The chemical composition, antimicrobial and antioxidant properties of the essential oils of *Tulbaghia violacea* Harv. and *Eucalyptus grandis* W. Hill ex Maiden.

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The chemical composition, antimicrobial and antioxidant properties of the essential oils of *Tulbaghia violacea* Harv and *Eucalyptus grandis* W.Hill ex Maiden.

By

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DECLARATION

The experimental work described in this dissertation was conducted in the Department of Biochemistry and Microbiology, Faculty of Science and Agriculture, University of Zululand between April 2010 – January 2012, under the supervision of Prof.A.R. Opoku and Prof. A.O. Oyedeji.

This study represents the original work by the author. Where use was made of the work of others, it has been duly acknowledged in the text.

I declare the above statement to be true.

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Prof. A.O. Oyedeji

DEDICATION

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

Mrs Modupe Olusola Soyingbe (1942 – 2005)

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.

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

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Am forever grateful to my family for their support, prayers and financial assistance. 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 tremediously. Thank you all and God bless u all.

ABSTRACT

Tulbaghia violacea Harv. and *Eucalyptus grandis* W. Hill ex Maidan are medicinal plants used by Zulu traditional healers for managing respiratory track diseases such as asthma and bronchitis. This study was designed to evaluate the chemical composition, antioxidant and antibacterial activities of the essential oils hydrodistilled separately from the rhizomes of *Tulbaghia violacea* and the leaves of *Eucalyptus grandis.*

Chemical profile of the oils were carried out using GC and GC-MS. The main constituents of the essential oils of *Tulbaghia violacea,* were found to be 2,4-Dithiapentane (51.04%), p- Xylene (4.43%),Chloromethylmethyl sulfide (8.62%), O-Xylene (6.08%), Thiodiglycol (6.17%), and p- xylol (5.88%); these together constituted 82.22% of the extracted oil. The main constituents (81.44%) of the essential oils of *Eucalyptus grandis* were m- Xylene (33.04%), Ethylbenzene (11.59%), Eucalyptol (15.50%), p- Xylene (9.61%), Limonene (3.48%), Operea 1(3.30%), p-cymene (2.75%) and Toluene (2.17%).

While the oils of *Tulbaghia violacea* showed very weak activity (≤ 50%) in the scavenging of DPPH and ABTS radicals, they strongly (63% and 61%) scavenged nitric oxide and chelated Fe^{2+} ions respectively. The essential oils of *Eucalyptus grandis,* on the other hand, had a better scavenging activity for DPPH and ABTS, and the other free radicals tested (≥ 50%), but poorly chelated Fe^{2+} ions.

The antimicrobial activity of the essential oils carried out on both Gram positive and Gram negative bacteria showed that the oils of *Tulbaghia violacea* were affective against 8 of the 16 microorganisms tested with minimum inhibitory concentration (MIC)

values ranging from 2.5 mg/ml - 5.0 mg/ml; the oils of *Eucalyptus grandis* were active against 13 of the 16 organisms tested with the MIC's ranging from 0.625 mg/ml – 5.0 mg/ml, and the minimum bactericidal concentration (MBC) value determined for 4 of the bacteria used, ranging from 2.5 mg/ml – 10 mg/ml. The essential oils of *Eucalyptus grandis* were also tested against 8 bacteria that were resistant to antibiotics (CIPRO: Levo, Clindamycin, Gentimicin, Penicillin, OxaClox, Oxameth, Cotrimoxazole and Ampicillin) and were seen to show high activity against 7 of the 8 with MIC ranging from 5 mg/ml – 10 mg/ml. The studies on the effect of the essential oils on the DNA of the susceptible microorganisms revealed that the oils could not damage the microbial DNA.

The cytotoxicity levels of the *T. Violacea* essential oils against HEK293 and HepG2 cell lines were low (IC_{50}values of 1218 µM and 1641 µM respectively).

It is apparent that the bioactivity of the essential oils of *T. violacea* and *E. grandis* contribute to the use of these plants in folk medicine.

LIST OF ABBREVIATIONS

AA Ascorbic acid

ABTS 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)

BHT Butylated hydroxytoluene

CCM Cell culture medium

DMSO Dimethyl sulfoxide

DPPH 1,1′-diphenyl-2-picrylhydrazyl

EDTA Ethylenediaminetetra-acetic acid

GC Gas chromatography

GC-MS Gas chromatography-mass spectroscopy

IC_{50} Inhibitory concentration with 50%

LC_{50} Lethal concentration with 50% inhibition

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

PBS Phosphate buffer 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

DNA Deoxyribonucleic acid

TAE Tris base, acetic acid and EDTA Buffer.

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Chapter 1

Introduction

Plants are the source of a large proportion of medicines. Estimates show that there are about 400,000 species of higher or vascular plants (Govaerts 2001; Thorne 2002). While some of these species are equally not useful, studies have shown, somewhere between a quarter and a third of all species have been used for their medicinal properties (Small and Catling, 2002).Plants are, therefore, an important source for drug discovery (Jean-Paul *et al.,* 2005). In recent years, there has been an upsurge of interest for natural substances as phytomedicines has resulted in a more thorough investigation of plant resources. Aromatic plants as well as the essential oils from these plants have been used time in memorial in folk medicine and for the preservation of food, are known sources of natural secondary metabolites having biological activity such as antimicrobial and antioxidant action among many others (Deans and Svoboda, 1990).

Essential oils are complex mixtures of biologically active substances that have been used as flavoring agents and constituents of a number of products. Many volatile compounds naturally found in essential oils have strong antibacterial activities (Cowan 1999); essential oils are, therefore, widely used in food production industries for preservation of food and are considered generally safer than inorganic chemicals. However, it has also been established that when essential oils are misused, they lead to adverse effects in human such as skin irritation, headache and nausea (Aromacaring

2004). Interest in essential oils has revived with the popularity of aromatherapy, a form of alternative medicine which uses specific aromas carried by essential oils for healing.

Tulbaghia violacea and *Eucalyptus grandis* are some of the plants which have been indicated by traditional healer in the treatment of respiratory tract infections, bronchial infections, asthma and cough. The investigation of the essential oil of these plants will help to verify the rationale behind the use of this plant as a cure for these illnesses.

Chapter 2

LITERATURE REVIEW

2.1. MEDICINAL PLANTS AND TRADITIONAL HEALERS

Higher plants are ‘treasure houses’ for a repertoire of phytochemicals which serve as valuable drugs used in combating several fatal illnesses the world over (George 2001). The reliance on the use of indigenous medicinal plants has a long history (Cunningham 1998). However, “during the last 20 years, there has been a revival of interest in herbals as remedies for self-medication in the Western countries that has been termed the ‘green boom’.”(Houghton 1999). Medicinal plants form a sizeable component of traditional medicine and are mainstay for 80% of the people in developing nations (George 2001). Estimates in 1996 showed that plant material make up the basis for 50% of western drugs (Robbers *et. al* 1996). It is estimated that three quarters of the medically active plant compounds were discovered after ethnomedical survey showed the use of the plant by traditional healers (Farnsworth and Soejarto1991).

The use of medicinal plants is a basic part of African culture (Hutchings *et al*., 1996) and it is one of the oldest and most diverse the world over (Van Wyk and Wink, 2004). Also in South Africa which is a developing nation, indigenous African medicine is used alongside, Western allopathic medicine (Van Wyk and Gericke 2003), which caters for different people of different cultures. Traditional healing which makes use of local herbs is widely practised in Zululand (Gumede, 1989).The medicinal effect of various plants traditionally used to cure different ailments has been well documented (George *et al*., 2001; Opoku *et al*., 2002; Jean-Paul *et al.,* 2005; Iwalewa *et al*., 2007; Bibhabasu Hazra a *et al.,*2008).

The therapeutic properties of medicinal plants have been rightly attributed to their constituent phytochemicals (e.g. essential oils).Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. They are nonessential nutrients, which imply that they are not need by the human body for everyday life. It is believed that plants produce these chemicals for their protection (against insect attacks and plant diseases), recent research demonstrates that they can also protect humans against diseases.

2.2. ESSENTIAL OILS

An essential oil is a concentrated, hydrophobic liquid containing volatile aroma compounds from plants. Essential oils also volatile or ethereal oils, or oil of the plant from which they were extracted, such as *oil of clove*. Oil is said to be "essential" because they carries a distinctive scent, or essence, of the plant they are gotten from. Essential oils don't need to have any specific chemical properties, apart from passing on characteristic fragrances. They are not essential fatty acids which humans and other animals must ingest because they are required by the body for a balanced diet, However, the body does not synthesise them; examples are α-linolenic acid (an omega-3 fatty acid) and linoleic acid (an omega-6 fatty acid).

The essential oils are stored in flowers, seeds, roots, barks and fruits of plants to mention but a few. Aliphatic hydrocarbons, monoterpenoids and sesqiterpenoids constitute the major component of essential oils.

2.2.1. USES OF ESSENTIAL OILS

Medicinal plants as well as essential oils has been used many years ago in food preservation, perfumery, aromatherapy, spices and for oral care products cause of their medicinal properties (Buchbauer, 2000). A wide range of essential oils have been used for their medicinally properties throughout history. Medical uses of essential oils have been claimed to treat a wide range of diseases from skin infections to cancer, which have been recorded in history. Importance of essential oils revived recently with the popularity of aromatherapy, a form of alternative medicine that uses specific aromas from essential oils for curative effects, by diffusing them in the air using a nebulizer or by burning over candle flame, or just as incense (Lehrner. *et al.,* 2005).

2.2.2. BIOLOGICAL ACTIVITIESOF ESSENTIAL OILS

Worldwide attempts are increasing in screening plants for the biological activities and also the activities of their essential oils, from chemical and pharmacological properties to their therapeutic properties.(Sonbolia *et al.,* 2005; Skaltsa *et al.*, 2003; Tzakou and Skaltsa, 2003).

Essential oils of varieties of plants have been seen over the years to posses' useful biological and pharmacological properties like antimicrobial (Kezemi *et al.,* 2011; Vale-Silva *et al.,*2009; Gulluce *et al*. 2006; Altanlar *et al*.,1999; Janssen *et al*.,1987and Kurita *et al.,* 1981), antinociceptive (Quintão *et al.* 2010; Sulaiman *et al.* 2009), anti – inflammatory (Maxia *et al.,* 2011; Chao *et al.,* 2005), vasorelaxant properties (Chiara *et al.,* 2010) and antioxidant properties (Kezemi *et al.* 2011; Kadri *et al.,*2011; Gulluce *et al*. 2006),

2.2.3. ANTIOXIDANTS

Free radicals are atoms or group of atoms with an odd (Unpaired) number of electrons which are formed when oxygen interact with certain molecules. Free radicals could be by-products of the normal ongoing biochemical processes in the body such as mitochondrial respiration and liver oxidases and xanthine oxidase activity. Atmospheric pollutants, drugs and xenobiotics contribute to the production of free radicals (Saha *et al*., 2008). Free radicals can be classified as reactive oxygen species (ROS) (e.g. $\cdot O_2^-$, $OH^\cdot$) and reactive nitrogen species (RNS) (e.g. $NO^\cdot$, $\cdot OONO^-$). Superoxide (O_2^-) can give rise to a non-radical but potent oxidant hydrogen peroxide (H_2O_2), and the most reactive hydroxyl radical ($OH^\cdot$) (Hsu *et al*., 2007, Ambrosio *et al*., 1997)

Major damages occur when free radicals bind to cellular components such as DNA, or other cell membranes. Free radicals have been implicated to be the cause of some pathophysiological conditions such as neurodegenerative diseases, autoimmune diseases, arthritis, cardiovascular diseases and even aging (Moore and Roberts, 1998; Atawodi, 2005).

The human body has various defensive systems that copes with the devastating effects of free radicals; these consist of enzymes and some biomolecules (Gutteridge and Halliwell, 1988). These defensive systems prevent, slow or minimise the damage caused by the free radicals.

Antioxidants are molecules which interact with free radicals and neutralize the chain reaction before tissues and other organs are damaged. There are several other enzyme systems (superoxide dismutase, catalase, glutathione reductase, and glutathione

peroxidases) that scavenge free radicals within the body, other antioxidants are vitamin E, beta-carotene, and vitamin C, selenium. The body cannot manufacture these micronutrients, so they must be supplied in the diet (NEJM, 1994). Antioxidants not only inhibit the formation of free radicals, but also scavenge and reduce the existing free radicals. Antioxidants usually work by donating electron or proton to the free radical which in turn makes them more stable, and become harmless to other chemical and/or biological structures. Antioxidants activity could be the mechanism by which plants used by Zulu herbalist exert their healing properties (Opoku *et al*., 2000 2002; Lin *et al,* 1999). The common synthetic antioxidants include butylatedhydroxytoluene (BHT), butylatedhyroxyanisole (BHA) and Trolox which are commonly used in processed foods. They are however known to exhibit some side effects (Ito *et al.*, 1983). Hence, the research on new plant based antioxidants is of utmost importance.

Plant secondary metabolites are major sources of the natural antioxidants. Among the various plant secondary metabolites, polyphenolic compounds are the most powerful natural antioxidants. They are singlet oxygen quenchers, free radical scavengers, metal ion chelators and reducing agents (Akular and Odhav, 2008; Hsu *et al*., 2007). Antioxidant activities of essential oils have been studied widely and reported Ramzi Ahmed Mothan (2011) working with the essential oils of *Nepetade flersiana* growing in Yemen showed that the oil was able to reduce DPPH and to demonstrate a moderate antioxidant activity although, the observed low antioxidant activity could be associated with low content of phenolic compounds such as thymol and carvacrol in the investigated oil. The essential oils from guava stem bark were seen to be a weak proton donor in DPPH reaction. However, compared favorably with α-tocopherol a good

scavenger of hydroxyl radical (Fasola *et al*., 2011). Kadri *et al.* (2011) while working on the essential oil from aerial parts of *Artemisia herba-alba* grown in Tunisian semi-arid region postulated that antioxidant activities of the essential oil studied may be a potential source of natural antioxidants in foods in order to find possible alternative to synthetic antioxidant, and the pharmaceutical industry for the prevention and the treatment of various human diseases. Inès Hammami *et al.* (2011) discovered that the essential oil obtained from flowers of *G. sanguineum L*. possessed antibacterial and antioxidant activities. Antioxidant properties of essential oils may make them a very good candidate for use as natural antioxidants and also a model for new free radical scavenging drugs.

2.2.4. ANTIBACTERIAL ACTIVITY OF ESSENTIAL OILS

Antibiotic resistance of organisms is a global threat (Zinn *et al.,* 2004). There is a need to search for new compounds (that are not penicillin based) that inhibit microbial growth. Essential oils are known to exhibit antimicrobial properties that are lethal or static to the growth of bacteria, fungi, or virus (Oka *et al*., 2000; Janssen *et al*., 1987; Kurita *et al*., 1981). Essential oils are used in the prevention and treatment of infections, with respect to their preservative and antimicrobial properties, in food products, in cosmetics and as disinfectants (Palevitch, D 1994, Suppakul *et al.,* 2003a, b). Seenivasan *et al*. (2006) while working on the *in vitro* antibacterial activity of six plant essential oils observed that Cinnamon, clove and lime oils inhibited antibacterial activity on both gram-positive and gram-negative bacteria. The minimum inhibitory and bactericidal concentration values were reported for the essential oils of *Eucalyptus globulus* as well as that of *Thymus algeriensis*, suggests that these oils have the potential to be used as natural agents in

preservatives for food and pharmaceutical products (Abdenour *et al.,* 2011). The essential oil of *Blumea megacephala* is a newly discovered potential source of natural antimicrobial compounds (Liang Zhu *et al.,* 2011). The study of Lalitha *et al.,* (2011) confirms that many essential oils as well as plant extracts possess *in vitro* antifungal and antibacterial activity. However, issues of safety and toxicity still need to be addressed seriously.

2.3.5. CYTOTOXICITY ASSAY

Cell line based bioassays are easy to perform and they are believed to provide reliable and valid results (Mire- Sluis *et al*., 1995)**.** They are also considered a better replacement of 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 after which the cell death are determined. Cell lines have also been mostly used in research and drug development as models of normal and cancer tissues. The cytotoxicity of the essential oils is carried out to ascertain the level of toxicity of the oils to damage tissues, cell wall and cell membrane. Essential oils easily pass through Cytoplasmic membrane, thereby making it easy to disrupt its structure and make it permeabilised. Cytotoxicity can cause damage to cell-membrane. Essential oils have ability to coagulate the cytoplasm, hence damaging lipids and proteins (Burt, 2004). Hence, the possibility of using essential oils as anticancer and antitumor can be verified with its cytotoxicity.

Essential oils obtained from various plant species are recently gaining much scientific and public interest because of their multifarious uses and diverse biological activities (Abdullah 2009)

Ethnobotanical survey (see Appendix c) carried out amongst traditional healer in the Empangeni region of Kwa Zulu Natal, South Africa, suggests that *Tulbaghia violacea* and *Eucalyptus grandis* are some of the medicinal plants which are widely used for the treatment of respiratory tract infections as well as Asthma, cough, colds, flu, sore throats and bronchitis. Current research at the University of Zululand focuses on the validation of the use of medicinal plants and essential oils by Zulu traditional healer for the treatment of various illnesses (Lawal and Oyedeji . 2009 a, b, c).

2.3. *Tulbaghia violacea* Harv.

Tulbhagia violacea, is also known commonly as wild garlic and has been in use customarily in the Southern African region for years, in combating fever, asthma, constipation, oesophageal cancer and hypertension (Hutchings et al., 1996; Van Wyk and Wink, 2004). *Tulbhagia violacea* is a bulbous plant with hairless leaves with a white, fleshy stalk (Van Wyk et al., 1997). (Figure 2.1) It has a very strong garlic and pungent smell when crushed, which has been ascribed largely to alliin, a compound found also in garlic (Van Wyk and Wink, 2004). “It has attractive mauve or purple flowers, which can be easily distinguished in the gardens of KwaZulu-Natal. *Tulbhagia violacea* is found in the Eastern Cape and Southern KwaZulu-Natal and is commonly wilde Knoffel (Afrikaans) and *isihaqa* (Zulu)” (Van Wyk *et al*., 1997).

Figure: 2.1. *Tulbhaghia violacea* H*arv.* (Floridata 2006)

2.3.1. TRADITIONAL USES OF *Tulbaghia violacea*

The crushed leaves are used for sinus and headaches also to eliminate moles from the garden. It repels insects when crushed on the skin and also used for coughs and colds. The bulbs are used for asthma and pulmonary tuberculosis and to combat intestinal worms. The Zulus eat the leaves and flowers as spinach and also for seasoning meat and potatoes. The bulbs are used as an aphrodisiac medicine, as a snake repellent. (Van Wyk *et al*., 1997).

2.4. *Eucalyptus grandis* W. Hill ex Maiden

Eucalyptus grandis is native to the east coast of Australia. Its common name is rose gum or flooded gum (a misnomer). It is a premier forest species in the Australian States of Queensland and New South Wales and it grows to 43 to 55 m tall (Figure. 2.2). (Hall *et.al.,* 1970). Massive planting programmes have been done in South Africa and Brazil. (Jacobs 1976).

Figure: 2.2. *Eucalyptus grandis* W. Hill ex Maiden (www.forestryimages.org)

2.4.1. TRADITIONAL USES OF *Eucalyptus grandis* W. Hill ex Maiden

Traditional healer use Eucalyptus to treat many illnesses such as infections, colds, flu, sore throats, bronchitis, pneumonia, aching, stiffness, neuralgia (Hutching *et al*., 1996) and as an antibiotic (Hopkins-Broyles 2004). Vivik (2008) reported its use as an antifungal agent for some skin infections. Sisay Feleke (2010) reported that the essential oils of *Eucalyptus globules* and *Eucalyptus citriodora,* which have 70% of their constituent to be 1,8 cineol (Eucalyptol) stimulate respiration, relieve coughing, helps to expel mucus, relax the respiratory muscles, and it is thus used for the management of bronchitis, asthma, catarrh, sinusitis and throat infections.

2.5. EXTRACTION OF ESSENTIAL OILS

Essential oils are usually extracted by means of distillation. Other processes used are expression and solvent extraction.

2.5.1. DISTILLATION

Most essential oils, such as lavender, peppermint, and eucalyptus are distilled. Plant material like flowers, leaves, wood, bark, roots, seeds, or peel, is put into an alembic (distillation apparatus) over water. As the water heats steam passes through the plant material, vaporising the volatile compounds. The vapours is then condensed in a condenser and collected in an amber bottle. (Figure 2.3.).

Fig. 2.3. Hydrodistillation using a Clevenger type apparatus

2.5.2. EXPRESSION

Most citrus peel oils are extracted by expression, or cold-pressed, due to the large quantities of essential oils in the peel. It is a physical process whereby the oils are extracted by crushing and breaking opens the peels of the citrus.

2.5.3. SOLVENT EXTRACTION

Solvent such as hexane or supercritical carbon dioxide is used to extract the essential oils from their source. Extracts from hexane and other hydrophobic solvent are called concretes, which is a mixture of essential oil, waxes, resins, and other lipophilic plant material.

2.6. ANALYSIS OF ESSENTIAL OILS

2.6.1. PHYSICOCHEMICAL PROPERTIES

Physicochemical properties are used to determine the quality of essential oils extracted. (Fabiane *et al.,* 2008), and Sensory evaluation and refractive index have been some of those used.

2.6.2. REFRACTIVE INDEX

Refractive index or index of refraction of a substance or medium is a measure of the speed of light in that medium (Freedman and Hugh. 2008) expressed as speed of light in vacuum relative to that in the considered medium.

n = speed of light in a vacuum / speed of light in medium.

The refractive index is used to distinguish water from other solvent and also to confirm the purity of Essential oils.

2.6.3. Sensory Evaluation

This is carried out by analysing the essential oils by its smell and the sense of smell is crucial in determining this physical properties. The sensory evaluation is done

immediately after extraction and a number of panelists are used 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 is also used (Minh Tu *et al.,* 2003) to determine the smell of essential oils used.

2.7. GAS CHROMATOGRAPHY

Gas chromatography or (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. A mobile and a stationary phase are required. The mobile phase which is a carrier gas uses inert gas e.g. helium, argon, nitrogen, etc. The stationary phase consists of a packed column. (Eiceman *et al.,* 1994).

2.7.1. GAS CHROMATOGRAPHY-MASS SPECTOMETERY. (GC-MS)

Gas chromatography–mass spectrometry (GC-MS) is a method that combine, gas-liquid chromatography and mass spectrometry in other to identify different substances in a compound. GC-MS is also used for identifying the complex properties of essential oils today and also in drug detection and identification of unknown samples. Molecules take different retention time to elute, from the gas chromatograph. The mass spectrometer breaks molecule into ionised fragments and thereby detecting these fragments using their mass to charge ratio.

Using the two components together, gives a better substance identification than either unit used separately. Combining the two processes helps a great deal in eliminating the possibility of error, as two different molecules would hardly act the same way when using gas chromatograph and a mass spectrometer (McLafferty *et al*., 1999).

2.8. AIMS & OBJECTIVES

Despite the many and varying pharmaceutical properties of *Tulbaghia violacea* and *Eucalyptus grandis* that are exploited by traditional healers, there has been little or no scientific verification of their therapeutic activities. To the best of the knowledge of this researcher, there is no mention of the composition, antioxidant and antibacterial activities of the essential oils of these two plants in literature. Such knowledge is essential for the complete exploitation (medicinal) of these two plants.

This project, therefore, aims to extract and investigate the composition and some bioactivities of the essential oils hydrodistilled from the rhizomes of *Tulbaghia violacea* and the leaves of *Eucalyptus grandis.*

2.8.1. OBJECTIVES

I. Collection and Identification of *Tulbaghia violacea* H*arv* and *Eucalyptus grandis* W. Hill ex Maiden
II. Phytochemical screening of the crude plant extracts of *Tulbaghia violacea* and *Eucalyptus grandis*
III. Extraction of essential oils from the Rhizomes of *Tulbaghia violacea* and from the leaves of *Eucalyptus grandis* by hydrodistillation.
IV. Investigation of the *in vitro* antioxidant activities of the extracted essential oils

V. Investigation of the Antimicrobial properties of the extracted essential oils

VI. Investigation of the microbial DNA Cleavage activity of the extracted essential oils.

VII. Evaluation of the cytotoxicity of the extracted essential oils.

Chapter 3

MATERIALS AND METHODS

Materials used in this research work are listed below and also a brief description of methods for the research is given; a full and detailed method of the reagent preparations and methods are presented in Appendix A and B respectively.

3.1. MATERIALS

3.1.1. CHEMICALS AND REAGENTS

(See Appendix A for reagent details)

1.1-diphenyl-2-picrylhydrazyl (DPPH), 2,2,-azino-bis 3-ethyl(benzthiazoline-6-sulfonic acid) (ABTS), Potassium persulfate, trichloroacetic acid (TCA), Ascorbic acid,Trolox, ferric chloride ($Fecl_3$), Iron (II) chloride tedrahydrate ($Fecl_2$), 3-(2-pyridyl)-5,6-Diphenyl-1,2,4-Triazine-4',4''-Disulfonic acid Sodium Salt (ferroxine), Sodium nitropruside, Sulphanilic acid, glacial acetic acid, naphthylethylenediamedihydrochloride (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, p-iodonitotetrazolium violet (INT), agarose gel and ethidium bromide were all obtained from Sigma-Aldrich Co. Germany.

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, Nutrient broth were all obtained from Merck.

Ampicillin and Neomycin were obtained from Oxiod. ZR Fungal/ Bacterial DNA MiniPrep™ Catalog No: D6005 supplied by Zymo research.

3.1.2. EQUIPMENT

Rotor evaporator (Heidolph—Laborota 4000)

Spectrophotometer (Spekol 1300)

Centrifuge (Eppendorf—5804 R)

pH meter (Hanna Instruments)

Incubator (Labcom)

BiotekElx 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)

UV transillumination EDAS 290 (Kodak)

3.1.3. BACTERIA STRAINS USED IN THE STUDY

Bacteria strains used in this study consisted of reference strains identified and obtained from University of Fort Hare (Microbiology Department): *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 19582), *Staphylococcus aureus* (ATCC 6538), *Staphylococcus faecalis* (ATCC 29212), *Bacillus cereus* (ATCC 10702), *Bacillus pumilus* (ATCC 14884), *Pseudomonas aeruginosa* (ATCC 7700), *Enterobacter cloacae* (ATCC 13047), *Klebsiella pneumonia* (ATCC 4352).

Also included in this study were environmental strains of; *Klebsiella pneumonia*, *Bacillus subtilis, Shigella flexineri, Salmonella* spp, *Staphylococcus epidermidis, Pseudomonas aeruginosa, Enterococcus faecalis, and Staphylococcus aureus.*

Antibiotic resistant strains of *Staphylococcus aureus* (B10808), *Staphylococcus aureus* (P12763), *Staphylococcus aureus* (P12702), *Staphylococcus aureus* (P12724), *Pseudomonas aeruginosa* (T3374), *Streptococcus viridians* S17141, *Klebsiella pneumonia* (S17298), *Klebsiella species* (S17302) were clinical isolates obtained from the Microbiology Department of the Bay Hospital (Richards Bay, SA).

The stock cultures were maintained at 4°C on Mueller-Hinton agar (Oxoid) slants.

3.2. **METHODOLOGY**

(See appendix B for details)

3.2.1. COLLECTION AND IDENTIFICATION OF PLANT MATERIAL

The plant, *Tulbaghia violacea* Harv. was collected from Leribe district, Lesotho. *Eucaylptus grandis* W. Hill ex Maiden, was collected from Mbakanathubana in Eshowe, Kwa Zulu Natal. The plants were taken to the Department of Botany, University of Zululand, KwaDlangezwa for identification and voucher specimens (OS.01/ OS.02 UZ) were deposited at the University Herbarium.

3.2.2. PHYTOCHEMICAL ANALYSIS

Phytochemical screening of the plant materials was done using standard procedures described by Harbone (1973); Odebiyi and Sofowora (1978); Sofowora (1984) for the determination of secondary metabolites.

3.2.3. EXTRACT PREPARATION

Freshly collected rhizomes of *Tulbaghia violacea* were washed and cut into small pieces (2cm), and the leaves of *Eucalyptus grandis* were picked from the stalk. The rhizomes (500g) of *Tulbaghia violacea* and the leaves (250g) of *Eucaylptus grandis* were separately subjected to more than three hour hydrodistillation using a Clevenger-type apparatus. The essential oils so obtained were dried over anhydrous sodium sulfate and then dissolved in methanol which was then stored at -5^{o}C until required.

3.2.4. PHYSICAL PROPERTIES

Physicochemical properties are useful methods in determining the quality of essential oils (Guenther *et al.,*1975). Quality assessment of essential oils requires different analysis some of which are sensory evaluation and refractive index. The sensory evaluation of the oils was by the panel of students in the Department of Biochemistry and Microbiology. The refractive index was determined with the Abbey refractometer.

3.2.5. ANTIOXIDANT ACTIVITY.

3.2.5.1. 2, 2'- DIPHENYL-1-PICRYLHYDRAZYL (DPPH) RADICAL SCAVENGING ACTIVITY.

The antioxidant activity of the essential oils of *Tulbaghia violacea* and *Eucalyptus grandis* were measured in terms of hydrogen donating or radical scavenging ability, using the stable

radical, DPPH (Brand-Williams, *et al* 1995). Methanolic solutions (2ml of 5mg/ml-100mg/ml) of the oils were incubated with DPPH (2ml, 2% in methanol) and absorbance (514nm) measured after one hour. The percentage scavenging activity was determined (see section 3.2.5.7).

3.2.5.2. 2, 2'- AZINOBIS (3-ETHYLBENZOTHIAZOLINE-6-SULFONATE) (ABTS) RADICAL SCAVENGINGACTIVITY.

The ABTS scavenging activity was done by the *in vitro* method of Re *et al*. (1999), which makes use of a pre-generated radical monocation 2,20-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS+). Methanolic solutions (1 ml of 5 mg/ml-100 mg/ml) of the essential oils were added to a 1 ml solution of ABTS mixed and allowed to stand for 6 min. The absorbance was read at 734nm and the percentage scavenging activity was calculated (see section 3.2.5.7).

3.2.5.3. NITRIC OXIDE RADICAL SCAVENGING ACTIVITY

The nitric oxide radical scavenging activity was determined using the method of Garret (1964). Methanolic solutions (0.5 ml of 5 mg/ml – 100 mg/ml) of the essential oils were added to a reaction mixture of sodium nitroprusside (2 ml of 10 mM) and phosphate buffer saline (0.5 ml of 0.01 M, pH 7.4), incubated for 150 min at 25^{o}C.Sulphanilic acid reagent (1 ml of 0.33%) was added to 0.5 ml of the incubated solution and allowed to stand for 5 min. Then napthylethylenediamine dihydrochloride (1 ml of 0.1%) was added and the mixture incubated at 25^{o}C after which absorbance was measured at 540nm and the percentage scavenging activity estimated (see section 3.2.5.7).

3.2.5.4. FE^{2+} CHELATION

The ferrous ion chelating activity of the essential oils was determined using the method of Decker and Welch (1990). Methanolic solutions of different concentrations of the essential oils (1 ml of 5 mg/ml-100 mg/ml) were added to a solution of deionised water (3.75 ml), $FeCl_2$ (0.1ml of 2mM) and ferrozine (0.2 ml of 5 mM). The mixture was allowed to stand for 10 min and absorbance read at 562nm and the percentage scavenging calculated (see section 3.2.5.7).EDTA and citric acid were used as positive control.

3.2.5.5. DETERMINATION OF THE REDUCING POWER

The reducing power of the essential oils was determined using the method of Oyaizu (1986). Methanolic solution of different concentrations of the essential oils (1 ml of 5 mg/ml - 100 mg/ml) were added to a solution of pottasuim ferricyanide (2.5 ml of 1%), Trichloroacetic acid (2.5 ml of 10%), ferric chloride (0.5 ml of 0.1%), and phosphate buffer (2.5 ml 0f 0.2 M, pH 6.6), which was then centrifuged (1000 rpm for 10 min at 37^{o}C) and absorbance read at 700nm against an appropriate blank solution. The higher the absorbance the greater the reducing power of the extract.

3.2.5.6. SH- SULPHYDRYL CONTENT

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

3.2.5.7. CALCULATION OF PERCENTAGE INHIBITORY EFFECT OF PLANT EXTRACTS

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 effect of the extract on each parameter was calculated as:

$$\% \text{ Inhibition} = \left(1 - {}^{At}/_{Ao}\right) \times 100$$

where, A_0 is the absorbance value of the fully oxidised 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.

3.2.6. ANTIMICROBIAL ACTIVITY OF THE ESSENTIAL OILS.

3.2.6.1. AGAR DISK DIFFUSION METHOD.

The essential oils antibacterial properties were done using the agar disk diffusion method (Van Vuuren and Vijoen, 2006). Bacteria were grown overnight in 20ml nutrient broth at 37^{o}C. The cultures were then diluted to the McFarland no.5 standard (1.0×10^8 CFU/ml). Standard Petri dishes containing nutrient agar were then inoculated with the bacteria suspension (1.0×10^8 CFU/ml). Sterile paper disk (6 mm) 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^{o}C for 24hr and the zone of inhibition measure using a ruler. Tests were performed in duplicates; Ampicillin and Neomycin were used as positive control.

3.2.6.2. MINIMUM INHIBITORY CONCENTRATION

The minimum inhibitory concentration (MIC) of the essential oils was determined as described by Eloff's (1998). Nutrient broth (50 µl) was added to all wells of the microtitre plate; 50 µl of 10 mg/ml of the essential oils in 10% DMSO was added to the well in row A and then serially diluted down the rows from row A, the remaining 50 ml was then discarded. Bacteria culture (50 µl) of McFarland standard was then added to all the wells and then incubated at 37^{o}C for 24hr. 20 µl of 0.2 mg/ml p-iodonitotetrazolium violet (INT) solution was then added to each well and incubated at 37^{o}C for 30 min. A reddish coloration which is as a result of INT being reduced by the metabolic active microorganisms to formazan indicated microbial activity. The MIC which is the lowest concentration at which no visible microbial growth is seen is then recorded.

3.2.6.3. MINIMUM BACTERICIDAL CONCENTRATION

The minimum bactericidal concentration (MBC) which is defined as the lowest concentration of the sample at which inoculated bacterial strains are completely killed 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^{o}C for 24hr.Bacteria treated with Ampicillin and neomycin were used as positive controls.

3.2.6.4. DNA DAMAGE (CLEAVAGE)

Fresh bacterial cultures were treated with the essential oils (MBC concentration). Treated as well as untreated cultures were centrifuged (10000 rpm, 1 min) and the pellets obtained were re-suspended in lysis solution (ZR fungal/bacterial DNA MiniPrep^{TM}) for 5 min and centrifuged (10,000 rpm for 1 min). Fungal / Bacterial DNA Binding buffer (ZR

fungal/bacterial DNA MiniPrep^{TM}) was then added to the suspension and centrifuged (10,000 rpm for 1 min). The supernatant collected was added to a DNA Pre Wash Buffer (ZR fungal/bacterial DNA MiniPrep^{TM}) and centrifuged (10,000 rpm for 1 min) after which Fungal/ Bacterial DNA Wash Buffer (ZR fungal/bacterial DNA MiniPrep^{TM}) washing buffer was added and then centrifuged (10,000 rpm for 1 min). The precipitated DNA was then eluted using the ZR fungal/bacterial DNA MiniPrep^{TM} DNA elution Buffer and centrifuged at 10,000 rpm for 30sec. Cleavage was analysed by agarose gel electrophoresis (150 V for 30 min) TAE buffer (45 mMTris base, 45 mM boric acid, 1 mM EDTA pH 8.0) along with standard DNA maker. The DNA was then visualised using a vilberlourmate Gel documentation system.

3.7. CYTOTOXITY ASSAY

3.7.1. MTT CELL PROLIFERATION ASSAY

The cells used for this assay were the Human embryonic kidney cells (HEK293) and Human hepatocellular carcinoma cells (HepG2). Cells were cultured in 25 cm^2 flask to confluenecy, which was then trypsinised and plated into 48 well plates at 2.5 x 10^4 seeding density per well . Cells were then incubated overnight at 37^oC. Fresh medium (MEN +Glutmax + antibiotic) were then added. The essential oils (50-350µg) were then added in triplicate and incubated for 4 hr. Medium was then removed and replaced by complete medium (MEM + Glutmax + antibiotics + 10% Fetal bovine serum). After 48hr cells were subjected to the MTT assay (Mosman, 1983).

3.8.1. CHEMICAL COMPOSITION OF ESSENTIAL OILS

3.8.2. GAS CHROMATOGRAPHY / MASS SPECTROMETER (GC/MS)

Gas Chromatography / Mass Spectrometer (GC/MS) of the essential oils was carried out using an Agilent Gas Chromatography (7890A) equipped with a capillary column of (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 programmed from 45^{o}C - 310^{o}C. Helium was used as the carrier gas at a flow rate of 5 ml / min with a split ratio of 1:20. 0.5 µl of the essential oil was diluted in hexane and 0.5 µl of the solution was manually injected into the GC/MS.

Chapter 4

Results

The results presented here were obtained when the essential oils (obtained through hydro-distillation) of *Tulbaghia violacea* rhizomes and the leaves of *Eucaylptus grandis* were characterised, assessed for antioxidant, antimicrobial, and cytotoxic activities.

4.1. Phytochemical screening of the crude plant materials of *Tulbaghia violacea* and *Eucalyptus grandis*

Table 4.1. Phytochemicals of the rhizomes of *Tulbaghia violacea* and the leaves of *Eucalyptus grandis*

Phytochemicals	*T. violacea*	*E. grandis*
Alkaloids	+	+
Saponins	-	+
Tannins	+	+
Anthraquinones	-	-
Cardiac glycosides	-	+
Flavonoids	+	+

Key: + present; - absent.

Table 4.1.Shows the phytochemicals present/absent in the 2 plants parts studied. The presence of Tannins, Alkaloids and Flavonoids were observed in the rhizomes of *Tulbaghia violacea* while Anthraquinones, Cardiac glycosides and Saponins were

absent. The leaves of *Eucalyptus grandis* on the other hand were positive for all the phytochemicals tested for except Anthraquinones.

4.2. Physicochemical properties of the essential oils

The physicochemical properties of the essential oils are summarised in Table 4.2, while the oil of *Tulbaghia violacea* had a strong pungent (garlic) smell; the *Eucalyptus grandis*' oil possessed a sweet minty smell.

Table 4.2. Physicochemical properties of the essential oils of *Tulbaghia violacea* and *Eucalyptus grandis*

Physicochemical properties.	***T. violacea***	***E. grandis***
Percentage Yield	0.15%	0.80%
Physical Appearance	Pale yellow	Light green
Smell	Strong Pungent smell	Sweet minty
Refractive index	1.5078	1.4865
(SH) sulfhydryl content	37.51µg/g (w/w)	9.00 µg/g (w/w)

4.3. Chemical composition of essential oils

The chemical (and percentage) compositions of the essential oils of the rhizomes of *Tulbaghia violacea* and the leaves of *Eucalyptus grandis* (identified according to their retention time, spectrometric electronic libraries (WILEY NIST)) are presented in tables 4.3 and 4.4 respectively (See Appendix C for the GC-MS spectra). A total of 16

constituents (representing 95.95%) were identified in the oil of *Tulbaghia. violacea*. The major components are 2,4-Dithiapentane (51.04%), ρ-Xylene (6.43%), Chloromethylmethyl sulfide (8.62%), σ- Xylene (7.38%), Thiodiglycol (6.17%), and ρ - Xylol (5.88%). The major compounds are Sulphur- containing compounds, allyl disulfide and allyl trisulfide. Similar compound (trisulfide-di-2-propenyl, disulfide, di-2-propenyl and trisulfide-methyl-2-propenyl) have been identified as the major constituents in the essential oil of *Allium sativum* (Dieumou *et al.,* 2009) and garlic (Kimbaris *et al.,* 2008). The major diallyl sulfide in *Tulbaghia Violacea* is 2,4- Dithiapentane which is 51.04% of the constituent of the essential oil.

Table 4.3. Volatile constituents of the essential oil of *Tulbaghia violacea*

Peak No.	Compounds	R.T[a]. (Mins)	Relative Abundance %
1.	Acetamide, 2-cyano	4.16	2.08
2.	Chlorodifluoro acetamide	4.20	2.70
3.	σ- xylene	4.24	7.08
4.	(E)-2-heptenoic acid	5.12	1.10
5.	ρ-xylol	5.87	5.88
6.	ρ-xylene	6.17	6.43
7.	Thiodiglycol	19.24	6.17
8.	2,4-Dithiapentane	23.11	51.04
9.	Chloromethylmethyl sulfide	28.78	8.62
10.	Acetamide	29.67	1.64
11.	phthalic acid 2-ethylhexyl isobutyl ester	29.71	0.92
12.	Phthalic acid	29.77	1.08
13.	phthalic acid, heptyl 2-methylallyl ester	29.87	0.32
14.	Nonadecane	36.98	2.21
15.	Heptacosane	38.70	3.02
16.	Tetracosane	40.33	1.96

[a]Retention time.

The chemical composition of *Eucalyptus grandis* (Table 4.4) revealed that a total of 22 constituents were identified, representing 95.95% of the major components of the essential oil. The main constituents included m-Xylene (33.04%), Ethyl benzene (11.59%), Eucalyptol (1,8-Cineole) (15.50%), p- Xylene (9.61%) and Limonene (3.48%). As in many other Eucalyptus species, *E. grandis* oil is characterised by a high 1, 8 cineole content. Damjanović-Vratnic *et al.,* (2011) reported that the major component of the essential oil of *Eucalyptus globules* Labill. from Montenegro, was 1,8-cineole (85.8%), while α-pinene (7.2%) and β-myrcene (1.5%) were minor main components. Other compounds identified in the oil obtained were β-pinene, limonene, α-phellandrene, γ-terpinene, linalool, pinocarveol, terpinen-4-ol, and α-terpineol. A total of thirty nine compounds representing 98% of total oil have been identified in the oil of *Eucalyptus loxophleba* (Rahimi-Nasrabadi and Batooli 2011) 1,8-cineole (39.4%), methyl amyl acetate (19.8%), aromadendrene (10.0%), viridiflorol (6.0%) and α-pinene (5.4%) were the major constituents. Forty three components were identified in the *Eucalyptus leocoxylon* oil where 1, 8-cineole (59.1%), cryptone (15.1%), α-pinene (9.9%), α-terpineol (5.1%) and globulol (5.0%) were the main components (Rahimi-Nasrabadi and Batooli 2011).

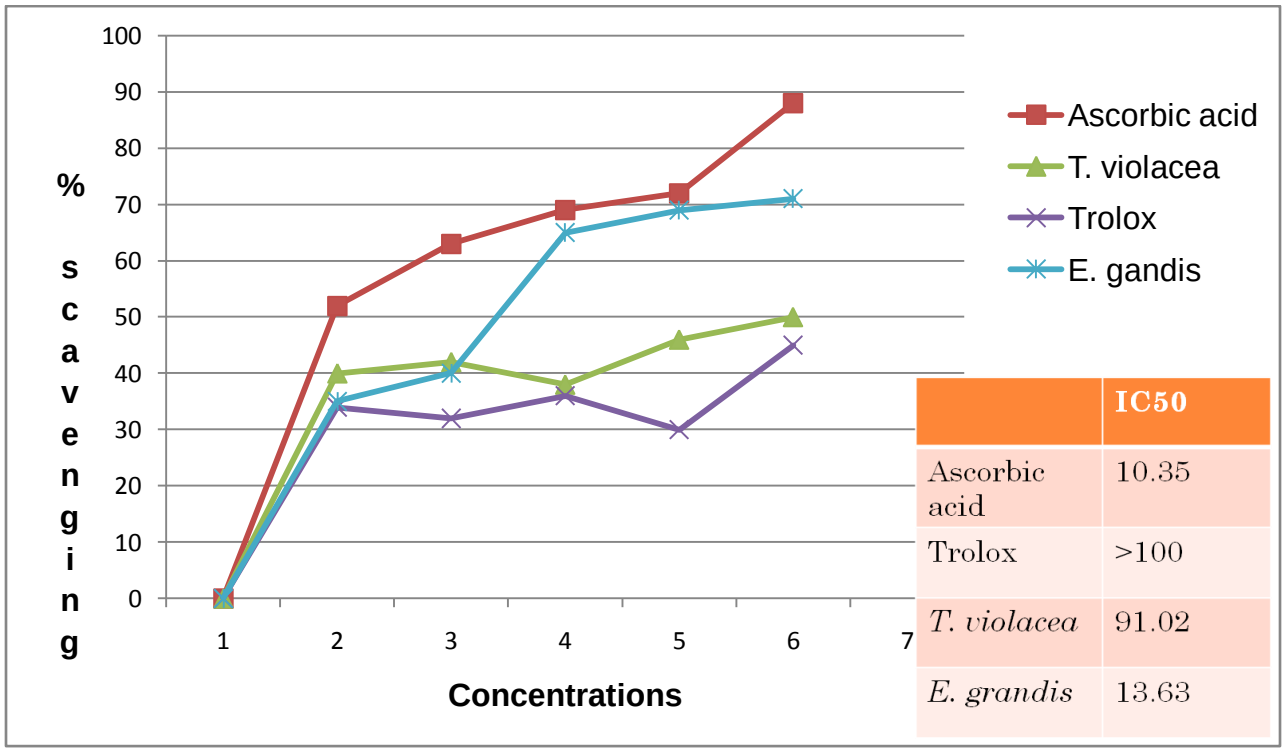
Table 4.4. Volatile constituents of the essential oil of *Eucalyptus grandis*

Peak No.	Coumpound	R.T[a]. (Mins)	Relative abundance %
1.	Dimefox	4.15	0.72
2.	Fluoxetine	4.17	1.13
3.	Acetic acid, butyl ester	4.42	1.52
4.	Ethylbenezene	5.41	11.59
5.	m-xylene	5.60	33.04
6.	ρ-xylene	6.17	9.61
7.	1R-alpha pinene	7.23	2.25
8.	Camphene	7.64	1.13
9.	Isocumene	7.76	0.71
10.	Toluene	7.99	2.17
11.	Mesitylene	8.17	1.29
12.	Hemellitol	8.17	1.29
13.	ρ-cymene	9.78	2.75
14.	Limonene	9.90	3.48
15.	Eucalyptol (1,8-Cineole)	9.99	15.50
16.	ɣ-Terpinene	10.78	1.06
17.	Gougerotin	14.24	0.96
18.	1H-pyrazole,1,3-Dimethyl-	14.52	0.86
19.	Operea 1	24.68	3.30
20.	Methoxyphenamide	24.83	1.38
21.	Phthalic acid, decyl Isobutyl ester	30.76	1.83
22.	4-nitrophenyl ester	30.78	0.57

[a] Retention time

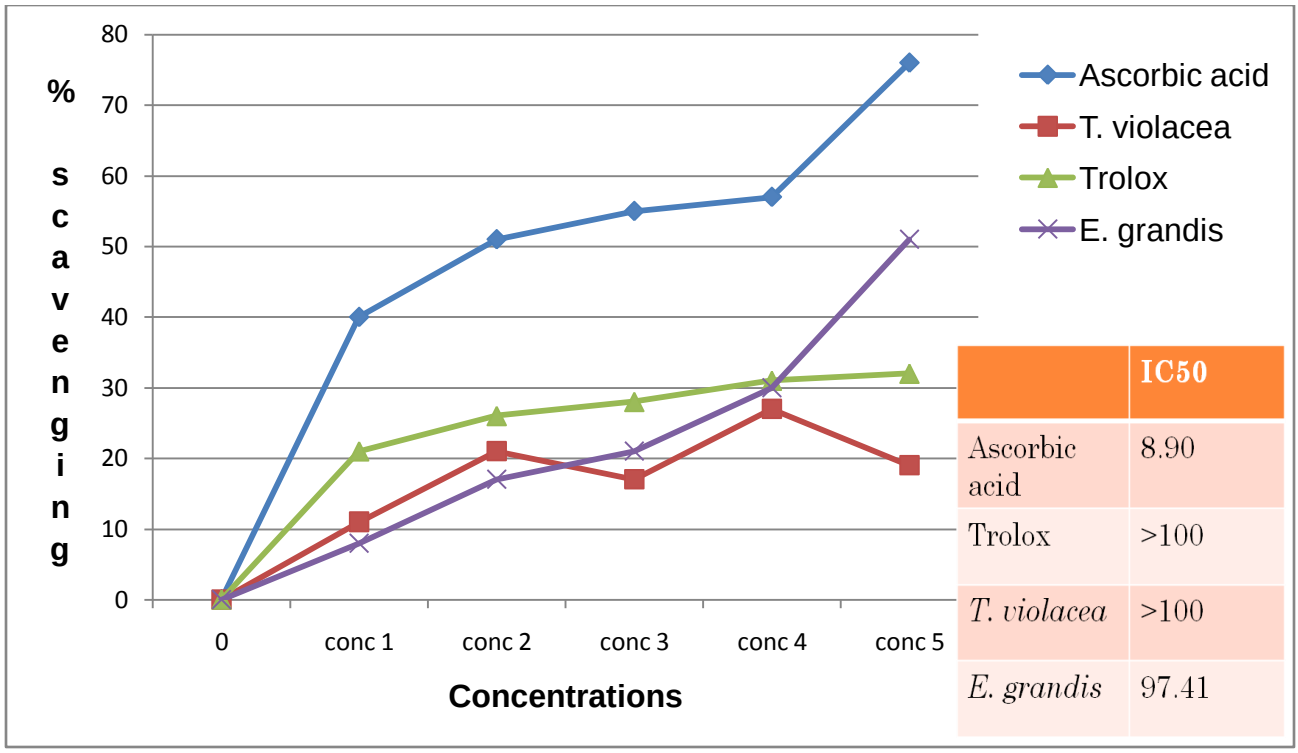
4.3. Antioxidative activities

The results presented in Figs. 4.1- 4.5 show the free radical scavenging activities of the essential oils of *T. violacea* and *E. grandis.*



	IC50
Ascorbic acid	10.35
Trolox	>100
T. violacea	91.02
E. grandis	13.63

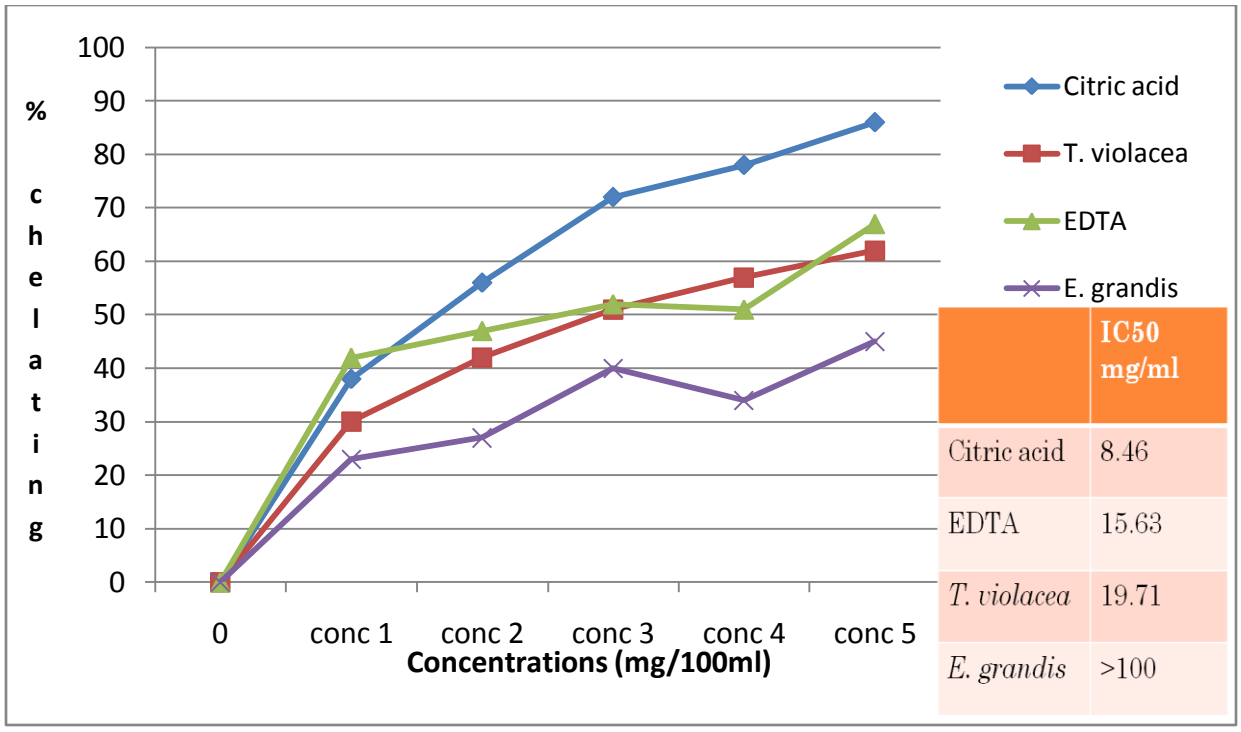
Figure 4.1. DPPH Scavenging activities of the essential oils of *Tulbaghia violacea* and *Eucalyptus grandis.* conc. 1 = 5 mg/ml, conc. 2 = 10 mg/ml, conc. 3 = 20mg/ml, conc. 4 = 50 mg/ml conc. 5 = 100 mg/ml



	IC50
Ascorbic acid	8.90
Trolox	>100
T. violacea	>100
E. grandis	97.41

Figure 4.2. ABTS scavenging activity of essential oil of *Tulbaghia violacea* and *Eucalyptus grandis.* 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

Figure 4.1 and 4.2 show the scavenging activities of the essential oils of *Tulbaghia violacea* and *Eucalyptus grandis* on DPPH and ABTS, respectively. The results indicate a poor (less than 50%) but a concentration dependent scavenging activity as compared to the standards Ascorbic acid and BHT for *Tulbaghia violacea,* and a relatively high and concentration depended scavenging activity for the essential oils of *Eucalyptus grandis* as compared with the standards.



	IC50 mg/ml
Citric acid	8.46
EDTA	15.63
T. violacea	19.71
E. grandis	>100

Figure 4.3. Chelating activity of essential oils of *Tulbaghia violacea* and *Eucalyptus grandis.* conc. 1= 5mg/ml, conc. 2=10mg/ml, conc. 3=20mg/ml, conc. 4=50mg/ml conc. 5=100mg/ml.

Figure 4.3 shows the chelating activity of the essential oil of *Tulbaghia violacea* as well as that of *Eucalyptus grandis.* The Fe^{2+} chelating activity of the essential oil is also concentration dependent and compared favorably to the standards as regards *Tulbaghia violacea* and a relatively low chelating activity for that of *Eucalyptus grandis* with IC_{50} greater than 100.

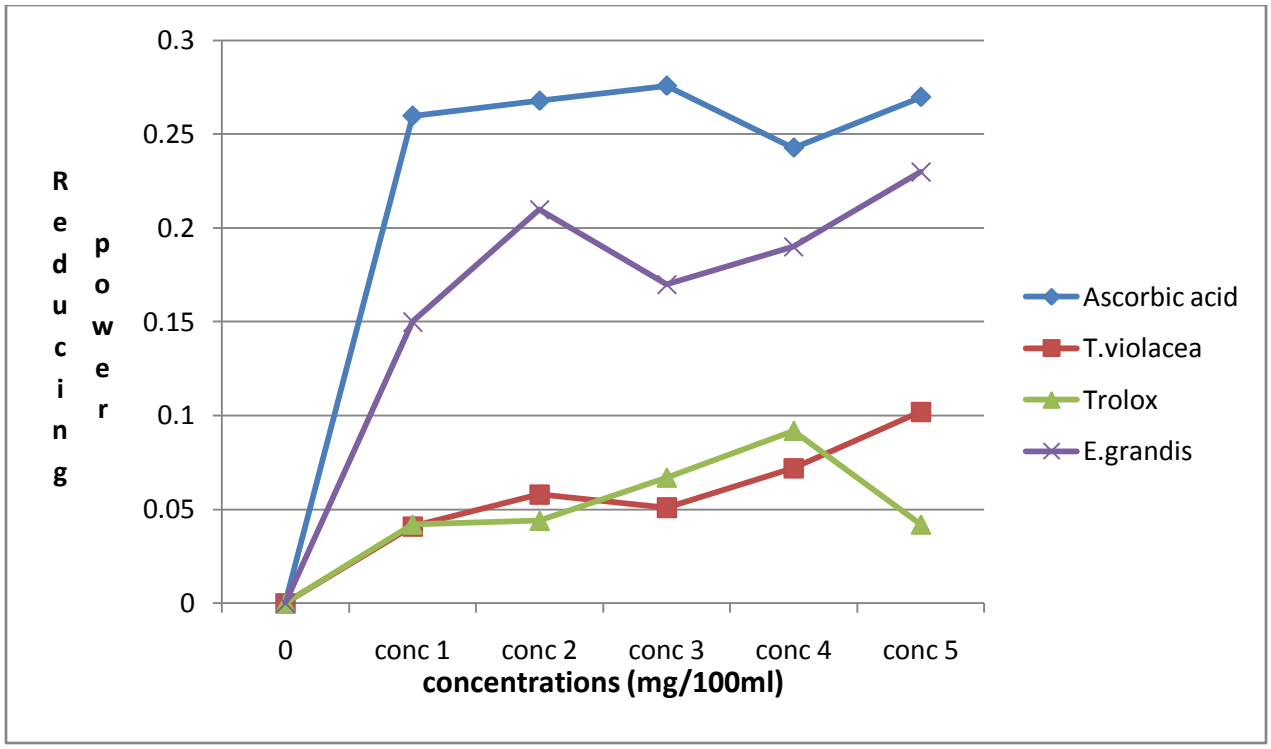
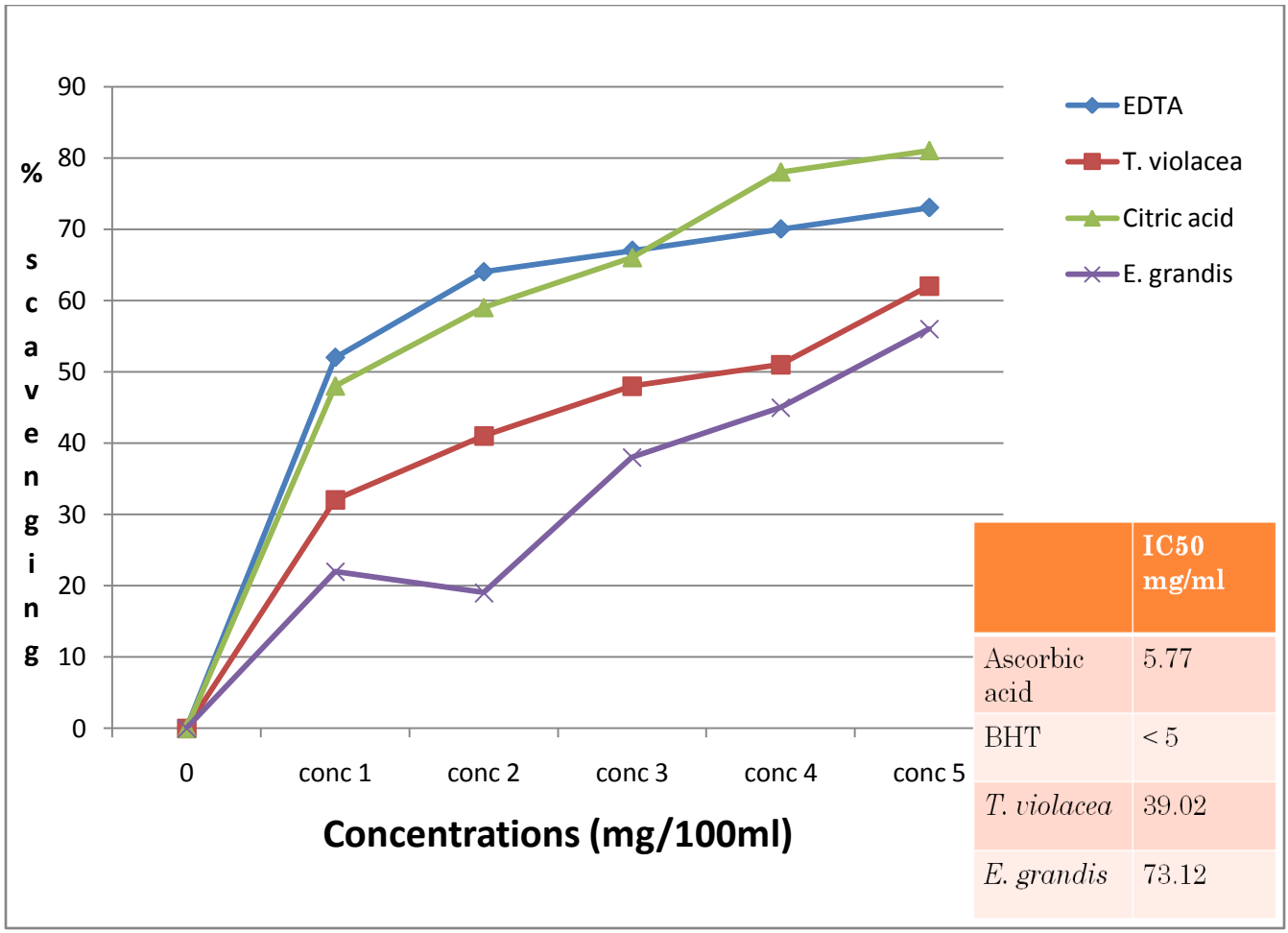


Figure 4.4. Reducing power of essential oils of *Tulbaghia violacea* and *Eucalyptus grandis.* conc. 1= 5mg/ml, conc. 2=10mg/ml, conc. 3=20mg/ml, conc. 4=50mg/ml conc. 5=100mg/ml.

Figure 4.4 shows a concentration dependent reducing power of the essential oils. The profile is similar to those of the standards, ascorbic acid and Trolox with *Eucalyptus grandis* having a relatively high reducing power while Tulbaghia violacea's reducing power was quite low.



	IC50 mg/ml
Ascorbic acid	5.77
BHT	< 5
T. violacea	39.02
E. grandis	73.12

Figure 4.5. Nitric oxide radical scavenging activity of essential oils of *Tulbaghia violacea* and *Eucalyptus grandis.* 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 nitric oxide radical scavenging activity of the essential oils presented in Figure 4.5 shows a concentration dependent and a high scavenging ability for the essential oil of *Tulbaghia violacea* and a low scavenging ability for the essential oil of *Eucalyptus grandis* with IC_{50} values of 39.02 and 73.12 for *Tulbaghia violacea* and *Eucalyptus grandis* respectively.

4.4. Antimicrobial activity of the essential oils of *Tulbaghia violacea* and *Eucalyptus grandis*

The *in vitro* antibacterial activities of the essential oils of *Tulbaghia violacea* and *Eucalyptus grandis* against the reference strains and antibiotic resistant strains are summarized in Tables 4.5 - 4.7. The results showed the essential oils of *Tulbaghia violacea* to be active against 10 of the 16 organism tested and 4 of the 8 antibiotic resistant organisms; the essential oils of *Eucalyptus grandis* were active against 14 of the 16 reference strains organisms tested and 7 of the 8 antibiotic resistant organisms tested.

4.4.1. Zones of inhibition.

Taking ≤ 9.0 mm in diameter as indicative of good activity, (see Figure 4.6), then it is apparent that the essential oils of *Tulbaghia violacea* and *Eucalyptus grandis* showed appreciable antibacterial activity against the organisms tested (Table 4.5).

(a) Clear Zone of inhibiton for Neomycin

(b) Clear Zones of inhibition of Essential oils

Figure 4.6: Zone of inhibition of the Essential oils and Standard Neomycin

Table 4.5. Antibacterial activities of the essential oils of *Tulbaghia violacea* and *Eucalyptus grandis* (Zones of inhibition)[a]

	Zones of inhibition (mm) ± SD						
Bacteria strain	Neomycin	Ampicillin	5mg/ml of the essential oils of *T. violacea*	10mg/ml of the essential oils of *T. violacea*	5mg/ml of the essential oils of *E. grandis*	10mg/ml of the essential oils of *E. grandis*	DMSO
Esherichia coli (ATCC 8739)	11.0 ± 2	10.0 ± 0	NA	NA	10 ± 0.1	20.0 ± 0	NA
Psuedomonas aeruginosa (ATCC 19582)	15.3± 1.5	9.0 ± 0.1	11.0± 0.1	12.0 ± 0.1	11.0± 0.1	19.0 ± 0	NA
Staphylococcus aureus (ATCC 6538)	16.0± 8.2	13.0 ± 0	7.0 ± 0.1	6.0 ± 0	7.0 ± 0.1	19.0± 1.3	NA
Streptococcus faecalis (ATCC 29212)	13.3± 0.6	11.0±0.2	9.0 ± 0	11 ± 0.1	10.0 ± 0	11.0± 0.1	NA
Bacillus cereus (ATCC 10702)	14.7± 2.5	14 ±0.2	6.5 ± 0.1	6.2 ± 0.1	17.0± 0.1	19.0± 0.1	NA
Bacillus Pumilus (ATCC 14884)	14.3± 1.2	13.0 ± 0	NA	NA	10.0± 0.1	14.0 ± 0	NA
Psuedomonas aeruginosa (ATCC 7700)	12.0 ± 1.2	10.0 ± 0	7 .0 ± 0.1	9.0 ± 0.1	7.0 ± 0.1	9.0 ± 0.1	NA
Enterobacter cloacae (ATCC 13047)	11.3± 1.5	11.0± 1.5	NA	NA	10.0± 0.2	14.0± 0.1	NA
Klebsiella pneumonia (ATCC 10031)	12.3± 0.6	11.0± 0.1	NA	NA	10.0 ± 0	15.0± 0.6	NA
Serratia marcescens (ATCC 6830)	15.7± 0.8	8.0 ± 0.2	NA	NA	NA	NA	NA
Acinetobacter calcoaceticus anitratus (CSIR)	14.3± 0.3	11.0± 0.1	9.0 ± 0.1	10.0 ± 0.1	NA	NA	NA

Bacillus subtilis (KZN)	11.3± 1.5	11.0 ± 0	11.0± 0.1	11.0 ± 0	14.0± 0.1	12.0 ± 0	NA
Shigella flexineri (KZN)	11.2± 1.1	10.0 ± 1	12 ± 0.1	9.0 ± 0.1	11.0± 0.1	14.0 ± 0	NA
Salmonella spp.(KZN)	17.0 ± 1	10.0 ± 0	8.0 ± 0.1	9.0 ± 0.1	11.0± 0.1	15.0± 0.1	NA
Staphylococcus epidermidis (KZN)	10.7± 1.5	9.0 ± 0.1	NA	NA	11.0± 0.1	18.0 ± 0	NA
Enterococcus faecalis (KZN)	15.3± 3.1	13.0 ± 0	11.0± 0.1	11.0 ± 0	8.0 ± 0.1	12.0 ± 0	NA

[a]Inhibition zone diameters (mm) including diameter of the sterile disc (6mm): values are given as mean ± SD (3 replicates). ND= not determined; NA= not active. ATCC= American type culture collection, USA. CSIR= Council of scientific and industrial research. S.A. KZN= Kwa Zulu Natal S.A. DMSO= Dimethyl sulfoxide. SD= standard deviation.

While *Esherichia coli* (ATCC 8739), *Bacillus Pumilus* (ATCC 14884), *Enterobacter cloacae* (ATCC 13047), *Klebsiella pneumonia* (ATCC 10031), *Serratia marcescens* (ATCC 6830) and *Staphylococcus epidermidis* (KZN) were resistant to the oil of *Tulbaghia violacea* at the concentration tested, only *Serratia marcescens* ATCC 6830 and *Acinetobacter calcoaceticus anitratus* (CSIR) showed resistance to the oil of *Eucalyptus grandis*

4.4.2. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values ranging from 0.625 mg/ml to 10 mg/ml and 2.5 mg/ml to 10 mg/ml respectively revealed a wide range of activity for the essential oils of *Tulbaghia violacea* and *Eucalyptus grandis* against reference strains organisms tested (Table 4.6). The results shows that while the essential oils of *Tulbaghia violacea* only inhibit the growth of the bacteria tested against, the essential oil of *Eucalyptus grandis* had both inhibitory and bactericidal properties against the organism tested.

(a) 96 well plates showing MIC (b) Petri dish showing MBC (c) MBC not determined

Figure 4.7. MIC and MBC activities of the essential Oils of *Tulbaghia violacea* and Eucalyptus *grandis*

Table 4.6. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the essential oils of *Tulbaghia violacea* and *Eucalyptus grandis.*

Bacteria Strains	MIC of essential oil *T. violacea* 10 mg/ml	MIC of essential oil of *E. grandis* 10 mg/ml	MIC of Ampicillin 10 mg/ml	MBC of essential oil of *T. violacea* 10 mg/ml	MBC of essential oil of *E. grandis* 10 mg/ml	MBC of Ampicillin 10 mg/ml	DMSO
Esherichia coli (ATCC 8739)	NA	0.625	1.25	NA	2.5	ND	NA
Psuedomonas aeruginosa (ATCC 19582)	2.5	10	5	>10	>10	ND	NA
Staphylococcus aureus (ATCC 6538)	10	1.25	2.5	>10	>10	ND	NA
Streptococcus faecalis (ATCC 29212)	5	2.5	5	>10	>10	ND	NA
Bacillus cereus (ATCC 10702)	10	0.625	5	>10	>10	ND	NA
Bacillus Pumilus (ATCC 14884)	NA	0.625	2.5	NA	10	ND	NA
Psuedomonas aeruginosa (ATCC 7700)	5	0.625	5	>10	>10	ND	NA
Enterobacter cloacae (ATCC 13047)	NA	1.25	1.25	NA	10	ND	NA
Klebsiella pneumonia (ATCC 10031)	NA	1.25	2.5	NA	>10	ND	NA
Serratia marcescens (ATCC 6830)	NA	5	NA	NA	>10	ND	NA
Acinetobacter calcoaceticus anitratus (CSIR)	5	2.5	NA	>10	>10	ND	NA
Bacillus subtilis (KZN)	2.5	2.5	0.625	>10	10	ND	NA
Shigella flexineri (KZN)	10	2.5	5	>10	>10	ND	NA
Salmonella spp.(KZN)	10	5	5	>10	>10	ND	NA

Staphylococcus epididirmis (KZN)	NA	10	10	NA	>10	ND	NA
Enterococcus faecalis (KZN)	5	2.5	5	>10	>10	ND	NA

MIC values given as mg/ml for essential oils, ND= not determined NA= not active, DMSO = Dimethyl sulfoxide

The activity of the essential oils on antibiotic resistant organisms tested is presented in Table 4.7. The MBC of the essential oils of *Tulbaghia Violacea* and *Eucalyptus grandis* were greater than 10 mg/ml

Table 4.7. Zone of inhibition, minimum inhibitory concentration and minimum bactericidal concentration of the essential oil of *Tulbaghia violacea* and *Eucalyptus grandis* on antibiotic resistant microorganism.

Antibiotic resistant Bacteria strains	Antibiotic resistant to	10mg/ml of essential oil of *T. violacea*	10mg/ml of essential oil of *E. grandis*	MIC of essential oil of *T. violacea* 10mg/ml	MIC of essential oil of *E. grandis* 10mg/ml	MBC of essential oil of *T. violacea* 10mg/ml	MBC of essential oil of *E. grandis* 10mg/ml
Staphylococcus aureus P12702	-	9 ± 0.1	19 ± 0	10	2.5	ND	ND
Staphylococcus aureus P12763	-	8 ± 0	14 ± 0.1	>10	10	ND	ND
Staphylococcus aureus P12724	CIPRO: Levo Clindamycin	9 ± 0.1	14 ± 0.1	10	10	ND	ND
Staphylococcus aureus B 10808	Oxa:Clox, Oxa: meth, Gentamicin, Penicillin	NA	14 ± 0.1	>10	10	NA	ND
Str.Viridans S17141	Oxa: meth, OxaClox	7 ± 0.1	18 ± 0.1	>10	5	ND	ND
Psuedomonas aeruginosa T 3374	Cotrimoxazole	NA	NA	NA	NA	NA	NA
Klebsiella Spp. S 17302	Ampicillin	NA	20 ± 0	>10	2.5	NA	ND
Klebsiella pnuemonia S 17298	Ampicillin	NA	21 ± 0.1	NA	2.5	NA	ND

Inhibition zone diameters (mm) including diameter of the sterile disc (6mm): values are given as mean ± SD (3 replicates). ND= not determined; NA= not active. DMSO= Dimethyl sulfoxide .

4.6 DNA Cleavege

The effect of the essential oil of *E. grandis* on microbial DNA (of 4 organisms that showed lowest MBC values i.e *Esherichia coli* (ATCC 8739), *Bacillus Pumilus* (ATCC 14884), *Enterobacter cloacae* (ATCC) 13047 and *Bacillus subtilis* (KZN) was assessed. DNA samples extracted from treated and untreated organisms were analyzed electrophoresis and observed. The Gel electrophoresis (Figure 4.8) reveals that the oil could not significantly damage the DNA of the organisms (there was no significant change in the molecular weight of treated and untreated DNA); it is probable that the oil kills the bacteria through the damaging of the cell membrane of the organisms rather than the DNA.

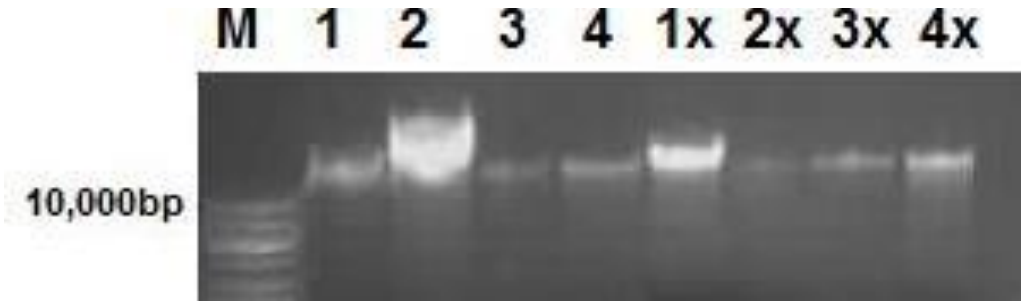


Figure 4.8. DNA cleavage activity of the essential oil of *Eucalyptus grandis* against *Esherichia coli* (ATCC 8739), *Bacillus Pumilus* (ATCC 14884), *Enterobacter cloacae* (ATCC 13047) and *Bacillus subtilis* (KZN). M= DNA maker lane 1- 4 are untreated DNA respectively and lane 1x- 4x are treated DNA respectively.

4.5. Cytotoxicity

The cytotoxicity of the essential oils of *Tulbahia violacea* and *Eucalyptus grandis* were assessed against HEK293 and HepG2 cell lines. The results thus obtained are presented in Table 4.8.

Table 4.8. Percentage inhibition of HEK293 and HEPG2 by the essential oils of *Tulbaghia violacea* and E*ucalyptus grandis.*

T. violacea			E*. grandis*		
(µg/200µl)	HEK293	HEPG2	(µg/200µl)	HEK293	HEPG2
0	0.00 ± 2.59	0.00 ± 2.23	0	ND	ND
50	0.30 ± 3.08	4.00 ± 4.30	50	ND	ND
100	-2.00 ± 1.11	12.70 ± 4.39	100	ND	ND
150	0.50 ± 0.08	10.80 ± 1.90	150	ND	ND
200	2.00 ± 0.93	17.00 ± 1.95	200	ND	ND
250	7.00 ± 2.06	20.60 ± 3.00	250	ND	ND
300	27.00 ± 1.91	27.20 ± 0.96	300	ND	ND
350	48.00 ± 2.63	35.20 ± 2.52	350	ND	ND
IC_{50} µg/ml	1218	1641		ND	ND

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

The cytotoxic activity of the essential oil of *Tulbaghia violacea* showed a weak concentration dependent cytotoxic activity with IC_{50} values for both HEK293 and HEPG2 as high as 1218 µg/ml and 1641 µg/ml respectively. The cytotoxicity of the oils of E*ucalyptus. grandis* was not determined.

Chapter 5

Discussion

For centuries, essential oils have been used in aroma-therapy. Interest in essential oils has been revitalized in recent years with the hope that oils with newer therapeutic activities might be discovered.

It is apparent that the major components of the essential oils of the rhizomes of *Tulbaghia violacea* do not contain the usual terpenoides associated with essential oils. Rather the oil is made up of sulfur-containing compounds that are similar to those found in *Allium sativa* (Garlic) (Kimbaris *et al*., 2009; Dieumou*et al.,* 2009; El-meleigy *et al.,* 2010; Martinez-Velazquez et al., 2011). The presence of the sulphur compounds could be the reason behind its pungent smell and could also contribute to the plants medicinal uses. Most of garlic's health benefits come from sulfur compounds (Imani *et al.,*2002).

Unlike most other Eucalyptus essential oils whose main component is 1, 8-cineole (Damjanović-Vratnic *et al.,* 2011; Rahimi- Nasrabadi and Batooli, 2011), *Eucalyptus grandis* essential oil's major component was m-xylene (33.04%) with 1,8-Cineole only making up 15.5% (Eucalyptol) of its contents. Cineol rich eucalypt oil are mainly used traditionally to treat influenza and colds, 1,8-Cineole is also found in products like cough syrups, lozenges, ointments and inhalants and in the treatment of bronchial infections(Santos and Rao*,* 2000; Pereira *et al.,* 2005; Salari *et al.,* 2006; Sisay Feleke 2010).The presence of 1,8-Cineol in the *Eucalyptus grandis* scientifically validates its traditional use for the treatment of Bronchial infections as well as Asthma and cough.

In the search for new antioxidants to combat the damaging effect of free radicals, medicinal plants are being screened for components with potential antioxidants properties. The antioxidant potential of the essential oil of *Tulbaghia violacea* showed a weak scavenging activity for free radicals (DPPH and ABTS), but a high Fe^{2+} chelating activity. H_2O_2 can react with reduced Fe^{2+} and Cu^{+} to produce highly toxic $OH^{\cdot}$, and these uncharged $OH^{\cdot}$ is able to penetrate membranes (Moller 2001; Thirupathi *et al.,* 2011). These reactive oxygen species that are formed are known to cause a variety of diseases in living tissues such as atherosclerosis, cancer and asthma (Lee *et al.,* 2004). The results indicate that *Tulbaghia violacea* oil may not necessary scavenge pre-existing free radical but, it does show the potential to prevent the generation of free radicals through Fe^{2+} chelating. The essential oils were also seen to have a high nitric oxide radical scavenging activity which is possibly as a result of the high SH content. Nitric oxide (NO) reacts with SH groups forming *s*-nitroso derivatives (Galigniana *et al.,* 1999). NO is an important signaling molecule, in a wide range of biological processes, as well as immunity against pathogens, signal transduction and protection from oxidative stress.

The role of NO has been controversial as both protective and harmful effects. For example a 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 researches (Stamler *et al.,* 1992; Galigniana *et al.,* 1999; Lee *et al.,* 2004). The essential oil showed a very low reducing power and thus might not have the potential to donate electrons to free radical in a bid

to make them stable. In general the essential oil of *Tulbaghia violacea* is a weak antioxidant as compared to ascorbic acid (vitamin C) which is a very strong antioxidant.

The essential oil obtained from *Eucalyptus grandis,* on the other hand showed a high scavenging activity for free radicals (DPPH and ABTS) and a low Fe^{2+} and Nitric oxide scavenging activity but had a high reducing power which shows its potential to donate electron to free radical thereby making them stable.

The ability of plants to exhibit antimicrobial activities supremacy lies within the volatile oil fraction (Lahlou, 2004; Panizzi *et al*., 1993). The essential oils of both *Tulbaghia violacea* and *Eucalyptus grandis* showed antimicrobial activity. The essential oils of *Eucalyptus grandis* exhibited activity comparable to the standards and was seen to be broad spectrum in nature as it affected both Gram positive and Gram negative bacteria. It is also worth noting that the oil effectively inhibited the growth of antibiotic-resistant organisms. The oils antimicrobial activity could be attributed to the presence of compounds like 1,8 cineole, α and β pinene and limonene which have been reported (Raju and Maridas 2011) to have antimicrobial properties. It is apparent from the DNA cleavage studies (fig 4.8) that, unlike cajanol (Liu *et al.*, 2011) and monoterpenoidindole alkaloid (Wu *et al.*, 2011) that were able to damage microbial DNA, the *E. grandis* oil did not affect the DNA of the organisms studied. Its effect on microbial cell membrane could be investigated.

Eucalyptus oil is used for its antimicrobial properties for dental care (Nagata et al., 2008). The essential oil of *T. violacea* was not as effective as the oil of *E. grandis* or the standards in inhibiting microbial growth. *Allium sativum,* a close relative of *Tulbaghia*

violacea, exhibits strong fungi toxicity (Lalitha *et al.,* 2011). The antifungal activity of the oil of *T. violacea* could be investigated. The ethno-pharmacological interview of Zulu traditional healers confirmed that medicinal plants can be used for more than one purpose. For example, plants used to treat malaria may be used for other medicinal purposes (i.e. to treat other diseases as well). Thus, even though the two plants under study (*Tulbaghia violacea* and *Eucalyptus grandis*) are used to treat respiratory tract diseases, they might have other therapeutic functions. Their essential oils have different compositions, and their biological activities (antioxidant and antimicrobial) were different. These oils could, however, be exploited as alternative source of natural bactericides for the management of human bacteria.

Biochemical instability of the alliicin, thiosulfonates and related compounds, as well as its strong odor have probably limited the use of *T. violacea* as a potential for food additive or food preservative (Benkebli, 2004). However, the essential oil of *Tulbaghia violacea* was seen to have a low cytotoxicity on cell lines.

Chapter 6

Conclusion

The results of this study suggest that the essential oils of both *Tulbaghia violacea and Eucalyptus grandis* have the potential to be exploited as good sources of antioxidant and antimicrobial agents of plant origin (with less side effects when used for drug based therapy and as preservatives for food against microbes). The therapeutic properties of garlic which is a close relative of *Tulbaghia violacea* has been ascribed to its Sulfur compounds (Imani *et al.,* 2002), which are also abundant in *Tulbaghia violacea* and thus its therapeutic properties, *Allium sativum* (Garlic) is not readily available and also a bit expensive as compared to the indigenous forms (*Tulbaghia violacea*), which is more within the means of people in the rural communities (Thamburan *et al.,* 2006). *Eucalyptus grandis* contained mostly monoterpenes, which have been reported in literature to have antimicrobial activities and antioxidant properties, and also the presence of 1,8 cineole which have been widely reported to treat respiratory tract infections, asthma and bronchial infection. This study thus adds value to the ongoing exploration for new and safer plant based compounds which are readily available and also as models for new drug discovery, towards effective and yet affordable antioxidant and antimicrobial agents. The results thus obtained supports the rationale for the use of *Tulbaghia violacea* and *Eucalyptus grandis* folk medicine.

6.1. Suggestion for further studies

The following are suggested for further studies on the study materials;

- The major compounds should be isolated and tested for bioactivity.
- The activities of the oils on the cell membrane and cell wall should be done so as to ascertain their mechanism of action.
- The antifungal activities be carried out.
- The antiasthma, anti-cough etc. should be investigated.

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APPENDIX A

(PREPARATION OF REAGENTS)

A.1. MAYER'S REAGENT:

1.36g of mercury (II) chloride was dissolved in 60ml of distilled water; 5.0g of potassium iodide was dissolved in 20ml of distilled water. Both solutions were mixed and the mixture was made up to 100ml with distilled water.

A.2. DRAGENDORFF'S REAGENT:

0.85g of subnitrate bismuth and 20ml of glacial acetic acid were dissolved in 40ml of distilled water. 0.8g of potassium iodide was dissolved in 20ml of distilled water. Solutions were stored separately in dark bottles. Just before use, 5ml of first solution and 5ml of the second solution and glacial acetic acid were mixed and up to 100ml with distilled water in a volumetric flask.

A.3. PHOSPHATE BUFFER (pH 6.6, 0.2M):

18ml of 0.2M KOH and 50ml of 0.2M KH_2PO_4 were mixed and made up to 100ml with distilled water.

A.4. ABTS*

1 tablet (10mg) of ABTS was dissolved in 3ml of distilled water and 0.002g of potassium persulphate was added to the solution. The mixture was incubated at room temperature in the dark for 16hrs. ABTS was diluted with methanol (1ml ABTS: 60ml methanol).

A.5. DPPH:

2mg / 100ml DPPH was prepared in methanol.

A.6. Glutathione preparation

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

A.7. EDTA

EDTA (ethylenediaminetetraacetic acid) 93.05 g EDTA disodium salt was dissolved in 400 ml deionized water, pH was adjusted to about 8.0 with NaOH then made up to 500 ml. with distilled water.

A.8. 5xTAE (TRIS-ACETATE-EDTA) BUFFER

5x TAE was prepared by weighing 24.2 g Tris base and dissolving in approximately 750 ml deionized water. Then 5.71ml glacial acetic acid and 10 ml of 0.5 M EDTA (pH 8.0) then made up to 1l with deionized water. The pH of TAEbuffer was then adjusted 8.5.with NaOH.

APPENDIX B

(DETAIL OF METHODOLOGY)

B.1. EXTRACTION:

The rhizomes of *Tulbaghia violacea* were washed free of adhering soil, cut into small pieces and allowed to dry at room temperature while the leaves of *Eucalyptus grandis* were plucked from the stuck respectively. 500g of the root, and 250g of the leaves were then weighed and hydrodistilled using a Clevenger type apparatus for 3hrs in a round bottom flask with deionized water. The oils were collected over water and dried over anhydrous sodium sulphate and then reconstituted with methanol using the ratio of 1mg/ml. the solution was stored in an amber bottle and stored in the fridge.

B.2. PHYTOCHEMICAL SCREENING

B.2.1. TEST FOR SAPONINS:

0.5g of the raw plant extracts extracted with boiling water. After cooling, the extract was shaken vigorously to froth and was then allowed to stand for 15- 20min and classified for saponin content as follows: no froth = negative (no saponins) and froth less than 1cm = weakly positive (Saponins present): froth 1.2cm high = positive; and froth greater than 2cm high= strongly positive.

B.2.2. TEST FOR TANNINS:

0.5g of the raw plant extracts were boiled with 10ml of distilled water for 15min, filtered and made up to 10ml. few drops of 0.1 % $FeCl_3$ solution were added to 2ml of the filtrate. Black-

blue, green or blue green precipitate was taken as preliminary evidence of the presence of tannins.

B.2.3. TEST FOR STEROIDS:

2ml of acetic anhydride and 2ml of concentrated sulphuric acid were added to 0.5ml of extract. A colour change from violet to blue or green was taken as evidence of the presence of steroids.

B.2.4. TEST FOR TERPENOIDS (SALKWOSKI TEST):

0.5ml of methanolic solution of extract was mixed with 2ml chloroform and 3ml of concentrated sulphuric acid was carefully added to form a layer. A reddish- brown colouration of the interface was taken as evidence of the presence of terpenoids.

B.2.5. TEST FOR ALKALOIDS:

0.5ml of methanolic solution of the plant materials was acidified with 5ml 1% HCL (aq). The solution was stirred on the steam bath and filtered. 1ml of the filtrate was treated with Mayer's reagents. A precipitate was taken as preliminary evidence of the presence of alkaloids. Another 1ml of the same filtrate was treated with dragendorff's reagent and turbidity or precipitate was also taken as the evidence of the presences of alkaloids.

B.2.6. TEST FOR ANTHRAQUINONES:

0.5 of methanolic solution of plant extract was dissolved and shaken with benzene. The mixture was filtered and 5ml of 10% ammonium solution was added to the filtrate. After shaking, the presence of a pink, red, or violet colour in ammonia solution (lower phase) was taken as evidence of the presence of anthraquinones.

B.2.7. TEST FOR FLAVONOIDS:

Three tests were used to determine the presence of cardiac glycosides in the extract as follows:

B.2.7.1. LEAD ACETATE TEST:

1ml of methanolic solution of plant extract was mixed with 1 ml of 10% lead acetate. A reddish-brown colouration or precipitate was taken as an evidence of the presence of flavonoids.

B.2.7.2. FERRIC CHLORIDE TEST:

1ml of methanolic solution of plant extract was mixed with 1ml of $FeCl_3$. A dark brown or dirty brown precipitate was taken as evidence of the presence of flavonoids.

B.2.7.3. SODIUM HYDROXIDE TEST:

1ml of methanolic solution of plant extract was mixed with 1ml of dilute NaOH. A golden yellow precipitate was taken as evidence of the presence of flavonoids.

B.2.8. TEST FOR CARDIAC GLYCOSIDES:

Three tests were used to determine the presence of cardiac glycoside in the extract as follows:

B.2.8.1. LIEBERMAN'S TEST:

0.5ml of methanolic solution of plant extract was mixed with 2ml of acetic acid. This was cooled well in ice and 1ml of concentrated sulphuric acid was carefully added down the sides. Colour change from violet to blue to green was taken as an indication of the presence of steroidal nucleus i.e. the aglycone portion of the cardiac glycoside.

B.2.8.2. SALKOWSKI TEST:

0.5ml of methanolic solution of plant extract was mixed with chloroform and 2ml of concentrated sulphuric acid was carefully added to form a lower layer. A reddish- brown colour at inter phase was taken as an indication of the presence of steroidal ring i.e. aglycone portion of the cardiac glycoside.

B.2.8.3. KELLER-KILIANI TEST:

0.5ml of methanolic solution of plant extract was dissolved in 2ml of glacial acetic acid containing 1 drop of 10% of FeCl3 solution and this was underplayed with 1ml of concentrated sulphuric acid. A brown ring at interphase was taken as an indication of the presence of deoxy sugar characteristic of cardenolides. Also a violet ring below the brown ring (i.e. in the acetic acid layer) and gradually spread through the layer was as an indication of cardiac glycosides.

B.2.9. TEST FOR PHLOBATANNINS:

2.5ml of methanolic solution of plant extract was added to water. The mixture was boiled with 1% HCL (aq). Deposition of a red precipitate was taken as evidence for the presence of phlobatannins.

B.3. *IN VITRO* ANTIOXIDANT ACTIVITIES OF THE ESSENTIAL OILS

B.3.1. DPPH FREE RADICAL SCAVENGING:

DPPH: 2mg / 100ml DPPH and different concentration of methanolic solution of the essential oils (5mg /ml, 10mg /ml, 20mg /ml, 50mg /ml, and 100mg /ml) were prepared. Six test tubes were set in triplicate for each concentration (18 tubes). 2ml of DPPH was put into each test tube and 2ml of each concentration was added into the corresponding test tubes. This was

mixed and let to stand for 30 – 60 min with interval mixing in the dark. Absorbance was read at 517nm. Methanol was used as blank and percentage scavenging activity was calculated;

% scavenging $=\left(\mathbf{1} - {}^{\boldsymbol{At}}/_{\boldsymbol{Ao}}\right) \times \mathbf{100}$

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

B.3.2. ABTS FREE RADICAL SCAVENGING:

7mM of ABTS was prepared in water and also different concentrations of methanolic solution of the essential oils (5mg /ml, 10mg /ml, 20mg /ml, 50mg /ml, and 100mg /ml) were prepared. Test tubes were set as in DPPH experiment. 1ml of ABTS was put into each tube and 1 ml of each concentration was added into the corresponding test tubes. This was mixed and let to stand for 6mins. Absorbance was read at 734nm with methanol used as blank and percentage scavenging activity was calculated;

% scavenging $=\left(\mathbf{1} - {}^{\boldsymbol{At}}/_{\boldsymbol{Ao}}\right) \times \mathbf{100}$

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

B.3.3. REDUCING POWER:

The different concentrations of methanolic solution of the essential oils oil were prepared. Also PB, 1% PF, TCA and 0.1 $FeCl_3$ were prepared. Test tubes were set as in DPPH experiment. 1ml of methanolic solution of oil was mixed with 2.5ml PB and 2.5ml PF. The mixture was incubated for 20min and 2.5ml of TCA was added. This was mixed and after 5 – 10min, themixture was centrifuged at 1000rpm for 10min. 2.5ml of supernatant was collected and diluted with 2.5ml of dilute water. This was mixed with 0.5ml $FeCl_3$. Absorbance was read at

700nm and distilled water was used as blank. The graph of absorbance (nm) versus concentration of oil (mg/ ml) was constructed.

B.3.4. CHELATING ACTIVITY ON FE^{2+}:

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

% scavenging $= \left(\mathbf{1} - {\boldsymbol{At}}/{\boldsymbol{Ao}}\right) \times \mathbf{100}$

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

B.3.5. 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. 2ml of sodium nitropruside, 0.5ml saline phosphate buffer and was added to 0.5ml methanolic solution of the essential oil. The mixture was incubated a 25^{o}C for 150mins, then 0.5ml of the reaction mixture was pipette into different test tube and 1ml of sulphanilic acid was added mixed and left to stand for 5mins after which 1ml of 0.1% naphthylethyleledіamedihydrochloride was added, mixed and left to stand in

diffused light for 30minsThe absorbance was read at 540nM using a plate reader with deionised water used as blank and percentage scavenging activity was calculated;

% scavenging $=\left(\mathbf{1}-{}^{\boldsymbol{At}}/_{\boldsymbol{Ao}}\right)\times\mathbf{100}$

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

B3.6. SH- SULPHYDRYL CONTENT

Different concentrations of Glutathione red (GSH) 0.1-10µg/ml was prepared in iced cold distilled water containing 30µm/ml of EDTA. The methanolic solution of the essential oils were also prepared with EDTA solution as above (1:5) Test tubes were set up (blank, GSH different concentrations and essential oils different concentration). 0.1M sodium phosphate buffer of pH 8 was prepared and 1% σ-phthaladehyde (OPT) in methanol was also prepared. To 2ml of GSH and essential oils in test tubes, 0.5ml sodium phosphate bufferwasadded(to the blank, 2.5ml of sodium phosphate buffer). Themixture was allowed to stand for 15-20mins at room temperature and the Flourescence at 420nm read.The standard graph was plotted with GSH (Figure B1) and the SH content in the essential oils were estimated from the graph.

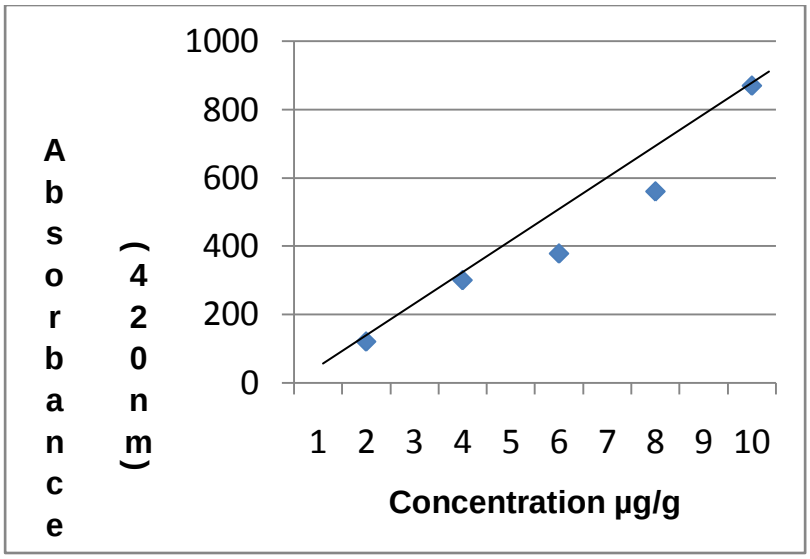


Figure B1. Standard graph of GSH

B 3.7. XANTHINE OXIDASE ACTIVITY:

Themethanolicsolutions of different concentration of the essential oils as well as 21µm of xanthine were prepared along with 10 units of xanthine oxidase. Test tubes were set up as in DPPH experiment, 1ml of xanthine was added to 0.5ml of the essential oils, incubated for 5mins at 20^{o}C, after which 0.2ml of xanthine oxidase was added to the mixture and incubated for 10mins at 20^{o}C.The absorbance was read at 295nMwith methanol used as blank and percentage scavenging activity was calculated;

% scavenging =$\left(\mathbf{1} - {\boldsymbol{At}}/{\boldsymbol{Ao}}\right) \times \mathbf{100}$

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

B.4. ANTIMICROBIAL ASSAY

B. 4.1. AGAR DISK DIFFUSION METHOD.

Bacteria were grown in 20ml nutrient broth at 37^{o}C over night. 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 disk were placed on the inoculated plates and 10 µl of 10mg/ml of the essential oils in 10% DMSO were added to the paper disk the plates then incubated at 37^{o}C for 24hrs and the zone of inhibition measure using a ruler. Tests were performed in duplicates using Ampicillin and Neomycin as positive control.

B. 4.2. MINIMUM INHIBITORY CONCENTRATION

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

B.4.3. MINIMUM BACTERICIDAL CONCENTRATION

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

B.4.3. DNA DAMAGE (CLEAVAGE)

B.4.3.1. DNA ISOLATION

Fresh bacterial cultures were treated with the essential oils (MBC concentration). Treated as well as untreated cultures were centrifuged 10000rpm, 1mins, and the pellets obtained were re-suspended in lysis solution (ZR fungal/bacterial DNA MiniPrep^{TM}) for 5mins and centrifuged 10,000rpm for 1min. Fungal / Bacterial DNA Binding buffer (ZR fungal/bacterial DNA MiniPrep^{TM}) was then added to the suspension and centrifuged 10,000rpm for 1minThe supernatant collected was added to a DNA Pre Wash Buffer (ZR fungal/bacterial DNA MiniPrep^{TM}) and centrifuged 10,000rpm for 1min after which Fungal/ Bacterial DNA Wash Buffer (ZR fungal/bacterial DNA MiniPrep^{TM}) washing buffer and then centrifuged at 10,000rpm for 1min. The precipitated DNA was then eluted using the (ZR fungal/bacterial DNA MiniPrep^{TM})DNA elution Buffer and centrifuged at 10,000rpm for 30secs.

B 4.3.2. AGAROSE GEL ELECTROPHORESIS

DNA was visualized on 1.5 % agarose, 0.8g agarose was suspended in 0.5x TBE buffer (45 mMTris base, 45 mM boric acid, 1 mM EDTA pH 8.0). The agarose was heated for60 – 90 seconds to dissolve. Theresultantagarose gel was then cooled before adding ethidium

bromide 10µl. The agarose gel was then poured into a casting tank with a comb to make the gel wells. The gel was placed into an electrophoresis tank and 0.5x TBE buffer added to a level sufficient to cover the gel after which the DNA samples were loaded. The electrophoresis tank was connected to a power source ensuring that the DNA moves towards the anode, with 100 volts. The DNAsamples was then visualized by UV transillumination on a 1D Kodak (EDAS 290) imaging system.

B. 5. CYTOTOXITY ASSAY.

B.5.1. MTT CELL PROLIFERATION ASSAY

An MTT solution of 5mg/ml was prepared in a Phosphate buffer. Then cells were cultured in 25cm^2 flask to confluency, which was then trysinised and plated into 48 well plates at specify seeding densities. Cells were then incubated overnight at 37^{o}C. Fresh medium (MEN +Glutmax + antibiotic) were then added. The essential oils were then added in triplicate and incubated for 4 hrs. Medium was then removed and replaced by complete medium (MEM + Glutmax + antibiotics + 10% Fetal bovine serum). After 48hrs medium was removed from cells in the multiwall plates, then 200µl of MTT solution as well as 200µl of medium to each well containing cell the plates incubated for 4 hrs thereafter, the medium and MTT solutions were removed from the well. 100/200/400µl of DMSO was then added to each well to stop the reaction and dissolve insoluble formazan crystal. The plates were then read in a plate read at 570nM. Graph of cell survivals were then plotted against concentration of extract.Data was evaluated through regression analysis using QED statistic program and from linear equation the LC50 values representing the lethal concentration for 50% cytotoxicity mortality.

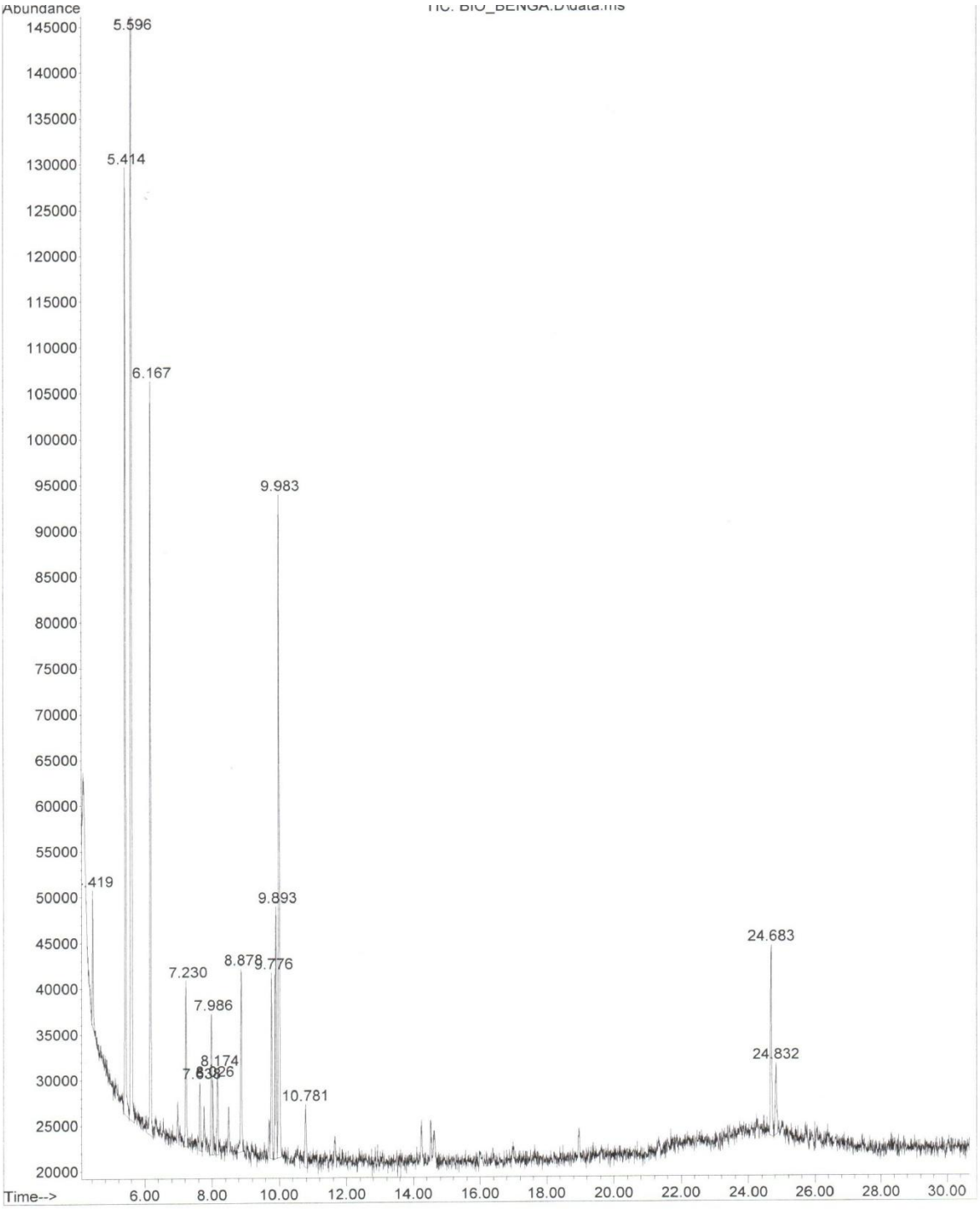
B.6. CHEMICAL ANALYSIS OF ESSENTIAL OILS

B.6.1. GAS CHROMATOGRAPHY / MASS SPECTROMETER (GC/MS)

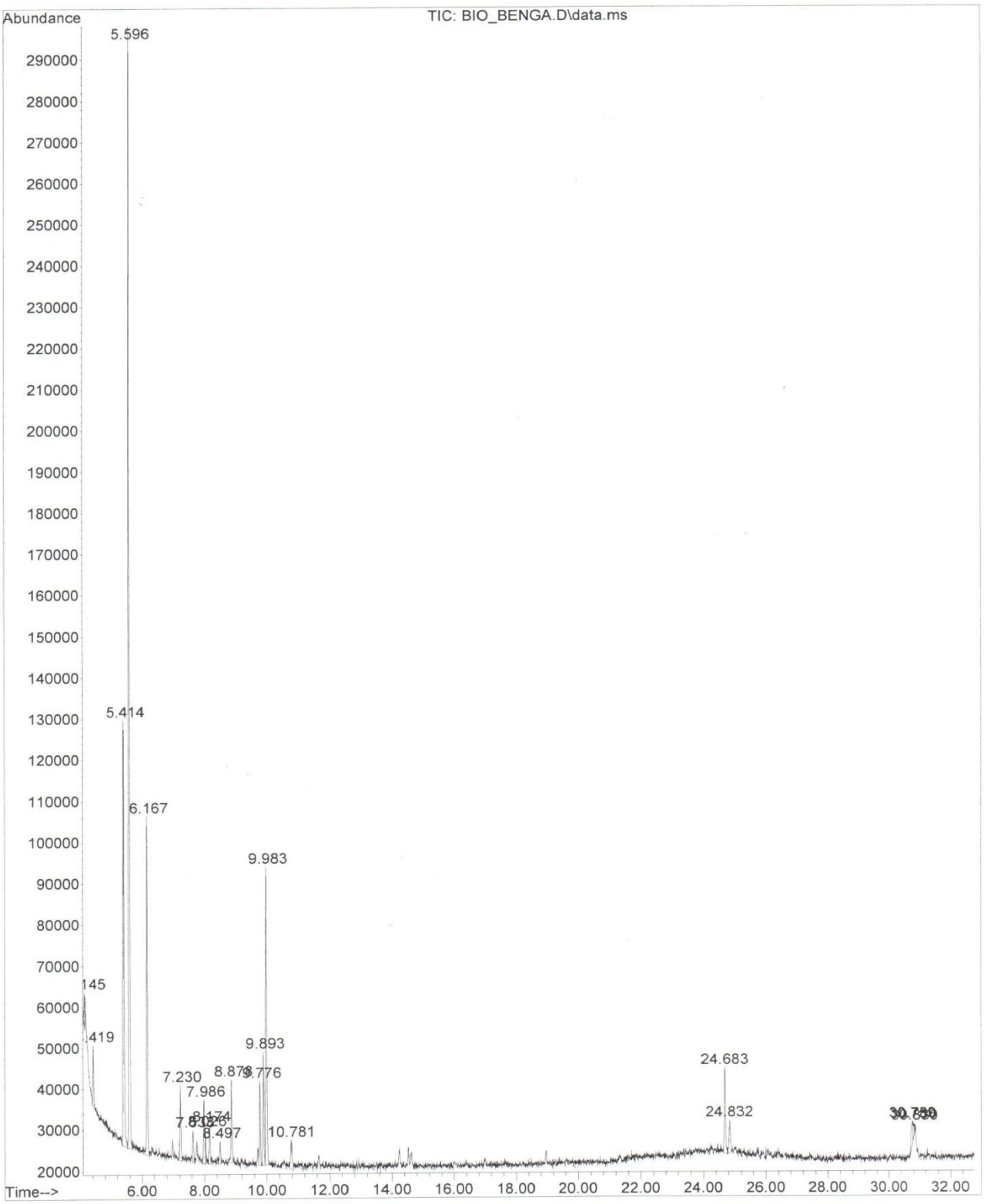
Gas Chromatography / Mass Spectrometer (GC/MS) of the essential oils was carried out using an Agilent Gas Chromatography (7890A) equipped with a capillary column of (Agilent 190915, 30m × 250µm × 0.25µm calibrated) attached with an Agilent mass spectrometer system (5975C VL MSD with Triple Axis Detector). The oven temperature was programmed from 45^{o}C - 310^{o}C. Helium was used as the carrier gas at a flow rate of 5 ml / min with a split ratio of 1: 20. 0.5µl of the essential oil was diluted in hexane and 0.5 µl of the solution was manually injected into the GC/MS.

Appendix C

GC-MS Spectra for *Tulbaghia violacea*



GC-MS Spectra for the essential oils of *Eucalyptus grandis*



Appendix D

Interview of 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	
Female	

15-24	
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	

Ethics Committee
Faculty of Science and Agriculture
University of Zululand
C/O Mr L Vivier
Department of Zoology
University of Zululand
Private Bag 1001
KwaDlangezwa
3886
Tel: 035 – 902 6741
Email: lvivier@pan.uzulu.ac.za

26 May 2010

To whom it may concern

ETHICS EVALUATION OF RESEARCH PROJECT PROPOSAL

This letter serves to confirm that **O. S. Soyingbe** (Student No **200906101**), registered for a MSc Degree in the Department of Biochemistry and Microbiology at the University of Zululand, in accordance with appropriate rules submitted a research project proposal to the Ethics Committee of the Faculty of Science and Agriculture at the University of Zululand. The research project will investigate: **The chemical composition, antimicrobial and antioxidant properties of the essential oils of *Tulbaghia violacea* and *T. alliacea***. Based on the research protocol stipulated, this committee could find no reason from an ethical standpoint to reject the proposed research.

Yours sincerely

Mr L Vivier
Chairperson
Ethics Committee
Faculty of Science and Agriculture
University of Zululand