* are presented in Table 4.4.

Table 4.4 Antioxidant activities of the essential oils from the fresh and dry leaves of *Eucalyptus grandis*

	^a Superoxide anion O ₂ ⁻	^b Nitric oxide NO [·]	^c Fe ²⁺ Chelating	^d Hydroxyl ·OH	^e TBARS	^f NADH	^g Sulfhydryl content
Essential oils of the fresh leaves of <i>E. grandis</i>	>5	4.34	>5	>5	32.76	0.8	9.00
Essential oils of the Dry leaves of <i>E. grandis</i>	>5	3.65	4.93	>5	41.51	2.1	13.14
Trolox	-	-	-	-	-	-	-
Ascorbic acid	4.02	2.88	-	-	-	-	-
BHT	-	>5		>5	57.56	-	-
EDTA	-	-	> 5	-	-	-	-
Citric acid	-	-	3.46	-	-	-	-

^{a,b,c,d} IC50 –inhibitory concentration, (O₂⁻) –Superoxide anion radical scavenging; (.OH) –Hydroxyl radical scavenging; (NO[·]) –Nitric oxide radical scavenging, - (Fe²⁺) Chelating. ^e Percentage inhibition-TBARS ^f pm/g-NADH, ^g μg/g(w/w)-Sulfhydryl content conc. 1 = 5 mg/ml, conc. 2 = 10 mg/ml, conc. 3 = 20 mg/ml, conc. 4 = 50 mg/ml conc. 5 = 100 mg/ml.

The essential oil obtained from the fresh and dry leaves of *Eucalyptus grandis* showed a similar antioxidant activity. They both showed a relatively low tendency to chelate Fe²⁺, and scavenge free radicals O₂⁻, .OH and Nitric oxide. This indicated a lack of potential to donate electrons to free radicals, and possibly prevent lipid peroxidation.

4.6 ANTI-ASTHMA ACTIVITY OF THE ESSENTIAL OILS

The results presented in Figure 4.3 show the anti-asthma activities of the essential oils, the isolated 1,8-cineole and the control drug aminophylline. The time in delaying the wheezing associated with asthma after rats were exposed to the irritant agents, histamine and acetylcholine is presented. The rats which were administered the control drug aminophylline delayed longer in developing wheezing after being exposed to the irritants, while the groups which were administered with the essential oils showed a concentration dependent activity in delaying wheezing associated with asthma after exposure to the irritants.

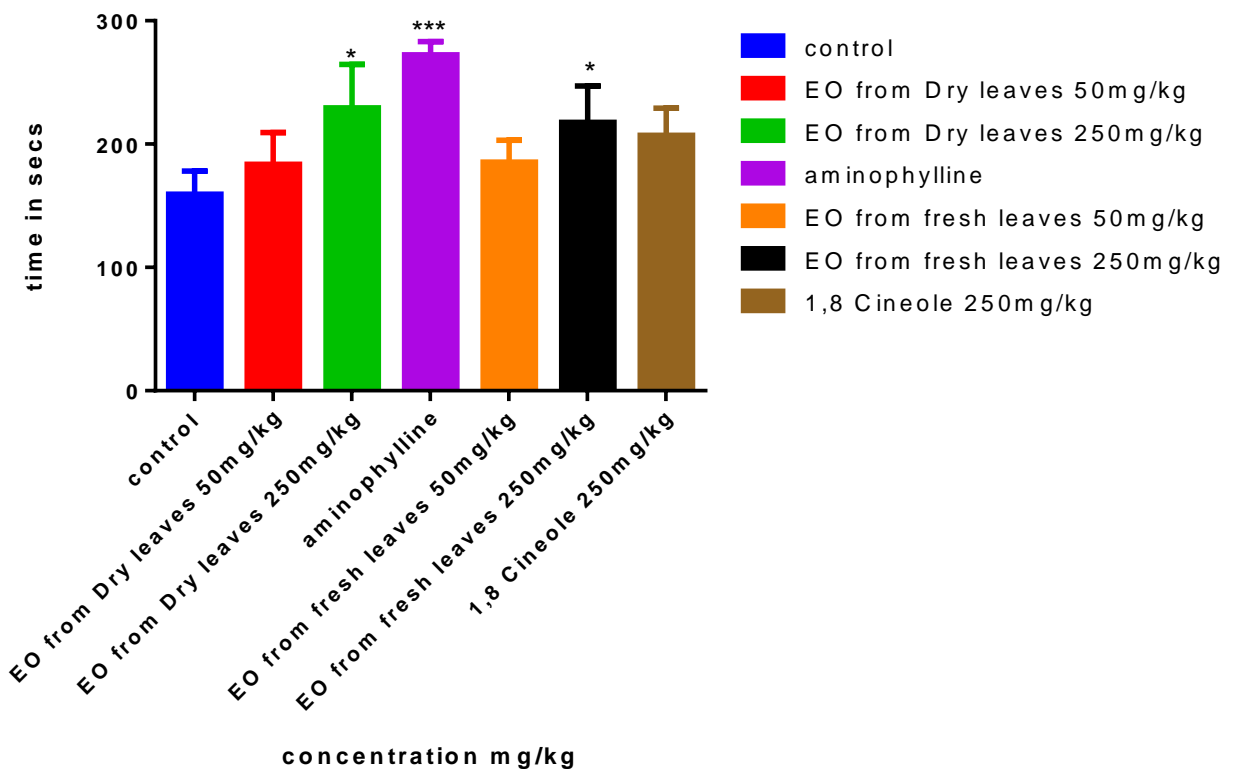


Figure 4.3 Anti-asthma activity of the essential oils, 1,8-cineole and the control aminophylline. Values expressed as mean \pm SD, (n=5)

4.7 ANTI-COUGH ACTIVITY OF THE ESSENTIAL OILS

The results presented in Figures 4.4 and 4.5 show the anti-cough activities of the essential oils, the isolated 1,8-cineole and the control drug dextromethorphan. The time in delaying the cough associated with asthma and the number of coughs in 2 minutes after 5 seconds exposed to ammonia is presented. The experimental groups showed a concentration dependent activity; as the concentration increased the delay in coughing also increased, though they were not as effective as that of the control drug dextromethorphan.

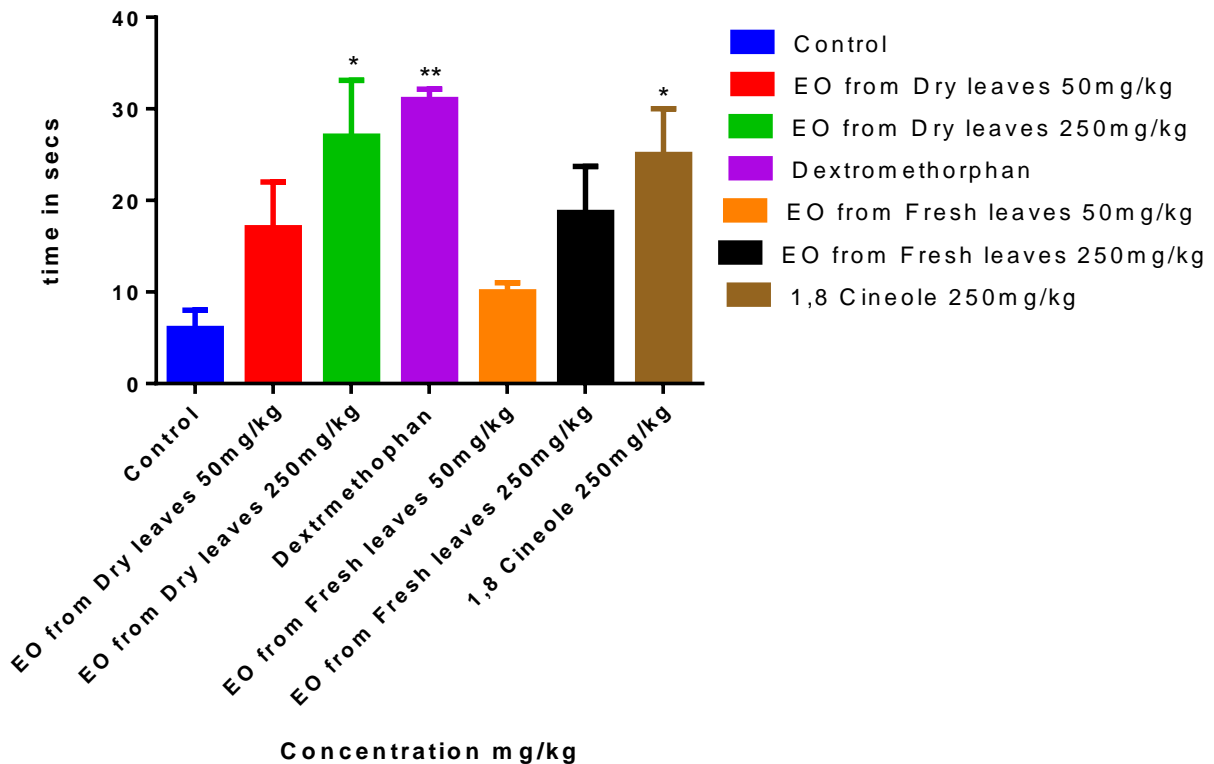


Figure 4.4 Anti-cough activity of the essential oils, 1,8-cineole and the control dextromethorphan. Values expressed as mean \pm SD, (n=5)

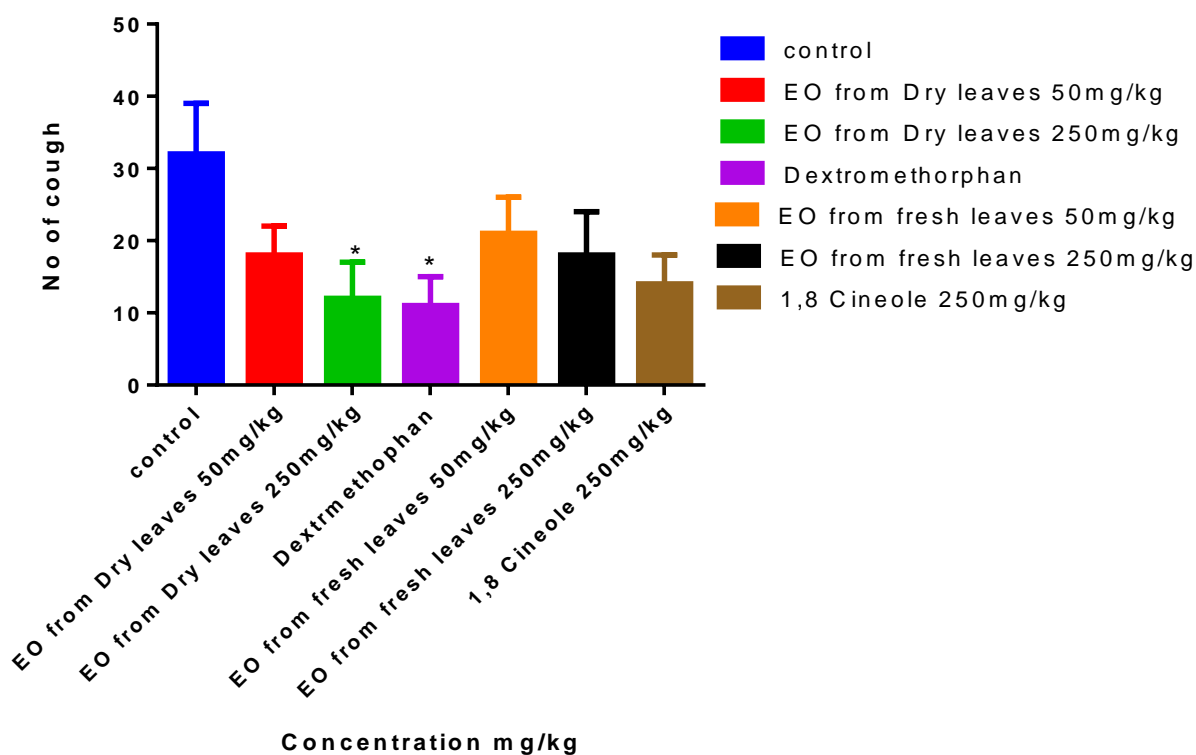


Figure 4.5: The activity of the essential oils, 1,8-cineole and the control dextromethorphan on the number of coughs for 2 minutes after 5 seconds of exposure to ammonia. Values expressed as mean ± SD, (n=5)

The anti-cough activity of the essential oils showed a concentration dependent activity for both the number of coughs recorded in a 2 minute period after exposure to ammonia and the delay in coughing after exposure. The delay in the time it took to develop a cough was longer at the highest concentration of the essential oils for both the fresh and dry leaves and also for 1,8-cineole, as compared to the control group with no drugs. The number of coughs counted for 2 minutes was also seen to decrease as the concentration increased.

4.8 ACETYLCHOLINESTERASE ACTIVITY

The percentage inhibition of acetylcholinesterase is presented in Table 4.5 for the essential oils from both the fresh and dry leaves of *Eucalyptus grandis*. The oils showed less than 50% acetylcholinesterase inhibition activity (fresh leaves show a 35.66 ± 1.06 % inhibition, while dry leaves exhibited 36.65 ± 3.04 % inhibition). The essential oils were not as effective as the standard tacrine (96.90 ± 0.63 % inhibition).

Table 4.5 Percentage inhibition of acetylcholinesterase

% INHIBITION OF ACETYLCHOLINESTERASE.		
Essential oils from fresh leaves	Essential oils from Dry leaves	Standard Tacrine
35.66 ± 1.06 (%)	36.65 ± 3.04 (%)	96.90 ± 0.63 (%)

values expressed as mean \pm SEM, (n=3)

4.9 ANTIMICROBIAL ACTIVITIES OF THE ESSENTIAL OILS FROM THE FRESH AND DRY LEAVES OF *EUCALYPTUS GRANDIS*.

The antibacterial activities of the essential oils were carried out against respiratory tract infection bacteria strains. The MIC and MBC are summarized in Table 4.6 and the membrane damage activity summarized in Table 4.7.

The essential oils from the fresh leaves showed MIC and MBC values ranging from 0.3125- 1.25 mg/ml and 0.626 - 2.5 mg/ml respectively. The MIC and MBC values for the dry leaves were 5 mg/ml for MIC and >5 mg/ml for the MBC. These values suggest that the oils are more bacteriostatic than bactericidal.

Table 4.6 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the essential oils from the fresh and dry leaves of *Eucalyptus grandis*.

Bacterial strains	MIC of E. oils fresh leaves 5mg/ml	MIC of E. oils Dry leaves 5mg/ml	MIC Neomycin 20µg/ml	MBC of E. oils fresh leaves 5mg/ml	MBC of E. oils Dry leaves 5mg/ml	MBC – Neomycin 20µg/ml
<i>S.aureus</i> (ATCC 25925)	1.25	5	20	2.5	> 5	ND
<i>K.pneumoniae</i> (ATCC31488)	0.3125	5	20	0.625	> 5	ND
<i>M. catarrhalis</i>	1.25	5	20	2.5	> 5	ND

MIC values given as mg/ml for essential oils, ND= not determined

The results presented in Table 4.7 show the percentage release of cytosolic LDH from bacteria cells. The low (8% - 24%) release of the LDH indicates that the observed antibacterial activities of the oils were not due to bacterial membrane damage.

Table 4.7 LDH release (membrane damage) activity.

LDH		
Bacterial strain	Fresh leaves	Dry leaves
<i>S. aureus</i> (ATCC 25925)	11%	8%
<i>K. pneumoniae</i> (ATCC 31488)	13%	24%
<i>M. catarrhalis</i>	11%	20%

LDH release (membrane damage) activity % LDH release in comparison to Triton X-100

4.9.1 EFFLUX PUMP INHIBITORY ACTIVITIES

The efflux pump inhibitory activities of the essential oils of the fresh and dry leaves of *Eucalyptus grandis* presented in Table 4.8 show the percentage accumulation of R6G inside the cells, while Figures 4.6, 4.7 and 4.8 show the accumulation of R6G in the bacteria cells over time.

Table 4.8 Percentage increase of R6G accumulation. The results show the percentage accumulation of R6G inside the cell after exposure to glucose, plant extract and standard inhibitor berberine. The value of the drug accumulation in the presence of glucose alone was taken as the control 0%.

Bacterial strain	Standard	Essential oils	
	Beberine	Oils (fresh leaves)	Oils (dry leaves)
<i>S. Aureus</i> (ATCC 25925) ^a	98%	53%	11%
<i>K. Pneumoniae</i> (ATCC 31488) ^a	74%	9%	3%
<i>M. catarrhalis</i> ^b	67%	25%	4%

% Values of drug accumulation in the presence of glucose. Glucose alone was taken as the control 0%. ATCC = American Type Culture Collection, USA. ^a - Environmental strains; ^b - Clinical isolates.

Table 4.9 Accumulation of R6G against respiratory tract infectious bacteria^a

Microorganism	FLEO	DLEO	Plus Glucose	No Glucose	Beberine
<i>S. aureus</i>	2.01 ± 0.50**	2.43 ± 0.75**	0.75 ± 0.20****	4.30 ± 1.00	2.50 ± 0.80**
<i>K. pneumonia</i>	3.24 ± 0.50*	2.30 ± 0.5***	0.89 ± 1.00****	4.75 ± 0.90	2.37 ± 0.60***
<i>M. catarrhalis</i>	2.84 ± 1.00	2.22 ± 1.00*	1.38 ± 0.50**	4.00 ± 0.50	2.75 ± 1.00

^a(n = 3, mean ± S.D); FLEO – fresh leaf essential oil; DLEO – dry leaf essential oil

Table 4.9 show the accumulation of R6G in bacterial cells over time. The results are of R6G concentration inside the cell after 30mins of incubation against the sample used (essential oils, ampicillin and beberine). Glucose was used to provide energy for the efflux pump in the form of ATP. Error bars denote the standard deviation from the mean (N=4) and statistical significant difference with the control (glucose) (P<0.05).

The results show that R6G was bacteria strain specific as observed in Table 4.9 where R6G absorption was greater for *K. pneumoniae* and *M. catarrhalis* for *S. aureus* in the presence of the essential oil from the fresh leaves, while *Staphylococcus aureus* had a high accumulation with the dry. For *M. catarrhalis* and *K. pneumonia*, the uptake of R6G by oil of the fresh leaf was even higher than that of the standard used, Beberine, while for *Staphylococcus aureus* the essential oils from the dry leaves were more effective, which showed that plant extracts increase R6G concentration. It is also noted that *S. aureus* had a higher percentage accumulation than the other organisms in Table 4.8, because gram positive organisms have a single layer of cell wall (making them more

susceptible to antibiotics than gram negative organisms which have a double membrane), making them less susceptible to antimicrobial agents.

4.10 ANTI-INFLAMMATORY ACTIVITY

The results obtained from the anti-inflammatory activity of the essential oils and the isolated 1,8-cineole on the cotton pellet-induced granuloma are presented in Figure 4.9.

The extracts showed a concentration dependent anti-inflammatory activity.

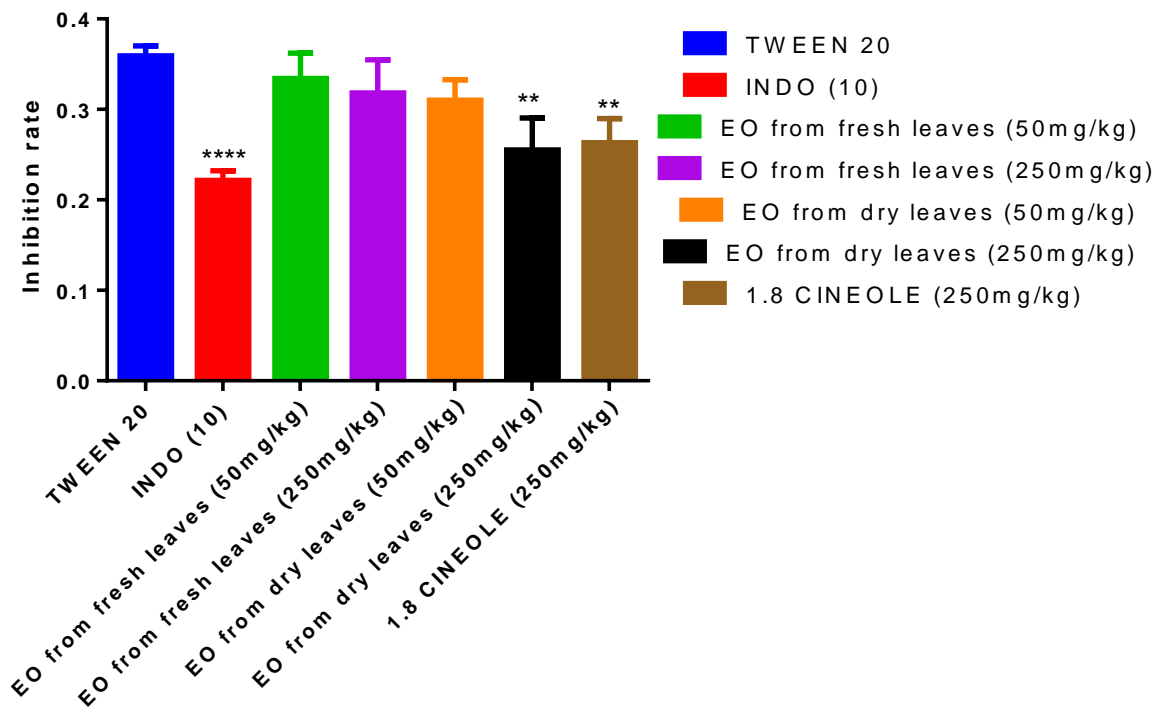


Figure 4.9 Anti-inflammatory activity of the essential oils and the isolated 1,8-cineole on the cotton pellet-induced granuloma. Values expressed as mean \pm SD, (n=5)

4.11 BIOCHEMICAL ESTIMATION OF ENZYMES

The biochemical estimation of some inflammatory enzymes; catalase, superoxide dismutase and cyclooxygenase (COX 1 and COX 2) are presented in Figures 4.10, 4.11 and 4.12 respectively.

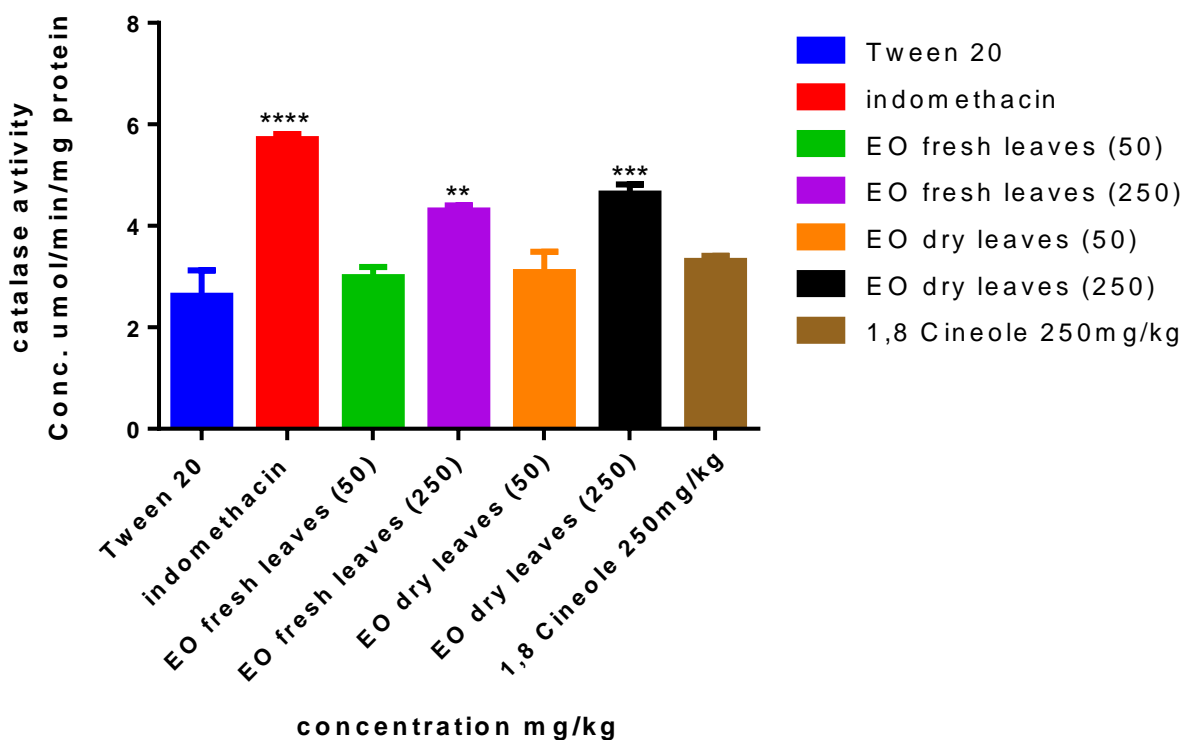


Figure 4.10 Catalase activity of the essential oils and the isolated 1,8-cineole. Values expressed as mean \pm SD, (n=5)

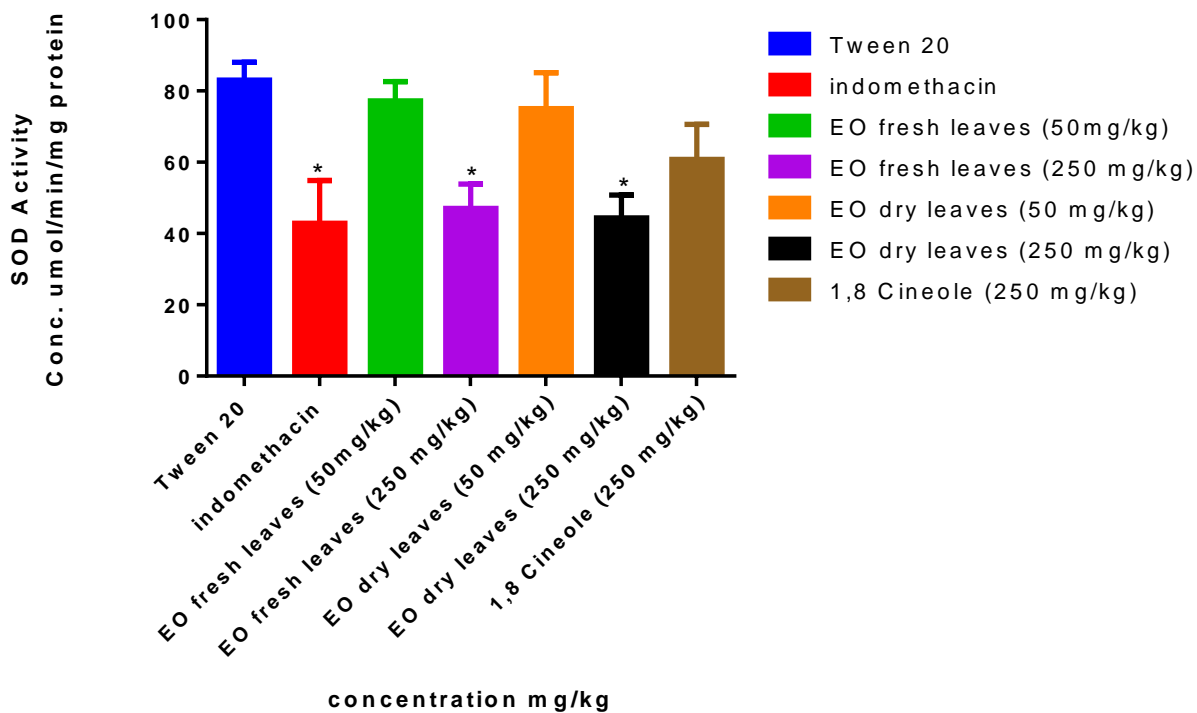


Figure 4.11 SOD activity of the essential oils and the isolated 1,8-cineole. Values expressed as mean \pm SD, (n=5)

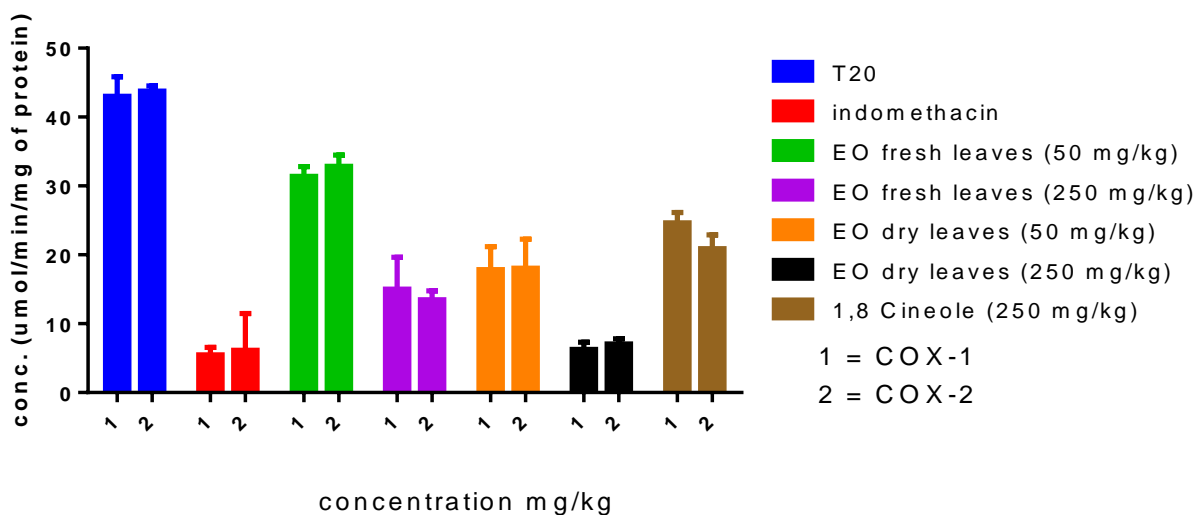


Figure 4.12 COX 1 and COX 2 activities of the essential oils and the isolated 1,8-cineole. Values expressed as mean \pm SD, (n=5)

4.12 INTERLEUKIN 6 (CYTOKINE ELISA)

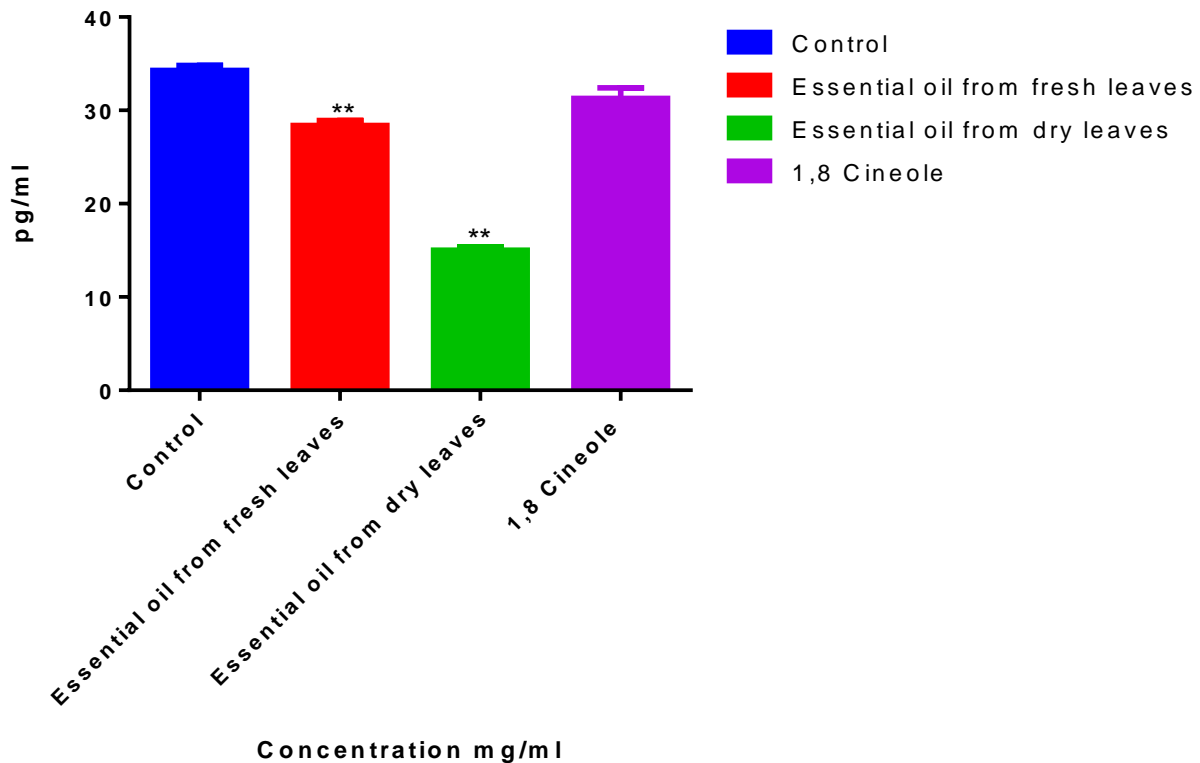


Figure 4.13 IL 6 (Cytokine activity) of the essential oils and 1,8-cineole activities on A549 lung cell cultures. Values expressed as mean \pm SD, (n=5)

The effects of the essential oils and isolated 1,8-cineole on inflammatory enzymes show their ability to activate the inflammatory enzymes SOD and catalase. They also inhibited the effect of COX 2 with little effect on COX 1, which is needed for physiological functions, as well as IL 6, which is produced during inflammation.

4.13 HISTOPATHOLOGICAL EVALUATION

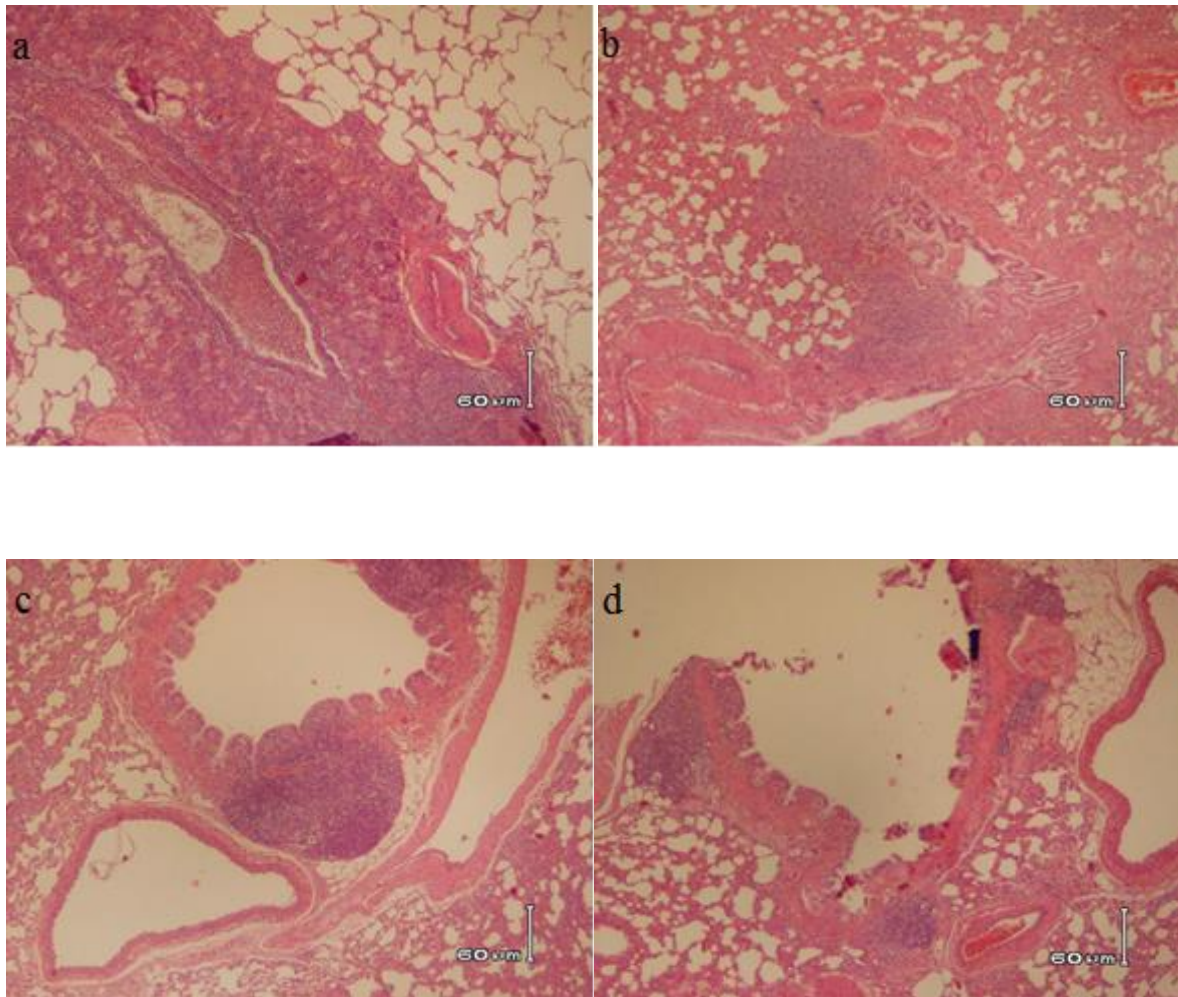


Figure 4.14 Micrographs of lung tissues (200x) of rats induced with LPS observed under a microscope. Group a: lung from blank group; Group b: lung from carrier solvent (10% tween-20); Group c: lung from essential oil derived from fresh leaves (250 mg/ml) and Group d: lung from essential oil derived from dry leaves (250 mg/ml).

Group a is the control group to which the other groups are compared. These were the rats which were normal and in an apparently healthy condition. Group b showed mild peribronchiolar lymphocytic accumulates (peribronchiolar associates lymphoid tissues or

BALT). BALT form part of the normal defence mechanism or immune system cell hyperplasia and peribronchiolar inflammation with neutrophils, lymphocytes, plasma cells and macrophages. There was purulent exudate in the bronchial lumen, which may suggest secondary bacterial infection or bronchopneumonia. Groups c and d, which were given the essential oils, showed a few lesions of mild thickening of the alveolar septal walls, with scant mononuclear cellular infiltrates and mild alveolar atelectasis. Though this is a non-specific finding and can be seen in, for instance, chronic irritation caused by abnormally high levels of uric acids. It is a common finding, especially in laboratory rodents.

4.14 MUSCLE CONTRACTION STUDIES (VASCULAR REACTIVITY ON AORTIC SMOOTH MUSCLE)

The effects of the essential oils and isolated 1,8-cineole on aortic smooth muscle are presented in Figures 4.15 - 4.19. Figure 4.15 shows the concentration response curve for the essential oils compared with noradrenaline. The curves were evidence of the concentration dependent vasorelaxant activity of the oils. Figure 4.16 shows the concentration dependent vasorelaxant activity of the essential oils and 1,8-cineole at different concentrations on the aortic ring isolated from the rats. Figures 4.17 - 4.19 shows the individual vasorelaxant activities of the essential oils and 1,8-cineole on the aortic rings isolated from rat muscles which had previously been stimulated by noradrenaline.

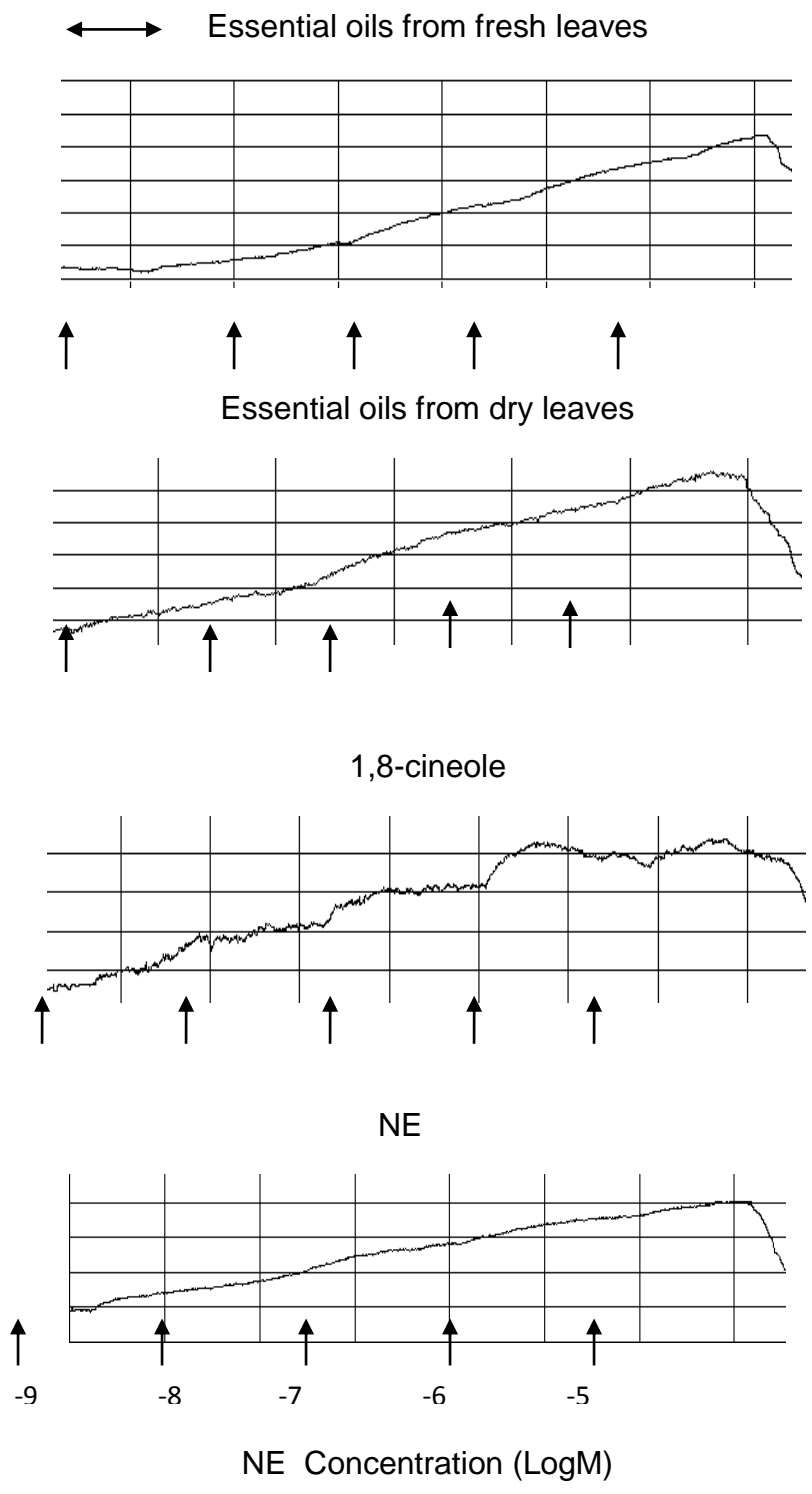


Figure 4.15 Typical tracing showing: concentration response curve of NE

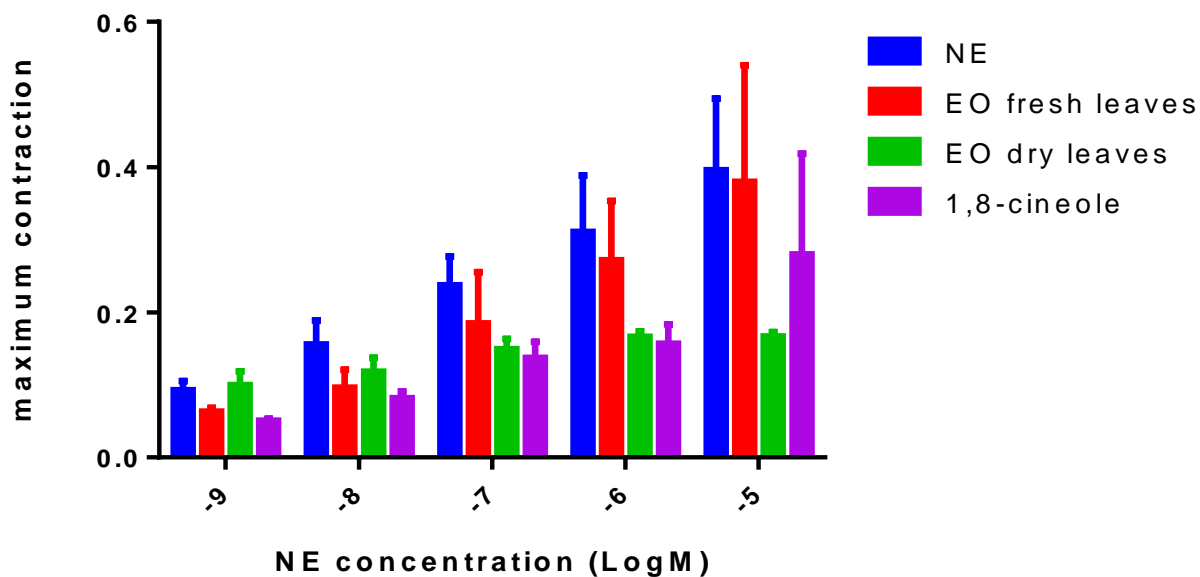


Figure 4.16 Concentration response for noradrenaline in aortic rings incubated with and without essential oils and 1,8-cineole (0.5mg/ml). n= 6.

The essential oils and 1,8-cineole extracted from the essential oils showed a concentration dependent muscle contraction activity on the aortic ring muscle isolated from rats. Figures 4.17 - 4.19 show the individual curve of each sample and the effects on muscle contraction.

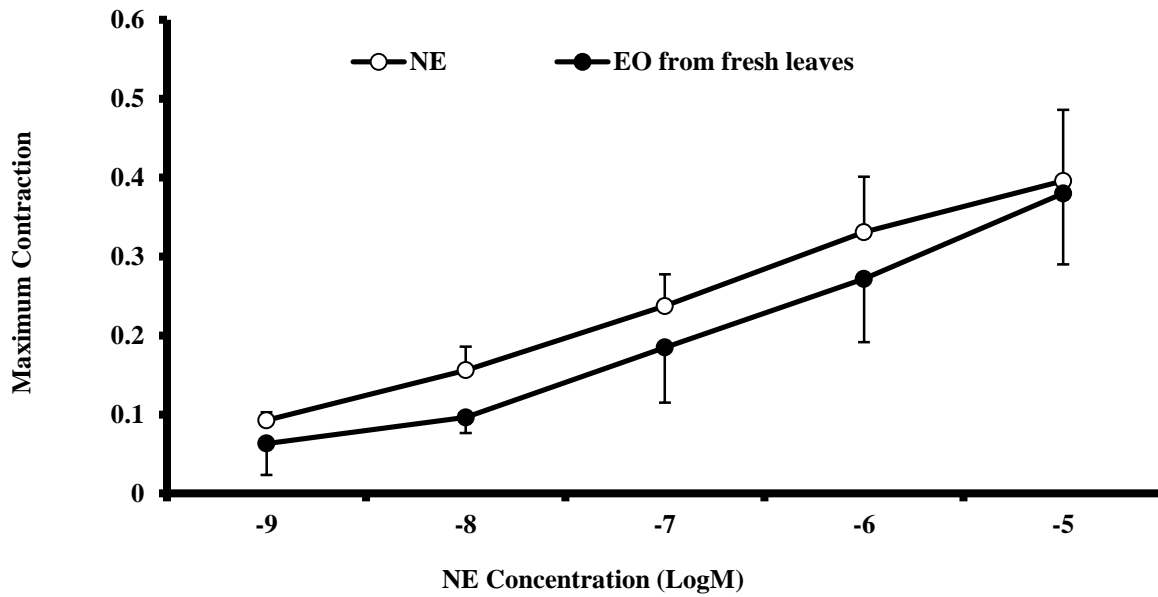


Figure 4.17 Concentration response curve for noradrenaline in aortic rings incubated with and without the essential oil from the fresh leaves (0.5 mg/ml). n=6

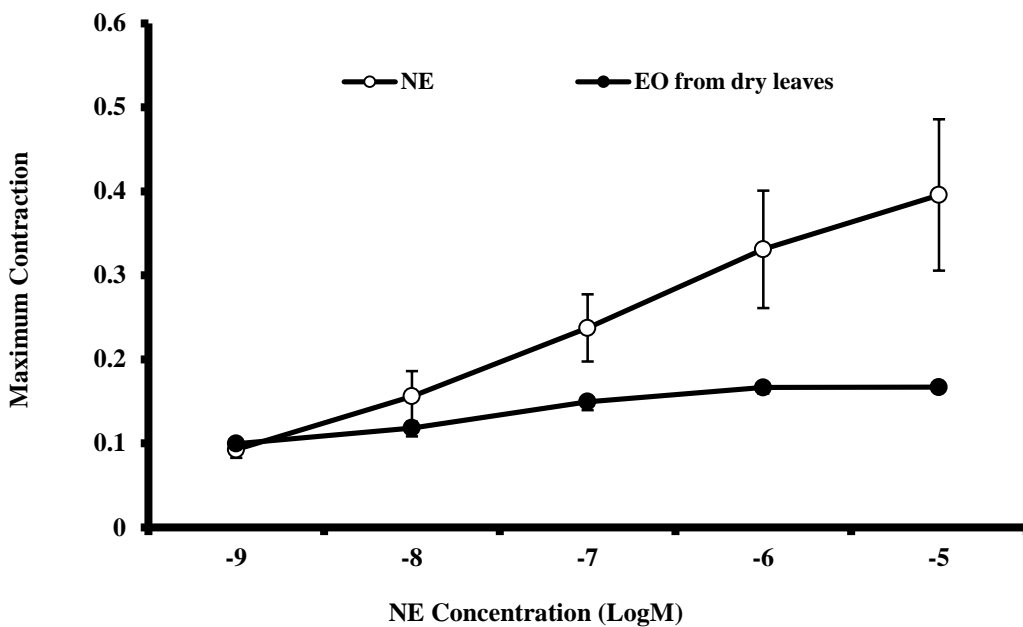


Figure 4.18 Concentration response curve for noradrenaline in aortic rings incubated with and without the essential oil from the dry leaves (0.5 mg/ml). n=6

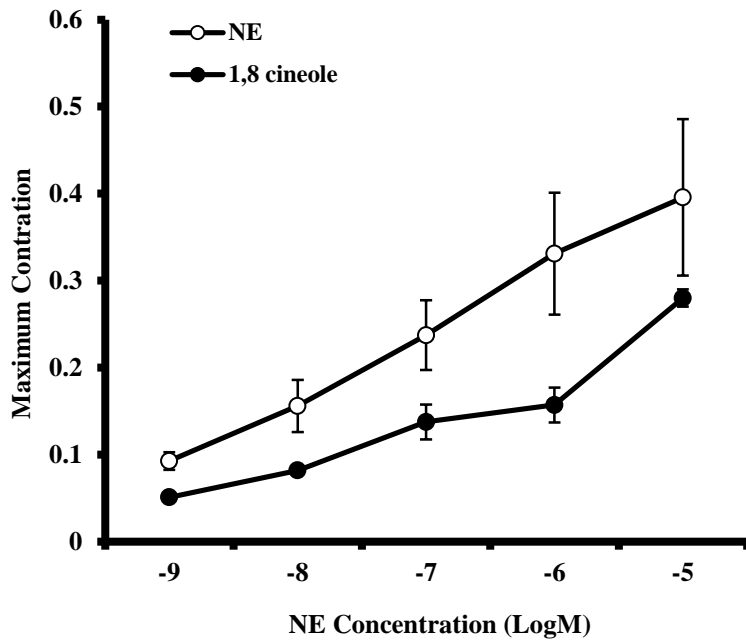


Figure 4.19 Concentration response curve for noradrenaline in aortic rings incubated with and without the isolated 1,8-cineole (0.5 mg/ml). n=6

Chapter 5

DISCUSSION

Asthma is a chronic inflammatory disease characterized by acute exacerbation of coughing, dyspnea, wheezing and chest tightness (Pan *et al.*, 2010; Clark *et al.*, 2010). The search for an effective drug to manage asthma should be directed towards agents that are antioxidant, antimicrobial, anti-inflammatory, anti-allergic, and immune-boosting in nature.

The anti-asthma activity (Figure 4.3) of the essential oils from both the fresh and dry leaves of *Eucalyptus grandis* indicated a concentration dependent activity in delaying asthma *in vivo* in rats after the rats had been exposed to the irritant agents histamine and acetylcholine. For the first time, a clinical study showed the anti-inflammatory activity of the terpenoid oxide 1,8-cineole, which is also abundantly present in the essential oils from *Eucalyptus grandis* (Juergens *et al.*, 2003).

A study on the use of organic essential oil of lavender reported its ability to assist with resistance to asthma attack triggers by strengthening the immune system. Lavender oil also assists in treating other respiratory problems such as bronchitis, sinus congestion, laryngitis, tonsillitis and also drug-resistant infections and fungal infections (Stevenson, 2013).

The physicochemical properties of the essential oils from the fresh and dry leaves of *Eucalyptus grandis* were assessed using the method as described in the British Pharmacopoeia (1988). The commercial importance of oils mostly depends on these physicochemical properties, which provide baseline data to determine their suitability for

consumption (Bamgboye and Adejumo 2010; Parthiban *et al.*, 2011; Barkatullah *et al.*, 2012). Yield depends on the period of harvest, the part of the plant used and the extraction process (Yentema *et al.*, 2007). The yield results of the essential oil of *Eucalyptus globulus*, extracted from Eucalyptus leaves by means of the hydro distillation method, showed an oil yield of 3.5% in the month of July and the lowest oil yield was 0.08%, found in the month of December. The refractive index of the oil was in agreement with that of our study (Astao *et al.*, 2012).

Drying of plant material has been reported to increase essential oil yields and accelerate distillation by improving the heat transfer (Whish and Willam, 1998). Other advantages of drying include the reduction of microbial growth and the inhibition of some biochemical reactions in the dried materials (Baritoux *et al.*, 1992; Combrinck *et al.*, 2006). This is evident in the high yield of essential oil obtained from the dry leaves as compared with that of the fresh leaves. The concentration of the oils with the removal of water by drying increases the pH of the essential oil derived from the dry leaves as compared with that of the fresh leaves (Table 4.1).

The chemical composition (Table 4.2) of the essential oil from fresh leaves revealed 31 compounds, which were about 99.25% of the essential oil. The compounds most abundant in the oil were α -pinene (29.67%), p-cymene (19.89%) and 1,8-cineole (12.80%). In the essential oil of the dry leaves, 14 compounds were identified which together constituted 92.63% of the essential oil, of which the major compounds were found to be 1,8-cineole (47.44%), d-limonene (13.34%), α -pinene (7.49%) and spathulenol (7.13%).

Concentrations of various volatile substances have been observed to increase in numerous species of plants when leaves are dried and have been attributed to the breakdown of glycosylated forms, dehydration reactions, and oxidation reactions (Moyler, 1994; Bartley and Jacob, 2000). The increase in concentrations may also be due to ruptures in plant cells where the volatile compounds are stored. Some compounds also arise from dehydration of oxygenated compounds which could have occurred during the process of drying (Combrinck *et al.*, 2006). It is obvious that the drying process does not only affect the composition of the oils of *Eucalyptus grandis*, but the concentration of the components as well. For example, while the concentrations of α -pinene and *p*-cymene decreased in the dry leaf, the concentration of 1,8-cineole and limonene increased in the dry leaf; only β -pinene was observed to be present in the dry leaf but was not found in the essential oil of the fresh leaf. As in most other *Eucalyptus* essential oils whose main component is 1,8-cineole (Damjanović-Vratnic *et al.*, 2011; Rahimi-Nasrabadi and Batooli, 2011), the essential oils from the dry leaves of *Eucalyptus grandis* 47.44% of its component comprised 1,8-cineole, while the main components of the essential oil from the fresh leaves of *Eucalyptus grandis* were α -pinene with 29.67% and 1,8-cineole, making up just about 12.80% (Eucalyptol) of its contents.

Cineol rich *Eucalyptus* oils have been used in traditional medicines to treat influenza and colds. 1,8-Cineole can now also be found in products like cough syrups, lozenges, ointments and inhalants and is used in the treatment of bronchial infections (Santos and Rao, 2000; Pereira *et al.*, 2005; Salari *et al.*, 2006; Sisay, 2010). The presence of 1,8-Cineol in the essential oils of both the fresh and dry leaves of *Eucalyptus grandis*

scientifically validates its traditional use for the treatment of bronchial infections as well as asthma and coughs.

Free radicals have been implicated as a major cause of various diseases arising in living tissues and their overproduction during inflammation makes them a major factor in asthma which is due to oxidative stress in the bronchia. Lipid peroxidation has been documented to play a major role in disease development (Keenoy *et al.*, 2001) and in fatigue syndrome (Vecchiet *et al.*, 2003). Hydroxyl radicals are the most reactive and predominant radicals, which are generated endogenously during aerobic metabolism (Waling, 1975). They have a very short half-life due to their high activity and are more effectively scavenged only at high concentrations (Rathee *et al.*, 2007). In addition, the role of NO has been controversial as it has both protective and harmful effects. For example, the dual role of NO has been implicated in many neurological disorders of the body. Its roles in the pathogenesis of major depression and modulatory activity of various antidepressants have been indicated by recent research (Rathee *et al.*, 2007; Simelane *et al.*, 2014). Reductones have peroxide formation preventive mechanism by reacting with certain precursors of peroxide (Rathee *et al.*, 2007). Oxidative damage could be effectively neutralized with antioxidants and it is apparent that a good anti-asthma drug should be a good free radical scavenger, it is also a good source of natural antioxidant that helps to prevent lipid peroxidation and thereby prevent oxidative damage (Moure *et al.*, 2001; Simelane *et al.*, 2014). The antioxidant activities of the essential oils presented in Table 4.4 show the ability of the essential oils to prevent the generation of free radicals with considerable Fe^{2+} chelating activity and a considerable nitric oxide radical scavenging activity which was seen to be concentration dependent.

This was also observed with the superoxide radical anion and hydroxyl radical scavenging activity which indicated high IC₅₀ values. Soyingbe (2012) reported that the essential oil from *Eucalyptus grandis* exhibited a rather high antioxidant activity in scavenging pre-existing free radical DPPH and ABTS, and also a high reducing power which indicated the potential of the oils to donate electrons to free radicals, making them stable. The essential oils probably contain reductones as seen in the percentage of TBARS from Table 4.4 thereby inhibiting lipid peroxidation. NADH therapy has been seen to be beneficial in patients with chronic fatigue syndrome (Santaella *et al.*, 2004). NADH contents (Table 4.4) indicate a potential for anti-lipoperoxidative activity.

The human system has an effective mechanism to prevent and neutralize free radical induced damaged (Sarma *et al.*, 2010). This is accomplished by a set of antioxidants such as SOD and CAT, but when the balance between the production of free radicals (Reactive Oxygen Species) and antioxidant defenses is not regulated, oxidative stress results (Sarma *et al.*, 2010) which can cause damage to DNA, lipids and proteins, and slowly destroy healthy tissues leading to rapid aging. The biochemical estimation of the antioxidant enzymes (catalase, and superoxide dismutase) and the inflammatory enzymes, cyclooxygenase (COX 1 and COX 2) presented in Figures 4.10, 4.11 and 4.12 respectively show the ability of the essential oils as well as the isolated 1,8-cineole in activating these enzymes and neutralizing the effect of free radicals produced during chronic inflammation.

Acetylcholine is a major compound which helps nerve impulses to transmit from nerve cell to nerve cell. Acetylcholinesterase is the principal enzyme involved in the hydrolysis of acetylcholine (Fujiwara *et al.*, 2010). At cholinergic synapses, acetylcholinesterase

breaks down into choline and acetate, regulating nerve impulse transmission across cholinergic synapses (Lopez and Villalobos, 2010). Acetylcholinesterase inhibition has been considered a promising strategy for the treatment of neurological disorders such as Alzheimer's disease in which a deficit in cholinergic neurotransmission is involved (Mukherjee *et al.*, 2007). Anticholinergic agents may help block irritant induced bronchospasm (Mukherjee *et al.*, 2007). But, studies have shown that acetylcholinesterase may provoke bronchospasm by increasing acetylcholine at parasympathetic nerve terminals (Hirshman, 1992). Thus, the use of cholinesterase inhibitors should be avoided in asthma if possible. The acetylcholinesterase inhibition activities of both the essential oils from the fresh and dry leaves presented in Table 4.5 show only a moderate inhibitory activity.

It is apparent that the antibacterial properties of the oil from the fresh leaves show higher activity than the oil of the dry leaves (Table 4.6). This could be attributed to the presence of compounds such α and β pinene, and limonene which are more abundant in the oil of fresh leaves than in the dry leaf oil. These compounds have been reported to possess antimicrobial properties (Raju and Maridas, 2011). In addition, monoterpene hydrocarbons which were dominant in dry leaves readily react with air and heat sources thereby reducing the antimicrobial activity of the dry leaf (Abdelmajeed *et al.*, 2012). The low 11-13% levels of cytosolic LDH released (Table 4.7) for the essential oils from the fresh leaves and 8-24% release for the essential oils from the dry leaves, does suggest that microbial cell membrane damage contributes very little to microbial death in the organisms exposed to the essential oils.

Living cells (including bacteria) have mechanisms that expel toxic substances. These systems (such as resistant-nodulation-division pumps) are mostly found in bacteria in which a pump structure (such as an efflux pump) is anchored to the inner membrane to release noxious substances, including antibiotics which are aimed to kill the bacteria (Wexler, 2012). The efflux pump confers bacterial resistance (Amusan *et al.*, 2007). It is therefore important that new antibiotics, specifically efflux pump inhibitors (EPIs) are developed to reduce the emergence of multidrug resistance (MDR). In this study, the essential oils of *E. grandis* were able to increase the accumulation of rhodamine 6G inside bacterial cells (Table 4.8 and 4.9), which revealed that the essential oils can apparently be used as efflux pump inhibitors. It is noted that *S. aureus* had the highest percentage accumulation over other organisms (Table 4.8); this could be because gram positive organisms have a single layer of cell wall (which makes them more susceptible to antibiotics) compared with gram negative organisms, which have a double membrane making them less susceptible to antimicrobial agents (Kaur and Arora, 2009).

The anti-inflammatory activity of the essential oils and 1,8-cineole presented in Figure 4.9 were carried out using the cotton pellet-induced granuloma models in rats. Cotton pellet-induced granuloma is broadly used to evaluate the transudative and proliferative components of chronic inflammation (Winter *et al.*, 1957). The weight of the wet cotton pellets correlates with transude material, whereas the weight of dry pellet correlates with the amount of granulomatous tissue. Verma *et al.* (2010) showed the anti-inflammatory activity of ethanolic root extract from *Aconitum heterophyllum* using cotton pellet-induced granuloma in rats. Inflammation was reduced by the extract as shown by decreased weight of the cotton pellet in cotton pellet-induced granuloma in rats. A

decrease in the weight of the cotton pellet was similarly observed in this study, suggesting that the essential oils do have anti-inflammatory activity.

Cyclooxygenase (COX) is an enzyme that is responsible for the production of important biological mediators called prostanoids, including prostacyclin, thromboxane and prostaglandins. COX converts arachidonic acid to prostaglandins which is responsible for inflammation, pain and fever. There are two types of cyclooxygenase enzyme COX-1 and COX-2. COX-1 is considered a constitutive enzyme that is required for daily physiological functions. COX-2 is an inducible enzyme that is produced during inflammation (Kurumbail *et al.*, 1996). The COX activity of the essential oils and 1,8-cineole presented in Figure 4.12 were seen to inhibit more of COX-1 than COX-2. The results obtained also showed a concentration dependent effect. The results thus indicate that the essential oils do have some anti-inflammatory activity. However, the inhibition of COX-1 has been shown to lead to drug toxicity and adverse side effects, whereas the inhibition of COX-2 accounts for the anti-inflammatory effect of the drug (Hawkey, 2001; Schachter, 2003). It is interesting to note that 1,8 cineole as well as the essential oils from the fresh leaves at 250 mg/kg were able to inhibit COX-2 more than it inhibited COX-1. This result is in line with previous studies that showed 1,8-cineole reduces inflammation and pain (Santos and Rao, 2000).

Interleukin (IL), is defined as any group of proteins or cytokines that occurs naturally and mediates the communication between cells. Similar to other cytokines, interleukins are not stored within cells but they are rapidly and briefly secreted in response to a stimulus (Sims *et al.*, 1988). Interleukins regulate differentiation, cell growth and motility; they also stimulate immune responses such as inflammation (Hirano *et al.*, 1986). Once an

interleukin has been produced, it travels to its target cell and binds to it through a receptor molecule on the cell's surface. Interleukins (1, 6, 13 and 17) are known to be involved in the inflammatory response (Minty *et al.*, 1993; Aggarwal and Gurney, 2002). Figure 4.13 shows the reduction in IL6 in cells that were treated with the essential oils with the essential oils from the dry leaves showing the lowest level of IL6 produced as compared to the control group. This is significant and shows the inhibition of inflammation by the essential oils with the reduced levels of IL6 produced.

Histopathological Micrographs of lung tissues observed under a microscope (200x) of rats induced with LPS are presented in figure 4.14. The thickening of the airway smooth muscle cells layer is the most common feature observed during airways hyperresponsiveness. This proliferation has been suggested as one of the main causes of hyperplasia (Aili *et al.*, 2005; Sun *et al.*, 2012). From the micrograph pictures the number of airway smooth muscle cells significantly reduced in the lungs treated with the essential oils compared to the tween-20 group. This is suggestive of the essential oils inhibiting the proliferation of airway smooth muscle cells.

The essential oils and 1,8-cineole isolated showed a concentration dependent vasorelaxant activity on the aortic ring from rats. The results show the contractile response to noradrenalin which was suppressed by the test samples (evident by the shifting of concentration response curve to the contractile agent to the right thereby depressing the maximum response to each agonist). The results in this study are similar to that observed by Salahdeen and Murtala (2012). The essential oils and 1,8-cineole had a direct dilatory effect on smooth muscle and since smooth muscle around the bronchial contracts during an asthma attack thereby obstructing the airways passage,

the dilatory effect of the oils validates the use of *Eucalyptus grandis* as an anti-asthmatic and anti-cough agent by traditional healers. Chinese formulations have also been employed in the treatment of asthma. Several active components of phenethyl alcohol were investigated in vitro and indicated an anti-asthmatic effect on the contraction of isolated tracheal smooth muscles in guinea pigs. This prevented histamine-induced bronchoconstriction, thereby corroborating the traditional use of this formulation as an anti-asthmatic agent (Chi *et al.*, 2009).

Cytotoxicity evaluations of compounds with an IC₅₀ value less than 30 µg/ml are considered to display significant activity (Suffness and Pezzuto 1990). The essential oil from the dry leaves appears to contain a bit more toxic materials than the oil from the fresh leaves; the IC₅₀ values suggest that the essential oils are not cytotoxic. The studies carried out by Doll-Boscardin *et al.* (2012) demonstrated that the essential oils of *Eucalyptus benthamii* show improved cytotoxic potential compared to the isolated terpenes α-pinene and γ-terpinene and can pave the way for the future development of therapeutic opportunities against cancer

Chapter 6

CONCLUSION

Asthma is characterized by inflammation of the airways, causing airway dysfunction with widespread airflow obstruction and also causes an associated increase in airway responsiveness to a variety of stimuli (Patel *et al.*, 2009). The underlying inflammation is a complex biological response of vascular tissues against aggressive agents such as pathogens, irritants, or damaged cells (Ferrero-Miliani *et al.*, 2007). Asthma causes an attack accompanied by wheezing, shortness of breath, chest tightness and coughing (Bousquet *et al.*, 2008).

Many synthetic drugs are used to treat asthma, but they are not completely safe for long term use. Various inhaled bronchodilators and anti-inflammatory drugs are available, require long-term use and are associated with side effects. (Bryan *et al.*, 2000; Angsten, 2000).

This study showed the effect of the essential oils from both the fresh and dry leaves of *Eucalyptus grandis* and isolated 1,8-cineole possessing anti-asthmatic and anti-cough activities. These activities resulted from their considerable activities against respiratory tract bacteria and their considerable activity in relieving the oxidative stress which triggers an asthma attacks (Soyingbe *et al.*, 2013).

The essential oils and 1,8-cineole showed anti-inflammatory activity and also the ability to inhibit the inflammatory enzyme which triggers inflammation and subsequently asthma and coughing. They also showed the ability to delay the effect of wheezing and coughing associated with histamine and acetylcholine induced asthma which leads to severe bronchoconstriction, and have displayed relatively low cytotoxicity.

The ability of the essential oils and 1,8-cineole to directly dilate smooth muscle and relieve the stress of bronchial contraction and obstruction of the airways passage validates the use of *Eucalyptus grandis* as an anti-asthmatic and anti-cough agent by traditional healers.

It is concluded that the essential oils could be used as part of existing anti-asthma therapy. The rationale behind the use of the *Eucalyptus grandis* plant in the treatment of asthma by traditional healers and in Zulu folklore medicine has also been justified.

6.1 SUGGESTIONS FOR FURTHER STUDIES

It is suggested that further studies be carried out on the mechanism of action of the essential oils on asthma and cough, as follows:

- The mechanism of action of active compound should be carried out.
- The active compound should be tested for other biological activities.
- More pure compounds should be isolated and tested and should be prepared from active compound.
- Clinical trials should be considered.

REFERENCES

- Abdenour, A., Susana L., Mohammed B., Amin L., Carmen R., Antonio H., Rafael P., Pilar C. (2011). Chemical composition and antimicrobial activity of essential oils of *Thymus algeriensis*, *Eucalyptus globulus* and *Rosmarinus officinalis* from Morocco. *Journal of the Science of Food and Agriculture*. **91**(14): 2643–2651.
- Adams R.P. (1991). *Analysis of Juniper and other forest tree oil: In Essential oils and waxes*. Edited by Linskens H.F. and Jackson J.F. Springer-Verlag. Berlin Heidelberg, Germany. vol **12**: 41-61.
- Aggarwal S. and Gurney A.L. (2002). IL-17: prototype member of an emerging cytokine family. *J. Leukoc. Biol.* **71** (1): 1–8.
- Ahmad Muhammad Mushtaq, Salim-Ur-Rehman, Faqir Muhammad Anjum and Ehsan Elahi Bajwa. (2006). Comparative Physical Examination of Various Citrus Peel Essential Oils. *Int. J. Agri. Biol.* **8** (2): 187-190.
- Aili L., Lazaar M.D and Reynold A. (2005). Molecular mechanism in allergy and clinical immunology. *J Allergy Clin Immunol.* **116**: 488-495.
- Altanlar, N., Soner, O and Tanker, M. (1999). Antimicrobial activity of some volatile oils. *Journal of Turkish Microbiological Society.* **29**:169-172.
- Ambrose D. and Ambrose B.A. (1961). Gas chromatography. George Newnes Ltd., London. Pp. 9-11.

- Amusan OOG., Sukati NA., Dlamini PS., Sibandze FG. (2007). Some Swazi
Phytomedicine and their constituents. *Afr. J. Biotechnol.* **6**: 267-272.
- Angsten, J.M., 2000. Use of complementary and alternative medicine in the treatment of
asthma. *Adolescent Medicine*, **11**(3): 535-546.
- Aqil F, Ahmad I and Owais M. (2006). In: Modern Phytomedicine: Turning medicinal
plants into drugs. Wiley-VCH, Germany, pp 59-79.
- Archana, N . Paranjape and Anita A. Mehta, 2008. Investigation into the Mechanism of
Action,of *Abutilon indicum* in the Treatment of Bronchial Asthma. *Global Journal
of Pharmacology*, **2** (2): 23-30.
- Aromacaring (2004). Using Essential Oils with Vulnerable People. Bristol: Aromacaring
Publications.
- Astaq Mohal Khan, Seatara Khatun, M.K. Hossain and M. L. Rahman.(2012).
Characterization of the Eucalyptus (*E. globulus*) leaves oil. *Journal of
Bangladesh Chemical Society.* **25** (2): 97-100.
- Babushok V.I., Linstrom P.J., Reed J.J., Zenkevich I.G., Brown R.L., Mallard W.G. and
Stein S.E. (2007). Development of a database of gas chromatographic retention
properties of organic compounds. *Journal of Chromatography A.* **1157**: 414-421.
- Badovinac V., Trajkovic V., Mostarica-stojkovic M.,(2000) Nitric oxide promotes growth
and major histocompatibility complex – unrestricted cytotoxicity of interleukin-2-
activated rat lymphocytes. *Scand. J. Immunol.* **52**: 62-70.

- Bagheri H., Lhiaubet V., Montastruc J.L. and Chouini-Lalanne N. (2000).
Photosensitivity to ketoprofen: mechanisms and pharmacoepidemiological data.
Drug Safety. **22**: 339-349.
- Bamgboye, A.I. and O.I. Adejumo. (2010). Physicochemical properties of Roselle seed
oil. *Nutrul and Food Science*, **40** (2): 186-192.
- Baritoux O, Richard H, Touche J, Derbesy M (1992). Effects of drying and storage of
herbs and species on the essential oil. Part I. Basil, *Ocimum basilicum L. Flavour
Frag. J.* **7**: 267-271
- Barkatullah, Muhammad Ibrar, Abdur Rauf and Inyat-Ur-Rahman. (2012).
Physicochemical Characterization of Essential and Fixed Oils of *Skimmia
laureola* and *Zanthoxylum armatum*. *Middle-East Journal of Medicinal Plants
Research* **1**(3): 51-58.
- Barnes PJ. (1990). Reactive oxygen species and airway inflammation. *Free Rad Biol
Med.* **109**: 235-243.
- Barnes PJ, Chung KF, Page CP. (1998). Inflammatory mediators of asthma: an
update. *Pharmacol Rev.* **50**: 515-596.
- Bartley J.P. and Jacobs A.L. (2000). Effect of drying on flavor compounds in Australian
grown ginger (*Zingiber officinalis*). *J. Sci. food Agric.* **80**: 209-215
- Baser K.H.C. (1995). *Analysis and assessment of assessment of essential oils: In A*

manual on the essential oil industry. UNIDO, Vienna Austria. Edited by K.T. De Silva. PP 155-178.

Batugal Pons, Jayashree Kanniah, Lee Sok Young and Jeffrey T Oliver (eds). (2004).

Medicinal plant Research in Asia, Volume 1: The Framework and project Workplans. International Plant Genetic Resources Institute- Regional Office for Asia, the pacific and Oceania (IPGRI – APO) serdang, Selangor DE, Malaysia.

Beauchamp C, Fridovich I.(1971). Superoxide dismutase: Improve assays and an essay applicable to acetylamide gels. *Anal Biochem.* **44**:276-87.

Bibhabasu H., Santanu B. and Nripendranath M. (2008).Antioxidant and free radical scavenging activity of *Spondia spinnata*. *BMC complementary and Alternative Medicine.* **8**: 63 1-10.

Bisht R, Katiyar A, Singh R and Mittal P, 2009. Antibiotic resistance- a global issue of concern, Asian Journal of Pharmaceuticals and Clinical Research, vol **2** (2).

Boira H. and Blanquer A. (1998). Environmental factor affecting chemical variability of essential oils in *Thymus piperella L.* *Biochemical systematics and Ecology.* **26**: 811-822.

Bousquet J, Bousquet PJ, Godard P, Daures JP.(2008). The public health implications of asthma. *Bull World Health Organ* 2005; **83**:548-554.

British Pharmacopoeia. (1988). Part II: HMSO, London. 109-110.

Broide DH, Lotz M, Cuomo AJ. (1992). Cytokines in symptomatic asthmatic airways. *J*

- Allergy Clin Immunol.* **89**:958–67.
- Bryan, S.A., M.J. Leckie, T.T. Hansel and P.J. Barnes, 2000. Novel therapy for asthma. *Expert Opinion on Investigational Drugs*, **9**(1): 25-42.
- Buchbauer, G. (2000). The detailed analysis of essential oils leads to the understanding of their properties. *Perfumer & Flavourist*. **25**: 64-67.
- Burbott A.J. and Loomis W.D. (2000). Effects of light and temperature on the monoterpenes of peppermint. *Plants physiology*. **42**: 20-28.
- Burt, S. (2004). Essential oils: their antimicrobial properties and potential application in foods- A review. *International Journal of Food Microbiology*. **94**: 223-253.
- Centre for Disease Control and Prevention, (2013). Antibiotic resistance Threats in the United States.
- Chao Louis Kuoping, Kuo-Feng Hua, Hsien-Yeh Hsu, Sen-Sung Cheng, Ju-Yun Liu, and Shang-Tzen Chang (2005). Study on the Antiinflammatory Activity of Essential Oil from Leaves of *Cinnamomum osmophloeum*. *J. Agric. Food Chem.* **53**(18): 7274–7278.
- Chi, T.; Ji, X.; Xia, M.; Rong, Y.; Qiu, F.; Zou, L.(2009). Effect of six extractions from Wuhu decoction on isolated tracheal smooth muscle in Guinea pig. *Zhongguo Shiyan Fangjixue Zazhi*. **15**, 52–55.
- Chiara Baccelli, Anneloes Martinsen, Nicole Morel and Joelle Quentin Leclercq. (2010).

- Vasorelaxant Activity of Essential oils from *Croton zambesicus* and some of their Constituents. *Planta Med.* **76** (14): 1506-1511.
- Chung KF, Pavord ID (April 2008). "Prevalence, pathogenesis, and causes of chronic cough". *Lancet* **371** (9621):1364–74.
- Cimanga K.K., Kambu T.L., Apers S., De Bruyne T., Hermans N., Totte J., Pieters L., and Vlietinck A.J. (2002). Correlation between chemical compositions and antibacterial activity of essential oils of some aromatic medicinal plants growing in the Democratic Republic of Congo. *Journal of Ethnopharmacology.* **79**: 213-220.
- Clark, A. T., Skypala, I., Leech, S. C., Ewan, P. W., Dugué, P., Brathwaite, N., et al. (2010). British society for allergy and clinical immunology guidelines for the management of egg allergy. *Clin Exp Allergy.* **40**: 1116–1129.
- Cohen, V.H and Lyle, J. (1966). A fluorometric assay for glutathione. *Anal Biochem.* **14**: 434-440.
- Combrinck S., Bosman AA., Botha BM, Plooy WD. (2006). Effect of post-harvest drying on the essential oil and glandular trichomes of *Lippia scaberrima* Sond. *J. Essent. Oil Res.*, **18**: 80-84.
- Connor D.E. and Beuchat L.R. (1984). Effects of essential oils from plants on growth of food spoilage yeast. *Journal of Food Science.* **49**: 429-434.
- Cowan, M.M. (1999). Plant products as antimicrobial agents. *Clin. Microbiol. Rev.* **12**: 564–582.

- Crespo M.E., Jimenez J. and Navarro C. (1991). *Special methods for the essential oils of the genus Thymus: In Essential oils and waxes*. Edited by Linskens H.F. and Jackson J.F. Springer-Verlag, Berlin Heidelberg, Germany. vol **12**: 41-61.
- Crouch, N., Symmonds, R., Spring, W., Diederichs, N. (2006). Fact Sheets for Growing Popular Medicinal Plant Species. In: *Commercialising Medicinal Plants. A Southern African Guide*, 1st ed. Sun, Stellenbosch, South Africa.
- Da Silva C.M., Bolzan A.A., Heinzmann B.M. (2006). Pyrrolizidine alkaloids from *Senecio* species. *Quimica Nova*. **29**: 1047-1053.
- Damjanović-Vratnica B., Tatjana D., Danijela Š. and Jovanka D. (2011). Antimicrobial Effect of Essential Oil Isolated from *Eucalyptus globulus* Labill. from Montenegro. *Czech J. Food Sci.* **29** (3): 277–28.
- Deans S.G. and Ritchie G.A. (1987). Antibacterial activity of plants essential oils. *International Journal of Food Microbiology*. **5**: 165-180
- Deans, S.G., and Svoboda, K.P. (1990). Biotechnology and bioactivity of culinary and medicinal plants. *Ag Biotech News Info*. **2**: 211–216.
- Dikshit A. and Husain A. (1984). Antifungal action of some essential oils against animal pathogens. *Ethnopharmacology*. **72**: 111-117.
- Doelman CJA, Bast A (1990). Oxygen radicals in lung pathology. *Free Rad Biol Med*. **9**: 381-400.
- Döll-Boscardin Patrícia Mathias, Adilson Sartoratto, Beatriz Helena Lameiro de Noronha

- Sales Maia. (2012). "In Vitro Cytotoxic Potential of Essential Oils of *Eucalyptus benthamii* and Its Related Terpenes on Tumor Cell Lines," Evidence-Based Complementary and Alternative Medicine, vol. 2012, pp. 8, doi: 10.1155 /2012/342652.
- Dorman H.J.D. and Deans S.G. (2000). *In vitro* evaluation of antioxidant activity of essential oils and their components. *Flavour and Fragrance Journal*. **15**: 12-16
- Downs SH, Marks GB, Sporik R, Belosouva EG, Car NG, et al. (2001). Continued increase in the prevalence of asthma and atopy. *Arch Dis Child* **84**: 20-23.
- Dubois R.N., Abramson S.B., Crofford L., Gupta R.A., Simon L.S., Van de Putte L.B. and Lipsky P.E. (1998). Cyclooxygenase in biology and disease. *Faseb j*. **12** (12):1063-1073.
- Ebeigbe A B and Aloamaka C P. (1985). Mechanism of hydralazine-induced relaxation of arterial smooth muscle, *Cardio Res*. **19**: 400.
- Eiceman, G.A.; Hill, H.H., Jr.; Davani, B. (1994). Gas Chromatography. *Analytical Chemistry*. **66** (12): 621R-33R.
- Ellman, G. L., Courtney, K. D., Andres, V. J.R. and Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biodiem. Plutrmac*. **7**: 88-95.
- Eloff JN (1998). A sensitive and quick method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica* **64**: 711-713.

Ernest J.P. (1921). The chemistry of essential oils and artificial perfumes. The Aberdeen University Press, LTD. Aberdeen, U.K.

Fabiane, K.G., Ferronato, R., Dos Santos, A.C., Onofre, S.B. (2008). Physicochemical characteristics of the essential oils of *Baccharis dracunculifolia* and *Baccharis uncinella* D.C. (Asteraceae). *Brazilian Journal of Pharmacognosy*. **18**: 197-203.

Fasola, R. T., Oloyede, G.K. and Aponjolosun, B.S. (2011). Chemical composition, toxicity and antioxidant activities of essential oils of stem bark of Nigerian species of guava (*psidium guajava* linn.). *EXCLI Journal*. **10**: 34-43.

Ferrero-Miliani, L.; Nielsen, O.H.; Andersen, P.S.; Girardin, S.E. (2007). Chronic inflammation: Importance of NOD2 and NALP3 in interleukin-1beta generation. *Clin. Exp. Immunol.* **147**: pp 227–235.

Fogarty and Britton (2000). The role of diet in the aetiology of asthma. *Clinical & Experimental Allergy*. **30** (5): 615–627.

Ford-Hutchinson A.W., Bray M.A., Cunningham F.M., Davidson E.M. and Smith M.J. (1981) Isomers of leukotriene B₄ possess different biological potencies. *Prostaglandins*. **21** (1): 143-152.

Fujiwara, M.; Yagi, N.; Miyazawa, M. (2010). Acetylcholinesterase inhibitory activity of volatile oil from *Peltophorum dasyrachis* Kurz ex Bakar (Yellow Batai) and bisabolene-type sesquiterpenoids. *J. Agric. Food Chem.* **58**, 2824-2829.

Garrat, D.C. (1964). *The Quantitative Analysis of Drugs*, Vol 3. Japan: Chapman and Hall, pp. 456-458.

- Gautam Raju, Kailis V. Karkhile, Kamlesh K. Btutani and Sanjay M. Jachak (2010). Anti-inflammatory cyclooxygenase (COX)-2, COX-1 inhibitory, and Free Radical Scavenging Effects of *Rumex nepalensis*. *Planta Med.* **76**: 1564–1569.
- Gedney J.J., Glover T.L. and Fillingim R.B. (2004). Sensory and affective pain discrimination after inhalation of essential oils. *Psychosomatic Medicine.* 66: 599-606
- George, J., Laing, M.D., Drewes, S.E. (2001). Phytochemical research in South Africa. *Journal of Science South Africa.* **97**: 93-105.
- Gosset P, Tsicopoulos A, Wallaert B (1991). Increased secretion by tumor necrosis factor and interleukin 6 by alveolar macrophages consecutive to the development of the late asthmatic reaction. *J Allergy Clin Immunol*; **88**:561–71.
- Gosset P, Tsicopoulos A, Wallaert B, (1992). Tumor necrosis factor α and interleukin-6 production by human mononuclear phagocytes from allergic asthmatics after IgE dependent stimulation. *Am Rev Respir Dis*; **146**:768–74.
- Govaerts, R. (2001). How many species of seed plants are there? *Taxon.* **50**: 1085 – 1090.
- Guenther E. (1949). *The Essential oils*. Vol. 3, D. Van Nostrand Company, New York, U.S.A.
- Guenther E., Gilbertson G., and Koenig R.T. (1971). Essential oils related products. *Analytical Chemistry.* **47**: 39-157.
- Gulluce, M., Sahin, F., Sokmen, M., Ozer, H., Daferera, D., Sokmen, A., Polissious, M., Adiguzel, A. and Ozkan, H. (2006). Antimicrobial and antioxidant properties of the essential oils and methanol extract from *Mentha longifolia* L. ssp. *Longifolia*. *food chemistry.* **103**. 1449-1456

- Gumede, M. V. (1989). In *Traditional Healers: A Medical Doctors Perspective*.
Johannesburg: Skotaville press.
- Halliwell B. and Gutteridge J.M. (1998). The deoxyribose assay: an assay both for 'free' hydroxyl radical and for site-specific hydroxyl radical production. *Biochem Journal*. **253** (3): 932 – 933.
- Hawkey C.J. (2001). COX-1 and COX-2 Inhibitors. *Best Pract Res Clin Gastroenterol*. **15** (5):801-820.
- Herent M., De Bie V., and Tilquin B., (2007). Determination of new retention indices for quick identification of essential oils compounds. *Journal of Pharmaceutical and biomedical Analysis*. **43**: 886-892.
- Herschman H.R. (1996). Prostaglandin synthase 2. *Biochim Biophys Acta*. **1299** (1):125-140.
- Hirano T., Yasukawa K., Harada H., Taga T., Watanabe Y., Matsuda T., Kashiwamura S., Nakajima K., Koyama K. and Iwamatsu A. (1986). Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature*. **324** (6092): 73–76.
- Hirshman CA. (1992). Anesthesia for Patients with Reactive Airway Disease. ASA Refresher Course Lectures; 221
- Hopkins-Broyles, D., Rieger. Y., Grim, A., Nihill, D., Jones, M., Damiano, R., Warren,

- D.K., Fraser, V.J. (2004). Risk Factors for *Staphylococcus aureus* Colonization in a Cardiac Surgery Population. *American Journal of infection control*. **32**: 119.
- Hwang SY, Lin HW, Chern RH, Lo HF, Li L.(1999). Reduced susceptibility to water logging together with highlight stress is related to increase in superoxide dismutase and catalase activity in sweet potato. *Plant Growth Regul.* **27**:167-72.
- Hutchings, A., Scott, A.H., Lewis, G., Cunningham, A., (1996). Zulu Medicinal Plants. An Inventory., Scottsville: University of Natal Press.
- Inès Hammami, Mohamed Ali Triki and Ahmed Rebai (2011).Chemical compositions, antibacterial and antioxidant activities of essential oil and various extracts of *Geranium sanguineum* L. flowers. *Arch. Appl. Sci. Res.* **3** (3):135-144.
- Iwalewa, E.O., McGaw, L.J., Naidoo, V. and Eloff J.N. (2007). Inflammation: the foundation of disease and disorders. A review of Phytomedicines of South African origin used to treat pain and inflammatory conditions. *African Journal of Biotechnology.* **6** (25): 2868-2885.
- Jacobs, Max. (1976). Eucalypts for planting. Food and Agriculture Organization of the United Nations, Rome, Italy. 398 p.
- Janssen, A. M., Sheffer, J and Baerheim - Svendsen, A. (1987).Antimicrobial activity of essential oils *Planta Med.* **53**: 395- 398.
- Jean-Paul Anthony, Lorna Fyte and Huw smith (2005).Plant active components- a resource for antiparasitic agents? *Trends in Parasitology.* **21** (10): 442-468.
- Juergens U.R. , U.Dethlefsen, G. Steinkamp, A. Gillissen, R. Repges and H. Vetter.

(2003). Anti-inflammatory activity of 1.8 -cineole (eucalyptol) in bronchial asthma: a double-blind placebo-controlled trial. *Respiratory Medicine*. **97**: 250-256

Jung Won-Kyo, Inhak Choi, Sangtaek Oh, Sae-Gwang Park, Su-Kil Seo, Soo-Woong

Lee, Dae-Sung Lee, Soo-Jin Heo, You-Jin Jeon, Jae-Young Je, Chang-Bum Ahn, Jin Soo Kim, Kwang Soo Oh, Young-Mog Kimh, Chisook Moon, Il-Whan Choi (2009). Anti-asthmatic effect of marine red alga (*Laurencia undulata*) polyphenolic extracts in a murine model of asthma. *Food and Chemical Toxicology* **47**: 293–297.

Kadri A., Ines, B. C., Zied, Z., Ahmed, B., Néji, G., Mohamed D. and Radhouane, G.

(2011). Chemical constituents and antioxidant activity of the essential oil from aerial parts of *Artemisia herba-alba* grown in Tunisian semi-arid region. *African Journal of Biotechnology*. **10**(15): 2923-2929.

Kariuki S. and Hart A. (2001). *Curr Opin Infect Dis*, vol **14**: 579-586.

Kaur G.J., Arora DS (2009). Antibacterial and phytochemical screening of *Anethum graveolens*, *Foeniculum vulgure* and *Trachysprum ammi*. *BMC Complement Altern Med*, vol **9**: 30.

Kazemi Masoud, Mohammad Dakhili, Abolfazi Dadkhah, Zahra Yasrebifar and Kambiz

Larijani (2011). Composition, antimicrobial and antioxidant activities of the essential oil of *Artemisia Kermanensis* Podl., an endemic species from Iran. *Journal of Medicinal Plant research*. **5** (18): 4481-4486.

Keenoy Begoña Manuel y, Greta Moorkens, Jan Vertommen and Ivo De Leeuw.

(2001). Antioxidant status and lipoprotein peroxidation in chronic fatigue syndrome, *Life Sciences*. **68** (17):, pp. 2037-2049, ISSN 0024-3205, [http://dx.doi.org/10.1016/S0024-3205\(01\)01001-3](http://dx.doi.org/10.1016/S0024-3205(01)01001-3).

Kilani S., Ben Sghaier M., Limem I., Bouhlel I., Boubaker J., Bhourri W., Skandrani I.,

Neffatti A., Ben Ammar R., Dijoux-Franca M.G., Ghedira K., and Chekir-Ghedira L. (2008). *In vitro* evaluation of antibacterial, antioxidant, cytotoxic and apoptotic activities of the tubers infusion and extracts of *Cyperus rotundus*. *Bioresource technology*. **99**: 9004-9008.

Knox AJ. (1994). Airway re-modelling in asthma: Role of airway smooth muscle. *Clin Sci* **86**:647–652.

Koedam A. (1987). *Some aspects of Essential oil preparation, In capillary gas chromatography in essential oil analysis*. Edited by Sandra P. and Bicchi C. Huethig, 13-27.

Kurita, N., Miyaji M., Kurane, R. and Takahara, Y. (1981). Antifungal activity of components of essential oils. *Agric. Biol Chem*. **45**: 945- 952.

Kurumbail R.G., Stevens A.M., Gierse J.K., McDonald J.J., Stegeman R.A., Pak J.Y., Gildehaus D., Miyashiro J.M., Penning T.D., Seibert K., Isakson P.C. and Stallings W.C. (1996). Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents. *Nature*. **384** (6610): 644-648.

Kuwano K, Boskev CH, Pare´ PD, Bai TR, Wiggs BR and Hogg JC (1993) Small

- airways dimensions in asthma and chronic obstructive pulmonary disease. *Am Rev Respir Dis.* **148**:1220–1225.
- Lahlou Mouhssen (2004). Essential oils and fragrance compounds: bioactivity and mechanisms of action. *Flavour Fragr. J.* **19**: 159–16.
- Lahlou Mouhssen (2004). Methods to Study the Phytochemistry and Bioactivity of Essential Oils. *Phytother. Res.* **18**: 435–448.
- Lalitha, V., Kiran, B. and Raveesha, K. (2011). Antifungal and antibacterial potentiality of six essential oils extracted from plant source. *International Journal of Engineering Science and Technology (IJEST).* **3** (4): 3029- 3038.
- Lawal, O.A. and Oyedeji, A.O. (2009a). Chemical Composition of the Essential Oils of *Cyperus rotundus* L. from South Africa. *Molecules.* **14** (8): 2909-2917.
- Lawal A.O. and Oyedeji O.A. (2009). Chemical Composition of the Essential Oils of the Flowers, Leaves and Stem of Two *Senecio polyanthemoides* Sch. Bip. Samples from South Africa. *Molecules.* **14**: 2007-2086; doi:10.3390/molecules14062077.
- Lawal, O.A. and Oyedeji, A.O. (2009c). The composition of the essential oil from *Cyperus distans* rhizome. *Nat Prod. Commun.* **4** (8): 1099-102.
- Levine SJ (1995) Bronchial epithelial cell-cytokine interactions in airway inflammation. *Invest Med.* **43**: 241-249.
- Levy SB. (2002). Active efflux, a common mechanism for biocide and antibiotic

- resistance. *J Appl Microbiol*, vol **92** :6555-7155.
- Liang Zhu, Ying-Juan Tian, Li Yang, Jian-Gou Jiang (2011). Chemical composition and antimicrobial activities of essential oil of *Blumea megacephala*. *EXCLI Journal*. **10**: 62 – 68.
- Lin, J., Opoku, A.R., Geheeb-Keller, M., Hutchings, A.D., Terblanche, S.E., Jager, A.K. Van Staden, J. (1999). Preliminary screening of some traditional Zulu medicinal plants for anti-inflammatory and anti-microbial activities. *J. Ethnopharmacology*. **68**: 267-274.
- Lindell Rebecca M., MD Thomas E. Hartman, MD Hassan F. Nadrous, MD Jay H. Ryu, MD (2005). Pulmonary Cryptococcosis: CT Findings in Immunocompetent Patients. *Radiology*. **236**:326 –331.
- López, M.D.; Pascual-Villalobos, M.J. (2010). Mode of inhibition of acetylcholinesterase by monoterpenoids and implications for pest control. *Ind. Crop Prod.* **31**, 284-288.
- Loziene K., and Venskutonis P.R. (2005). Influence of environmental and genetic factors on the stability of essential oil composition of *Thymu pulegiodes*. *Biochemical Systematics and Ecology*. **33**: 517-525
- Lehrner, J., Marwinski, G., Lehr, S., Jöhren, P. and Deecke, L. (2005). Immunological and Psychological Benefits of Aromatherapy Massage. *Evid Based Complement Alternate Med.* **2** (2): 179.
- Li HZ. and Nikaido H. (2004). Efflux mediated drug resistance in bacteria, *Drug*, vol **64**:159-204.
- Lin J., Opoku A.R., Geheeb-Keller M., Hutchings A.D., Terblanche S.E., Jager A.K. and

van Stadan J. (1999). Preliminary screening of some traditional Zulu medicinal plants for anti-inflammatory and anti-microbial activities. *Journal of Ethnopharmacology*. **68**: 267-274.

Lomovskaya O and Bostian KA, 2006. Practical application and feasibility of efflux pump inhibitors in the clinic-A vision for applied use. *Biochem Pharmacol*, vol **71**:910-918.

Machaba, K. E., Cobongela, S. Z., Mosa, R. A., Oladipupo, L. A., Djarova, T. G., &

Opoku, A. R. (2014). *In vivo* anti-hyperlipidemic activity of the triterpene from the stem bark of *Protorhus longifolia* (Benrh) Engl. *Lipids in Health and Disease*, **13**(1), 131. doi:10.1186/1476-511X-13-131

Maddox L, Schwartz DA (2002). "The pathophysiology of asthma". *Annu. Rev. Med.* **53**: 477–98.

Maesaki S, Marichal P, Bossche HV, Sangland D and Kohno S, 1999. Rhodamine

6G efflux for the detection of CDR1-overexpressing azole-resistant *Candida albicans* strains. *Journal of Antimicrobial Chemotherapy*, vol **44**: 27-31.

Mak J.C.W., Chang-Yeung M.W. (2006) Reactive oxidant species in asthma. *Curr Opin Pulm Med*. **12**: 7-11.

Maroyi Alfred. (2013). Traditional use of medicinal plants in south-central Zimbabwe:

review and perspectives. *Journal of Ethnobiology and Ethnomedicine*.
9:31 doi:10.1186/1746-4269-9-31.

Martinez, F. D. (1999). Recognizing early asthma. *Allergy*. **54**: 24–28.

doi: 10.1111/j.1398-9995.1999.tb04384.x

Martinez-Velazquez M., R. Rosario-Cruz, G. Castillo-Herrera, J. M. Flores-Fernandez,

A. H. Alvarez and E. Lugo-Cervantes (2011). Acaricidal Effect of Essential Oils from *Lippia graveolens* (Lamiales: Verbenaceae), *Rosmarinus officinalis* (Lamiales: Lamiaceae) and *Allium sativum* (Liliales: Liliaceae) Against *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae). *Journal of Medical Entomology*. **48** (4):822-827.

Masoli M, Fabian D, Holt S, Beasley R.(2004). The global burden of asthma: executive summary of the GINA Dissemination Committee report. *Allergy*; **59**: 469-478.

McLafferty, F. W., Hertel, R. H. and Villwock, R. D. (1999). Probability based matching of mass spectra. Rapid identification of specific compounds in mixtures. *Organic Mass Spectrometry*. **9** (7): 690–702.

Meier R., Schuler W. and Desaulles P. (1950). L-usnic acid: tumor inhibitor isolated from Lichens. *Experimentia* **6**: 469-471.

Minh Tu N.T., Onishi, Y., Choi H.S., Kondo, Y., Ukeda H. and Sawamura M. (2003).

Characteristic odour components of *Citrus sp.* (Kiyookadaidai) cold-pressed peel oil. *Flavour Fragr. J.* **18**: 515–52.

Minty A., Chalon P., Derocq J.M., Dumont X., Guillemot J.C., Kaghad M., Labit C.,

Leplatois P., Liauzun P. and Miloux B. (1993). Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses. *Nature*. **362** (6417): 248–250.

Mire-Sluis A.R., Page L. and Thorpe R. (1995). Quantitative cell line based bioassays for human cytokines. *Journal of Immunological Methods*. **187**(2): 191-199.

Mohamad, S., Zin, N.M., Wahab, H.A., Ibrahim, P., Sulaiman, S.F., Zahariluddin, A.S.M., Noor, S.S.M., (2011). Antituberculosis potential of some ethnobotanically selected Malaysian plants. *Journal of Ethnopharmacology* **133**: 1021-1026.

Mohamoud A., Chevalier J, Libert-Franco S, Kern WV and Pages JM. (2007). Antibiotic efflux pump in gram negative bacteria: the inhibitory response strategy. *J. Antimicrob Chemother*, vol **59**:1223-1229.

Mosa Rebamang A., Mandlakayise L. Nhleko, Thandeka V. Dladla and Andy R. Opoku. (2014). Antibacterial activity of two triterpenes from stem bark of *Protorhus longifolia*. *Journal of Medicinal Plant Research*. **8**(18): pp. 686-702, DOI: 10.5897/JMPR2013.5259

Mosmann T.(1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**: 55–63.

Moure A., Cruz J.M., Franco D., Domínguez J.M., Sineiro J., Domínguez H., Núñez M.J.

and Parajó J.C. (2001). Review : Natural antioxidants from residual sources. *Food Chem.* **72** : 145-171.

Mukherjee, P.K.; Kumar, V.; Mal, M.; Houghton, P.J. (2007). Acetylcholinesterase inhibitors from plants. *Phytomedicine.* **14**, 289-300.

Mulaudzi R.B., Ndhlala A.R., Kulkarni M.G. and Van Staden J. (2012). Pharmacological properties and protein binding capacity of phenolic extracts of some Venda medicinal plants used against cough and fever. *Journal of Ethnopharmacology.* **143**:(1) pp 185-193.

Murray and Nadel's Textbook of Respiratory Medicine, 4th Ed. Robert J. Mason, John F. Murray, Jay A. Nadel, 2005, Elsevier pp. 334.

Nagai, T. Sakai, M. Inoue, H. Suzuki, N. (2001). Antioxidative activities of some commercially honeys, royal jelly, and propolis. *Food Chem.* **75**: 237-240.

Nester, E.W., Anderson, D.G., Robert, C.E., Pearsall, N.N., Nester, M.T.,(2001). *Microbiology: A Human perspective*, 3rd ed. McGraw, New York, U.S.A.

Nevas M., Korhonen A., Lindstrom M., Turkki P and Korkeala H. (2004). Antibacterial efficiency of Finnish spice essential oils against pathogenic and spoilage bacteria. *Journal of Food Protection.* **67**: 199-202.

News.brown.edu/pressreleases/2011/11/bacteria. 03 December 2014 @16h00.

Nikaido H, 1996. Multidrug efflux pumps of gram-negative bacteria. *Journal of*

Bacteriol, vol **178**:5853-5859.

Niki LR, Emiel FM, Yvonne MWJ (2007) Modulation of Glutaredoxin-1 Expression in a mouse model of allergic airway disease. *Am. J. Respir Cell Mol. Biol.* **36**: 147-151.

Nunez M, Peacock JE and Chin R, 2000. *Pulmonary Cryptococcosis* in the immunocompetent host. Therapy with oral fluconazole. A report of four cases and a review of the literature. *Chest*, vol **118**:527-534.

Obiefuna P C M, Ebeigbe A B, Sofola O A and Aloamaka C P (1991). Altered responses of aortic smooth muscle from Sprague-Dawley rats with salt-induced hypertension. *Clin Exp Pharmacol Physiol*, **18**: 813

Ojeikere O, Usifoh CO and Ebeigbe AB (2003) Vascular effects of 2-methyl-3-propynylquinazolin-4-(3H)-one in isolated porcine tail artery, *Scientia Pharmaceutica*, **71**: 321.

Opoku, A.R., Maseko, N.F. Terblanche, S.E. (2002).The *in vitro* antioxidative activity of some traditional Zulu medicinal plants. *Phytotherapy Research*. **16**: S51-S56.

Osawa, T. Kawakishi, S. Chung, S.K. (1997). Hydroxyl radical scavenging effects of species and scavengers from Brown Mustard (*Brassica nigra*). *Bioscience Biotechnology Biochemistry*. **61**: 118-123.

Özbek Hanefi , Mehmet Karaca , Aydın Him, Mehmet Tütüncü , Hasan Altan Akkan

- and Veysel Kaplanoğlu.(2007). Investigation of Anti-inflammatory Activity of Bergamot Oil. *Eur J Gen Med.*, **4** (4):176-179.
- Pan, S. J., Kuo, C. H., Lam, K. P., Chu, Y. T., Wang, W. L., & Hung, C. H. (2010). Probiotics and allergy in children—an update review. *Pediatr Allergy Immunol.* **21**, 659–666.
- Panizzi, L., flamini, G., Cioni, P.L., Morelli, I. (1993).Composition and antimicrobial properties of essential oils of for Mediterranean Lamiaceae. *Journal of ethnopharmacology.* **39**: 167-170.
- Parthiban, K.T., P. Selvan, M. Paramathma, S.U. Kanna, P. Kumar, V. Subbulakshmi and S. Vennila. (2011). Physico-chemical characterization of seed oil from *Jatropha curcas* L. genetic resources. *Journal of Economic and Natural Environment*, **3**(5): 163-167.
- Patel, P.K., K.V. Patel and T.R. Gandhi, 2009. Evaluation of Effect of *Taxus baccata* Leaves Extract on Bronchoconstriction and Bronchial hypersensitivity I n Experimental Animals. *Global Journal of Pharmacology*, **3**(3): 141-148.
- Penduka, D., Mosa, R., Simelane, M., Basson, A., Okoh, A., & Opoku, A. (2014). Evaluation of the anti-*Listeria* potentials of some plant-derived triterpenes. *Annals of Clinical Microbiology and Antimicrobials*, **13**: 37. doi:10.1186/s12941-014-0037-1.

Pereira S.I., Freire C.S.R., Neto C.P., Silvestre A.D., Silva A.M.S.(2005).Chemical composition of the essential oil distilled from the fruits of *Eucalyptus globulus* grown in Portugal. *Flav Fragr J* **20**: 407-409.

Piddock LV. (2006). Clinical relevant chromosomally encoded multidrug resistance efflux pump in bacteria. *Clin Microbiol Rev*, vol **19**:382-402.

Pinto S., Rao, A.V., Rao, A. (2012) Erythrocyte and Plasma Antioxidant in Bronchial Asthma before and after Homeopathic treatment. *J. Homeopat Ayurv Med.* **1**: 13.

Poole K. (2005). Efflux mediated antimicrobial resistance. *J. Antimicrob. Chemother.*, vol **56**:20-51.

Prabuseenivasan Seenivasan, Manickkam Jayakumar and Savarimuthu Ignacimuthu (2006). *In vitro* antibacterial activity of some plant essential oils. *BMC Complementary and Alternative Medicine.* **6**: 39

Punjani BL and Kumar V. (2002) *J. Nat. Remed.* **2**(1): 84-87.

Quintão Nara L. M., Gislaine F. Da-Silva, Carla S. Antonialli, Lilian W. Rocha,

Valdir Cechinel Filho and José F. Ciccio (2010).Chemical Composition and Evaluation of the Anti-Hypernociceptive Effect of the Essential Oil Extracted from the Leaves of *Ugnimyricoides* on Inflammatory and Neuropathic Models of Pain in Mice. *Planta Med.* **76** (13): 1411-1418.

- Rahimi-Nasrabadi Mehdi and Hossein Batooli (2011). Chemical composition of essential oils of two cultivated *Eucalyptus* species from the central Iran. *Journal of Plant Breeding and Crop Science*. **3** (13): 380 – 382.
- Rahman I, MacNee W (2002) Reactive oxygen species. In: Asthma and COPD, edited by Barnes PJ, Drazen J, Rennard S, London, Academic Press 243-254.
- Raju, G. And Maridas, M. (2011). Composition, Antifungal and Cytotoxic activities of Essential oils of *Piper barberi* fruits. *International Journal of Biological Technology*. **2** (2): 100-105.
- Ramzi Ahmed Mothana (2011). Chemical Composition, Antimicrobial and Antioxidant Activities of the Essential Oil of *Nepetade flersiana* Growing in Yemen. *Rec. Nat. Prod.* **6** (2): 189-19.
- Rathee, J.S. Hassarajani, S.A. Chattopadhyay, S. (2007). Antioxidant activity of *Nyctanthes arbor-tristis* leaf extract. *Food. Chem.* **103**: 1350-1357.
- Roger FG., Greenwood D., Norby SR., Whitley RJ.(2003). Antibiotic and Chemotherapy. The problem of resistance, 8th ed. Churchill Livingstone pp. 25-47.
- Roussis V., Tsoukatou M., Chinou I.B. and Harvala C. (2002). Composition and antibacterial activity of the essential oils of two *Helichrysum stoechas* varieties growing in the island of Crete. *Journal of Essential Oil Research*. **14**: 459-461
- Sahoo A. (2005). Drug safety in the post- viox era new legislation, regulation and company strategies in Europe and the US. *Business Insight Ltd.* **8**(3): 24-45.

- Salahdeen H M and Murtala B A. (2012). Vasorelaxant effects of aqueous leaf extract of *Tridax procumbens* on aortic smooth muscle isolated from the rat, *J Smooth Muscle Res*, **48** : 37.
- Salari, M.H., G. Amine, M.H. Shirazi, R. Hafezi, and M. Mohammadypour.(2006). Antibacterial effects of *Eucalyptus globulus* leaf extract on pathogenic bacteria isolated from specimens of patients with respiratory tract disorders. *Clinical Microbiology and Infection* **12**:194-196.
- Santos F. A. and Rao V. S. (2000). Anti-inflammatory and anti-nociceptive effects of 1.8-Cineole, a terpenoid oxide present in many plant essential oils. *Phytotherapy Research*.**14** (4): 240–244.
- Santaella, M.L. Font, I. Disdier, O.M. (2004). Comparison of oral nicotinamide adenine dinucleotide (NADH) versus conventional therapy for chronic fatigue syndrome. *PRo Health. Sci. J.* **23**: 89- 93.
- Sarma A.D., Mallick A.R. and A. K. Ghosh (2010). Free Radicals and Their Role in Different Clinical Conditions. *International Journal of Pharma Sciences and Research.* **1** (3):185-192.
- Sawamura M., Song H., Choi H., Sagawa K and Ukeda H. (2001). Characteristic Aroma Components of Tosa-buntan (*Citrus grandis* Osbeck forma *Tosa*) Fruits. *Food Science and Technology Research.* **7**: 45-49.
- Schachter M. (2003). COX-2 Inhibitors and Cardiovascular Risk. *Br J Cardiol.* **10**

(4):288-292.

Schultz, J. (2002). Secondary Metabolites in Plants. Biology. Encyclopedia. com. 19/07/2010.

Scott J., Anthony G., W. Abdullah Brooks, J.S. Malik Peiris, Douglas Holtzman and E.

Kim Mulhollan. (2008). Pneumonia research to reduce childhood mortality in the developing world. *J Clin Invest.* **188** (4): 1291 -1300.

Scott R.P.W. (1958). *Principles and practice of chromatography*. Edited by Desty D.H.

Butterworths Scientific Publications, London. pp. 12-74

Shibamoto T. (1987). *Retention Indices in Essential Oil Analysis. In Capillary gas*

chromatography in essential oils analysis. Edited by Sandra P. and Bicchi C. Huethig. Pp. 259-274

Simelane Mthokozisi BC , Addmore Shonhai , Francis O Shode , Peter Smith,

Moganavelli Singh, Andy R Opoku.(2013). Anti-plasmodial Activity of some Zulu Medicinal Plants and of some Triterpenes isolated from them. *Molecules*, **18**: 12313-12323.

Sims J.E., March C.J., Cosman D., Widmer M.B., MacDonald H.R., McMahan C.J.,

Grubin C.E., Wignall J.M., Jackson J.L. and Call SM. (1988). cDNA expression cloning of the IL-1 receptor, a member of the immunoglobulin superfamily. *Science.* **241** (4865): 585–589.

Shinha, A.K (1971). Colorimetric assay of catalase. *Anal Biochem.*, **47**: 389-394.

- Sisay Feleke. (2010). Gil, L., Tadesse, W., Tolosana, E. & López. R., Eds. *Eucalyptus Species Management, History, Status and Trends in Ethiopia. Proceedings from the Congress held in Addis Ababa. September 15th-17th, 2010.*
- Skaltsa, H. D., Demetzos, C., Lazari, D., and Sokovic, M. (2003). Essential oil analysis and antimicrobial activity of eight *Stachys* species from Greece. *Phytochemistry*. **64**: 743-752.
- Small, E. and P.M. Catling. 2002. Blossoming treasures of biodiversity: 7. Ginkgo biloba - brainfood from a living fossil. *Biodiversity* **4**(1): 29-31.
- Sonbolia, A., Salehib, P., Mohammad, R. K., and Samad, N. E. (2005). Antibacterial and Antioxidant Activity and Essential Oil Composition of *Grammosciadium scabridum* Boiss. from Iran. *Z. Naturforsch.* **60c**: 534-538.
- Soyingbe O.S. (2012). The chemical composition, antimicrobial and antioxidant properties of the essential oils of *Tulbaghia violacea* Harv. and *Eucalyptus grandis* W. Hill ex Maiden. Department of Biochemistry and Microbiology, Faculty of Science and Agriculture, University of Zululand, KwaDlangezwa, South Africa
- Soyingbe O.S., Oyedeji A. O., Basson A. K. and Opoku A. R. (2013). The Essential Oil of *Eucalyptus grandis* W. Hill ex Maiden Inhibits Microbial Growth by Inducing Membrane Damage. *Chinese Medicine*. **4**: 7-14 doi:10.4236/cm.2013.41002
- Soyingbe O.S., Oyedeji A. O., Basson A. K., Singh M. and Opoku A. R. (2013). Chemical composition, antimicrobial and antioxidant properties of the essential oils of *Tulbaghia violacea* Harv L.F. *Afr. J. Microbiol. Res.* **7** (18), pp 1787-1793, 30.

- Starvi M, Piddock LJW and Gibbons S, 2007. Bacterial efflux pumps from natural sources. *J Antimicrob Chemother*, vol **59**:1247-1260.
- Stern, N. Hochman, A. Zemach, N. Weizman, N. Hammel, I. Shiloh, Y. Rotman, G. Barzilai, A. (2002). Accumulation of DNA damage and reduced levels of nicotine adenine dinucleotide in brains of Atm-deficient mice. *J Biol Chem*. **277**: 602-608.
- Stevenson Heidi (2013). "Lavender Is Effective Against Drug Resistant Staph Infection," ,GreenMedInfo.com, Reposted by Health Impact News.
- Suffness M. Pezzuto J.M.(1990). Assays related to cancer drug discovery. In *Methods in Plant Biochemistry: Assays for Bioactivity*; Hostettman, K., Ed.; Academic Press: London, UK, 71–133.
- Sulaiman Mohd' Roslan, Tengku Azam, Shah Tengku, Mohamad Wan Mastura ,Shaik Mossadeq, Saidi Moin, Mazina Yusof, Ahmad Fauzi Mokhtar, Zainul Amiruddin Zakaria, Daud Ahmad Israf and Nordin Lajis (2009). Antinociceptive Activity of the Essential Oil of *Zingiber zerunbet*. *Planta Med*. **79**: 107-112.
- Sun Jing, Lulu Li, Wu JinFeng, Liu Baojun, WeiYi Gong, Lv YuBao, Luo Qingi, Duan XiaoHong and Dong JingCheng. (2012). Effect of Baicalin on Airway remodeling in Asthmatic Mice. *Planta Medica*; **79**: 199-206
- Sunilson J. Anbu Jeba, K. Anandarajagopal, Abdullah khan, Khaja Pasha, Qusro Bin Hassan and Puspa V. Kuna Raja (2010). Antihistaminic evaluation of formulated polyherbal cough syrup. *Journal of Medicinal Plants Research*. **4** (14). 1482-1485.

- Sylvestre M., A. Pichette, A. Longtin, F. Nagau, and J. Legault (2006). Essential oil analysis and anticancer activity of leaf essential oil of *Croton flavens* L. from Guadeloupe, *Journal of Ethnopharmacology*. **103**: (1), pp. 99–102.
- Tabuti, J.R.S., Kukunda, C.B., Waako, P.J. (2010). Medicinal plants used by traditional medicine practitioners in the treatment of tuberculosis and related ailments in Uganda. *Journal of Ethnopharmacology* **127**: 130-136.
- Temelli F., Chen C.S and Braddock R.J. (1988). Supercritical fluid extraction in *Citrus* oil processing. *Food Technology*. 145-150
- Thorne, R. F. (2002). How many species of seed plants are there? *Taxon* **51**: 511–512
- Tippets B, Guilbert TW (2009). "Managing Asthma in Children: Part 1: Making the Diagnosis, Assessing Severity". *Consultant for Pediatricians*. **8** (5).
- Trelle S., Reichenbach S., Wandel S., Hildebrand P., Tschannen B., Villiger P.M., Egger M. and Jüni P. (2011). Cardiovascular safety of non-steroidal anti-inflammatory drugs: network meta-analysis. *British Medical Journal (Clinical research ed)*. **342** (7789): 154-165.
- Tzakou, O. and Skaltsa H. (2003). Composition and anti- bacterial activity of the essential oil of *Satureja par-nassica* subsp. *parnassica*. *Planta Med*. **69**: 282-284.
- Vale-Silva L., André M., José G., Carlos C., Lígia S. and Eugénia P. (2010). Antifungal

Activity of the Essential Oil of *Thymus x viciosoi* against *Candida*, *Cryptococcus*, *Aspergillus* and *Dermatophyte Species*. *Planta Med.* **76** (9): 882-888.

Van Vuuren S.F., Viljoen A.M. (2006). A comparative investigation of the antimicrobial properties of indigenous South African aromatic plants with popular commercially available essential oils. *Journal of Essential Oil Research*, **18**: 66–71

Van Wyk, B., Gericke, N., (2003). Peoples Plants. A Guides to Useful of Southern Africa. Briza Publications, South Africa.

Van Wyk, B.-E., Van Oudtshoorn, B., Gericke, N., 2009. Medicinal Plants of South Africa. 2nd ed. Briza, Pretoria, South Africa.

Van Wyk, B., Wink, M. (2004). Medicinal Plants of the World. Briza Publications, South Africa.

Vecchiet, J. Cipolin, F. Falasca, K. Mezzetti, A. Pizzigallo, E. Bucciarelli, T. De Laurentis, S. Affaitati, G. De Cesare, O. Giamberardino, M.A. (2003). Relationship between musculoskeletal symptoms and blood markers of oxidative stress in patients with chronic fatigue syndrome. *Neurosci. Lett.* **335**: 151-154.

Verma S., Ojha S. and Raish M. (2010). Anti-inflammatory activity of *Aconitum heterophyllum* on cotton pellet-induced granuloma in rats. *Journal of Medicinal Plants Research.* **4**(15):1566-1569.

Vernin G., Petitjean M., Metzger J., Fraisse D., Soun K.N and Scharff C.(1987). *Gas*

chromatography/mass spectrometry of essential oils. Part 1: Computer matching techniques. In capillary gas chromatography in essential oil analysis. Edited by Sandra P. and Bicchi C. Heuthig. Pp. 287- 328.

Vijoen A.M. van Vuuren S.F., Gwebu T., Demirci B., Baser K.H.C.(2006). The geographical variation and antimicrobial activity of African wormwood (*Artemisia afra* Jacq.) essential oils. *Journal of Essential Oil Research.* **18**: 66-71.

Vivek, K., Bajpai, S. S. and Sun, C. K. (2008). Chemical composition and antifungal activity of essential oil and various extract of *Silene armeria* L. *Bioresource technology.* **99**: 8903-8908.

Waling, C. (1975). Fenton's reagent revisited. *Acc. Chem. Res.* **8**: 125- 131.

Webber MA., Piddock LVJ. (2003). The importance of efflux pumps in antibiotic resistance. *J. Antimicrob Chemother*, vol **51**: 9-11.

Wexler HM (2012). Pump it up: Occurrence and regulation of multi-drug efflux pumps in *Bacteriodes fragilis*. *Journal of Molecular biology, genetics and biotechnology.* **18**: 200-208.

Whish JPM, Willam RR (1998). Effects of post-harvest drying on the yield of the tea tree oil *Melaleuca alterriba*. *J. Essent. Oil Res.*, **8**: 47-51.

Winter C.A. and Porter C.C. (1957). Effect of alterations in the side chain upon anti-inflammatory and liver glycogen activities of hydrocortisone esters. *J. Am. Pharm. Assoc. Sci. Educ.* **46**(9):515-519.

Winter C. A., Risley E. A., Nuss G. W. (1962) *Proc. Soc. Exp. Biol. Med.* **111**: 544—552.

World Health Organisation Fact Sheet N 194, 2002.
(<http://www.who.int/mediacentre/factsheet/fs194/en>).

World Health Organisation (WHO), 2005a. Prevention and control of chronic respiratory diseases in low and middle income African countries: a preliminary report.
<http://www.who.int/respiratory/publications/WHO-NMH-CRA-04.1.pdf>
(accessed 10 February 2014).

Yentema O., Alioune O. and *Cymbopogon schoenanthus* Dorosso S.A. (2007) Chemical composition and physical characteristics of the essential oil of (*L.*) speng of Burkina Faso. *J Applied Sci.* **7**: 503-506

Young, H. and Freedman, R. (2008). University physics with modern physics. 12 ed. San Francisco, Pearson Addison-Wesley. 1632 p.

Zaiki L.L. (1989). Spices and herbs: their antimicrobial activity and its determination. *Journal of Food science.* **9**: 97-118

Zhu Z, Tang W, Ray A. (1996). Rhinovirus stimulation of interleukin-6 in vivo and in vitro: evidence for nuclear factor- κ B-dependent transcriptional activation. *J Clin Invest.* **97**:421–30.

Zechini Barbara and Versace Ilaria. (2009). Inhibitors of Multidrug Resistant Efflux Systems in Bacteria. *Recent Patents on Anti-Infective Drug Discovery.* **4**(1) pp. 37-50 (14). DOI: 10.2174/15748910978723625

APPENDIX A

REAGENT PREPARATION

A.1 PHOSPHATE BUFFER (pH 7.4, 0.2M)

40ml of 0.2 M of potassium hydroxide was prepared and mixed with 50ml of 0.2M potassium dihydrogen phosphate. The prepared mixture made up to 100ml.

A.2 Sulphanilic acid reagent

0.33 % of sulphanilic acid was prepared in 20 % glacial acetic acid.

A.3 Glutathione preparation

0.1-10 µg/ml was dissolved in ice cold distilled water containing 30 µmoles/ml EDTA.

A.4 Tris-buffer (pH 7.0)

7.88 g of Tris-HCl, 2.79 g of EDTA and 10.27 g of NaCl was dissolved in distilled water and the solution made up to 1 L.

A.5 TBA

50ml of glacial acetic acid and 50ml of distilled water was mixed and 1 g of TBA was added to the solution and the solution was made up to 100ml with distilled water.

A.6 Phosphate buffed saline

0.13 M NaCl, 2.68 M KCl, 0.01 M Na₂HPO₄, 1.76mM KH₂PO₄ dissolved in distilled water.

A.7 PBS buffer (pH 7.4)

27mM potassium chloride, 137mM sodium chloride, 4.3mM sodium hydrophosphate and 1.4mM potassium hydrophosphate was dissolved in distilled H₂O and autoclaved

A.8 Formalin

1.75 g of sodium dihydrogen orthophosphate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) and 3.25 g of di-sodium hydrogen orthophosphate (Na_2HPO_4) was dissolved in 25ml of boiling water. 50ml of 40 percent formalin was added and the resulting mixture made up to 400ml with distilled water.

APPENDIX B

DETAILED METHODOLOGY

B.1 Extraction

About 300 g of the fresh and dry leaves of *Eucalyptus grandis* were hydro-distilled using a cleverger type apparatus for three hours with deionized water. The oils were collected and stored in a brown vial bottle until required.

B.2 Compound Preparation

Compound preparation for a concentration of 50 mg/kg, 0.5 g of essential oil, 1.8 cineole and 0.5 g of betulinic acid were weighed and dissolved in 5 ml tween-20 which was then topped up to 50 ml with distilled water, separately. Preparation was made for a concentration of 250mg/kg, 2.5g of essential oil and 1.8 cineole and dissolved in 5 ml tween-20 which was then topped up to 50 ml with distilled water; this was also done separately. Indomethacin 10 mg/kg, 0.1g was weighed then dissolved in 5 ml tween-20 and topped up to 50 ml with distilled water. The compounds were administered to the rats with respect to their weight.

B.3 Determination of SOD content

Different concentrations of (0.1, 1, 5, 10 and 50 mg/ml) were prepared. Absorbance was read at 420nm against a reagent blank

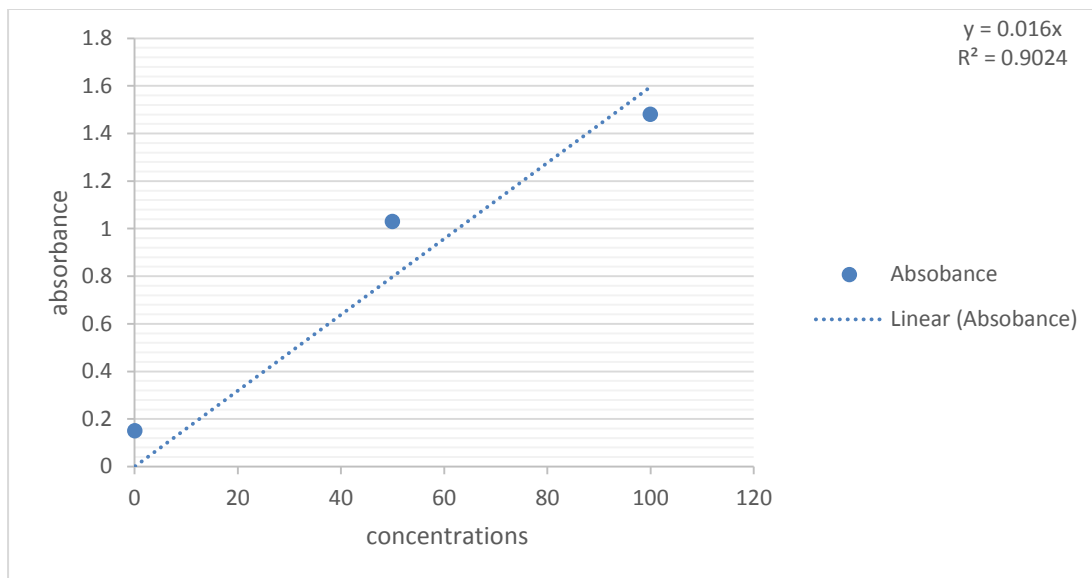


Figure 3.2 Calibration curve of SOD concentration (mg/ml) against absorbance (nm).

B.4 Chelating activity on FE^{2+} :

Different concentrations of methanolic solution of the essential oils were prepared. 2 mM $FeCl_2$ and 5 mM ferrozine were also prepared and test tubes were set as in DPPH experiment. 1ml of oil solution was diluted with 3.75 ml deionised water and this was mixed with 0.1 of $FeCl_2$ and 0.2 ml ferrozine. This was left to stand for 10 min with intermittent mixing. The absorbance was read at 562nm and with deionised water used as blank. Percentage scavenging activity was calculated as:

$$\% \text{ scavenging} = (1 - \frac{At}{Ao}) \times 100$$

Graphs of percentage scavenging activity versus concentration of oil (mg/ml) were constructed.

B.5 Nitric Oxide Radical Scavenging Activity:

Methanolic solutions of different concentrations of the essential oils were prepared. 10mM of sodium nitropruside, (pH 7.4) 0.01M saline phosphate buffer, 0.33% of sulphanilic acid reagent in 20% glacial acetic acid and 0.1% naphthylenediamedihydrochloride were also prepared. Test tubes were set as in DPPH experiment. 2 ml of sodium nitropruside and 0.5 ml saline phosphate buffer were added to 0.5 ml of methanolic solution of the essential oil. The mixture was incubated at 25°C for 150 mins, then 0.5 ml of the reaction mixture was pipetted into different test tubes and 1ml of sulphanilic acid was added, mixed and left to stand for 5 mins after which 1ml of 0.1% naphthylethylelediamedihydrochloride was added, mixed and left to stand in diffused light for 30 min. The absorbance was read at 540nM using a plate reader with deionised water used as blank and percentage scavenging activity was calculated as:

$$\% \text{ scavenging} = (1 - A_t/A_0) \times 100$$

Graphs of percentage scavenging activity versus concentration of oil (mg/ml) were constructed.

B.6 SH- Sulphydryl Content

Different concentrations of glutathione red (GSH) 0.1-10 µg/ml was prepared in iced cold distilled water containing 30 µm/ml of EDTA. The methanolic solution of the essential oils was also prepared with the EDTA solution as above (1:5). Test tubes were set up (comprising: blank, GSH of different concentrations and essential oils of different concentrations). A 0.1M sodium phosphate buffer of pH 8 was prepared and 1% σ-phthaladehyde (OPT) in methanol was also prepared. To 2 ml of GSH and essential oils in test tubes, 0.5 ml sodium phosphate

buffer was added (to the blank, 2.5 ml of sodium phosphate buffer). The mixture was allowed to stand for 15-20 min at room temperature and the fluorescence read at 420nm. The standard graph was plotted with GSH (Figure B1) and the SH content in the essential oils was estimated from the graph.

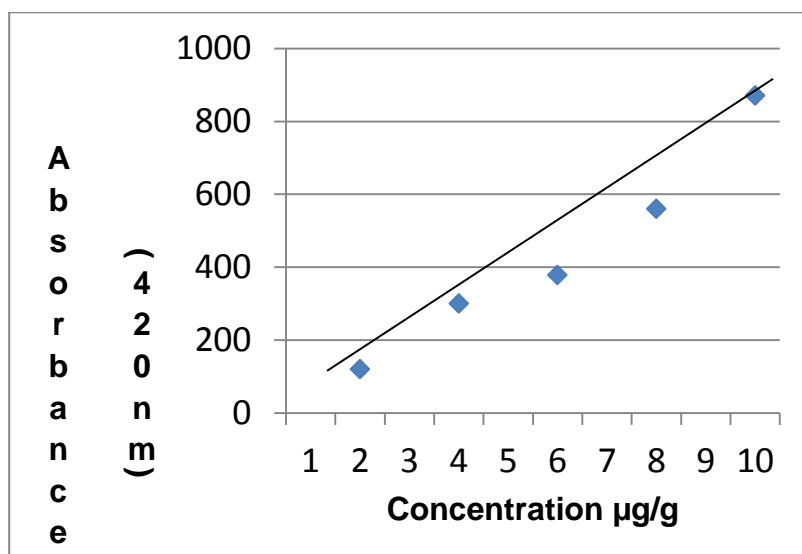


Figure B1. Standard graph of GSH

B. 7 Agar Disk Diffusion Method.

Bacteria were grown in 20 ml nutrient broth at 37°C overnight. The cultures were then adjusted to compare with the McFarland no.5 standard (1.0×10^8 CFU/ml). Standard Petri dishes containing nutrients agar were then inoculated with the bacteria suspension which had been adjusted to the McFarland standard. Sterile paper disks were placed on the inoculated plates and 10 µl of 10 mg/ml of the essential oils in 10% DMSO were added to the paper disk. The

plates were then incubated at 37 °C for 24 hrs and the zone of inhibition measured using a ruler. Tests were performed in duplicates using ampicillin and neomycin as positive control.

B.8 Minimum Inhibitory Concentration (MIC)

About 50 µl of nutrients broth was added to all wells of the microtitre plate. 50 µl of 10 mg/ml of the essential oils in 10% DMSO was then added to the well in row A, then serially diluted down the rows from row A. The remaining 50 ml was then discarded. 50 µl of bacteria culture of McFarland standard was then added to all the wells and then incubated at 37 °C for 24 hrs. About 20 µl of 0.2 mg/ml p-iodonitotetrazolium violet (INT) solution in distilled water was then added to each well and incubated at 37 °C for 30 mins. A reddish coloration which was a result of INT being reduced by the metabolic active microorganisms to formazan indicated microbial activity. The MIC measurement, which is the lowest concentration at which no visible microbial growth is seen, is then taken.

B.9 Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration (MBC) is defined as the lowest concentration of the sample at which inoculated bacterial strains are completely killed. The MBC was confirmed by reinoculating 10 µl of each culture medium from the microtiter plates which were used for MIC on nutrient agar plates and incubated at 37 °C for 24 hrs. The plates were then observed for growth.

B.10 Assay of hydroxyl radical (\cdot OH) scavenging activity

the assay is based on benzoic acid hydroxylation method, as described by Osawa, et al (1997). In a screw-capped tube 0.2 ml of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (10 mM) and 0.2 ml EDTA (10 mM)

were added. Then, 0.2 ml extract (0-5 mg/100 ml), 0.2 ml DNA (10 mM) and 1 ml phosphate buffer (pH 7.4, 0.1 mol.) were added. Finally, 200 µl of an H₂O₂ solution (10 mM) was added. The reaction mixture was then incubated at 37⁰C for 4 hr (of internal mixing). After this, 1ml of TCA (2, 8%) and 1ml of TBA (1%) were added and the mixture was boiled for 10 min and allowed to cool on ice. Absorbance was determined at 520nm and the inhibition of lipid peroxidation by the extract was calculated.

B.11 Assay of NO- scavenging activity

About 2 ml of sodium nitropruside (10 mM) and 0.5 ml of phosphate buffer saline (0.01 M; pH 7.4) was mixed with 0.5 ml extract (0-5mg/100ml) and incubated at 250 °C for 150 min. 0.5 ml of reaction mixture was pipetted into different test tubes. 1 ml sulphanic acid reagent (0.33% in 20 % glacial acetic acid) was added and incubated at room temperature for 5 min and 1ml 1-naphthylamine (5 %) was added and allowed to stand for 30 min in diffused light. The absorbance was determined spectrophotometrically at 540nm (Garrat, 1964).

B.12 Chelating Activity on Fe²⁺

The method reported by Decker and Welch (1990) was used to measure the chelating activity of plant extracts on Fe²⁺. Then 1 ml of plant extract (0-5 mg/100 ml) was diluted with 3.75 ml of deionised water. This was mixed with FeCl₂ (2 mM, 0.1 ml) and 4, 4, 1- [3-(2-pyridinyl)-1, 2, 4-triazine-5, 6-dryl] bisbenzene sulphonic acid (ferrozine) (5 mM, 0.2 ml), and after 10 min the absorbance was measured. Ethylenediaminetetra-acetic acid (EDTA) and citric acid were used as standards.

B.13 NADH

A modified method for determining NADH by Stern *et al* (2002) was used to determine the concentration of NADH in the plant extract. Phosphate buffer (containing 20 mM sodium dihydrogen carbonate, 100 mM sodium carbonate and 10mM nicotinamide) was used to extract plant powder (1 g in 5 ml). The supernatant was divided into two portions. The NAD⁺/NADP⁺ of one portion was destroyed by incubating extract at 60 °C for 30 min on a dry heating block and promptly chilling to 0 °C. Both (heated and unheated) extracts were combined with 1ml of reaction mixture (10 µM tris-HCl, pH 8, 2 µM phenazineethosulfate, 0.5 µM tetrazolium bromide and 0.2 µg alcohol dehydrogenase). The mixture was incubated for 5 min at 37 °C; 0.1 ml ethanol (600 µM) was added and the mixture was reincubated for 10 min at 37 °C. The absorbance was measured at 570nm and the concentration was determined using the standard graph.

B.14 SH determination

Powdered plant material was extracted (1:5 w/v) with EDTA solution (ice cold water containing 30 µM/ml EDTA) and filtered. 2 ml of extract (0-5 mg/100ml) was mixed with 0.5 ml phosphate buffer (0.1 M, pH 8.0) and 0.1 ml of 1% σ - phthaldehyde. The mixture was allowed to stand at room temperature for 15-20 min. The fluorescence was measured at 420nm (activation at 350nm). A standard graph for GSH (0.1-10 µg) was plotted (Figure B3) and -SH content of the sample was estimated from the graph.

B.15 MTT cell proliferation assay

Human embryonic kidney (HEK293) and human hepatocellular carcinoma (HepG2) cells were all grown to confluency in 25 cm² flasks. This was then trypsinised and plated into 48 well plates at specific seeding densities. Cells were incubated overnight at 37⁰C. Medium was then removed and fresh medium (MEM + Glutmax + antibiotics) was added. Extracts were then added in triplicate and incubated for 4 hrs. Thereafter the medium was removed and replaced by complete medium (MEM + Glutmax + antibiotics +10 % Fetal bovine serum). After 48 hr cells were subjected to the MTT assay (Mosman, 1983). At the end of the incubation period (48 hr), medium from cells in the multiwell plate was removed. 200 µl of MTT solution as well as 200 µl of medium was added to each well containing cells. A multiwell plate was incubated at 37⁰C for 4 hr. Thereafter, medium and MTT solution were removed from the wells. 100/200/400 µl of DMSO was added to each well (this stops the reaction and dissolves insoluble formazan crystals). The plate was read in a plate reader at 570nm (Mosman, 1983). Data was evaluated through regression analysis using a QED statistics program and from the linear equation; the LC₅₀ values representing the lethal concentration for 50 % mortality.

B.14 A549 LUNGS CELL CULTURES

A549 lungs cells were cultured (37 °C, 5% CO₂) to confluency in 25 cm³ culture flasks in complete culture media (CCM). CCM was made up of eagle's minimum essential medium (EMEM), 10% foetal calf serum, 1% L-glutamine and 1% penstrepfungizone. Growth of cells was monitored and confluent flasks were trypsinized using 1 ml trypsin. Cell numbers were counted with the aid of trypan blue dye stains. The cytotoxicity effect of the essential oils was determined using (MTT) assay. A549 cells (15,000 cells/ well) were seeded into a

96-well microtitre plate and incubated at 37 °C (with 5% CO₂) and a range of essential oil dilutions, in triplicates, for 24 hr. A549 cells incubated with CCM only were used as positive control. 120 µl of CCM/MTT salt solution at a concentration of 5mg/ml was added to each well and incubated for 4 hr at 37 °C. Following incubation, the supernatant was removed and 100 µl DMSO was added to each well and incubated for 1 hour. The optical density of the formazan produced was measured at 570nm, with a reference wavelength of 690nm. The percentage cell viability was determined (% viability = average OD of treatment/average OD of untreated control × 100).

B.15 INDUCTION OF INTERLEUKIN (IL-6) SECRETION (CYTOKINE ASSAYS)

The levels of cytokines interleukin 6 (IL-6) in A549 cells was determined by enzyme-linked immunosorbent assay. (ELISA) IL-6 secretion was induced by the addition at time zero of 10 ul of a stimulatory agent to the test wells. Supernatants were removed from triplicate wells for each stimulus at the four experimental time points. The triplicates were combined into one sample and immediately frozen at -20°C. A control sample obtained from unstimulated cells was treated similarly. Samples from a single experiment were analyzed in the same IL-6 assay.

B.16 MUSCLE CONTRACTION STUDIES

Healthy, young adult, male and female Wistar albino rats weighing 250-300 g were used. The animals were kept and maintained under conventional laboratory conditions of temperature, humidity and light, and allowed free access to a standard pellet diet and water *ad libitum*. All the animals used were fasted for 18 h, but still allowed access to water before the commencement of the experiments.

B.17 PREPARATION AND MOUNTING OF AORTIC RINGS

The rats were sacrificed by cervical dislocation. The thoracic aorta was quickly removed, freed of connective tissue and placed in a petri-dish containing physiological salt solution (PSS). The aortic lumen was gently flushed with PSS and sectioned into 2mm ring segments. Each aortic ring was suspended in a 60ml jacketed tissue bath containing PSS with the following composition (Ebeigbe and Aloamaka, 1987; Obiefuna et al., 1991) (in mmol/L): NaCl, 118.0; KCl, 4.7; KH_2PO_4 , 1.2; MgSO_4 , 1.2; NaHCO_3 , 15.0; CaCl_2 , 1.6 and glucose, 11.5. The temperature of the bath was maintained at 37°C and the solution bubbled with a 95% O_2 , 5% CO_2 gas mixture (pH 7.35-7.40). Each ring was mounted between a stainless steel hook connected to the base of the bath, and a stainless steel rod anchored to a force transducer (model 7004; Ugo Basile Varese, Italy) connected to a data capsule acquisition system (model 17400) for recording of isometric contractions. An initial tension of 2 g was applied to all arterial rings. This level of initial tension produces maximal active contractions in aortic rings stimulated with noradrenaline (NA) or a depolarizing solution (Ojeikere et al., 2003). An equilibration period of 60-90 min was allowed before the start of experiments, and during this time it was stimulated thrice with 10^{-6} M noradrenaline for 5 min at 30 min intervals (Salahdeen and Murtala 2012). At the end of the equilibration period, the vessel was subjected to the following procedures. Cumulative doses of noradrenaline (NA) were added to the PSS to obtain concentrations of 10^{-9} to 10^{-5} M and the concentration–response curves (CRCs) were determined. Following incubation with essential oil of the extract CRCs were determined for (NA).

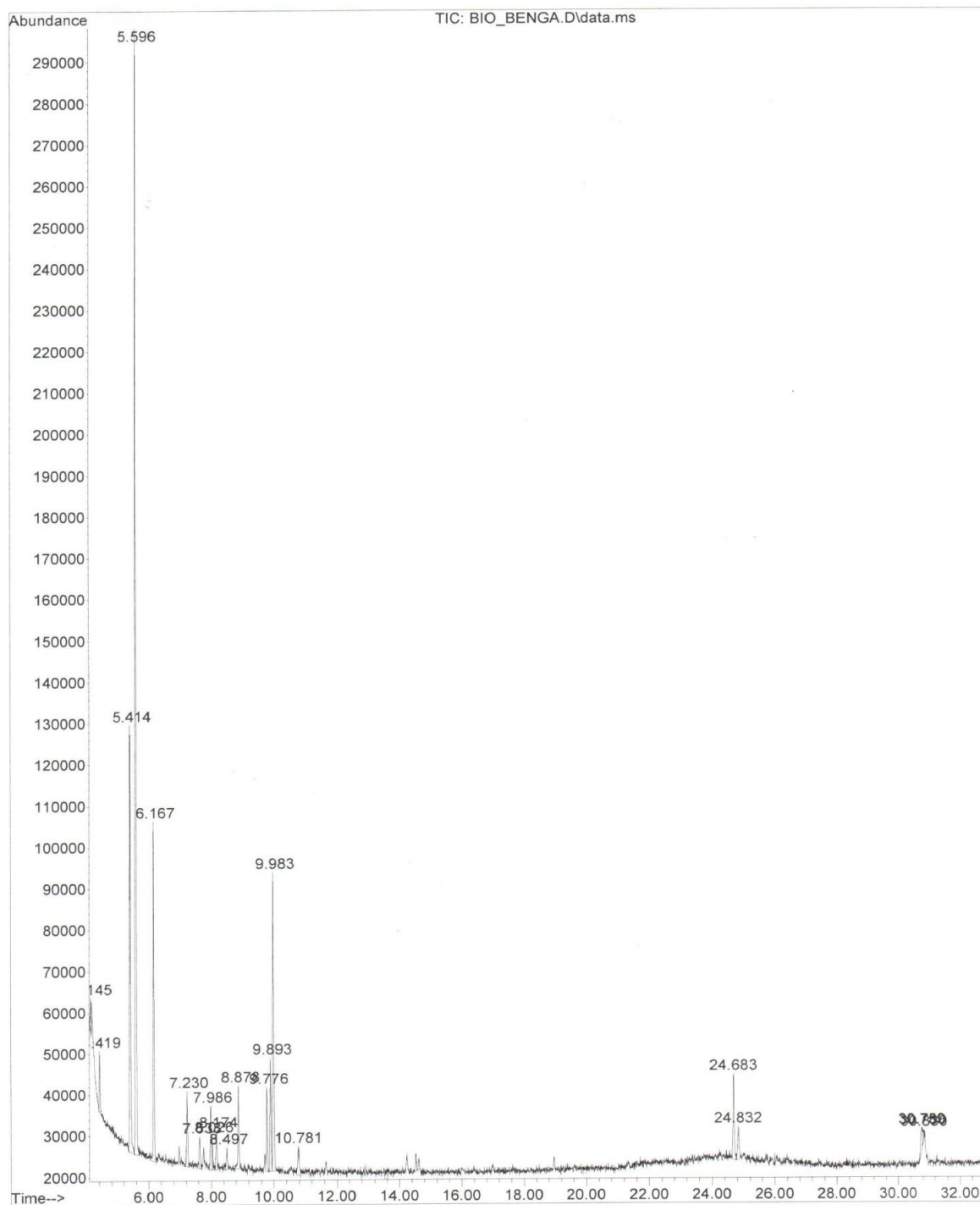
Data is expressed as means \pm SE, where n equals the number of animals from which blood vessels were isolated. The data is analyzed using two-way ANOVA. The Student-Newman-Keuls post hoc test was used to identify differences between individual means. The confidence interval was set at 95%, so that in all cases, results with a value of $P < 0.05$ were considered to indicate statistical significance.

B 18 LACTATE DEHYDROGENASE (LDH) RELEASE ASSAY (MEMBRANE DAMAGE)

The microbial cultures were then centrifuged (5000 g; 5 mins). The supernatant (100 μ l) was then mixed with 100 μ l of a lactic acid dehydrogenase substrate mixture of 54mM lactic acid, 0.28mM of phenazinemethosulfate, 0.66mM p-iodonitrotetrazolium violet, and 1.3mM NAD⁺. The pyruvate-mediated conversion of 2,4-dinitrophenyl-hydrazine into visible hydrazone precipitate was measured on an auto microplate reader (BiotekELx 808) at 492nm. The total loss of membrane integrity resulting in complete loss of cell viability was determined by lysing the cells of untreated organisms with 3% triton X-100 and using this sample as a positive control. The cytotoxicity in the LDH release test was calculated using the formula: $(E-C)/(T-C) \times 100$, where E is the experimental absorbance of the cell cultures, C is the control absorbance of the cell medium, and T is the absorbance corresponding to the maximal (100%) LDH release of Tri- ton X-100 lysed cells (positive control).

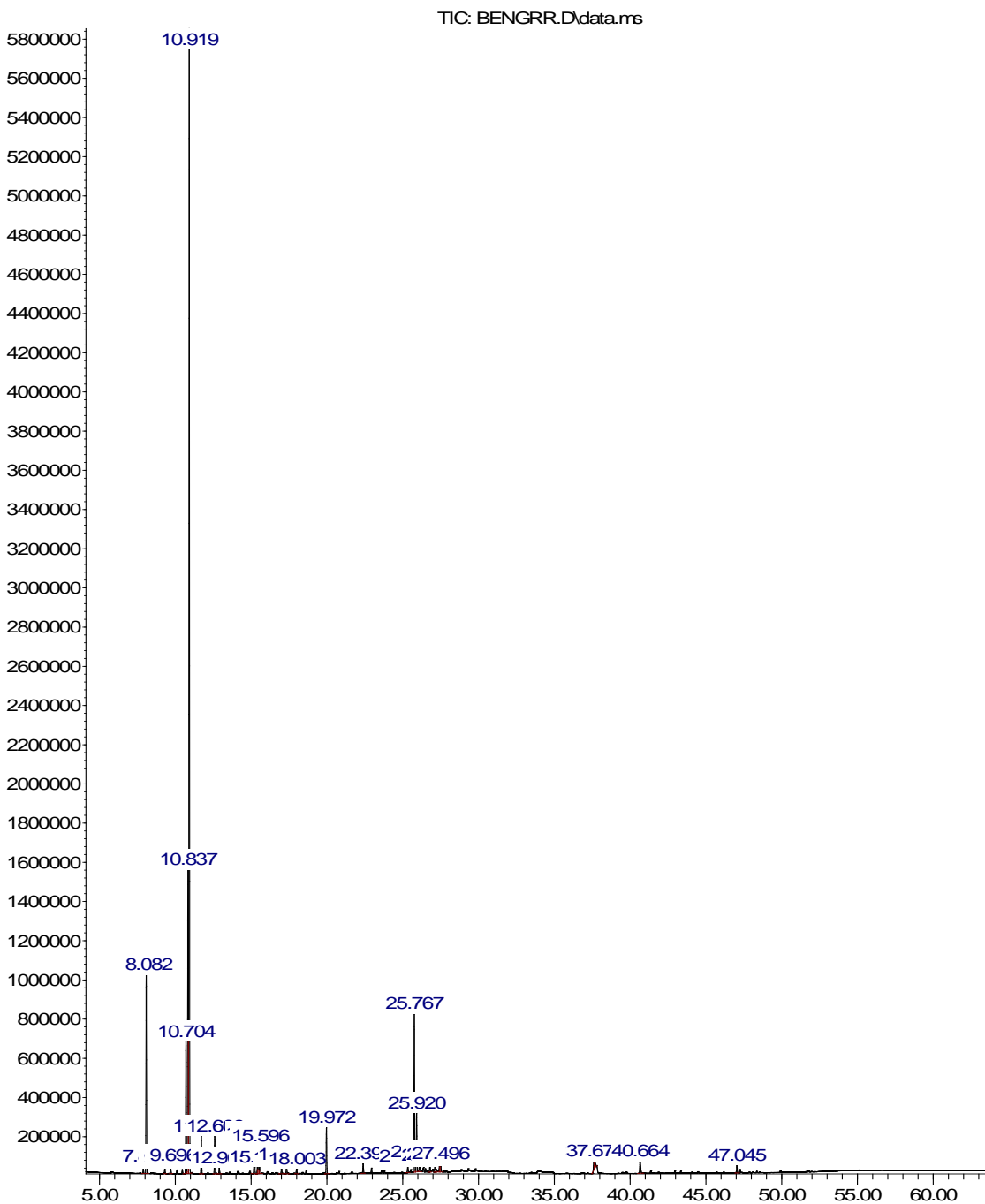
APPENDIX C ADDITIONAL DATA

C.1 GC-MS Spectra for the essential oil of the fresh leaves of *Eucalyptus grandis*.



C.2 GC-MS Spectra for the essential oil of the dry leaves of *Eucalyptus grandis*.

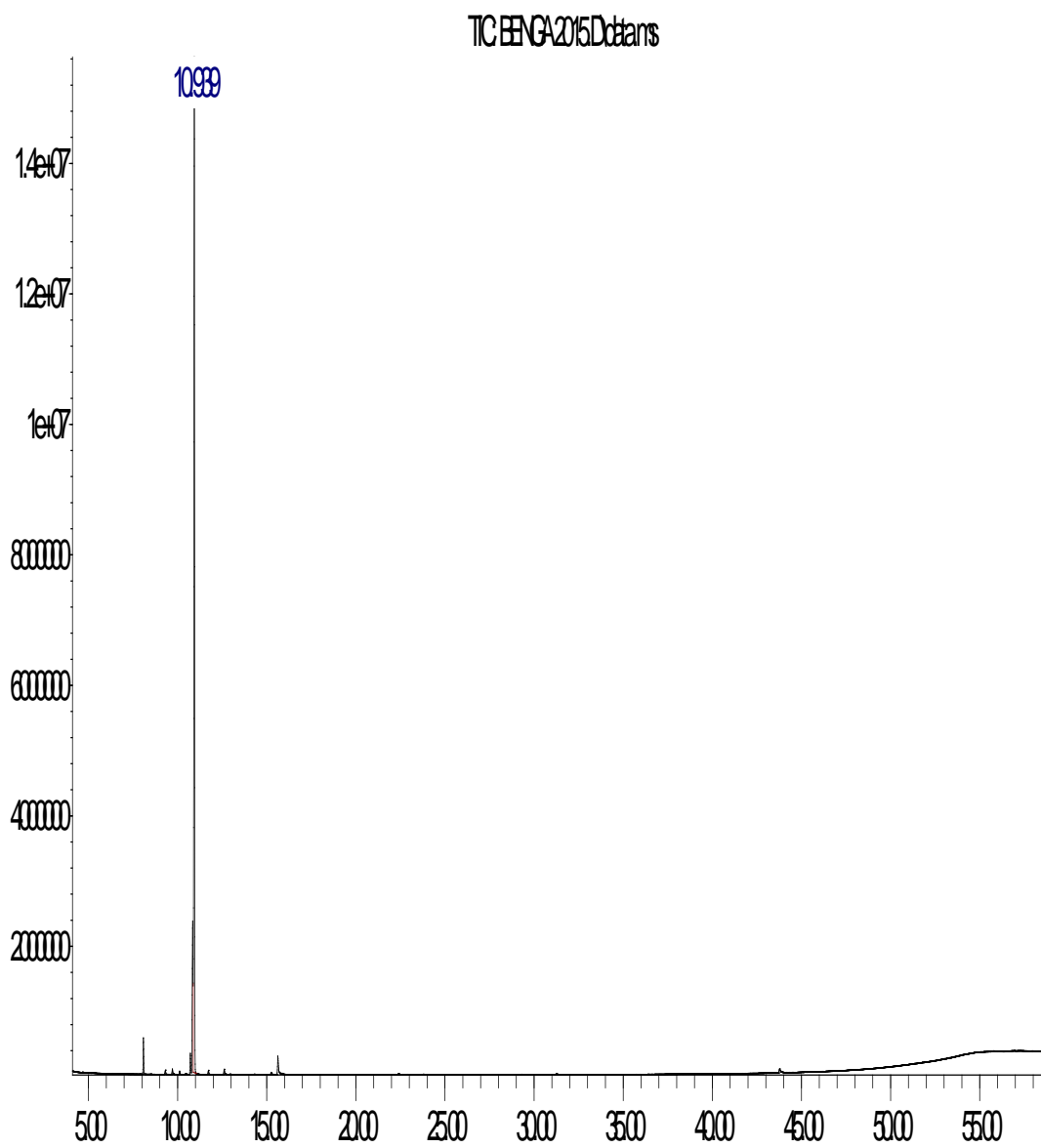
Abundance



Time-->

C.3 GC-MS Spectra of the isolated 1,8-cineole.

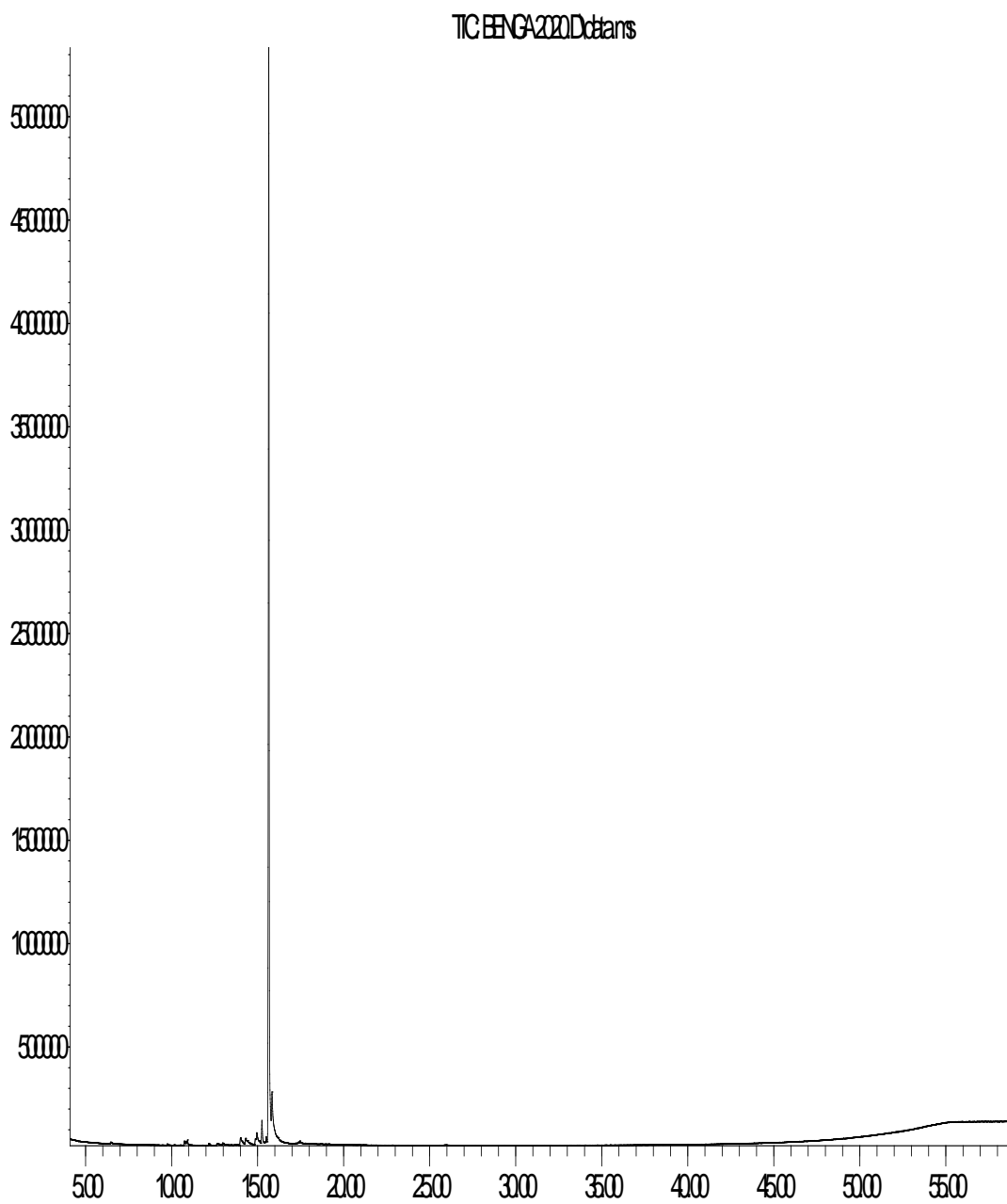
Abundance



Time→

C.4 GC-MS Spectra of the isolated terpinen-4-ol

Abundance



Time->

Appendix D

Ethical clearance.

**UNIVERSITY RESEARCH ETHICS
COMMITTEE**
(Reg No: UZREC 171110-30)



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ETHICAL CLEARANCE CERTIFICATE

Certificate Number	UZREC 171110-030 PGD 2013/26							
Project Title	Anti-asthmatic and anti-cough activities of the essential oil of <i>Eucalyptus grandis</i>							
Principal Researcher/ Investigator	OS Soyingbe							
Supervisor and Co- supervisor	Prof. Opoku							
Department	Biochemistry							
Nature of Project	Honours/4 th Year	<input type="checkbox"/>	Master's	<input type="checkbox"/>	Doctoral	<input checked="" type="checkbox"/>	Departmental	<input type="checkbox"/>

The University of Zululand's Research Ethics Committee (UZREC) hereby gives ethical approval in respect of the undertakings contained in the above-mentioned project proposal and the documents listed on page 2 of this Certificate. Special conditions, if any, are also listed on page 2.

The Researcher may therefore commence with the research as from the date of this Certificate, using the reference number indicated above, but may not conduct any data collection using research instruments that are yet to be approved.

Please note that the UZREC must be informed immediately of

- Any material change in the conditions or undertakings mentioned in the documents that were presented to the UZREC
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research

The Principal Researcher must report to the UZREC in the prescribe format, where applicable, annually and at the end of the project, in respect of ethical compliance.

The table below indicates which documents the UZREC considered in granting this Certificate and which documents, if any, still require ethical clearance. (Please note that this is not a closed list and should new instruments be developed, these may also require approval.)


Documents	Considered	To be submitted	Not required
Faculty Research Ethics Committee recommendation	X		
Animal Research Ethics Committee recommendation			X
Health Research Ethics Committee recommendation	X		
Ethical clearance application form	X		
Project registration proposal	X		
Informed consent from participants			X
Informed consent from parent/guardian			X
Permission for access to sites/information/participants			X
Permission to use documents/copyright clearance			X
Data collection/survey instrument/questionnaire			X
Data collection instrument in appropriate language		Only if necessary	
Other data collection instruments		Only if used	

Special conditions: Documents marked "To be submitted" must be presented for ethical clearance before any data collection can commence.

The UZREC retains the right to

- Withdraw or amend this Certificate if
 - Any unethical principles or practices are revealed or suspected
 - Relevant information has been withheld or misrepresented
 - Regulatory changes of whatsoever nature so require
 - The conditions contained in this Certificate have not been adhered to
- Request access to any information or data at any time during the course or after completion of the project

The UZREC wishes the researcher well in conducting the research.


 Professor Rob Midgley
 Deputy Vice-Chancellor, Research and Innovation
 Chairperson: University Research Ethics Committee
 06 May 2013

PROF. JR MIDGLEY
 DEPUTY VICE-CHANCELLOR
 RESEARCH & INNOVATION

06 MAY 2013

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APPENDIX E CONTRIBUTION TO KNOWLEDGE.

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Scientific
Research

The Essential Oil of *Eucalyptus grandis* W. Hill ex Maiden Inhibits Microbial Growth by Inducing Membrane Damage

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ABSTRACT

Eucalyptus grandis is a medicinal plant which has been indicated by Zulu traditional healer in the treatment of respiratory tract infections, bronchial infections, asthma and cough. The investigation of the essential oil of this plant could help to verify the rationale behind the use of the plant as a cure for these illnesses. Essential oil was hydro-distilled from the fresh leaves and characterised for the chemical constituents and bioactivity. The main constituents of the oil of the *E. grandis* are α -Pinene (29.69%), p-Cymene (19.89%), 1,8-cineole (12.80%), α -Terpineol (6.48%), Borneol (3.48%) and D-Limonene (3.14%). The essential oil of *E. grandis* showed high scavenging of DPPH and ABTS radicals, and was active against 13 of the 16 organisms tested with the MIC ranging from 0.625 mg - 5.0 mg/ml; the MBC value ranged from 2.5 mg - 10 mg/ml. The essential oil also inhibited the growth of 7 of the 8 antibiotic resistant bacteria tested, with MIC ranging from 5 mg/ml - 10 mg/ml. The DNA extracted from the affected microorganisms did not show any damage however, there was an increase of released cytosolic LDH activity. We conclude that the antibacterial activity of the essential oil was exhibited through cell membrane damage rather than the damage of the DNA. It is apparent that the bioactivity of the essential oil of *E. grandis* plays an important role in the plants' use in folk medicine for the treatment of respiratory tract illnesses.

Keywords: Essential Oil; Antioxidant; Antimicrobial Activity; LDH

1. Introduction

Medicinal plants form a sizeable component of traditional medicine and are mainstay for about 80% of the people in developing nations [1]. The use of medicinal plants is a basic part of African culture [2] and it is one of the oldest and most diverse the world over [3]. In South Africa indigenous African medicine is used alongside Western allopathic medicine [4], which caters for different people of different cultures. Traditional healing which makes use of local herbs is widely practised in Zululand [5]. The medicinal effect of various plants traditionally used by the Zulus to cure different ailments has been well documented [1,6-9].

E. grandis (Figure 1) belongs to the Myrtaceae family previously native to Australia. Massive planting programs have been carried out in the Republic of South Africa, Zambia and Zimbabwe. The leaves are leathery in texture, hang oblique or vertically, and are studded with glands containing a fragrant volatile oil.

The flowers in bud are covered with a cup-like membrane (whence the name of the genus, derived from the Greek word "eucalyptus" meaning well covered), which is thrown off as lid when the flower expands. Eucalyptus trees are quick growers and many species reach great heights [10].



Figure 1. *Eucalyptus grandis* W. Hill ex Maiden (www.forestryimages.org).

*Corresponding author.

Full Length Research Paper

Antimicrobial and efflux pumps inhibitory activities of *Eucalyptus grandis* essential oil against respiratory tract infectious bacteria

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Essential oils obtained by hydrodistillation from the fresh and dry leaves of *Eucalyptus grandis* were analyzed by gas chromatography (GC) and gas chromatography mass spectrometry (GC-MS). The main components of the fresh leaf oil were α -pinene (29.6%), *p*-cymene (19.8%), 1,8-cineole (12.8%) and α -terpineol (6.4%). While, the dry leaf oil had 1,8-cineole (47.4%), limonene (13.3%), α -pinene (7.5%) and spathulenol (7.1%). The antimicrobial activities of the essential oils were tested against respiratory tract infectious microorganisms (*Klebsiella pneumoniae*, *Staphylococcus aureus* and *Moraxella catarrhalis*) using the microdilution-broth methods. The minimum inhibitory concentration and minimum bactericidal concentration values of the oils ranged between (0.31 to 1.25) mg/ml and (0.63 - >5) mg/ml respectively. The minimum bactericidal concentration values caused the release of cytosolic lactate dehydrogenase (membrane damage) which ranges from 8 to 24% in comparison with Triton X-100. The accumulation of rhodamine 6G in bacterial cells showed that the essential oils were effective as efflux pump inhibitors. The results of this study support the use of the plant in folk medicine.

Key words: *Eucalyptus grandis*, myrtaceae, essential oil, antimicrobial activity, efflux pump, R6G.

INTRODUCTION

Eucalyptus (Myrtaceae), previously native to Australia, now grows in both tropical and subtropical climates round the world. Different species of these plants are known, but *Eucalyptus globulus* is the most studied (Nagpal et al., 2012). In many countries around the world, traditional healers reportedly use the leaves (fresh and dry) of different species of the genus *Eucalyptus* for asthma,

cough colds, flu, sore throats, bronchitis, pneumonia, aching, stiffness, neuralgia, and as an antibiotic (Bajpai et al., 2008; Hutchings et al., 2006; Hopkins-Broyles et al., 2004). The dry leaves are also consumed as teas or used in bathing (Chen et al., 2006). Sisay (2010), reported that the essential oils of *E. globules* and *Corymbia citriodora*, which have 70% of their constituent to be 1.8 cineole

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APPENDIX F

Interview with Traditional Healers

Research Questionnaires

Date:

Questionnaire No.

Name of the Interviewer:

Particulars of the area

GPS reading:

Name of the Area:

Name of the Village (Precise place):

Sociodemographic data

Gender:

Age:

Male		15-24	
Female		25-34	
		35-44	
		45-54	
		55-64	

Plant Species particulars

Zulu name:

Plant

1: _____

Plant

2: _____

Plant

3: _____

Plant

4: _____

Scientific name:

Plant

1: _____

Plant

2: _____

Plant

3: _____

Plant

4: _____

English name:

Plant

1: _____

Plant

2: _____

Plant

3: _____

Plant

4: _____

Source of plant material:

Collected from the wild	
Cultivated (home-garden)	

What are the other uses of the plant?

Plant usage and collection

Question	Usage
Which part(s) is used?	
Are the plants sold?	
In which state are the plants sold? (fresh or dry)	
If collected from the wild, when? (season)	

Any specific time of collection during the day?	
What places does the plant prefer to grow in? (wetland, dry land, forests, old fields, as weeds among the plants)	

Preparation Method:

a) How is the medicine taken (e.g. by mouth or as enema)?

b) How is the medicine prepared?

Storage Method:

Dosage:

a) What is the dosage (e.g. one cup three times a day)? _____

b) For how many days is the medicine taken? _____

c) Are there any known side effects? _____

d) Where did the knowledge come from (e.g. grandmother, relative)?

Age Group:

Infants	
Children	
Adults	