

***In vitro* evaluation of antimicrobial and antioxidant activities of *Olea Europaea* subsp. *africana* and *Euryops brevipapposus* used by Cala community folkloric medicine for the management of infections associated with chronic non-communicable diseases**

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DECLARATION

I hereby declare that the dissertation I have submitted to the University of Fort Hare for the degree of Master of Science in Microbiology is my original work, and has not been previously submitted for any degree at this institution or any other university.

Signature/date _____

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Prof C.L. Obi (Supervisor)

DEDICATION

I dedicate this dissertation to GOD Almighty for who He is and who He can never be.

“Who is he that saith, and it cometh to pass when the Lord commanded it not”

Lamentations 3:37 (KJV)

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SUMMARY

Chronic non-communicable diseases are a global public health challenge that continuously threatens the development and health of humans. Risk factors such as unbalanced diet-the high consumption of processed food or food from animal origin are responsible for NCDs. NCDs result in weakened immune system, making the host susceptible to opportunistic infections. Thus, the NCDs burden is most times chronic and multiple with the illness and suffering of the affected person numerous. The lack of cure for NCDs, the high cost of drugs, their high side-effects, and the emergence of multiple drug resistance has given rise to the investigation of other sources for therapeutic cure such as medicinal plants.

The ethanol, n-hexane and ethyl acetate extracts of *Olea europaea* were analysed for their antioxidant and antimicrobial activities. The essential oil was also analysed for their chemical constituents. The n-hexane extracts of *O. europaea* exhibited no inhibition against all of the microorganisms tested, while the ethyl acetate and ethanol extracts exhibited inhibition, with minimum inhibitory concentration values between 0.625 mg/ml to 1.25 mg/ml. The ethanol leaf and ethyl acetate stem extracts exhibited significant activity in the inhibition of 2, 2'-azinobis-(3-ethylbenzothiazolin - 6-sulfonic acid diammonium salt (ABTS) free radical, the n-hexane leaf extract had the overall significant lipid peroxidation inhibition activity, while in the inhibition of 2, 2'-diphenyl-1-picrylhydrazyl radical (DPPH), the ethanol and ethyl acetate leaf extracts had strong activity. Nonanal, phytol, α -Pinene, α -Phellandrene, spatulenol and farnesol were some of chemical components identified after the GC-MS analysis of *O. europaea* oil.

In the final part of the dissertation, *Euryops brevipapposus* essential oil was assessed for the antioxidant activities using free radical scavenging assays. In addition to this, the antimicrobial activities were assessed and the chemical composition was analysed using GC-MS. The essential oil demonstrated significant antioxidant activity against 2, 2'-diphenyl-2-picryl-hydrazyl free radical (DPPH), 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and lipid peroxides with IC₅₀ value of 0.0000000671 mg/ml, 1.05 mg/ml, and 1.170 mg/ml respectively. The essential oil also showed significant activity against all microorganisms tested with minimum inhibitory concentration (MIC) values between 0.055 mg/ml to 0.5 mg/ml. α -pinene, α - Phellandrene, germacrene D, β -pinene, trans- β -Ocimene, bicyclogermacrene and β - Phellandrene were some of the chemical compounds identified in *E. brevipapposus* oil.

The study has shown that *E. brevipapposus* and *O. europaea* are abundant in phytochemical compounds which were thought to be the root cause for the activities demonstrated. Therefore, these therapeutic properties observed validate and elucidate the traditional usage of the both plants in the treatment /management of diseases.

TABLE OF CONTENTS

| | |
|---|-----------|
| DECLARATION | ii |
| DEDICATION | iii |
| ACKNOWLEDGEMENT | iv |
| SUMMARY | v |
| TABLE OF CONTENTS | vii |
| LIST OF TABLES | ix |
| LIST OF FIGURES | x |
| LIST OF ACRONYMS | xi |
| CHAPTER 1 GENERAL INTRODUCTION | 1 |
| 1.1 Statement of the Problem | 4 |
| 1.2 Hypothesis..... | 5 |
| 1.3 Aims and Objectives of the research..... | 5 |
| 1.4 References..... | 6 |
| CHAPTER 2 LITERATURE REVIEW | 11 |
| 2.1 Medicinal Plants..... | 11 |
| 2.2 Chronic Non-Communicable Diseases (NCDs)..... | 15 |
| 2.3 Diabetes Mellitus | 16 |
| 2.4. Current Approaches for the Management of Diabetes..... | 26 |
| 2.5 Free Radicals, Oxidative Stress, Antioxidants and Diabetes Mellitus | 32 |
| 2.6 Importance of Medicinal Plants in Diabetes Mellitus..... | 38 |
| 2.7 <i>Olea europaea</i> subspecies <i>africana</i> and <i>Euryops brevipapposus</i> | 39 |
| 2.8 References..... | 44 |
| CHAPTER 3 The Bioactive Potentials of <i>Olea Europaea</i> subsp. <i>africana</i>, A Medicinal Plant Commonly Used in Folkloric Medicine Among Xhosa Tribe Eastern Cape Province, South Africa | 65 |
| 3.1. Introduction..... | 66 |
| 3.2 Materials and Methods..... | 68 |
| 3.3. Results..... | 73 |
| 3.4 Discussion | 108 |
| 3.5 Conclusion | 112 |
| 3.6 References..... | 112 |

| | |
|--|------------|
| Chapter 4 <i>In vitro</i> Free-Radical-Scavenging and Antimicrobial Activities of <i>Euryops brevipapposus</i> Essential Oils | 118 |
| 4.1 Introduction..... | 119 |
| 4.2 Materials and Methods..... | 121 |
| 4.3 Results..... | 127 |
| 4.4 Discussion | 134 |
| 4.5 Conclusion | 137 |
| 4.6 References..... | 137 |
| Chapter 5 General Conclusion..... | 142 |

LIST OF TABLES

Table 3.1: Solvent extraction yield efficiencies of *Olea europaea subsp. africana*

Table 3.2: The chemical composition of bioactive compounds of *O. europaea* essential oil

Table 3.3: Growth inhibition effect of *Olea europaea subsp. africana* extracts

Table 3.4: The minimum inhibitory concentration values for *O. europaea* extracts

Table 4.1: Results obtained for extraction yield and percentage yield of extraction values

Table 4.2: Antioxidant activity of essential oils of *Euryops brevipapposus*

Table 4.3: Growth inhibition effect of *E.brevipapposus* oil

Table 4.4: MIC and MBC values for *E. brevipapposus*

Table 4.5: Major composition of bioactive compounds identified using GC–MS

LIST OF FIGURES

- Figure 3.1:** Gas chromatogram of *O. europaea* essential oil
- Figure 3.2:** ABTS dose response curve for each of the extracts tested.
- Figure 3.3:** ABTS radical scavenging activity by different *O. europaea* leaf extracts and the standard antioxidant, vitamin C.
- Figure 3.4:** ABTS radical scavenging activity by different *O. europaea* stem extracts and the standard antioxidant, vitamin C.
- Figure 3.5:** Lipid Peroxide radical scavenging activity of *O. europaea* leaves extract and the standard antioxidant, vitamin C.
- Figure 3.6:** Lipid peroxide radical scavenging activity of different *O. europaea* stem extracts and the standard antioxidant, vitamin C
- Figure 3.7:** DPPH dose response curve for *O. europaea* leaves extracts tested
- Figure 3.8:** Comparison of the DPPH radical scavenging effect of different *O. europaea* leaves extract and the standard antioxidant, vitamin C.
- Figure 3.9:** Comparison of DPPH radical scavenging effect of the different *O.europaea* stem extract, and the standard antioxidant, vitamin C.
- Figure 3.10:** Comparison of the antioxidant activity of the different *O.europaea* extracts with vitamin C.
- Figure 4.1:** The map illustrating the study area of Cala Community
- Figure 4.2:** Map showing local municipality in which Cala community is located
- Figure 4.3:** DPPH radical scavenging by *E. brevipapposus* essential oil and the standard antioxidant, vitamin C.
- Figure 4.4:** ABTS radical scavenging by *E. brevipapposus* essential oil and the standard antioxidant, vitamin C.
- Figure 4.5:** Lipid peroxide radical scavenging by *E. brevipapposus* essential oil and the standard antioxidant, vitamin C.
- Figure 4.6:** GC-MS chromatogram of *E. brevipapposus* essential oil

LIST OF ACRONYMS

| | |
|--------------------|--|
| AEMREG: | Applied and Environmental Microbiology Research Group |
| ABTS: | 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) |
| ADA: | American Diabetes Association |
| ANOVA: | Analysis of Variance |
| ATCC: | American Type Culture Collection |
| CAM: | Complementary or Alternative Medicine |
| CDC: | Centers for Disease Control and Prevention |
| CLSI: | Clinical Laboratory Standards Institute |
| DPPH: | 2, 2-Diphenyl-1-picryl hydrazyl radical |
| GC-MS: | Gas chromatography–mass spectroscopy |
| IC ₅₀ : | Extract concentration capable of inhibiting by 50% |
| IDF: | International Diabetes Federation |
| MAR: | Multiple Antibiotic Resistant |
| MIC: | Minimum Inhibitory Concentration |
| NCDs : | Non-Communicable Diseases |
| NIST: | National Institute Standard and Technology |
| RNS: | Reactive Nitrogen Species |
| ROS: | Reactive Oxygen Species |
| SAMRC: | South Africa Medical Research Council |
| THPs: | Traditional Health Practitioners |
| WHO: | World Health Organization |

CHAPTER 1

GENERAL INTRODUCTION

The increase in the prevalence of non-communicable diseases (NCDs) forms one of the main problems of the 21st century development (United Nations, 2011), and this has led to the increase in the attention placed on NCDs in recent years (WHO, 2011). The term Chronic non-communicable diseases (NCDs) describes “diseases or conditions which usually affect or can occur in people over a long term, for which there are no recognized etiologic agents that are passed from one affected person to another” (Daar et al., 2007).

In developed nations, NCDs contribute immensely to their burden of diseases while they are also becoming increasingly prevalent in developing nations due to urbanization - urban influenced lifestyle of people and demographic transitions (WHO, 2011; Ueda, 2013). The widespread increase of NCDs in emerging economies is seen as an unfavourable aftermath of socio-economic development due to the rapid urbanization and economic growth connected with an increase in modern lifestyles such as drinking and an ageing population (Alwan et al., 2010; Jones and Geneau, 2012; WHO, 2013a; Vellakkal et al., 2014).

One particular type of NCDs responsible for the third cause of mortality in the world and other increasing associated bacterial infections is Diabetes mellitus (DM) (Chauhan et al., 2010). In certain age groups, diabetics have an increase of two-fold in the risk of stroke (Boden-Albala et al., 2008) and a threefold increase in the risk of tuberculosis (Jeon and Murray, 2008). While in both developing and developed nations, it is also a major cause of blindness, visual impairment (Yau et al., 2012) and renal failure (Icks et al., 2009). Diabetic patients are susceptible to a variety of infections as a result of their immunocompromised state. These infections include urinary tract infections, foodborne illnesses and those peculiar to people with diabetes (Peleg et al., 2007; Casqueiro et al., 2012). Hence, diabetics' need two to three times of the healthcare resources compared to individuals without diabetes, and this care may account for up to 13% of national healthcare budgets (Zhang et al., 2010).

Despite the fact that antimicrobial agents can inhibit pathogens, it has been reported that majority of these agents designed to combat these pathogens are not as effective as they used to be in the

treatment of infectious diseases (CDC, 2013) as the pathogenic microorganisms have developed drug resistance (Hancock, 2007; Demain and Sanchez, 2009). Thus, the development of antimicrobial resistance in microorganisms has become a huge challenge and in consequence of this, more researches have emerged in the quest for new antimicrobial agents from other natural sources to mitigate the situation (Demain and Sanchez, 2009). Before the 20th century, plants and their products were the main methods of chemotherapy (Lahlou, 2013), and being the common origin of medications, they have invariably been used either as pure active substances or as traditional preparations (NarasingaRao and Kaladhar, 2014). These plant-based systems have consistently played a significant role in the healthcare sector and approximately 80% of the world's population depends solely on trado-medicines for their primary health care (Owolabi et al., 2007).

Medicinal plants are a rich biological resource of drugs usually employed in systems such as modern medicine, intermediate and chemical entitled for synthetic drugs, folk medicine, pharmaceuticals, and nutraceuticals (Ncube et al., 2008; Verpoorte, 2009). There are diverse species of medicinal plants that abound worldwide which are being utilized as antibacterial, antiviral and antifungal agents (Ramasamy and Charles, 2009; Mohamed et al., 2010; Bhalodia and Shukla, 2011; Cock and van Vuuren, 2015).

The observation of the ethnomedicinal use of the plants led to the discovery of 74% of pharmacologically active plant-derived components and an estimate of 14 to 28% of the species of higher plants being applied medicinally (Ncube et al., 2008). Some parts of medicinal plants are utilized as antimicrobial agents, in the form of infusions, powdered or fluid extracts, oral administration or decoctions (Remington and Remington, 2008). Medicinal plants are known to bring about definite physiological effects in the body of humans (Kaladhar et al., 2010), if after administration of the plants, and the toxicity level is found to be low, the introduction of such drugs for therapeutic purposes are possible. Generally, drugs derived from plant and herbal formulations are thought to be less toxic and free from side effects than synthetic ones (Tiwari et al., 2008; Pandey et al., 2015). Plants have the capacity to produce a wide range of secondary metabolites such as phenols, saponins, alkaloids and many others that have bioactive potentials (Vasu et al., 2009; Prabha et al., 2013).

Despite the current countless chemical structures available for screening for their therapeutic value, natural products especially those of plant origin are still the most significant sources of new drugs (Odugbemi and Akinsulire, 2006). There are still ongoing researches by scientists of divergent fields on recently discovered plants so as to serve as a substitute for existing drugs and also for their usefulness as antimicrobials (Ajiboye et al., 2015). Medicinal plants and their diverse bioactive compositions present a promising resource for antimicrobial agents with both specific and general antimicrobial activity (Nair and Chanda, 2007; Senthil and Reetha, 2009). Unlike the available synthetic drugs, antimicrobials derived from plants are not usually linked with numerous side effects (Chanda et al., 2010; Habbal et al., 2011) and have tremendous medicinal potentials in the treatment of various infectious diseases. Those plant derived compounds that act by killing or inhibiting the growth of microorganisms having little or no toxic effect on the host cells are good candidates for the development of new antimicrobial drugs (Madani and Jain, 2008).

Antibiotic resistance can spread across borders easily, and they contribute to the already burdened healthcare systems. Many at times, the infections that results from this antibiotic resistance necessitate long-term and expensive treatments, healthcare use, and prolonged hospital admissions, leading to severe consequences including mortality and disability in patients as compared to infections that can be treated easily with antibiotics (Roberts et al., 2009). As the antibiotic resistance spreads, antibiotics that were previously employed in the treatment of infections would either not function optimally or stop having any effect on the microorganisms. The consequence of which also affects the treatment of infectious complications in people with other diseases. Therefore, the majority of the advancement made in the medical treatment of chronic diseases such as asthma and diabetes depends on the ability of antibiotics to fight infections caused by microorganisms. And if the loss of this ability occurs, it would result in the loss of life-improving and lifesaving modern medical advantages (CDC, 2013). Therefore, the various advancements in the medical field which were made possible by the availability of effective drugs are endangered in today's world by the continuous rise of bacterial resistance to all the currently available antibiotics (Paphitou, 2013).

The solution to the antibiotic resistance of microorganisms is the continual development of novel drugs that meet the numerous medical needs (Mossialos et al., 2009). Despite this pressing need

to replace ineffective antibiotics with new antimicrobials, pharmaceutical industries are reluctant to beef up their research and discover new drugs (Wright, 2012), as antibiotic resistance raises the demand for new drugs and simultaneously reduces their estimated life, thereby adversely diminishing the long-term potential of the drugs to return profits. In addition, a new drug to which there is no resistance would be minimally used and these are not the type of products pharmaceutical companies, which are also profit making companies, would like to create (Cooper and Shlaes, 2011), as the production of new drugs is laborious and expensive (Dickey et al., 2010).

The release of novel drugs may spawn enormous profits, however, 30% of most of these drugs fail at phase III of their clinical trials implying that these costly projects oftentimes would reach a standstill without generating any returns, and to which microbial resistance may emerge (Cooper and Shlaes, 2011). By reason of this, pharmaceutical industries are unenthusiastic about investing large sums in the development of drugs except there is the assurance that there will be dividends on the investment made as all business including that of saving lives and treating infections and diseases, is concerned with realizing as many profits as possible (RocíoAcuña, 2013).

1.1 Statement of the Problem

Currently, South Africa has been ranked as a nation with the second highest number of people suffering from type 2 diabetes in sub-Saharan Africa (Popkin et al., 2012) as around 2.28 million people are affected by diabetes (IDF, 2015), with the largest concentration of the ailment occurring among the low income group (Mayosi et al., 2009). Despite the advances in medical care, there is still no available allopathic cure for diabetes (Dewanjee et al., 2009).

With this in mind and other challenges peculiar to the use of drugs such as their high cost, side-effects and the emergence of multiple drug resistance by human pathogenic microorganisms, has led to untreatable infections and the unwillingness of pharmaceutical companies to beef up their research and discover new drugs (Wright, 2012; Cushnie et al., 2014), the demand for new, non-toxic, lower side-effect and cheaper therapeutic cure has become more imperative than ever and

thus, the need to find alternative chemotherapeutic agents is shifting to plants (Cushnie et al., 2014).

The plants investigated in this study have been reported to be used in traditional medicine. It has therefore become very expedient that the efficacies of these medicinal plants as claimed by these traditional health practitioners be analysed scientifically so as to prove their claims. Considering the dearth of information on the therapeutic activities of these medicinal plants, it is necessary that a study of this type is conducted as information that will emanate from this *in vitro* determination of the active components, antioxidant and antimicrobial activity of these plants, *Olea europaea* subsp. *africana* and *Euryops brevipapposus* could be useful in the development of antimicrobial agents.

1.2 Hypothesis

The study hypothesizes that *Olea europaea* subsp. *africana* and *Euryops brevipapposus* used in Cala community folkloric medicine for the management of infections associated with NCDs do not possess antioxidant and antimicrobial properties.

1.3 Aims and Objectives of the research

Aim

This study aimed at evaluating the antioxidant and antimicrobial activities of *Olea europaea* subsp. *africana* and *Euryops brevipapposus* for the management of infections associated with NCDs using modern biological evaluation techniques.

Objectives

To achieve this, the following objectives were set:

- 1) To collect *O. europaea* and *E. brevipapposus* used by Cala Community in the management of NCDs in particular diabetes and associated bacterial infections.
- 2) To extract bioactive constituents of *O. europaea* and *E. brevipapposus*
- 3) To determine the antioxidant and the antimicrobial properties of the plants.

- 4) To characterise the bioactive molecules using gas chromatography and mass spectrometry.

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CHAPTER 2

LITERATURE REVIEW

2.1 Medicinal Plants

Sofowora et al. (2013) described medicinal plants as any plant which one or more of its parts are composed of substances which can be used for therapeutic purposes or can be used as a precursor for the synthesis of useful drugs. Medicinal plants can also be described as any plants employed medicinally in galenical preparations (e.g. decoctions, infusions, etc.) or used for extraction of pure compounds either for the hemi-synthesis of medicinal compounds or for direct medicinal use or food, spice, and perfumery plants used medicinally (Sofowora, 2008; Evans, 2008). They represent a consistent part of the natural biodiversity endowment of many countries in Africa as well as the world at large (Okigbo et al., 2008; Pradhan et al., 2011). They include: *Pilostigma reticulatum*, *Anogeissus leiocarpa*, *Enantia chlorantha*, *Senna occidentalis* and *Azadirachta indica* (Agbabiaka et al., 2010).

2.1.1 General Uses of Medicinal Plants

Plants with medicinal value are also significant to the human health and the communities in which they can be found. They gain further importance in regions where modern medical health facilities are either unavailable or are not easily accessed as they contain some bioactive substances that bring about a definite physiological action in the human body (Kaladhar et al., 2010; Yadav and Agarwal, 2011; Ahmed, 2012). Medicinal plants secrete compounds that act as antioxidants and antimicrobial agents and are auxiliaries in medicine and pharmacy which can inhibit microorganisms by a different mechanism other than that of the currently used drugs, as they are capable of sustaining pharmacological activity and therapeutic efficacy (Sengul et al., 2009). Medicinal plants that are indigenous are occasionally used to supplement food substances prepared for nursing and expectant mothers for therapeutic purposes (De Boer and Lamxay, 2009). The majority of these medicinal plants also serve as food plants and spices (Muchuweti et al., 2007).

Medicinal plants are usually used in folkloric medicine, otherwise known as traditional medicine. Traditional medicine has been defined as the total summation of practices and knowledge

(whether applicable or not), used in preventing or eliminating a physical, social or mental disease and which may depend exclusively on past experience and observations passed down from generation to generation either in written or verbal form (Agbabiaka et al., 2010). Indigenous societies for a long time have carefully maintained the traditions of collecting, processing and applying plant-based medications (Patil et al., 2011). Other populations aside from the indigenous cultures have also adopted traditional medicine and it is often times referred to as complementary or alternative medicine (CAM) (WHO, 2008).

The African traditional medicine has been described often as the cradle of mankind, it is the oldest medicinal system and perhaps the most diverse of all as the use of herbal medicine precede the existence of modern drugs and antibiotics in Africa (Ojo et al., 2010). The African traditional medicine comes in various forms and is holistic because it involves both the body and the mind. Traditional health practitioners usually diagnose and treat the psychological cause of an illness prior to the prescription of medicinal plants to treat the symptoms (Gurib-Fakim et al., 2010; Gurib-Fakim and Mahomoodally, 2013). Medicinal plants, being the most important constituents of traditional medicines are sold in marketplaces or prescribed by traditional healers in their homes (Ajibesin et al., 2008). A considerable number of the people of South Africa practice traditional medicine to meet with demands ranging from their psychological to their health needs. They do not regard traditional medicine as an inferior substitute to modern medicine but consider it has a necessity for the treatment of a wide range of health issues that modern medicine treats inadequately (Sobiecki, 2012).

2.1.2 Pharmacological properties of Medicinal Plants

There are diverse species of medicinal plants that abound worldwide that are being utilized for their antibacterial, antiviral and antifungal activities (Akrayi and Abdullrahman, 2013; Sivananthan, 2013). Their roles as sources of anti-infective compounds have been extensively documented (Ndhlala et al., 2013; Ngameni et al., 2013). For example, Abera et al. (2011) reported that the aqueous and ethanol extracts of eight different plants species (including *Hogenia abyssinica*, *Allium sativum*) had antifungal potential *in-vitro* and *in-vivo* against *Colletotrichum kahawae*. In another study, the essential oils of cinnamon, lemon grass, Japanese mint, ginger grass, geranium and clove oils at concentrations ranging from 0.01 to 0.15% were

all observed to show activity against *Candida albicans* (Hammer and Carson, 2011). Though pathogenic fungi have similarities with their hosts being eukaryotes making them difficult to combat (Routh et al., 2011), however, a large number of medicinal plants have been reported to contain bioactive compounds which have antifungal action (Kumar et al., 2007).

Plants generally, including those used as traditional medicine, synthesize a diverse variety of chemical substances (Hartmann, 2007; Jenke-Kodama et al., 2008). These chemical substances are referred to as secondary metabolites, and they include saponins, tannins, oxalates, phytates, trypsin inhibitors, cyanogenic glycosides and many others (Soetan and Oyewole, 2009). The most significant amongst these chemical plant components are phenolics and flavonoids (Wojdylo et al., 2007), as different studies have proven that plants having a high content of phenols have a great capacity to act as an antioxidant (Shan et al., 2007; Wojdylo et al., 2007).

Studies have suggested that majority of these antioxidant compounds exhibit anti-inflammatory, anti- carcinogenic, antibacterial, antitumor, or antimutagenic effects in cells (El-Chaghaby et al., 2011; Aggarwal et al., 2011; Chanda and Nagani, 2013; Xiong et al., 2013), as they contain a wide range of free-radical scavenging molecules (Agati et al., 2012). These metabolites mostly have prominent effects on the animal systems and microbial cells (Tahara, 2007). They are known to possess therapeutic properties (Rahman et al., 2007), which can be used and have been used to treat human and animal diseases globally (Soetan and Oyewole, 2009).

Secondary metabolites have shown their potentials as antimicrobials when used alone and as synergists of other antimicrobial agents. They often act through different mechanisms other than that used by conventional drugs and it is expected that they will be active against drug-resistant pathogens. These compounds have been found also to act by either killing pathogens or inhibiting their growth while having little or no toxic effect on host cells and are considered prospects for the development of antimicrobial drugs (Rajendran and Ramakrishnan, 2009; Abreu et al., 2012).

Unlike conventional drugs, medicinal plants usually contain different chemicals which work together catalytically and synergistically to generate a combined effect that would exceed the total effect of the individual components. The combined effect of these compounds leads to the

increased activity of the main medicinal components by accelerating and delaying its assimilation in the body. They minimize the rate of undesired side effects, and have an additive or antagonistic effect. It has been suggested that the different chemical structures contained in plants are not waste products. As an individual plant may, for instance, possess alkaloids which improve the mood, phenols that act as an antioxidant; tannins that can act out as natural antibiotics, and anti-inflammatory compounds that reduce swellings and pain (WHO, 2008; Chintamunnee and Mahomoodally, 2012; Nunkoo and Mahomoodally, 2012; Shohawon and Mahomoodally, 2013).

2.1.2.1 Antioxidant Compounds in Medicinal Plants

Plants supply a very remarkable range of antioxidant compounds such as carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid, tocopherols and tocotrienols to prevent oxidation of their susceptible substrates (Ozsoy et al., 2009). These plant-based dietary antioxidants, when taken by humans, have been reputed to play a crucial role in the maintenance of human health as our endogenous antioxidants may not sufficiently provide protection against the regular and unavoidable challenge of reactive oxygen species (ROS) (Chen et al., 2012). Apart from being effective in the inhibition and reduction of free radicals, these natural antioxidants have been established as safe (Morten et al., 2012; Camila et al., 2013), as studies have suggested that their synthetic counterparts cannot be re-used neither can they be recycled once their electron has been donated thereby becoming deleterious metabolic by-products that elevate the entire load of oxidative stress (Wang et al., 2008).

2.1.3 Solvents Used in Extraction Process of Medicinal Plants

Extraction is an important process carried out first when screening medicinal plants for their pharmacological properties. This step is required in the analysis of plants in order to obtain their desired constituents for further separation and characterization (Sasidharan et al., 2011). There exist various extraction methods; however, the conventional methods are the most commonly used in plants extraction. And in conventional extraction, the release of the desired compounds usually needs soaking and maceration in mild solvents (Chan et al., 2012).

The solvent selected for extraction process depends greatly on the nature of the bioactive compounds targeted. There are different solvent available for extracting bioactive compounds from natural products (Sasidharan et al., 2011). Solvents have different polarities just like secondary metabolites. The three different polarity strengths of solvents are polar, non-polar and medium-polar. Polar solvents will extract polar chemicals while non-polar solvents will extract non-polar chemical compounds. Examples of polar solvents include water, ethanol, and methanol, non-polar solvents examples are chloroform, toluene and hexane and medium-polar solvents include dichloromethane, ethyl acetate and acetone. Therefore for a given sample, different solvents can be mixed for extraction or they can be used in sequence in the same sample material (Majekodunmi, 2015).

The plant material used as a source for secondary plant metabolites can be either fresh or dried while the solvent utilized should not interfere with the bioassay as the end product in extraction will contain traces of residual solvent (Ncube et al., 2008). Extraction from the plant is an empirical exercise in which different solvents are utilized under a variety of conditions such as time and temperature of extraction. The success or failure of the extraction process depends on the most appropriate assay (Doughari, 2012). A wide range of extractants as well as their combinations that have been utilized in studies screening plants for their antimicrobial properties, range from acetone (Lalli et al., 2008), methanol, water (Magee et al., 2007), dichloromethane, hexane (Masoko et al., 2007), hexane, ethyl acetate, ethanol, and water (Karthikumar et al., 2007), ethanol and ethyl acetate (Valarmathy et al., 2010).

2.2 Chronic Non-Communicable Diseases (NCDs)

NCDs are diseases that are not transmitted from one person to another (WHO, 2015). They are a set of conditions not generally induced by severe infections. They are incessant, occurring for a long time giving rise to an extensive period of health consequences, thereby creating a need for a long-term treatment and care (United States Department of Health and Human Services, 2014). The rise in the burden of NCDs in various nations of the world accounts for 38 million deaths per year (WHO, 2015). Globally, NCDs is a major cause of disability (Kruk et al., 2015). They have been reported to kill more individuals annually than any other causes of death combined (WHO, 2014), thus making them a leading threat to the development and health of individuals

worldwide (WMA, 2009). In 2014, these diseases resulted in 608,000 deaths suggesting that after tuberculosis and HIV and AIDS, NCDs should be given a top priority (WHO, 2014).

2.2.1 Features of Chronic Non-Communicable Diseases

NCDs occur as a result of modifiable and preventable lifestyle-related risk factors. The modifiable risk factors refer to those that can be changed; they include, living and working conditions, socio-cultural factors while the non-modifiable factors refer to those which cannot be controlled by the person affected such as hereditary factors (Puoane et al., 2008). Risk factors of NCDs include the use of tobacco, physical inactivity, unhealthy diet, indiscriminate consumption of alcohol, high blood cholesterol, obesity and high blood pressure (Puoane et al., 2008; WHO, 2010; Ueda, 2013). These risk factors produce diverse lifelong disease processes resulting in high mortality rates which can be attributed to heart attacks, tobacco-induced cancers, strokes, obstructive lung diseases (Puoane et al., 2008).

2.3 Diabetes Mellitus

Diabetes gotten from Greek word “Diab” which means passing through and this refers to the constant urination and the cycle of heavy thirst. While “mellitus” is a Latin word meaning “sweetened with honey” and this refers to the presence of sugar in the urine (Warjeet, 2011). Diabetes mellitus (DM) is a chronic disorder of metabolism that affects humans worldwide in terms of their physical, social and psychological health. It can simply be defined as a group of disorders characterized by a high level of glucose in the bloodstream (hyperglycemia), a modified metabolism of proteins, lipids and carbohydrate (Patel et al., 2011a; Warjeet, 2011).

The body normally converts consumed food into glucose during digestion, which the insulin hormone aids to reach the body cells. In diabetics, however, the glucose is retained in the bloodstream as a result of the insufficient insulin and its resultant action (Warjeet, 2011). Diabetes is, therefore, the consequence of the loss of the balanced effect of these hormones, it usually occurs when the body cannot utilize effectively the insulin it produces or when the pancreas does not produce enough insulin (Warjeet, 2011). Insulin is a hormone that regulates blood sugar and it is secreted by the pancreatic beta cells of the islets of Langerhans (Khan,

2003; CDC, 2011). Hyperglycemia gradually results in damage of the body systems, in particular, the blood and the nerve systems (WHO, 2015). Diabetes is fast rising to become a third major cause of mortality in humans alongside with the other main types of NCDs (Chauhan et al., 2010). The widespread of diabetes is anticipated to increase by 4.4% in 2030 having a high occurrence in China, USA, and India (Balaraman et al., 2010).

2.3.1 World Status of Diabetes Mellitus

Drawing on the report from the study carried out by the International Diabetes Federation (IDF), over 415 million individuals in 2015 had diabetes mellitus and this figure is still expected to rise to 640 million by 2040 (IDF, 2015) as against the report carried out in 2013 which showed that nearly 382 million adults had diabetes (IDF, 2013). This shows that in most countries, there is a rapid rise in the proportion of individuals living with type 2 diabetes and nearly 75% of these adults are living in low and middle-income countries. The highest percentage of people living with diabetes falls between the ages of 40 and 59 years (IDF, 2015). Diabetes does not exempt children as more than 20.9 million births were affected in the course of pregnancy in 2015 while over 542,000 children have type 1 diabetes in 2015 (IDF, 2015).

2.3.2 Diabetes Mellitus in South Africa

According to the South African epidemiological data on diabetes mellitus, there is an increase in the incidence of diabetes in urban areas compared to rural areas (Erasmus et al., 2012; Peer et al., 2012). Reports have also stressed the strong genetic component in Xhosa-speaking people in the Eastern Cape for developing diabetes (Molleutze and Levitt, 2005). The diabetes prevalence in the South African population is about 7%, which is around 2.28 million people affected by diabetes (IDF, 2015). The people who suffer from diabetes have steadily been increasing over the past decades. Popkin et al. (2012) reported that South Africa holds the second highest number of people suffering from type 2 diabetes in sub-Saharan Africa, with the largest number of diabetes cases found among the low-income groups (Mayosi et al., 2009).

Numerous reports have described the high rate of mortality as a result of diabetes particularly among the black populations (IDF, 2015). This is due to the fact that there are no promising treatments for diabetes; the various oral hypoglycemic drugs and insulin injection that abound

cannot cure diabetes mellitus (Dewanjee et al., 2009; Venkatesh et al., 2010). Besides, these drugs are characterized by unavailability to rural dwellers, high cost, and high level of toxicity. As a result, many South African diabetics find relief from traditional health practitioners who utilize herbal preparations for treatment (Oyedemi et al., 2009; Semanya and Potgieter, 2014). Nevertheless, it must be noted that 50-85% of people suffering from diabetes (particularly in rural areas) remain undiagnosed (Amod, 2012).

2.3.3 Clinical Manifestation and Risk Factors of Diabetes Mellitus

In diabetes mellitus, the primary symptoms that may present include blurring of vision, weight loss, increased thirst and increase in output of urine (polyuria). Whenever any of these symptoms occur or there is a history of diabetes in the family, diabetes should be questioned (ADA, 2009; Warjeet, 2011). Symptoms of chronic diabetes include susceptibility to certain infections and growth impairment in both children and young adults (Giannini et al., 2014). The most severe clinical symptoms of uncontrolled diabetes are hyperglycemia with the non-ketotic hyperosmolar syndrome or ketoacidosis which may result in coma, stupor or death in the absence of treatment. However, the symptoms are not usually present or may not be severe, which may lead to non-routine screening and no treatment inducing functional and pathological alterations for an extended period of time until a proper diagnosis is made (ADA, 2009, 2010).

Risk factors that increase the susceptibility for diabetes are mainly associated with family history of diabetes, obesity, smoking, age, physical inactivity, and ethnicity. Diabetes and their related complications are important causes of the high rates of mortality and morbidity among diabetic people and this leads to a huge economic burden on any healthcare system. Nephropathy leading to renal dysfunction, lower-extremity amputation, peripheral neuropathy with risk of foot ulcers, autonomic neuropathy leading to genitourinary, gastrointestinal, and cardiovascular symptoms, sexual dysfunction and retinopathy with potential blindness are diabetes-related complications which occur as a result of their long-term relativity (Deshpande et al., 2008; CDA, 2008; ADA, 2011). Diabetic patients have a high incidence of cardiovascular and cerebrovascular disease, as well as abnormalities of lipoprotein metabolism and hypertension (ADA, 2009).

2.3.4 Classification of Diabetes Mellitus

It is difficult to decide which class of diabetes to assign people with diabetes, as the present circumstance as at diagnosis time is used to attribute a specific type of diabetes to a person (ADA, 2014). Diabetic patients may need insulin treatment at any stage of their disease and this application of insulin does not, of itself, permit aetiological classification (ADA, 2013), also a majority of them may not fit easily into a single class (ADA, 2014). Therefore, it is more important to understand the pathogenesis of diabetes and effectively treat it in a patient by the clinician than to identify the particular type of diabetes (ADA, 2014). Individuals who are developing or currently have diabetes can be classified by the clinical characteristics of the clinical stage, even when the information on the underlying causes is not present (Amod, 2012). Diabetes mellitus can be divided into 4 clinically different types (Deshpande et al., 2008) which include:

I. Type 1 Diabetes Mellitus

This type of diabetes previously referred to as Insulin Dependent Diabetes Mellitus or Juvenile-Onset diabetes is as a result of cellular mediated autoimmune pancreatic beta-cell destruction (ADA, 2009; Warjeet, 2011). People affected by this form of diabetes do not produce insulin and need to take insulin every day to strain glucose out of their blood into their cells as fuel. There are times when the pancreas, which supplies insulin, gets damaged (Warjeet, 2011). This type of disease accounts for 5 to 10% of diabetic cases, it usually occurs in childhood and adolescence, though it can occur at any age, even in the 8th and 9th decades of life (ADA, 2009; Amod, 2012).

The rates at which cells are destroyed varies in type 1 diabetes, it can be fast in some persons, majority being infants and children, and it can be slow in other people (the majority being adults). In some diabetic individuals, the clinical manifestation may firstly be ketoacidosis while some may have modest fasting hyperglycemia that can quickly be altered to severe hyperglycemia and/or ketoacidosis when infection or stress sets in. Some diabetic patients, especially adults, may reserve beta-cell function which would be enough to prevent ketoacidosis for many years; however, they tend to be at the risk of ketoacidosis and also depend on insulin for their survival. There is little or no insulin secretion, at the terminating stage of this disease, which is made evident by the undetectable levels of plasma C-peptide (ADA, 2009).

II. Type 2 Diabetes Mellitus

This type of diabetes previously referred to as adult-onset diabetes or non-insulin dependent diabetes mellitus (NIDDM), includes people who commonly have relative (rather than absolute) insulin deficiency (i.e. disorders of insulin action and secretion) and insulin resistance (disorders of insulin action). This form of diabetes is the most common type of diabetes mellitus as it accounts for more than 90% of diabetes cases (ADA, 2009; Amod, 2012). People affected by this form of diabetes do not produce sufficient amount of insulin neither do their cells respond to the presence of insulin. Type 1 diabetes is more severe while type 2 diabetes occurs more particularly in developing countries (Warjeet, 2011). There are no specific causes of type 2 diabetes, as patients do not have any of the other causes of type 1 diabetes and neither does the autoimmune destruction of beta-cells occur.

However, the majority of the patients with type 2 diabetes are obese and this, to a certain degree, induces insulin resistance. While those who are not obese, may have a high amount of body fat predominantly distributed in the regions of the abdomen. Ketoacidosis rarely occurs in this form of diabetes, and even when it does occur, it is associated with the stress of an infection. Type 2 diabetes, usually for many years proceeds undiagnosed and this is due to the gradual development of hyperglycemia and it is not sufficiently severe for the patient to take note of any of the symptoms of diabetes. In such patients, there is a high risk of developing microvascular and macrovascular complications, and their insulin level may be elevated or normal, although values if their beta-cell function been normal, higher blood glucose levels would be expected to lead to an increase in insulin level (ADA, 2009).

III. Gestational Diabetes Mellitus (GDM)

Gestational Diabetes Mellitus (GDM) is defined as any form of glucose intolerance with onset during pregnancy. This is applicable if insulin or only diet modification is used for treatment or if the disease condition continues after pregnancy. It, however, does not rule out the possibility that unrecognized glucose intolerance may have begun concomitantly with the pregnancy or be antedated. Depending on the population studied, the prevalence of gestational diabetes ranges from 1 to 14% and it represents almost 90% of diabetes complication in pregnancy. The decline

in glucose tolerance usually occurs during pregnancy, especially in the 3rd trimester (ADA, 2009).

The risk of developing gestational diabetes is higher in obese women, women who have had gestational diabetes in a previous pregnancy, women with a family history of diabetes and women from ethnic minority groups in particular the Hispanic and Asian women (Lawrence et al., 2008). Therefore, the management and strict glycemic control are essential to prevent complications in birth and in the developing infant. It is also important to note that women who have had gestational diabetes have a 20% to 50% increased risk of developing type 2 diabetes at a later stage in life (ADA, 2009).

IV. Other Types Of Diabetes Mellitus

This encompasses all the types of diabetes caused by:

I) Specific Genetic Defects of Beta-Cell Function

This type of diabetes is usually identified by the early onset of hyperglycemia (mostly before 25 years of age). They are usually referred to as maturity-onset diabetes of the young (MODY) and are denoted by impaired insulin secretion with minimal or no defects in insulin action (ADA, 2009).

II) Drugs or Chemicals Induced Diabetes

There are several drugs that can damage the secretion of insulin and these may trigger diabetes in people with insulin resistance but may not cause diabetes themselves. In cases like this, the classification is not clear as the sequence or relative significance of beta cell dysfunction and insulin resistance is unknown (ADA, 2009).

III) Genetic Defects in Insulin Action

These are rare causes of diabetes which result from genetically determined abnormalities of insulin action and it is connected with the insulin receptor mutation ranging from modest hyperglycemia and hyperinsulinemia to severe diabetes (ADA, 2009).

2.3.5 Common Infections of Diabetes Mellitus and Their Management

Diabetes patients have increased susceptibility to infections and these infections, as well as the consequences linked with its infectivity, may elicit DM complications such as hypoglycemia and ketoacidosis (Casqueiro et al., 2012). Common infections of diabetes mellitus include both the most prevalent infections in addition to those that constantly affect only people with diabetes, for instance, rhinocerebral mucormycosis (Peleg et al., 2007).

I. Skin Infections

Skin infections occur frequently in approximately 79.2% of individuals living with diabetes (CDC, 2011) as some pathogens of bacterial and fungal origins are usually found on the mucosal and skin membrane of diabetics, especially *Candidia albicans* and *Staphylococcus aureus*. Reports have shown that there is an increased prevalence of the carriage of *Staphylococcus* in people with diabetes (Lipsky et al., 2010); however, this finding has not been consistent (Duran et al., 2006). In a clinical study involving 750 diabetic patients, it was shown that the most frequent dermatological complaints were inflammatory skin diseases (20.7%), xerosis (26.4%), and cutaneous infections (47.5%) (Demirseren et al., 2014).

Diabetic patients having type 1 diabetes are less likely to develop cutaneous infections than those with type 2 diabetes. Cutaneous manifestations either develop during the course of the disease diabetes or as the first sign of diabetes. They include dermatophytosis, bacterial infections, and candidiasis (Duff et al., 2015). Hyperglycemia elevates the risk of developing candidiasis which in turn supports the proliferation of *C. albicans* (Kalus et al., 2012). The most frequent of candidiasis infection is candidal vulvovaginitis while perianal candidiasis is usually prevalent in both females and males (Kalus et al., 2012). Treatment of choice for this condition in diabetics consists of systemic or topical antifungal medications such as Clotrimazole vaginal 1% cream, Miconazole 2% cream, Fluconazole 150 mg \times 1 dose, Ticonazole vaginal 6.5% ointment, Butoconazole vaginal 2% cream (Duff et al., 2015). Cutaneous bacterial infections also frequently occur in individuals with diabetes and are more life-threatening. The most occurring bacterial infections in patients with uncontrolled diabetes are skin abscesses or staphylococcal folliculitis. Treatment options include surgical drainage and antibiotics which they respond well to (Duff et al., 2015).

II. Soft Tissue Infections

Soft tissue infections (STIs) are inflammatory microbial invasion of the epidermis, dermis and subcutaneous tissues (Dryden, 2010). They also include infections such as infected ulcers, major abscesses, Furuncles and foot infections, in which underlying disease such as diabetes mellitus, complicates the response to treatment (Dryden, 2010; FDA, 2010). Foot infections as a result of diabetes usually lead to hospitalization with grave consequences such as amputations and osteomyelitis (Pendsey, 2013). This occurs because the majority of the non-severe diabetic foot infections may lack systemic manifestations such as chills, leukocytosis or fever and they have a painless nature in diabetics with peripheral sensory neuropathy (Sumpio, 2012). These manifestations are not reliable in diagnosing foot infections as they appear to be much reduced in diabetic patients (Richard et al., 2011). The diagnosis of diabetic foot infections should therefore be based on clinical findings and not the microbiological analysis of wound cultures since foot infections are colonized by microorganisms (Jeffcoate et al., 2008; Lipsky, 2008).

It also can be demanding to differentiate between the colonizing microorganisms and pathogens from a culture of swabs of foot lesions in diabetic patients (Martin et al., 2010). Findings suggest that a result of an infected wound culture identifies the bacterial load and not the virulence potential of the bacteria isolated, to therefore, quantify the bacterial burden, a tissue biopsy would be needed (Richard et al., 2011). Antimicrobial therapy is needed for nearly all clinically infected diabetic foot wounds, however, they are not required for clinically uninfected wounds (Lipsky et al., 2015) as the roles played by antimicrobials for clinically uninfected wounds continues to be a topic of debate (Arzt et al., 2012). Appropriate antibiotic therapy should promptly be given in order to improve the chances of limb salvage since most diabetic foot infections are true exigencies (Benwan et al., 2012). Antimicrobial agents such as ceftobiprole, dalbavancin, linezolid, ertapenem, doripenem, daptomycin or tigecycline have proven to be active systemically against isolated bacteria from foot infections (Sotto et al., 2008; Goldstein et al., 2008).

III. Pulmonary Tuberculosis

Pulmonary tuberculosis has had a recognized linkage with diabetes for more than a century, but in spite of the fact that this susceptibility to tuberculosis in diabetic patients has been known, it is

the confrontation by the present type 2 diabetes epidemic that has led to the potential mechanisms and public health impact of this association being addressed (Restrepo et al., 2008).

The nature of the association between diabetes and tuberculosis is not well known, but a number of pathways have been proposed (Young et al., 2009). As reports of studies have shown that tuberculosis being connected with the incidence of diabetes is possible as a result of extended insulin resistance induced by the administration of ATT medication—rifampicin. The inflammatory modifications associated with chronic infectious conditions might likewise be accountable for this association particularly neuropathy (Brostrom, 2010).

Studies have shown that diabetic people have a high risk of tuberculosis pneumonia (25-75%) (Kornum et al., 2008) as a result of hyperglycemia which weakens their innate immune system (Stegenga et al., 2008; Dooley and Chaisson, 2009). A review of 13 observational studies showed that diabetes was associated with a 1.2-7.8 increased risk of tuberculosis (Jeon and Murray, 2008). Leegaard et al. (2011) also found diabetes to be consistently associated with an increased tuberculosis risk. Although another report suggested that only diabetic patients having poorly controlled glycemia were more susceptible to tuberculosis (Restrepo et al., 2008).

Wang et al. (2009) found that individuals with diabetes have a more severe type of tuberculosis. Similar findings were reported by Baker et al. (2011), who, in addition, found that after being treated, diabetic patients have an increased risk of relapse or relative risk of dying during treatment of tuberculosis. Some studies also reported that diabetes is connected with the high death rate in patient with tuberculosis (Dooley et al., 2009; Wang et al., 2009). These studies demonstrated that the risk of death was six times higher in diabetic patients having tuberculosis.

The presence of diabetes makes the management of tuberculosis arduous, although conversely, the persistent stimulation of the inflammatory system caused by tuberculosis adversely affects the treatment of diabetes (Wang et al., 2009). Moreover, tuberculosis and its treatment may also worsen the glycemic control in a diabetic patient, which may support the development of diabetes complications particularly neuropathy (Brostrom, 2010).

Recent studies have also shown that the spread of diabetes pandemic has an impact on the outcomes of tuberculosis, and an estimate of about 1.5 million deaths and 7.8 million cases

attributed to tuberculosis maybe prevented in the next 10 years if interventions can decrease diabetes by 35% (Pan et al., 2015).

IV. Urinary Tract Infections

Urinary tract infections (UTI) occur commonly in people with diabetes especially those with type 2 diabetes (Geerlings, 2008), although, the exact relationship between the cause-effect has not been established (Johnsson et al., 2013). The varieties of urinary tract infections include lower UTI (cystitis), severe urosepsis, asymptomatic bacteriuria (ASB) and pyelonephritis. And the severe complications of urinary tract infections occur more in patients with type 2 diabetes than in other diabetic patients (Kofteridis et al., 2009; Mnif et al., 2013). There are numerous factors that predispose people with diabetes to urinary tract infections; they include glucosuria, neurogenic bladder, hyperglycemia etc (Geerlings, 2008; Rackley, 2011).

Microorganisms such as *Klebsiella*, *Proteus*, *Pseudomonas* and *Escherichia coli* are amongst the frequently isolated pathogens from the urine sample of diabetic patients with UTI (Kolawole et al., 2009). Nevertheless, individuals with diabetes are more susceptible to having resistant pathogens such as vancomycin-resistant *Enterococci* (Papadimitriou-Olivgeris et al., 2014), fluoroquinolone-resistant uropathogens (Wu et al., 2014), carbapenem-resistant *Enterobacteriaceae* (Schechner et al., 2013), and extended-spectrum β -lactamase-positive *Enterobacteriaceae* (Inns et al., 2014) as the cause of their urinary tract infections (UTIs). The pathogenesis of UTIs is greatly influenced by factors such as poor metabolic control of diabetes, the reduction of the urinary bladder sensitivity as a result of autonomic neuropathy and other impairments in the immune system (Truzzi et al., 2008; Fünfstück et al., 2012).

V. Foodborne Infections

Diabetic patients are immunocompromised hence they have a heightened risk of getting foodborne infections (Shah and Hux, 2003). Foodborne infections otherwise known as foodborne illnesses are prevalent, and even preventable global public health issues. They occur as a result of the consumption of contaminated foods, water or beverages. According to the Centre for disease control and prevention (CDC), approximately 48 million people get sick with foodborne

poisoning, 128000 get hospitalized and 3000 deaths occur annually in the United States (CDC, 2016). The majority of people affected are young children, older adults, or those with weakened immune systems who may be unable to fight infection. The foodborne diseases are caused by different agents, they include parasites, viruses and pathogenic microorganisms such as *Salmonella*, *Camphylobacter*, *Listeria*, *E. coli*. (CDC, 2016).

2.4. Current Approaches for the Management of Diabetes

The primary management approach used for diabetes mellitus is the reduction of high blood sugar level without inducing abnormal reduction (hypoglycemia) by diet management, nutritional dietary aid, and scheduled exercise; using oral anti-diabetic drugs and insulin administration (Chawla et al., 2013).

2.4.1. Diet Management and Scheduled Exercise

According to a study by Boffetta et al. (2011), there have been substantial decreases in type 2 diabetes incidence through lifestyle and diet modifications. Around 20-50% of individuals affected by type 2 diabetes regulate their blood sugar levels by only modification of diet (WHO, 2004). Over 50% of the calorific value of human diet is actualized by starch based foods. Starch comprises of amylopectin and amylose in the ratio of 3:1. Amylopectin contains extensive branching while amylose has linear long chains that usually hinder digestion, consequently playing a role in the formation of resistant starch (Goni et al., 2007).

The mechanisms and processes used to exert disease control properties by foods rich in fibre and resistant starch are not well understood. A part of these factors is believed to have impacts on digestion and absorption rate. They include physical nature and source of food material, the presence of enzyme inhibitors, food components, anti-nutrients as well as processing methods (Goni et al., 2007). Starches containing high amylose have the potential to control high-fat-diet-induced obesity by modulating hepatic fatty acid oxidation (Shimotoyodome et al., 2010), while resistant starches induces elevation in the levels of anti-diabetic hormones such as glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) (Severijnen et al., 2007; Zhou et al., 2008).

Therefore, food having high water content e.g. lettuce, tomato etc. and high fibre content such as flax, celery, oat, bran etc. and low sugar e.g. papaya, bitter gourd, cranberries etc. are recommended for the management of diabetes (Wheeler and Pi-Sunyer, 2008). Glucose burden on the body can be minimised by just diet, by so doing, delay insulin resistance and prevent the overuse of anti-diabetic approaches. Notwithstanding the physiological response owing to glucose homeostasis linked with energy currency attributes, exercise or physical work in daily schedule is important in order to channel the energy and revitalize the body cells also. When exercise and diet, both lifestyle modifications, are combined they can either minimise or delay by more than 50% diabetes incidence (Joshi et al., 2008).

2.4.2 Pharmacotherapy for Diabetes Mellitus

2.4.2.1 Oral Hypoglycemic Agents

Oral hypoglycemic agent is a term used to describe drugs used orally which are effective in lowering the glucose level in the blood (Wadkar et al., 2008). Treatment of diabetes is designed to lower insulin resistance and to restore the pancreatic α - and β -cells function. β -cell dysfunction degenerates gradually resulting in the need for insulin replacement therapy.

2.4.2.1.1 Classification of Oral Hypoglycemic Agents:

Oral hypoglycemic drugs are classified into two based on their mechanism of action; drugs that assist insulin action otherwise known as “Sensitizers” (biguanides, α -glucosidase inhibitors, and thiazolidinediones) and drugs that augment insulin supply (sulfonylurea, non-sulfonylurea secretagogues, and insulin) or “Secretagogues”. Meanwhile, there are five distinct classes of oral hypoglycemic drugs and they include the Sulfonylureas (SUs), Meglitinides, Biguanides, Thiazolidinediones (TZDs)/glitazone, α -glucosidase inhibitors (Chawla et al., 2013).

(I) Sulfonylureas :

Sulphonylurea has been around for the last 50 years and have been used as a first-line oral hypoglycemic drug for type 2 diabetes (Heurgue et al., 2004). They cause the release of more stored insulin in response to glucose by activating the receptors on the β islet cells of the pancreas. Hence, they are ineffective in totally insulin deficient individuals as they do not

increase insulin formation (Wadkar et al., 2008). Sulphonylureas are generally classified into first- and second-generation based on their duration of action. The first-generation sulphonylureas include acetohexamide, tolbutamide and chlorpropamide. Chlorpropamide has a long duration of action, up to 48 hours, and even its metabolites have active hypoglycemic potential. Second-generation sulphonylureas include glipizide (Minidiab®), gliclazide (Diamicron®) and glibenclamide (Daonil®). Glimepiride (Amaryl®) is classified as a third-generation SU. Second-generation sulphonylureas have a greater potency and improved safety (Joshi and Joshi, 2008).

(II) Biguanides:

The principal drugs which belong to the biguanide class of compounds are phenformin and metformin. They do not cause insulin release but require the presence of some insulin to act. They enhance insulin binding to its receptors and stimulate insulin-mediated glucose disposal (Wadkar et al., 2008). Phenformin and buformin are older generations of oral anti-diabetic drugs but have both been withdrawn from the market, and for many years now their use has been discontinued. They have been replaced with metformin which has a lower associated risk of drug-induced lactic acidosis (The International Association of Forensic Toxicologists, 2010). In the United State and European Union, metformin is currently accepted as a first-line drug for type 2 diabetic obese patients due to the glucose-lowering effect, its economical nature, the less weight gain induced compared to other drugs and that it does not induce hypoglycemic attacks (Ito et al., 2010). Precautions should be taken when using an oral hypoglycemic agent as biguanide class of compounds should not be used in patients having renal diseases (Wadkar et al., 2008).

III) α -Glucosidase inhibitors

These refer to drugs that inhibit the α -glucosidase enzymes found on the border of the intestinal wall thereby delaying the breakdown and absorption of complex carbohydrates in the small intestine as they do not target any specific physiologic defect in diabetes. The inhibition brought about by the α -Glucosidase inhibitors is a competitive and reversible one, lasting up to the next major meal therefore dosing is needed before consuming every meal containing carbohydrate. α -

Glucosidase inhibitors have no hypoglycemic effects because they do not affect insulin secretion. Acarbose is a α -Glucosidase inhibitor which may be used as an adjuvant to diet in obese diabetics and their main side effect is flatulence (Wadkar et al., 2008; Joshi and Joshi, 2008).

2.4.3 Insulin

Diabetes is recognised as a wasting disease because of insulin deficiency in humans and insulin deficiency occurs in an individual due to the functional disorder of the pancreas (Wadkar et al., 2008). Basal insulin is known to regulate the metabolism of carbohydrate; it ensures that sufficient glucose for cerebral functions is maintained, while also reducing hepatic glucose production (Wadkar et al., 2008; Arnolds et al., 2010). It has been recognized that insulin is the therapy with the most potential to attain the glycemic target in diabetic patients (Philis-Tsimikas, 2009). Humalog and novolog are some of the different types of insulin (short-acting / long-acting) currently available for diabetes management (Wadkar et al., 2008). Insulin is usually recommended after oral hypoglycemic drugs have been futile, in oftentimes much later than is ideal (Philis-Tsimikas, 2009).

In people with normal glucose tolerance, the endogenous plasma insulin profile exhibits low but then constant insulin levels during fasting (Philis-Tsimikas, 2009), as the endogenous plasma insulin integrates the secretion of prandial insulin with the production of pulsatile basal insulin (Arnolds et al., 2010). However, after meals, there is a sharp rise in the glucose levels in the blood which is promptly sensed by the beta cells in the pancreas, leading to the insulin release across two phases, into the portal circulation and a quick rise in circulating insulin levels (Arnolds et al., 2010). When the increased insulin secretion is not required anymore, it will be followed by a gradual return to basal levels (Philis-Tsimikas, 2009). Therefore, exogenously administered insulin would have to perfectly and thoroughly mimic the healthy physiological pharmacokinetic insulin profile to prevent glycemic excursions (Philis-Tsimikas, 2009).

2.4.4 Plant-based anti-diabetic medicine

This refers to the medicinal plants that have been used in the treatment of diabetes. Plant-based medicine has been used cost-effectively for the treatment of diabetes globally. This may be the

form of therapy available in many parts of the world particularly in developing countries (Joseph and Jini, 2011). Traditional medicinal systems for the diabetes treatment have reported some plants used as herbal drugs. They contain bioactive compounds which have been described to possess pancreatic β cells regeneration, insulin release, and combat the problem of insulin resistance (Khan et al., 2012).

Plant-based anti-diabetic medicine or herbal medicines for diabetes can be classified based on their mode of action into: 1) Herbal medicine acting like insulin. Fruits and crude leaf extract of *Momordica charantia* have been used by diabetic patients and have shown hypoglycemic activity (Wadkar et al., 2008). It contains polypeptide-p or p-insulin, an insulin-like hypoglycemic protein, that have been suggested to reduce blood sugar levels in gerbils, langurs, and humans when injected subcutaneously (Tayyab et al., 2012). Polypeptide-p acts by mimicking the action of human insulin in the body and so it may be used as plant-based insulin replacement in type-1 diabetes patients (Paul and Raychaudhuri, 2010).

2) Medicinal plants stimulating insulin-secretion from beta cells, *Gymnema sylvestre* extract evokes stimulation of insulin secretion in various pancreatic beta cell lines (Oh, 2015). The bitter principle of *Aloe vera* mediates the stimulation of synthesis or release of insulin from the beta-cells of Langerhans producing a hypoglycemic effect in rats (Singh, 2011); 3) Herbal medicine acting by modifying glucose utilisation, in streptozotocin induced diabetic rats, the aqueous leaf extract of *Aegle marmelos* exhibit antihyperglycemic activity by increasing glucose utilization after 14 days treatment (Ayodhya et al., 2010). 4) Herbal drugs showing adrenomimeticism. 5) Pancreatic beta cell potassium channel blockers. 6) cAMP (2nd messenger) stimulators, the butanol extract of *Zizyphus spina-christ* treatment of diabetic rats significantly increases serum insulin and pancreatic cAMP levels (Bnouham et al., 2006).

7) Herbal drugs preventing oxidative stress that is possibly involved in pancreatic β -cell dysfunction found in diabetes, *Annona muricata* plays a significant role in reducing oxidative stress of pancreatic β -cells of streptozotocin induced diabetic rats (Malviya et al., 2010). 8) Herbal medicine regenerating and/or repairing pancreatic beta cells. 9) Effectors of size and number of cells in the islets of Langerhans, glycogenesis, and hepatic glycolysis stimulators

(Miura et al., 2001). 10) Drugs preventing pathological conversion of starch to glucose by inhibition of β -galactosidase, α -glycosidase and alpha-amylase. 11) Renal glucose resorption inhibitors (Jarald et al., 2008).

2.4.5 Complications Associated With the Pharmacotherapy of Diabetes

Every year, approximately 3.2 million deaths are attributed to diabetes complications with six deaths occurring per minute (WHO, 2010). The side effects related to the pharmacotherapy of diabetes can be categorised into three groups: i) common side effects which include elevation in “bad” cholesterol (LDL), edema (fluid in legs and ankles), gastro-intestinal side effects (nausea, diarrhea, abdominal pain, gassiness, vomiting, and bloating), and hypoglycemia (symptoms include profuse sweating, tremor, hunger, mental confusion, coma, shakiness, dizziness, and a rare risk of stroke or death); ii) uncommon side effects which include allergic reactions, anemia (low red blood cell counts) and congestive heart failure; iii) rare side effects/complications include leucopenia (low white blood cell counts), thrombocytopenia (low blood platelet counts, liver disease/liver failure, lactic acidosis (buildup of acid in the blood) and macular edema (eye problems) (Modi, 2007).

2.4.6 The Limitations of Current Available Pharmacotherapies in the Management of Diabetes Mellitus

There have been significant improvements recorded from the administration of the currently used therapies in the treatment of diabetes, despite this; several harmful side effects have been noticed during treatment when using these therapies. Studies have also shown that the oral hypoglycemic drugs most times tackle only the symptoms of diabetes instead of the underlying pathophysiology (Akinmoladun et al., 2014). The effectiveness of oral hypoglycemic drugs is also hindered by medical practitioners who tend to slow down the initiation and intensification of therapy. Oral hypoglycemic drugs are many times introduced late in the progression of diabetes, with the intensification delayed thereby subjecting the diabetic patient to hyperglycemia (Philis-Tsimikas, 2009).

Jellinger et al. (2007) suggested that when continuous titration of oral anti-diabetic drug monotherapy fails to achieve target HbA1c levels (ie, $\leq 6.5\%$), the combination therapy should be introduced. Another limitation is the use of oral anti-diabetic agents in the management of diabetes, particularly in the developing countries where the citizens can barely meet the expense of modern treatment, which brings about an additional economic burden (Akinmoladun et al., 2014). Popular traditional beliefs state that every disease has a cure. With this in mind, the depiction of diabetes mellitus as a chronic non-communicable disease reveals the limitations of allopathic medicine and the constant challenge to optimize the suitable anti-diabetic drug for effective glycemic control; cost of the therapy, adverse effect profiles etc, encouraging individuals who support these popular traditional beliefs to consult traditional health practitioners also showing the need to examine the anti-diabetic effects of medicinal plants and their continued role in diabetes management with a view to produce new and more effective drugs to curtail the surge of the ravaging epidemic of diabetes mellitus (Vijayakumar et al., 2010; Chinenye and Ogbera, 2013; Akinmoladun et al., 2014).

2.5 Free Radicals, Oxidative Stress, Antioxidants and Diabetes Mellitus

2.5.1 Free Radicals

A healthy cell has a fatal enemy known as the free radical (Suvetha and Shankar, 2014). The term “Free Radicals” is used to describe the highly reactive atoms, molecules or ions that have an unpaired electron in their outer orbit (Lü et al., 2010). The instability of this configuration produces energy that is released through reactions with nearby molecules such as nucleic acids, lipids, proteins, and carbohydrates (Lobo et al., 2010). These free radicals continually search for healthy cells and strike at their susceptible outer membranes (Hamid et al., 2010). They normally attack the nearest stable molecule removing its electron to attain stability. Following the reaction, the attacked molecule loses its electron becoming a free radical itself and the generation of the free radicals persist, giving rise to the disruption of the substance (Lippincott and Wilkins, 2008).

2.5.1.1 Sources of Free Radicals

Nitrogen, sulfur and oxygen are the elements free radicals are gotten from, therefore generating reactive nitrogen species (RNS), reactive sulfur species (RSS) and reactive oxygen species (ROS) (Lü et al., 2010). Free radicals can be generated steadily in the course of cellular metabolism or are periodically produced to neutralize foreign bodies by the immune system. Furthermore, they can also be generated in the human body by environmental factors like smoking, pollution, herbicides, radiation etc (Vega et al., 2013).

2.5.1.2 Functions of Free Radicals

ROS and RNS function in a number of cellular signaling systems. Their production by non-phagocytic NADPH oxidase isoforms plays a major part in the regulation of intracellular signaling cascades in different types of non-phagocytic cells including fibroblasts, endothelial cells and thyroid tissue (Pacher et al., 2007). They perform a critical part in the physiological process, although their levels have to be contained by the endogenous antioxidants due to their toxicity. An imbalance occurs when the production of the free radicals increases and the antioxidants are not present or inadequate enough to prevent the production. This causes cellular deterioration, serious damage to the body organs and tissues, some defects in the normal functioning of the DNA and RNA resulting in loss of function and form. These undesirable changes in the body result in a high number of chronic disease conditions (Singh et al., 2014). Thus, free radicals have a huge impact on living systems, and they are known for playing a double part as both beneficial and harmful species, since they can be either valuable or to damaging to living beings (Pham-Huy et al., 2008).

2.5.1.3 Free Radicals and Diabetes

Experimental evidence suggests that free radical-induced damage may contribute significantly to the onset of diabetes, the progression of diabetes complication and pathological consequences of diabetes (Naudi et al., 2012; Ayepola et al., 2014). Studies have also shown that scavengers of free radicals are effective in the prevention of type 1 and 2 diabetes in experimental diabetes in animal models and also in the reduction of the severity of diabetic complications (Ghamarian et al., 2012; Nishikawa and Araki, 2013). When hyperglycemia persists in individuals having

diabetes, it induces oxidative stress which increases with age due to i) the activation of polyol pathway; ii) overactivation of the hexosamine pathway; iii) the formation of advanced glycation end-products (AGE); and iv) PKC activation (Kowluru and Chan, 2007; Ramasamy and Goldberg, 2010).

2.5.2 Concept of Oxidative Stress

Oxidation reaction is a chemical reaction which transfers electrons to an oxidizing agent from a substance (Vafa et al., 2011; Sahni and Gupta, 2012; Tabesh et al., 2013). Despite the importance of oxidation reactions to life, they can also be deleterious as they can supply free radicals which begin chain reactions that damage cells (Halliwell, 2012). Antioxidants abort chain reactions by the removal of free radical intermediates and oxidize themselves in order to inhibit other oxidation reactions. Therefore, antioxidants are generally reducing agents such as thiols, polyphenols or ascorbic acid (Vafa et al., 2011; Halliwell, 2012). Despite the fact that oxidation reactions are important for life, they can similarly be harmful; and so animals and plants maintain complex systems of various types of antioxidants, such as glutathione, vitamin C and vitamin E along with enzymes such as catalase, superoxide dismutase and various peroxidases. Inhibition of the antioxidant enzymes or low levels of antioxidants induce oxidative stress and may harm or kill cells (Vafa et al., 2011; Halliwell, 2012; Rafieian-Kopaei et al., 2013).

The concept of 'oxidative stress' refers to a situation whereby the steady-state ROS concentration is transiently or chronically enhanced, disturbing cellular metabolism and its regulation and damaging cellular constituents (Lushchak, 2014). Oxidative stress may possibly be an essential part of many diseases that plague humans; hence the employment of antioxidants in pharmacology is studied extensively. Notwithstanding, it is not known whether oxidative stress is the aetiology of diseases (Bjelakovic et al., 2007). The diverse levels of antioxidant defence in a healthy human body, keeps the production of pro-oxidants in the form of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in check. However, whenever it is

subjected to harmful environmental, pathological or physicochemical agents like toxic chemicals, ultraviolet rays, atmospheric pollutants and radiation, this maintained balance shifts in favor of pro-oxidants leading to 'oxidative stress' (Sultan, 2014).

2.5.2.1 Role of Oxidative Stress in Diabetes Mellitus

Oxidative stress is considerably involved in the onset, progression and complications of diabetes as there are corroborative evidence available which support that oxidative stress aggravate the adverse effects of diabetes (Gupta et al., 2013; Rashid et al., 2013); as it is related to the high production of reactive oxygen species in the presence of compromised antioxidant defence systems, which prompt the modification in antioxidant enzymes, the impaired glutathione metabolism and the lipid peroxidation (Dewanjee et al., 2009). The production increase and ineffectual scavenging of reactive oxygen species may contribute to diabetes mellitus as the modification process in antioxidant enzymes reduced ascorbic acid levels and impaired glutathione metabolism are the main causes of the disturbance of antioxidants defence system in diabetes (Bagria et al., 2009; Patel et al., 2011b).

The primary diagnostic feature of diabetes mellitus is an increased blood sugar level due to the lack of regulation, which raises the oxygen level and releases superoxides which react easily with the available nitric oxide disabling its action as endothelial vasodilator (Haidara, 2010). There is the decrease in the cell synthesis and endothelium-dependent relaxation in the wall of blood vessels which would result in both micro-pathological and macro-pathological alterations (Aladag et al., 2009; Vikram et al., 2010). The elevated blood sugar level also results in sorbitol concentration and an elevated formation and accumulation of advanced glycation products (AGEs), which plays a crucial part in the complications of diabetes such as neuropathy, renal dysfunction and retinopathy (Ding et al., 2010).

2.5.3 Antioxidants

An antioxidant can simply be defined as any substance that is capable of inhibiting or preventing the oxidative damage of a susceptible molecule (Halliwell and Gutteridge, 2007). These substances protect the cells from the deleterious effects of free radicals, as they are able to

interact with, stabilize or deactivate the free radicals before they attack cells thereby preventing some of the harmful effects the free radicals might otherwise cause (Rahman, 2007). A highly elaborate antioxidant (enzymic and non-enzymic) system have been developed by humans which functions harmoniously and in combination with each other to protect the body cells and organ systems against the damage of free radical (Goodman et al., 2011).

2.5.3.1 Antioxidant Systems

Antioxidants can be derived exogenously either as dietary supplements or part of a diet (plants such as fruits and vegetables (Carlsen et al., 2010; Guerra et al., 2013) or be endogenous in nature (Goodman et al., 2011). These antioxidant compounds contained in plants act as quenchers of singlet oxygen formation, reducing agents and as free radical scavengers (Nweze and Okafor, 2010; Tuttolomondo et al., 2013). Certain dietary compounds cannot neutralize free radicals however they can boost endogenous activity and so could be categorized as antioxidants (Rahman, 2007).

An ideal antioxidant should be one that is easily absorbed and suppresses free radicals and chelate redox metals at suitable physiologically levels. It should function in aqueous and/or membrane domains effecting gene expression in a positive way (Rahman, 2007; Fahmi et al., 2013). The antioxidants that are endogenous in nature perform an important role in the optimal maintenance of cellular functions, systemic health and general wellbeing. Although, when undergoing conditions that encourages oxidative stress, these antioxidants may not be enough and may be supplemented with dietary antioxidants to ensure the cellular functions are maintained optimally (Ozben, 2015).

2.5.3.2 Classification of Antioxidants

According to their functions, endogenous antioxidants can either be classified as enzymatic and non-enzymatic (Goodman et al., 2011). The major enzymatic antioxidants include catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GRx) and glutathione peroxidase (GPx) (Genestra, 2007; Halliwell and Gutteridge, 2007). The non-enzymatic antioxidants can be subdivided into nutrient antioxidants and metabolic antioxidants. Nutrient antioxidants which

belong to exogenous antioxidants, are those compounds that have to be provided through foods such as carotenoids, trace elements (Se, Cu, Zn, Mn), vitamin E, vitamin C, as they cannot be produced in the body. Metabolic antioxidants include L-arginine, uric acid, lipoic acid, glutathione, bilirubin etc. (Seifried et al., 2007; Carocho and Ferreira, 2013). A few antioxidants can collaborate with other antioxidants thus reviving their original properties and making them more effective, this process is usually described as the “antioxidant network” (Brewer, 2011).

2.5.3.3 Roles Played By Antioxidants in Diabetes

Diseases such as cancer, diabetes and many others could be caused by free radicals and studies have shown that antioxidants from plants having the ability to scavenge free radicals have great prospects in improving these disease processes (Ramchoun et al., 2009). The normalization of the activities of oxidative stress markers (thiobarbituric acid reactive substances (TBARS), enzymes and free radicals), and the balance or removal of free radicals subsequently, portrays an effective method for the reduction of the adverse effects of free radicals (Webb and Falkowski, 2009). In the diabetic state, it has been suggested that there is an increased oxidative stress, and this coincides with the decrease in the platelet antioxidant status, thereby increasing the damaging effects of free radicals (Gibson et al., 2011). Studies have shown that diabetics with reduced antioxidant status are at an increased risk for diabetic complications (Lodovicia et al., 2008). Harding et al. (2008) suggested that a low content of lipid standardized plasma vitamin E or vitamin C is an elevated risk factor for the progress of type 2 diabetes and its complications.

Aktunc et al. (2010) showed in an experimental study of diabetes that N-acetyl-L-cysteine (NAC) when used in the treatment of alloxan-induced diabetic mouse model has the potential to decrease the endothelial damage which may normalize impaired angiogenesis in diabetes. In another study, the administration of antioxidants to rats with streptozotocin (STZ)-induced diabetes was shown to increase blood lipid profile, improved cardiac contractile function and reduce myocardial cell necrosis (Tappia et al., 2011). Hashemipour et al. (2009) reported the anti-oxidative activity of microelements such as zinc (Zn). Furthermore, it was recently reported that the multiple mechanisms through which physical exercise acts which includes up-regulating mechanisms governs physiological antioxidant generation (Venkatasamy et al., 2013).

Many drugs, for example N-acetylcysteine, that are in use for the treatment of diabetes in animals have antioxidant properties, besides their main pharmacological activity (Kamboj and Sandhir, 2011) and these antioxidant properties may be a key factor in the effectiveness of these drugs (Robertson, 2010; Kamboj and Sandhir, 2011). Furthermore, studies have shown that blocking the formation of free radicals with antioxidants is the goal and have suggested the need for the possible use of antioxidants in the treatment of DM. Also antioxidant therapies have been proposed to likely inhibit the onset of diabetes and in addition prevent the development of diabetes complications (Inoguchi et al., 2007; Sasaki and Inoguchi, 2012). Therefore, medicinal plants especially those with high content of antioxidants perform an integral part in the protection of the human body against harm by ROS and in the amelioration of diseases associated with oxidative stress such as diabetes mellitus (Mohamed et al., 2010). This implies that application of antioxidants as supplements can play a chemoprotective part in diabetes (da Silva et al., 2010).

2.6 Importance of Medicinal Plants in Diabetes Mellitus

Medicinal plants have always been a very good source of drugs and have been used to derive the currently existing drugs directly or indirectly. It has been suggested by available ethnobotanical information that around 800 medicinal plants may yet exhibit potential for anti-diabetic activity. These medicinal plants which have been evaluated using diverse experimental methods have revealed anti-diabetic activity (Ponnusamy et al., 2011). Some of the medicinal plants that have been used to treat diabetes include: *Azadirachta indica*, *Caseariato mentosa*, *Catharanthus roseus*, *Garuga pinnata*, *Ficus benghalensis*, *Hemidesmus indicus*, *Wattakaka volubilis*, *Pongamia pinnata* and *Premna latifolia* (Maruthupandian et al., 2011).

The administration of the ethanol extract, juice and oil of *Allium sativum* (garlic) orally has huge effect on lowering blood sugar in both normal and alloxan-induced diabetic rats models (Chauhan et al., 2010). Also, investigations showed that the constituents of the various *Asparagus racemosus* root extracts using organic solvents such as ethanol, hexane, chloroform and ethyl acetate extractants had insulinotropic activity (Hannan et al., 2007). Chloroform extracts of leaves of *Boerhaavia diffusa* were revealed to have anti-diabetic activity in streptozotocin induced diabetic rats and it acts by increasing insulin sensitivity and reducing blood glucose level (Malviya et al., 2010). When the ethanolic extracts of *Ricinus communis* was

administered at 500 mg/kg, p.o. for 20 days, it improved the lipid profile and body weight of the diabetic animal models and also increased insulin level significantly (Rao et al., 2010).

2.7 *Olea europaea* subspecies *africana* and *Euryops brevipapposus*

2.7.1 The plant *Olea europaea* subspecies *africana*

The plant *Olea europaea* subspecies *africana* (Mill) was previously referred to as *Olea africana* Mill and at the subspecies level it was known as subsp. *cuspidata* (Wall. ex G.Don) Cif. (Mabberley, 2008). *Olea europaea* subspecies *africana* is generally regarded as “African wild olive” meanwhile in South Africa, it is locally called “*motholoari*” (Sotho and Tswana people) and “*umnquma*” (Xhosa, Zulu and Ndebele people) (Long et al., 2010), “*Swartolien*, *swartoleen*, *swartolienhout*, *wilde-olyf*” (Khoe-San people) (De Vynck et al., 2016). It is an evergreen tree or shrub, with a height of about 18 m. The flowers and fruits are clustered on auxiliary panicles while the fruits are one seeded ranging from 0.5 to 1 cm in diameter and when ripe appear dark violet. *O. europaea* flowering and fruiting time usually occurs inbetween September and January. Dispersal is predominantly by frugivorous birds (Abiyu et al., 2016). It is naturally distributed in South Africa and has diverse habitats such as bush, forest, rocky ledges, rivers, mountain kloops and open grassveld (Green and Kupicha, 1976; Neuwinger, 1994). The *O. europaea* complex comprises of different wild and cultivated forms as the plant names are dependent on the taxonomy and nomenclature (Hamman-Khalifa et al., 2007).

2.7.1.1 Traditional uses of *Olea europaea* subspecies *africana*

Dold and Cocks (1999) named *Olea europaea* subspecies *africana* as the most significant plant employed in traditional medicine out of 120 plant species. In South Africa, Pappe (1857) reported the first use of wild *O. europaea* leaves as a styptic on fresh wounds. In several parts of Africa, the bark and roots are generally used for the treatment of urinary infections, colic, tapeworms, rheumatism and other infections. It is also been used as a folk remedy for combating kidney problems, eye infections, sore throat and backaches or headaches. Reports have also shown that it has the capacity to act as an emollient, hypotensive, styptic and febrifuge (Somova et al., 2003). Altinyay et al. (2011) reported the leaves to be effective in malaria treatment.

O. europaea is usually used as a hypotensive, tonic, emollient, diuretic, febrifuge and an anti-inflammatory agent in Europe and the Mediterranean region (Khan et al., 2007). Furthermore, south-eastern Morocco, the wild form leaves are used in the decoction form traditionally to treat diabetes and hypertension (Tahraoui et al., 2007). Khan et al. (2007) also suggested that the leaves may contain anti-spasmodic, vasodilator and anti-arrhythmic properties.

2.7.1.2 Components of *Olea europaea subspecies africana* plant containing bioactive compounds

The leaves are extensively investigated as they are vital for their secondary metabolites although the oil and fruits also serve as a principal portion of the daily diet of a large part of the world's population (Pereira et al., 2007). The quantity and type of phenolic compounds found in the leaves, fruits and seeds of *O. europaea* vary as the chemical composition depends on factors such as geographical location, climatic conditions, storage conditions, plant nutrition, proportion of branches on the tree and the level of contamination with soil and oils. Hence, the plant extract composition can be affected by the disparity due to these variations (Molina-Alcaide and Yáñez-Ruiz, 2008; Sudjana et al., 2009; Talhaoui et al., 2015). During the maturation period the phenolic composition of fruits and leaves changes significantly (Giannakopoulou et al., 2011). The plant extract composition can be affected by the disparity due to the differences such as geographical location, plant nutrition and cultivar (Sudjana et al., 2008).

In *O. europaea* leaves, the major groups of phenolic compounds found are oleuropein, flavones, flavonols, flavan-3-ols, and substituted phenols such as tyrosol, hydroxytyrosol, vanillin, vanillic acid, and caffeic acid (Sato et al., 2007; Ferreira et al., 2007). The phenolic compound generously found is oleuropein which is followed by hydroxytyrosol, the flavone-7-glucosides of luteolin and apigenin, and verbascoside. Hydroxytyrosol is a precursor of oleuropein, and verbascoside is a conjugated glucoside of hydroxytyrosol and caffeic acid (Liakopoulos et al., 2006; Makris et al., 2007). Olive leaves are the most abundant source of oleuropein and its derivatives, while others are present in lower quantities. The oleuropein is considered to be the main active compound responsible for the biological activities of the leaves (Luque de Castro and Japón-Luján, 2006; Khan et al., 2007; Papoti and Tsimidou, 2009).

2.7.1.3 Pharmacology of *Olea europaea*

i) Antioxidant Activity

Different studies have also been carried out to evaluate the antioxidant properties of the parts of the *O. europaea*. Jiang and Takamura (2013) investigated olive fruits for their radical scavenging activity, the olive fruits exhibited high antioxidant activity. In another study, the antioxidant activities of the leaf of *O. europaea* were determined, where the ethanol extracts showed a high activity against 2,2-Diphenyl-1-picryl hydrazyl radical (DPPH) radicals (Lafka et al., 2013). Other extracts that have been investigated include aqueous olive leaf extract (Hayes et al., 2011), leaves infusion (Goncalves et al., 2013).

ii)Antimicrobial Activity

Pereira et al. (2007) reported a broad antimicrobial activity of the leaf extracts of the *O. europaea* when assessed for their antimicrobial properties as the extracts inhibited all the tested microorganisms in a concentration-dependent manner with values of IC₂₅ lower than 1 mg/ml. The phenolic compounds hydroxytyrosol and oleuropein have been shown to inhibit the growth of pathogens, such as *Micrococcus* sp., *Salmonella typhi*, *Vibrio cholerae*, *Staphylococcus aureus*, *Vibrio parahemolyticus* and *Bacillus subtilis* (Sudjana et al., 2009).

iii)Anti-diabetic Activity

O. europaea leaves are well recognized as an antidiabetic and antihypertensive herbal drug traditionally used (Pereira et al., 2007). The oleuropein has a hypoglycemic effect using two mechanisms: 1) the potential to affect glucose-induced insulin release and 2) an effect of increase peripheral uptake of glucose (Al-Azzawie and Alhamdani, (2006). Part of the oleuropein effect on diabetes and its complication is gotten from its antioxidant activity (Sato et al., 2007). This also supports the findings of Al-Azzawie and Alhamdani (2006) which suggested that the relief in oxidative stress decreases serum glucose levels hence patients suffering from diabetes may be treated with good antioxidants. Eidi et al. (2009) investigated the potency of olive leaves extract in both normal and streptozotocin-induced diabetic rats. After the oral administration of olive leaves extracts at different doses of 100, 250, and 500 mg/Kg of body weight for 2 weeks. The total cholesterol, urea, serum glucose levels of the streptozotocin-

induced diabetic rats were significantly reduced, suggesting that olive leaf extract may be used as an anti-diabetic agent.

iv) Anti-cancer Activity

O. europaea has been described to be effective as chemopreventive and therapeutic agents against cancer as the components such as phenol have shown anticancer activities against different types of cancer (Casaburi et al., 2013). Fares et al. (2011) tested the antiproliferative activity of the ethanolic extract of the leaves of a Lebanese *O. europaea* on human leukemic cell line using WST-1 proliferation kit and (3 H)-thymidine incorporation method. Their findings reported the ethanolic extract had a concentration-dependent activity for inducing apoptosis. Wang et al. (2011) in a study of Inhibitory effect of oleanolic acid on hepatocellular carcinoma via ERK-p53-mediated cell cycle arrest and mitochondrial-dependent apoptosis described the oleanolic acid to have antitumor activities in hepatocellular carcinoma in the *in vitro* and *in vivo* models.

2.7.2.1 The family Asteraceae

The family *Asteraceae* is a large family of herbaceous plants and shrubs consisting of nearly 1,100 genera and 25,000 species extensively distributed in the arid and semi-arid regions of subtropical and lower temperate latitudes (Anderberg et al., 2007). According to Cowling et al. (1992), in the fynbos biome, the *Asteraceae* is the largest. The majority of traditional medicines used indigenously are gotten from plants that belong to this family. A lot of fynbos plants have been used as herbal remedies by descendants of the original inhabitants, the Khoi and San people for a long time (De Selincourt, 1992). The plants generally have hairy aromatic leaves with flat clusters of small flowers on top of the stem. Most of the family members are medicinal plants having therapeutic uses while a few of them are popular garden plants and serve as a vital economic source of food such as lettuce and artichokes (Anderberg et al., 2007; Achika et al., 2014).

2.7.2.2 The genus *Euryops*

The genus *Euryops* (Cass.) Cass. belongs to the *Senecioneae* tribe of the family *Asteraceae* and is made up of 97 completely described species plus another five awaiting descriptions or recently described (Nordenstam et al., 2009). *Euryops* is the third largest genus in the *Asteraceae*, in terms of species numbers in Southern Africa (Koekemoer, 1996). It is distributed all over Africa with the exception of one specie, *E. arabicus* Steud., which is represented in Arab and Socotra (Anderberg et al., 2007; Devos et al., 2010). *E. arabicus* is used to treat wounds in both Yemen and Saudi Arabia (Miller and Morris, 2004).

Notwithstanding the restriction of the genus to Africa, a greater portion (96 species) occurs in South Africa. Members of this genus are native to restricted areas in the karroo, fynbos shrublands of the Cape Floristic Region (CFR) and grasslands. Around eight species of *Euryops* are confined to the high mountains of tropical East Africa, Somalia, Ethiopia and south-western Arabia (Nordenstam, 1969; Pierson and McAuliffe, 1995). Out of all the species in the genus, only *Euryops arabicus* is represented in Saudi Arabia (Anderberg et al., 2007). The majority of the *Euryops* members are perennial, erect and multi-stemmed shrubs (with the exception of *E. annuus* Compt.), distinguishable by coriaceous leaves and yellow or orange flowered capitula on simple peduncles usually lacking leaves or bracts. The genus was split into six sections namely, *Euryops*, *Chrysops*, *Leptorrhiza*, *Angustifoliae*, *Brachypus* and *Psilosteum* (Nordenstam, 1968; Nordenstam et al., 2009; Devos et al., 2010).

The name resin bush is usually applied to all *Euryops* and the name literally translates the dutch name *Harpuis bosch*, derived from the Dutch word "hars" (resin) "puisje" (a small pimple), referring to the resinous secretion which exudes from the stem and branches in the form of small pimply drops on most *Euryops* species. The resin accumulates under the bushes of most species and was effortlessly collected as it was recognized during the colonial South Africa for its purported medicinal value (Smith, 1966). Several of the species are known locally by distinctive names.

2.7.2.3 Phytochemicals present in the genus *Euryops*

The members of the *Senecioneae* tribe are recognized for their distinct phytochemicals compared to other members of *Asteraceae* family and this characteristic has been used to differentiate taxonomically the genera of the *Senecioneae* tribe and the species found within each genera (Hegnaur, 1977). The phytochemicals usually found in the three important genera of the tribe are acetophenone, sesquiterpene lactones, diterpene derivatives, and furanoelemophilones. Most of the phytochemicals are very bitter and several are toxic so it is believed that when sesquiterpene lactone is produced, it acts as a toxicant in defense against parasites and herbivores. The nature of the distinctive phytochemistry of the entire tribe suggests that many of the chemicals produced serve a role in defense against herbivores and parasites, as chemical inhibitors involved in allelopathy, or both (Hegnaur, 1977).

2.7.2.4 Pharmacology of the genus *Euryops*

Mothana et al. (2011) studied the *in vitro* antimicrobial and radical scavenging effects of the essential oils from *Euryops arabicus* and phytochemical compounds such as secofuroermophilanes, furoermophilanes, eremophilanolides and flavonoids were identified. The flavonoids have been shown to increase the expression of the rate limiting enzyme in the synthesis of c-glutamylcysteine synthetase with a concomitant increase in the intracellular glutathione concentrations (Moskaug et al., 2005). *Euryops arabicus* has also been described to have a protective effect on the liver and kidneys and prevents the damaging effects of paracetamol in acute toxic doses while stimulating the endogenous antioxidant defense system (Hafez et al., 2015).

2.8 References

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Chapter 3

The bioactive potentials of *Olea europaea* subspecies *africana*, a medicinal plant commonly used in folkloric medicine among Xhosa tribe Eastern Cape Province, South Africa

Abstract

This study assessed the medicinal potential of the *Olea europaea* subspecies *africana* obtained from Cala community, Eastern Cape Province of South Africa by evaluating the antimicrobial and antioxidant efficacy of the extract as well as identifying the bioactive compounds from the oil. The ethanol leaf and ethyl acetate stem extracts showed significant activity in the inhibition of 2, 2-azinobis-(3-ethylbenzothiazolin - 6-sulfonic acid diammonium salt (ABTS) free radical, the n-hexane leaf extract had the overall significant lipid peroxidation inhibition activity, while in the inhibition of 2, 2- diphenyl-1-picrylhydrazyl radical (DPPH), the ethanol and ethyl acetate leaf extracts had strong activity. The antimicrobial efficacy were also assessed using both Gram positive (*S. marcescens*, *E. faecalis* and *P. vulgaris*) and Gram-negative bacteria (*K. pneumoniae*, *P. aeruginosa* and *S. flexneri*). The major antimicrobial activity was exhibited by the ethyl acetate extract with a MIC value of 0.625 mg/ml which was comparable to ciprofloxacin against *S. marcescens*, followed by the ethanol extract with an MIC value of 1.25 mg/ml against *K. pneumoniae* and *S. marcescens*. The ethanol leaves and stems extracts and ethyl acetate leaves and stem extracts were bacteriostatic while the n-hexane extract had no antimicrobial effect. Gas chromatography–mass spectroscopy studies on the oil revealed 61 compounds, accounting for 93.031% of the total oil. Nonanal, phytol and 2-isopropyl-5-methyl-9-methylene-bicyclo[4.4.0]dec-1-ene (5.65%) were the main chemical components identified.

Keywords: xhosa; *Olea europaea*; antioxidant activity; antimicrobial activity; gas chromatography-mass spectroscopy.

3.1. Introduction

The continuous rise of microbial resistance to currently available antibiotics and diverse chronic human pathologies caused by oxidative stress has become a huge challenge, and as a result more searches have emerged in the quest for novel antimicrobial agents and antioxidants from other origins such as plants, which have distinct modes of action other than that of the currently used drugs, with which to combat pathogens as well as protecting the human body against free radical induced damage and oxidative stress (Bjelakovic et al., 2007; Demain and Sanchez, 2009; Rajendran and Ramakrishnan, 2009; Sengul et al., 2009; Davies and Davies 2010; Duracková 2010; Mohamed et al., 2010; Abreu et al., 2012; Buffet-Bataillon et al., 2012; Paphitou, 2013).

Unlike conventional drugs, medicinal plants usually contain different bioactive principles which work together as catalyst and synergist to generate an integrated action that would exceed the aggregate action of individual components. The combined effect of these compounds lead to the increased activity of the main medicinal components by accelerating and delaying its assimilation in the body. It has been suggested that the different chemical structures contained in plants are not waste products. As an individual plant may, for instance, possess alkaloids which improves the mood and give a sense of well-being, phenols that function as a venotonics, and antibacterial, tannins functioning as diuretic substances that increase waste products and toxins elimination (WHO, 2008; Chintamunnee and Mahomoodally, 2012; Nunkoo and Mahomoodally, 2012; Shohawon and Mahomoodally, 2013). Notwithstanding the numerous benefits of plants, their potential as new drugs sources is still mostly unexplored as only a little out of the reported 500,000 plant species have been explored for biological or pharmacological activity (Mahesh and Satish, 2008; Fabricant and Farnsworth 2010).

The plant *Olea europaea* subspecies *africana* is classed under the family Oleaceae and it is generally regarded as “African wild olive” while in South Africa, it is locally called “*motholoari*” (Sotho and Tswana people), “umnquma” (Xhosa, Zulu and Ndebele people) (Long et al., 2010), “Swartolien, swartoleen, swartolienhout, wilde-olyf” (Khoes-San people) (De Vynck et al., 2016). *Olea europaea* tree is of about 18 m, its flowers and fruits are found clustering on auxiliary panicles while the fruits are one seeded ranging from 0.5 to 1 cm in diameter and when ripe appear dark violet (Mabberly, 2008; Abiyu et al., 2016). It is naturally

distributed in South Africa, northern Africa, India, Asia, south eastern Europe, Arabian Peninsula, northern Iran (Somova et al., 2003, Parvaiz et al., 2013) and has diverse habitats such as bush, forest, rocky ledges, rivers, mountain kloops and open grassveld (Green and Kupicha, 1976; Neuwinger, 1994).

In South Africa, Pappe (1857) reported the first use of wild *Olea europaea* leaves as a styptic on fresh wounds while later the fruits were employed in treatment of diarrhea by the early Cape settlers in South Africa (Watt and Breyer-Brandwijk, 1962). The plant is also used traditionally to treat kidney problems, eye infections, sore throat, backaches, headaches (Somova et al., 2003). The leaves are suggested to be potent in malaria and fevers treatment (Altinyay et al., 2011; Gentile and Uccella, 2014). A decoction of the wild form leaves is employed traditionally to manage hypertension and diabetes (Tahraoui et al., 2007).

Different studies have also been conducted to evaluate the antioxidant properties of the parts of the *O. europaea*. Jiang and Takamura (2013) investigated olive fruits for their radical scavenging activity, the olive fruits exhibited high antioxidant activity. In another study, the ethanolic extracts showed a high activity against DPPH radicals (Lafka et al., 2013). Other extracts that have been investigated include aqueous olive leaf extract (Hayes et al., 2011), leaves infusion (Goncalves et al., 2013), ethanolic extracts (Marwah et al., 2007). However a study reported that the olive leaf extract did not exhibit any *in vitro* inhibitory activity when tested against *S. aureus*, *B. subtilis*, *E. coli* and *K. pneumoniae* (McGaw et al., 1997).

O. europaea leaves have been shown to contain phenolic compounds, the major groups found are oleuropeoside, flavones, flavonols, flavan-3-ols, and substituted phenols (Sato et al., 2007; Ferreira et al., 2007). The oleuropein is considered to be the principal active compound responsible for the biological activities of the leaves, although some of the other phytochemical compounds have been observed to promote the biological activities (de Castro and Japón-Luján, 2006; Khan et al., 2007; Papoti and Tsimidou, 2009).

The quantity and type of phenols found in the leaves, fruits and seeds of *Olea europaea* vary as the chemical composition depends on factors such as geographical location, climatic

conditions, storage conditions, plant nutrition, proportion of branches on the tree and the level of contamination with soil and oils. Hence, the plant extract composition can be affected by the disparity due to these variations (Goldsmith et al., 2014; Talhaoui et al., 2015). Also, during the maturation period, the phenolic composition of fruits and leaves changes significantly (Giannakopoulou et al., 2011). The plant *O. europaea* are commonly used in the folklore medicine in Cala community, Eastern Cape especially in the management of chronic non-communicable diseases such as diabetes. This study therefore was aimed at assessing the medicinal potential of the *Olea europaea* subspecies *africana* growing in this community and validating its usage in traditional therapy by evaluating the antimicrobial and antioxidant efficacy of the extract and identifying the bioactive compounds from the essential oil using gas chromatography mass spectrometry (GC–MS) analysis.

3.2 Materials and Methods

3.2.1 Study Sites

Cala is a small rural town in the Sakhisizwe Local Municipality of Eastern Cape Province, majorly comprised of rural settlements (Caine et al., 2014). Cala is situated at geographical coordinates of 31.5230° S, 27.6980° E in the northern region of the Province (Statistics South Africa, 2012). It is reported to have a low Human Development Index (HDI) of 0.49, a high rate of unemployment and a high percentage of people living in poverty (75%) (CHDM, 2015). Hence, the people usually resort to traditional medications but it is either combined with modern drugs or used alone for the treatment of various diseases. The Xhosa tribe is the main ethnic group dwelling in this rural settlement.

3.2.2 Collection and identification of the plants

Ethical clearance was obtained from the University of Fort Hare, committee of research ethics, prior to the commencement of study. The leaves of *Olea europaea* subspecies *africana* were collected in May, 2016 from a mountain in Cala community. The plant was initially identified by its vernacular name “*umnquma*” and later authenticated in Selmar Schonland herbarium by Mr Tony Dold of Botany Department, Rhodes University.

3.2.3 Preparation of plant extract

Upon identification, the collected plant materials were washed thoroughly with sterile water to remove dust and dried at room temperature. The *O. europaea* was then crushed with Polymix (PX-MFC 90 D model) till finely powdered and stored airtight till further use. The extracts were prepared by soaking 75 g each of the dry powdered plant materials in 500 ml of organic solvents (n-hexane, ethanol and ethyl acetate) in 1000 ml glass bottles. Glass bottles were covered tightly to prevent spillage. The mixture was then shaken at 100 revolutions per minute for 72h at room temperature. The extract filtration was performed before concentrating with vacuum rotary evaporator (BÜCHI Rotavapor R-200/205, Model R205V800). Preparation of the stock solutions of crude n-hexane, ethanol and ethyl extracts was done by diluting each of the dried extracts with the solvents used for the extraction process. Prior to reconstituting the extracts with the extracting solvents, the extracts were stored in pre-weighed screw capped bottles and the yield of extracts was weighed and then screw capped bottles were kept in refrigerator.

3.2.4 Gas chromatography-mass spectrometry (GC–MS) Analysis

The oil was obtained by hydrodistillation using Clevenger apparatus. The oils were collected at 100°C in a cloud of steam, distilling into *n*-hexane before treating with anhydrous sodium sulfate PA to get rid of the leftover water. The oils were stored in dark colored vials and was used for the GC-MS analysis at the University of Fort Hare, Botany Department, using the HP 6890 GC-MS system coupled with a mass selective detector (HP5973). The various compounds contained in the oil were identified when the spectrum obtained through the GC-MS analysis was compared with the standards available or well known components spectrum saved on the database of National Institute Standard and Technology (NIST).

3.2.5. Antioxidant Assay

For the antioxidant assays, the *O. europaea* extracts were reconstituted in their respective solvents at concentrations between 0.03125–0.5 mg/mL and their antioxidant potency of the extract was compared with vitamin C.

I) 2, 2-azinobis-(3-ethylbenzothiazolin - 6-sulfonic acid) diammonium salt ABTS Assay

The *O. europaea* extracts' ability to scavenge ABTS radicals was determined according to decolorization reaction method described by Re et al. (1999). Briefly, potassium persulfate solution (2.45 mM) was used to oxidize ABTS solution (7.0 mM) in equal amount forming pre-formed ABTS monocation (ABT). The mixture was then left for 12 h in a dark cupboard at ambient temperature for complete reaction. Thereafter, 1 mL of the solution was mixed with 60 mL methanol to attain 0.705 ± 0.001 at 734 nm, which is the absorbance required in performing the assay. Then 100 μ l of methanol was added to the wells of the microtiter plates except the second and third rows respectively. Thereafter, 200 μ l of the plant extracts and standards prepared in methanol was then added into the third row in triplicates.

Starting from the first column in the third row, a twofold serial dilution was done by mixing the well contents and transferring 100 μ l into the second well of the same column and repeating this process until the 7th well of the same column with the remaining 100 μ l from the 7th well discarded. The two fold dilution method was used to prepare the 0.05- 0.5 mg/mL concentrations vitamin C and plant extracts in the wells. Then 100 μ l of the already prepared ABTS solution were added into all the wells. After seven minutes, the absorbance was recorded at 734 nm with the spectrophotometer. The % inhibition of ABTS by the extract and Vitamin C were evaluated using the following expression:

$$\text{Inhibition (\%)} = [((\text{Control}_{\text{Abs}} - \text{Extract}_{\text{Abs}}) / (\text{Control}_{\text{Abs}})) \times 100] \text{ ----- } (\times)$$

Where $\text{Control}_{\text{Abs}}$ =absorbance of DPPH+ methanol (control sample) and $\text{Extract}_{\text{Abs}}$ =absorbance of DPPH radical + extract or reference drug (various samples).

I. Lipid Peroxidation Test

A modified protocol previously reported by Badmus et al. (2011) was adopted using methanol for serial dilution of extracts in place of distilled water. Ten percent of 0.5 mL homogenate (in distilled water) of egg yolk, known to be lipid-rich was mixed with the extracts of the plants and essential oil at varying concentrations of 0.05-0.50 mg/ mL and brought up to 1.0 mL. To induce the lipid peroxidation, 0.05 mL of FeSO_4 (0.07 M) was mixed with the solution above and thereafter incubating for 30 min. Then, 10 % CH_3COOH (1.50 mL) of 3.50 pH adjusted with

NaOH, 0.80 % of 2-thiobarbituric acid (1.5 mL) made in sodium dodecyl sulphate 1.1 % and 20 % trichloroacetic acid (0.05 mL) was combined in Eppendorf tubes and was then allowed to complete reaction in water bath at 65 °C for one hour. On cooling, 1-butanol (0.5 mL) was mixed with individual tubes and subjected to centrifugation for ten minutes at three thousand revolutions per minute. Finally, at 532 nm organic layer absorbance of each was then recorded. Methanol was employed as the blank. The lipid peroxide % inhibition activity was obtained using the equation stated for ABTS assay. The results were in triplicates and average values computed.

II. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

This was carried out with the method of Odeyemi et al. (2015). The DPPH solution (2.7×10^{-6} M) was prepared using methanol solvent at 0.1 mM concentration and thereafter kept in the dark. Then 100 µl of methanol was added to the wells except the second and third rows respectively. Thereafter, 150 µl of methanol was added into the third row. 50 µl of the plant extracts prepared in methanol was then added into the third row in triplicates. Starting from the first column in the third row, a twofold serial dilution was done by mixing the well contents and transferring 100 µl into the second well of the same column and repeating this process until the 7th well of the same column with the remaining 100µl from the 7th well discarded. The two fold dilution method was used to prepare the 0.05- 0.5 mg/mL concentrations of vitamin C and plant extracts in the wells. Then 100 µl of the already prepared DPPH radical was added into all the wells. Afterwards, the reaction mixture was vortexed, left for half an hour at ambient temperature away from light. The DPPH percentage inhibition activity was obtained using the expressing (×) stated for ABTS test. The results were in triplicates and average values computed.

3.2.6 Antimicrobial Assay

3.2.6.1 Test Organisms

The antimicrobial activity was done using the American Test Culture Collection (ATCC) and clinical isolates. The microorganisms used were *Klebsiella pneumoniae* (ATCC 4352), *Serratia marcescens* (ATCC 9986), *Shigella flexneri*, *Pseudomonas aeruginosa* (ATCC 19582), *Proteus*

vulgaris, *Enterococcus faecalis* (ATCC 29212). These microorganisms were selected based on their importance as opportunistic pathogens of humans suffering from diabetes mellitus.

3.2.6.2 Preparation of Bacterial Suspensions (CLSI, 2014)

The bacteria were inoculated into different tubes of 7ml of Mueller Hinton Broth from their stock culture and incubation was for 24 hours at 37°C. After 24 h of incubation, the bacteria suspensions (inoculums) were diluted serially to an optical density of McFarland 0.5 using isotonic sodium chloride solution. Dilutions matching with 0.5 McFarland scale standards were selected. The reference drug used was ciprofloxacin.

3.2.6.3 Minimum Inhibitory Concentration (MIC)

This was determined by using agar dilution. Firstly, Mueller-Hinton agar was prepared and the sterilized media was cooled to about 50°C. Nineteen milliliters of the molten agar was transferred to sterile conical flasks, in addition to 1ml of different concentrations of the plant extracts to give a final concentration of 0.03125 to 0.5mg/ml, standard and the control (DMSO) to make a final volume of 20 ml. The contents of the flasks were then thoroughly mixed and gently poured into properly labeled sterile Petri dishes. The plates were inoculated with 0.5 McFarland suspensions before incubating for 24 hours at 37 °C. The lowest concentration which was able to inhibit apparent microorganism's growth was noted as the MIC (Christofilogiannis, 2001; Bonjar, 2004).

3.2.7 Statistical Analysis

The IC₅₀ value was calculated with linear regression analysis. MINITAB Release 17 statistical package was used for one way ANOVA (Analysis of variance). Microsoft Office Excel was used to plot bar charts and the standard deviation was represented by the error bars. $P < 0.05$ significance level was used.

3.3. Results

3.3.1 Effects of extracting solvent on the extracts yields from the medicinal plant materials

The ethanol extracts of both the stem and leaves of *O. europaea*, gave the highest yield of 8% and 21.04% respectively, while the n-hexane extracts gave the least yield of 2% and 3.68 % respectively (Table 3.1).

Table 3.1: Solvent Extraction yield Efficiencies of *Olea europaea subsp. africana*.

| Plants Part | Extraction yield (g) | | | Percentage yield of extraction (%) | | |
|-------------|----------------------|---------------|---------|------------------------------------|---------------|---------|
| | N-hexane | Ethyl acetate | Ethanol | N-hexane | Ethyl acetate | Ethanol |
| Leaves | 2.76 | 8.07 | 15.78 | 3.68 | 10.76 | 21.04 |
| Stem | 0.2 | 0.63 | 0.8 | 2 | 6.3 | 8 |

3.3.2 Chemical analysis of *O. europaea* essential oil

From the *O. europaea* essential oil, 61 compounds were identified, out of which 8 compounds were the most prevailing compounds (Table 3.2). The mass spectrum of *O.europaea* essential oil showed 9 prominent peaks including Nonanal, Phytol, 2-isopropyl-5-methyl-9-methylene-bicyclo[4.4.0]dec-1-ene, 4-tert-Butylcatechol, dimethyl ether, Octadecane, 2-Hexanal, (E)-, 5,9-Undecadien-2-one, 6,10-dimethy 1-, (E)-, Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, and Decanal, (E)- (Figure 3.1). The less prominent peaks having other retention times reveals the peak identities of the different compounds identified as seen in Figure 3.1 and Table 3.2.

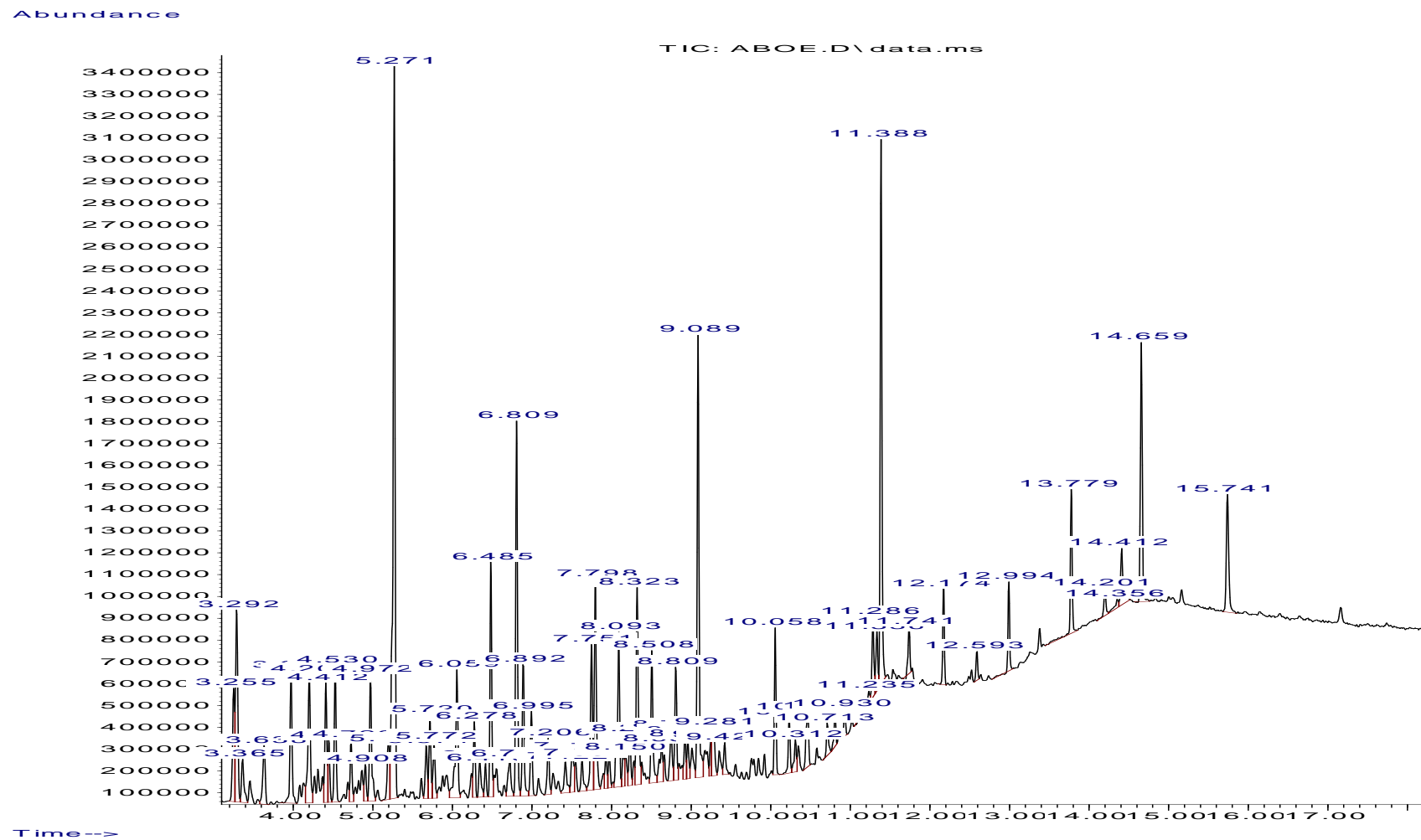

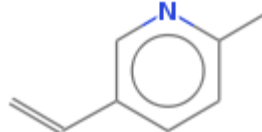

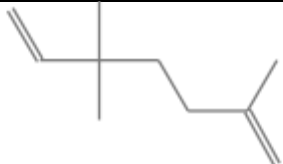
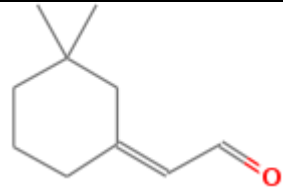
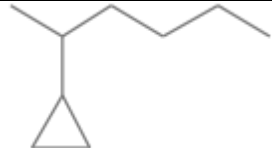

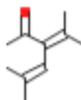

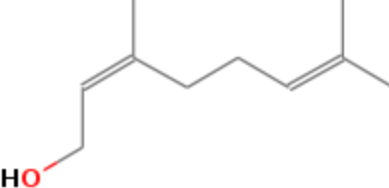


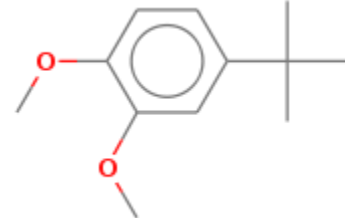
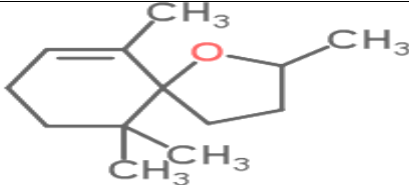

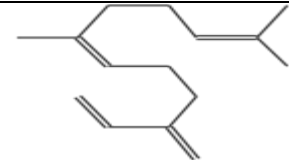
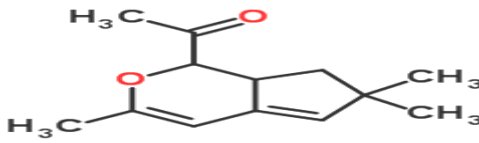
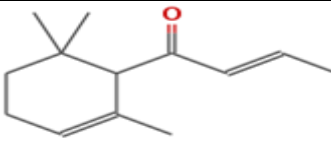
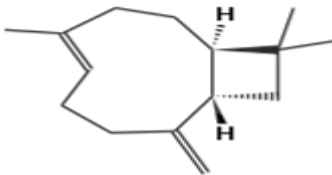


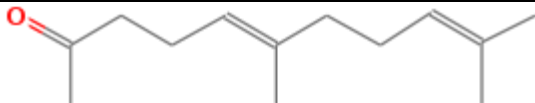
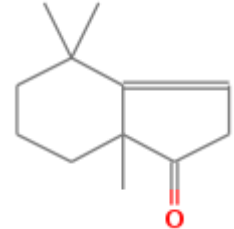
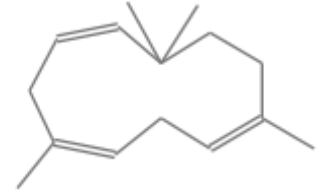
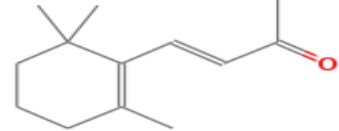
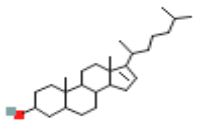
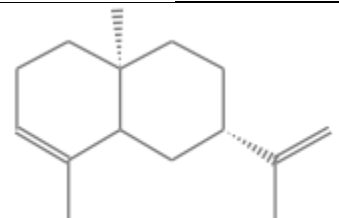
Figure 3.1 Gas chromatogram of *O. europaea* essential oil.

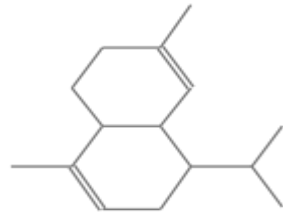
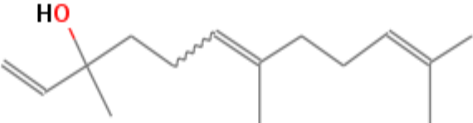

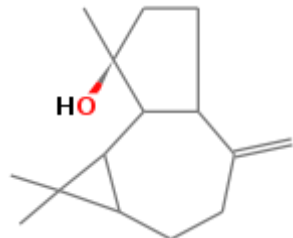
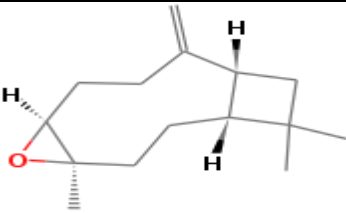
Table 3.2: The chemical composition of bioactive compounds of *O. europaea* essential oil

| S/N | Retention time (min) | Name of the Compounds | Molecular Formula | Structure | Molecular Weight | Peak Area % |
|-----|----------------------|--|----------------------------------|-----------|------------------|-------------|
| 1 | 3.255 | 2-Butenal, 2-ethenyl- | C ₆ H ₈ O | | 96.1271 | 1.56 |
| 2 | 3.292 | 2-Hexenal, (E)- | C ₆ H ₁₀ O | | 98.1430 | 2.95 |
| 3 | 3.365 | 1-Hexanol | C ₆ H ₁₄ O | | 102.1748 | 0.75 |
| 4 | 3.638 | Heptanal | C ₇ H ₁₄ O | | 114.1855 | 1.00 |
| 5 | 3.975 | (1S)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene | C ₁₀ H ₁₆ | | 136.2340 | 1.89 |
| 6 | 4.204 | Pyridine, 3-ethenyl- | C ₇ H ₇ N | | 105.1372 | 2.21 |
| 7 | 4.412 | 2,4-Heptadienal, (E,E)- | C ₇ H ₁₀ O | | 110.1537 | 1.58 |
| 8 | 4.450 | Octanal | C ₈ H ₁₆ O | | 128.2120 | 0.77 |
| 9 | 4.530 | .alpha.-Phellandrene | C ₁₀ H ₁₆ | | 136.2340 | 1.72 |
| 10 | 4.729 | (+)-4-Carene | C ₁₀ H ₁₆ | | 136.2340 | 0.79 |
| 11 | 4.908 | 2-Octenal, (E)- | C ₈ H ₁₄ O | | 126.1962 | 0.50 |

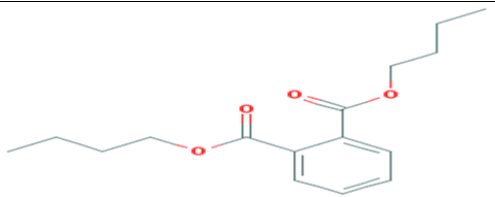

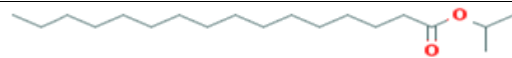

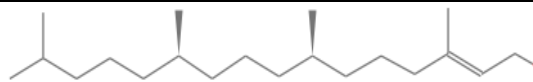



| | | | | | | |
|----|-------|--|-----------------------------------|---|-----------|-------|
| 12 | 4.972 | 1-Octanol | C ₈ H ₁₈ O |  | 130.2279 | 1.87 |
| 13 | 5.198 | Pyridine, 5-ethenyl-2-methyl- | C ₈ H ₉ N |  | 119.1638 | 0.88 |
| 14 | 5.271 | Nonanal | C ₉ H ₁₈ O |  | 142.2386 | 10.57 |
| 15 | 5.674 | 1,6-Heptadiene, 2,5,5-trimethyl- | C ₁₀ H ₁₈ |  | 138.2499 | 0.731 |
| 16 | 5.720 | Acetaldehyde, (3,3-dimethylcyclohexylidene)-, (E)- | C ₁₀ H ₁₆ O |  | 152.2334 | 1.07 |
| 17 | 5.772 | 1-Methylpentyl cyclopropane | C ₉ H ₁₈ |  | 126.2392 | 0.80 |
| 18 | 6.059 | Decanal | C ₁₀ H ₂₀ O |  | 156.2652 | 2.23 |
| 19 | 6.278 | 3-Isopropylidene-5-methyl-hex-4-en - 2-one | C ₁₀ H ₁₆ O |  | 152.23344 | 0.99 |
| 20 | 6.347 | Octane, 1-iodo- | C ₈ H ₁₇ I |  | 240.1250 | 0.57 |
| 21 | 6.418 | 2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)- | C ₁₀ H ₁₈ O |  | 154.2493 | 0.39 |
| 22 | 6.485 | 2-Decenal, (E)- | C ₁₀ H ₁₈ O |  | 154.2493 | 2.78 |

| | | | | | | |
|----|-------|--|--|---|----------|------|
| 23 | 6.724 | 2,4-Decadienal, (E,E)- | C ₁₀ H ₁₆ O |  | 152.2334 | 0.76 |
| 24 | 6.809 | 4-tert-Butylcatechol, dimethyl ether | C ₁₂ H ₁₈ O ₂ |  | 194.2701 | 4.63 |
| 25 | 6.892 | 9-Oxabicyclo[4.2.1]non-7-en-3-one | C ₈ H ₁₂ O | | 124.1803 | 1.80 |
| 26 | 6.995 | 1-Oxaspiro[4.5]dec-6-ene, 2,6,10,10-tetramethyl- | C ₁₃ H ₂₂ O |  | 194.313 | 1.10 |
| 27 | 7.206 | 2-Undecenal | C ₁₁ H ₂₀ O |  | 168.2759 | 0.78 |
| 28 | 7.422 | cis-.beta.-Farnesene | C ₁₅ H ₂₄ |  | 204.3511 | 0.60 |
| 29 | 7.510 | 1-(3,6,6-Trimethyl-1,6,7,7a-tetrahydrocyclopenta[c]pyran-1-yl)ethanone | C ₁₃ H ₁₈ O ₂ |  | 206.281 | 0.72 |
| 30 | 7.631 | 2-Buten-1-one, 1-(2,6,6-trimethyl-1-cyclohexen-1-yl)- | C ₁₃ H ₂₀ O |  | 192.2973 | 0.50 |
| 31 | 7.751 | Caryophyllene | C ₁₅ H ₂₄ |  | 204.3511 | 2.04 |

| | | | | | | |
|----|-------|--|-----------------------------------|---|----------|------|
| 32 | 7.798 | 5,9-Undecadien-2-one, 6,10-dimethyl-, (E)- 1- | C ₁₃ H ₂₂ O |  | 194.3132 | 2.91 |
| 33 | 7.931 | 1H-Inden-1-one, 2,4,5,6,7,7a-hexahydro-4,4,7a-trimethyl- | C ₁₂ H ₁₈ O |  | 178.2707 | 0.46 |
| 34 | 7.974 | 1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z- | C ₁₅ H ₂₄ |  | 204.3511 | 0.44 |
| 35 | 8.093 | trans-.beta.-lonone | C ₁₃ H ₂₀ O |  | 192.2973 | 2.39 |
| 36 | 8.150 | 21.xi-methyl-17-ischolest-16-en-3.beta.-ol | C ₂₇ H ₄₆ O |  | 386.6535 | 0.47 |
| 37 | 8.232 | Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-, [2R-(2α,4α,8αβ)]- | C ₁₅ H ₂₄ |  | 204.3511 | 0.69 |

| | | | | | | |
|----|-------|---|--|---|----------|------|
| 38 | 8.323 | Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)- | C ₁₅ H ₂₄ |  | 204.3511 | 2.91 |
| 39 | 8.508 | 1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-, [S-(Z)]- | C ₁₅ H ₂₆ O |  | 222.3663 | 1.82 |
| 40 | 8.631 | Benzoic acid, nonadecyl ester | C ₂₆ H ₄₄ O ₂ |  | 388.6264 | 0.79 |
| 41 | 8.750 | 1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1aα,4aα,7β,7aβ,7bα)]- | C ₁₅ H ₂₄ O |  | 220.3505 | 0.80 |
| 42 | 8.809 | Caryophyllene oxide | C ₁₅ H ₂₄ O |  | 220.3505 | 1.49 |
| 43 | 8.923 | 4,2,8-Ethanylylidene-2H-1-benzopyran, octahydro-4-methyl- | | | | 0.55 |

| | | | | | | |
|----|--------|--|--|--|----------|------|
| 44 | 8.961 | 1,4-Methano-1H-indene, octahydro-1,7a-dimethyl-4-(1-methylethenyl)-, [1S-(1 α ,3 $\alpha\beta$,4 α ,7 $\alpha\beta$)]- | C ₁₅ H ₂₄ | | 204.3511 | 0.52 |
| 45 | 9.089 | Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-9-methylene- | C ₁₅ H ₂₄ | | 204.3511 | 5.65 |
| 46 | 9.250 | Heptadecane | C ₁₇ H ₃₆ | | 240.4677 | 0.39 |
| 47 | 9.281 | 7-epi-cis-sesquisabinene hydrate | C ₁₅ H ₂₆ O | | 222.3663 | 0.94 |
| 48 | 9.424 | 2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl- | C ₁₅ H ₂₆ O | | 222.3663 | 0.46 |
| 49 | 10.058 | 2-Pentadecanone, 6,10,14-trimethyl | C ₁₈ H ₃₆ O | | 268.4778 | 1.66 |
| 50 | 10.235 | 1,2-Benzenedicarboxylic acid,bis (2-methylpropyl) ester | C ₁₆ H ₂₂ O ₄ | | 278.3435 | 0.72 |
| 51 | 10.312 | Eicosane | C ₂₀ H ₄₀ | | 282.5475 | 0.47 |
| 52 | 10.467 | 2,6,10,14,18-Pentamethyl-2,6,10,14,18-eicosapentaene | C ₂₅ H ₄₂ | | 342.601 | 0.97 |

| | | | | | | |
|----|--------|---------------------------|-------------------|---|-----------|------|
| 53 | 10.713 | Dibutyl phthalate | $C_{16}H_{22}O_4$ |  | 278.3435 | 0.43 |
| 54 | 10.810 | Eicosane | $C_{20}H_{42}$ |  | 282.548 | 0.42 |
| 55 | 10.930 | Isopropyl palmitate | $C_{19}H_{38}O_2$ |  | 298.50382 | 0.29 |
| 56 | 11.286 | Octadecane | $C_{18}H_{38}$ |  | 254.4943 | 1.03 |
| 57 | 11.336 | 2-Myristynoyl-glycinamide | | | | 0.77 |
| 58 | 11.388 | Phytol | $C_{20}H_{40}O$ |  | 296.531 | 6.40 |
| 59 | 12.174 | Eicosane | $C_{20}H_{42}$ |  | 282.548 | 1.09 |
| 60 | 14.356 | Silicic acid | H_4O_4Si |  | 96.115 | 0.20 |
| 69 | 14.659 | Octadecane | $C_{18}H_{38}$ |  | 254.4943 | 3.37 |

3.3.3 In vitro antioxidant activity

The percentage inhibition of scavenging activities of *O. europaea* extracts for ABTS, DPPH and Lipid peroxide radicals are shown in Figures 3.2 to 3.10.

3.3.3.1 ABTS

The quantitative determination of the radical scavenging activity of the ABTS free radical by *O. europaea* extracts involved measuring the disappearance of the coloured free radical, ABTS. Figure 3.2 represents the concentration response curve used for the indication of decreased ABTS concentration obtained as the sample concentration increased.

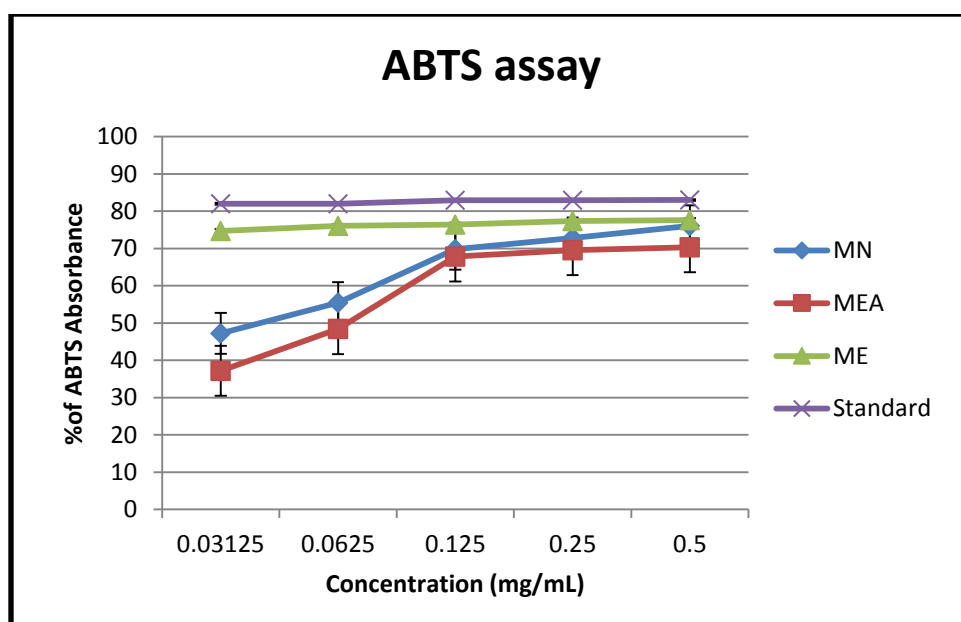


Figure 3.2: ABTS dose response curve for *O. europaea* leaves extracts tested. This indicates the relationship between the extracts concentration (mg/ml) and the % inhibition of ABTS after incubation. MEA-Ethyl acetate leaf extracts, MN-N-hexane leaf extracts, ME-Ethanol leaf extracts, Standard-Vitamin C.

The pattern of the percentage inhibitions in ABTS assay was slightly comparable to that of DPPH. The ethyl acetate and ethanol extracts showed high scavenging effect; however, the n-hexane leaf extracts at all concentrations tested demonstrated stronger inhibitory effect than the ethyl acetate extracts. Also, the high activity of the vitamin C was comparable to that of the ethanol extracts (Figure 3.3- 3.4). The trend of the inhibition of ABTS radicals by *O. europaea* stem extracts was considerably lower than that of vitamin C except at 0.125 mg/ml where ethanol stem extract inhibitory potential was significantly similar to vitamin C ($P < 0.05$) and at 0.25 mg/ml where both ethanol and ethyl acetate stem extracts inhibitory potentials were

significantly similar to vitamin C ($P < 0.05$) (Figure 3.4). The *O. europaea* n-hexane leaf extract was significantly similar to vitamin C ($P < 0.05$) in the inhibitory potential of ABTS radicals while both the ethanol and ethyl acetate leaf extracts were significantly lower than vitamin C except at 0.03125 mg/mL (Figure 3.3). The IC_{50} values of the *O. europaea* extracts and vitamin C were in the increasing order of: vitamin C < ethyl acetate stem extract < ethanol stem extract < n-hexane stem extract < ethanol leaf extract < ethyl acetate leaf extract < n-hexane leaf extract < aqueous extract (Figure 3.10).

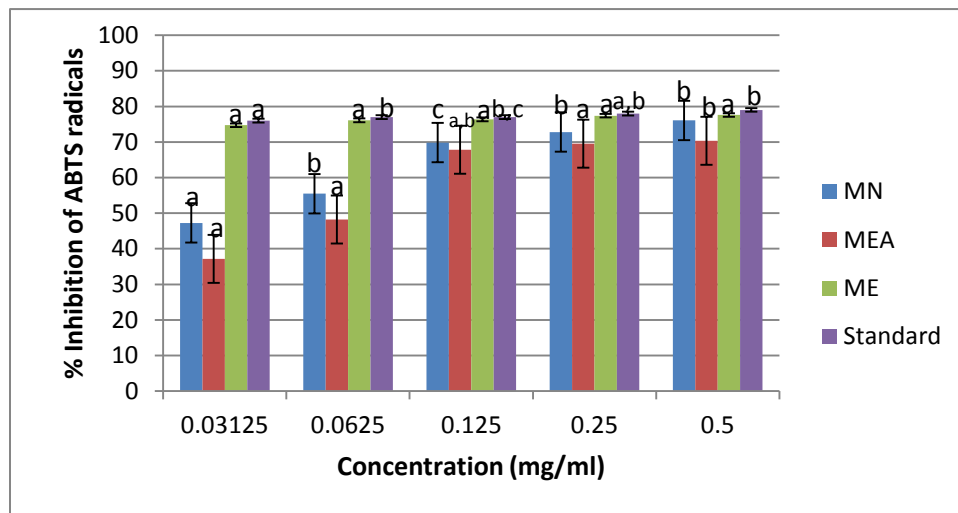


Figure 3.3 ABTS radical scavenging activity by different *O. europaea* leaf extracts and the standard antioxidant, vitamin C. Bar graphs with different letter superscript in the same concentration are significantly different ($P < 0.05$). MEA-Ethyl acetate leaf extracts, MN-N-hexane leaf extracts, ME-Ethanol leaf extracts, Standard-Vitamin C.

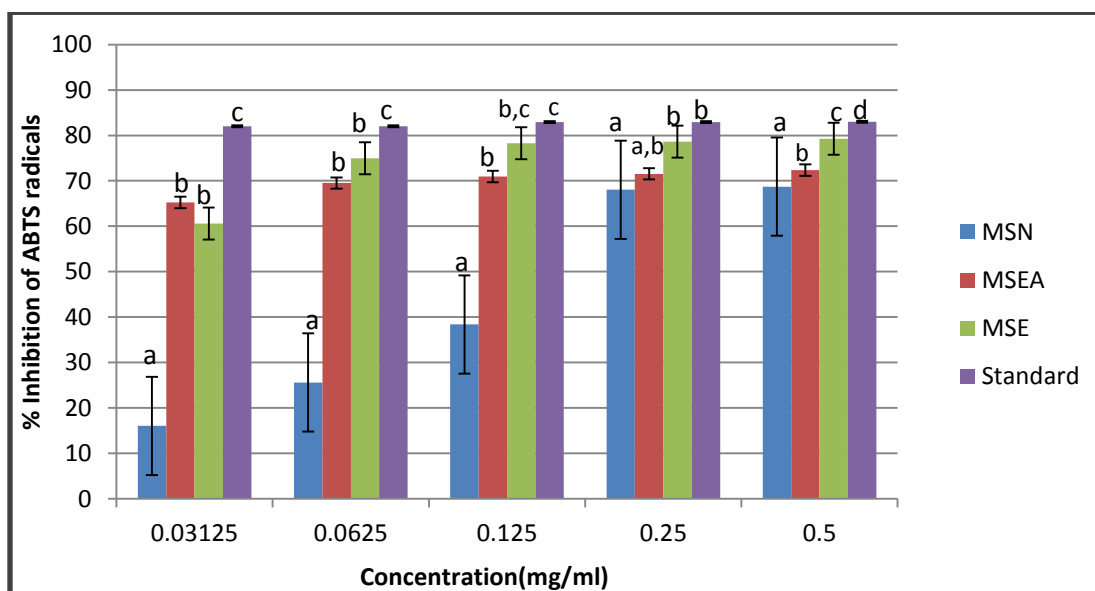


Figure 3.4 ABTS radical scavenging activity by different *O. europaea* stem extracts and the standard antioxidant, vitamin C. Bar graphs with different letter superscript in the same concentration are significantly different ($P < 0.05$). MSEA-Ethyl acetate Stem extracts, MSN-N-hexane Stem extracts, MSE-Ethanol Stem extracts, Standard-Vitamin C

3.3.3.2 Lipid Peroxidation

This was measured by the ability of *europaea* to scavenge lipid peroxides which were compared with the standard vitamin C. The *O. europaea* n-hexane leaf and stem extracts exhibited overall higher activity than the ethyl acetate and ethanol extracts with percentage inhibitions ranging from 52.05% to 67.25% (Figure 3.5 - 3.6). At 0.5 mg/ml, the scavenging activity of the ethanol and ethyl acetate leaf extracts reached 64.91% and 64.56% which was comparable to vitamin C with 64.91%. However, only the ethyl acetate extract of the stem was close to that of the vitamin C with 60.28% while the vitamin C was 64% at the same concentration. From the IC₅₀ value determination of the *O. europaea* leaf and stem extracts, it was indicated that n-hexane extract had the highest inhibitory capacity which was followed by ethyl acetate leaf extract and ethanol stem extract respectively ($P < 0.05$) (Figure 3.10).

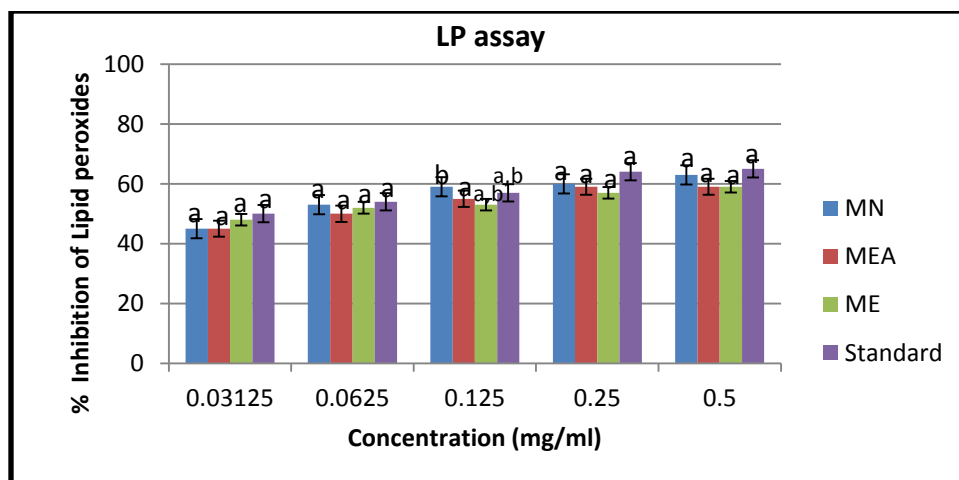


Fig. 3.5 –Lipid Peroxide radical scavenging activity of *O. europaea* leaves extract and the standard antioxidant, vitamin C. Bar graphs with different letter superscript within the same concentration are significantly different ($P < 0.05$). MEA-Ethyl acetate leaf extracts, MN-N-hexane leaf extracts, ME-Ethanol leaf extracts, Standard-Vitamin C.

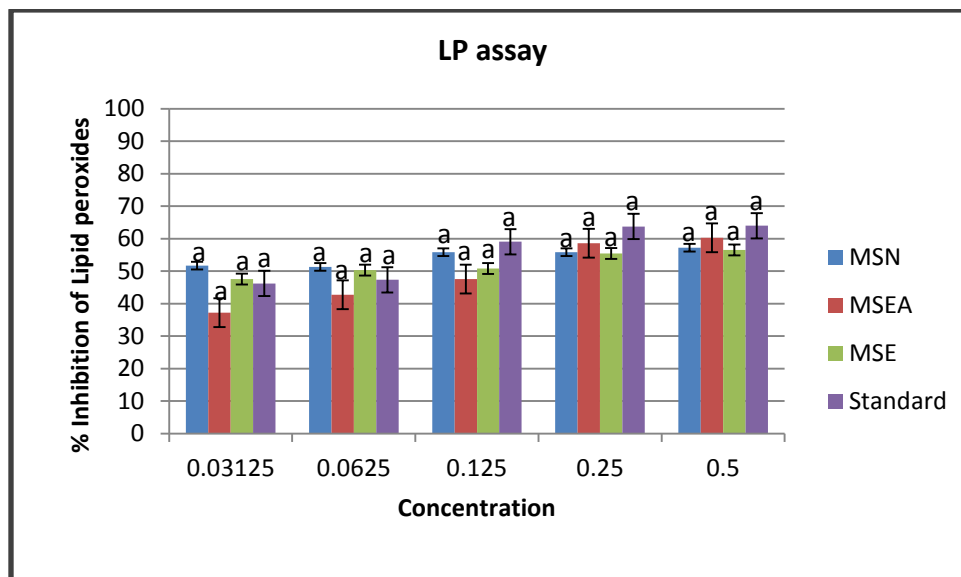


Fig. 3.6 Lipid peroxide radical scavenging activity of different *O. europaea* stem extracts and the standard antioxidant, vitamin C. Bar graphs with different letter superscript within the same concentration are significantly different ($P < 0.05$). MSEA-Ethyl acetate Stem extracts, MSN-N-hexane Stem extracts, MSE-Ethanol Stem extracts, Standard-Vitamin C.

3.3.3.3 DPPH

The DPPH radical scavenging activity was represented as a concentration response curve which indicated that the sample concentration increased as the DPPH concentration decreases (Figure 3.7). This enabled also the calculation of the IC 50 value, i.e the extract concentration capable of inhibiting by 50% of the DPPH radicals initial concentration of the DPPH radicals (Figure 3.10).

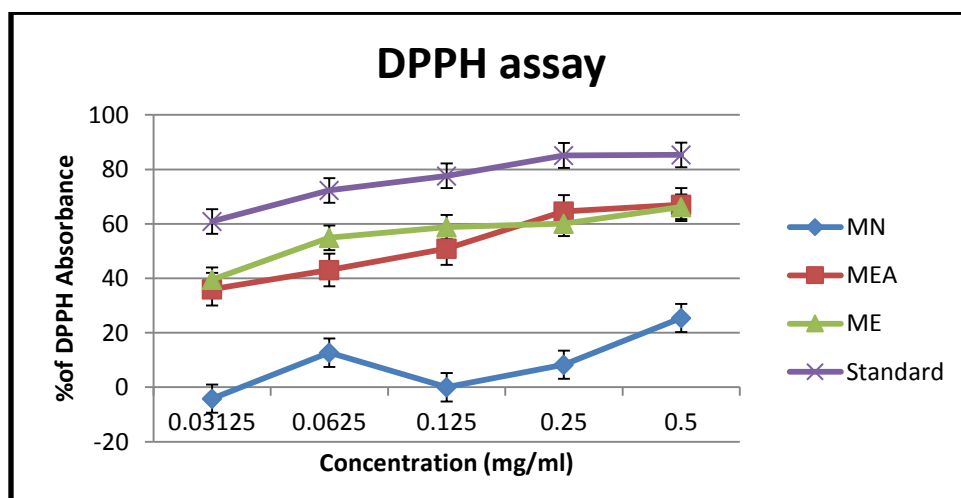


Figure 3.7: DPPH dose response curve for *O. europaea* leaves extracts tested. This indicates the relationship between the extracts concentration (mg/ml) and the % of DPPH after incubation. MEA-Ethyl acetate leaf extracts, MN-N-hexane leaf extracts, ME-Ethanol leaf extracts, Standard-Vitamin C.

The extract concentration and scavenging activity was observed to be positively correlated when the scavenging activity of each extract was compared. Most of the extracts effectively reduced DPPH radical with ME, followed by MEA extracts having high free radical scavenging activity. At 0.0625 and 0.5 mg/ml, both the leaves and stem of the ethanol and ethyl acetate extracts showed a stronger DPPH radicals scavenging activity. The percentage inhibition values ranged from 66% to 36% than the leaves of n-hexane extracts which at 0.5 mg/ml had the inhibitory value of 25%. The n-hexane stem extracts, however, had strong activity compared to the n-hexane leaf extracts of *O. europaea* (Figure 3.8- 3.9). The IC₅₀ values for the DPPH assay was significantly in the following order, vitamin C, the ethanol stem, followed by the ethyl acetate stem, n-hexane stem, and the ethanol leaf extracts ($P < 0.05$) (Figure 3.10).

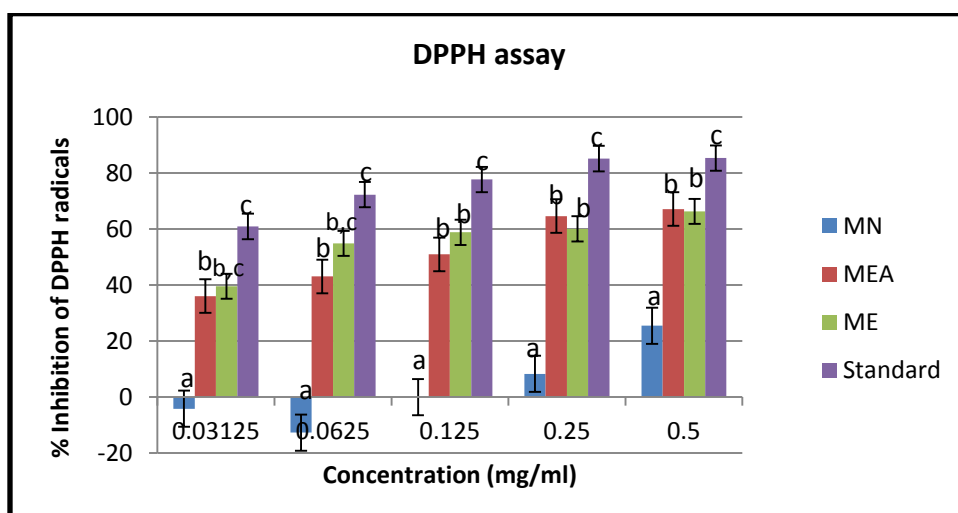


Fig. 3.8 Comparison of the DPPH radical scavenging effect of different *O. europaea* leaves extract where the indicated amount of extracts was added to the DPPH solution. MEA-Ethyl acetate leaf extracts, MN-N-hexane leaf extracts, ME-Ethanol leaf extracts, Standard-Vitamin C.

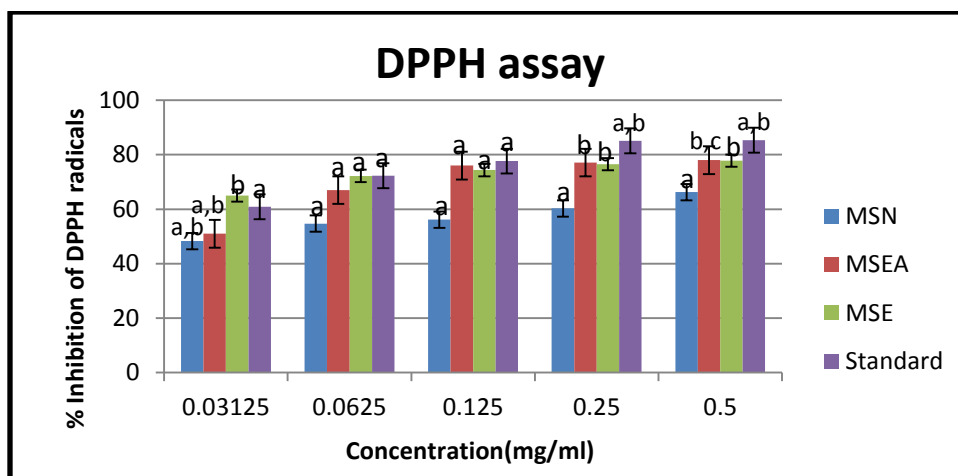


Fig. 3.9 Comparison of DPPH radical scavenging effect of the different *O. europaea* stem extract, where the indicated amount of extracts was added to the DPPH solution. MSEA-Ethyl acetate Stem extracts, MSN-N-hexane Stem extracts, MSE-Ethanol Stem extracts, Standard-Vitamin C.

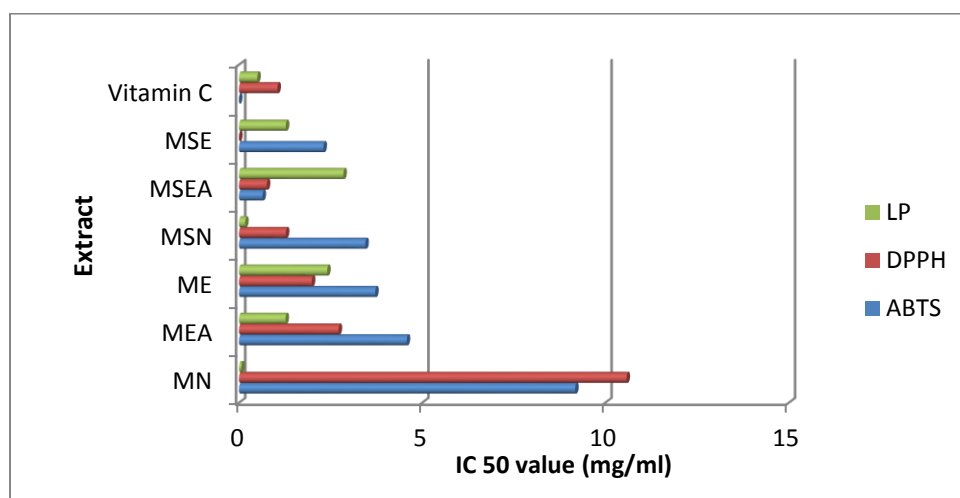


Figure 3.10 Comparison of the antioxidant activity of the different *O. europaea* extracts with vitamin C, indicated that for ABTS, MSEA followed by MSE had the highest scavenging activity, while for DPPH, MSE followed by MSEA had the highest scavenging activity, and MN followed by MSN had the highest scavenging activity of the extracts for LP of the extracts. LP (Lipid Peroxidation), DPPH (2,2-Diphenyl-1-picrylhydrazyl), ABTS (2, 2-azinobis-(3-ethylbenzothiazolin - 6-sulfonic acid) diammonium salt) determination in different solvent extracts of *O. europaea*. MN: n-hexane leaf, MEA: Ethyl acetate leaf, ME: Ethanol leaf, MSEA: Ethyl acetate stem, MSE: Ethanol stem, MSN: n-hexane stem,

3.3.4 Antimicrobial Activity of *O. europaea* Extracts

The bacteria were sensitive as the *O. europaea* extracts exhibited significant antimicrobial activity (Table 3.3). However, this activity was observed only in the ethanol and ethyl acetate extracts. Overall, ciprofloxacin had the highest activity when tested against the microbes compared with the extracts.

Table 3.3: Growth inhibition effect of *Olea europaea* subsp. *africana* extracts

| Microorganism | Extracts | | | | |
|--|----------|-------------|-----------|---------------|---------------|
| | Gram+/- | Ethanol | N-hexane | Ethyl Acetate | Ciprofloxacin |
| <i>Serratia marcescens</i> ATCC 9986 | + | Susceptible | Resistant | Susceptible | Susceptible |
| <i>Enterococcus faecalis</i> ATCC 29212 | + | Susceptible | Resistant | Susceptible | Susceptible |
| <i>Proteus vulgaris</i> | + | Susceptible | Resistant | Susceptible | Susceptible |
| <i>Klebsiella pneumoniae</i> ATCC 4352 | - | Susceptible | Resistant | Susceptible | Susceptible |
| <i>Pseudomonas aeruginosa</i> ATCC 19582 | - | Susceptible | Resistant | Susceptible | Susceptible |
| <i>Shigella flexneri</i> | - | Susceptible | Resistant | Susceptible | Susceptible |

3.3.4.1 Minimum Inhibitory Concentration (MIC)

The n-hexane extracts did not have antimicrobial effect on the microorganisms, Table 3.4 gives the MIC results for the ethanol extracts and ethyl acetate extracts. The *O. europaea* extracts inhibited both the Gram positive (*S. marcescens*, *E. faecalis* and *P. vulgaris*) and Gram-negative bacteria (*K. pneumoniae*, *P. aeruginosa* and *S. flexneri*) (Table 3.4). The extent of growth

inhibition of the microorganisms tested was concentration dependent. The ethyl acetate extract with a MIC value of 0.625 mg/ml had the highest activity which was significantly similar to ciprofloxacin (standard drug) against *S. marcescens* (Table 3.4). While the ethanol extract had the MIC value of 1.25 mg/ml against *K. pneumoniae* and *S. marcescens*. However, the ethanol and ethyl acetate extracts were only bacteriostatic at the concentrations tested.

Table 3.4: The minimum inhibitory concentration values for *O. europaea* extracts

| Microorganism | Culture collection and Ref. No. | Extracts (mg/ml) | | |
|-------------------------------|---------------------------------|------------------|---------|---------------|
| | | Ethyl Acetate | Ethanol | Ciprofloxacin |
| <i>Serratia marcescens</i> | (ATCC 9986) | 0.625 | 1.25 | <0.625 |
| <i>Enterococcus faecalis</i> | (ATCC 29212) | 10 | 10 | <0.625 |
| <i>Proteus vulgaris</i> , | AMEREG | 10 | 10 | <0.625 |
| <i>Klebsiella pneumonia</i> | (ATCC 4352) | 10 | 1.25 | <0.625 |
| <i>Pseudomonas aeruginosa</i> | (ATCC 19582) | 10 | 10 | <0.625 |
| <i>Shigella flexneri</i> | AMEREG | 10 | 10 | <0.625 |

3.4 Discussion

3.4.1 Effect of extraction solvent on the extract yields

The result presented in Table 3.1 shows that the extraction yield of *O. europaea* increased significantly as the polarity of solvents used in the extraction process changed from n-hexane (non-polar) to ethanol (polar). When ethanol was used, an extract yield of 15.78g and 0.8g for leaf and stem respectively was recorded which was the highest amount of recovered extract. The maximum amount of extract yield was recovered from the ethanol extract with a percentage yield of 8 % and 21.04 % for the stem and leaves respectively. This indicates that more polar compounds are present in the extracts and the different chemical components of the *O. europaea* may have resulted in the varied amounts of extraction yields.

3.4.2 GC–MS analysis

Previous studies have shown that in the investigation of the components of plant origin, the GC–MS analysis performs a vital part, as plant materials are usually very complex making the GC–MS analysis suitable for plant analysis due to its high selectivity and sensitivity. Hence, the GC–MS analysis is considered to be the gold standard in scientific analysis (Balamurugan et al., 2012). GC–MS has been used in different studies which investigated phytochemical screening worldwide (Dubey et al., 2014; Omoruyi et al., 2014; Doshi et al., 2015). The GC-MS analysis on the oil of *O. europaea* identified 61 compounds representing 93.031% of the total oil.

Nonanal (10.57%), Phytol (6.40%) and 2-isopropyl-5-methyl-9-methylene-bicyclo[4.4.0]dec-1-ene (5.65%) were revealed as the most prevailing phytoconstituents (Figure 3.1).

Phytol is a significant therapeutic molecule identified with known activity. It is a precursor for vitamins E and K, it has been reported to be an anticancer, anti-inflammatory and antimicrobial agent (Duke, 1992; Takashikomiya et al., 1999). It is able to reduce free radical production *in vitro* due to the activity of its hydroxyl group (Santos et al., 2013). The potent antioxidant activity exhibited could be linked with the phytol content of the *O. europaea*. Also according to previous studies, it has been reported that the main active constituent of *O. europaea* leaves is oleuropein and it is responsible for activities such as antioxidant, antimicrobial, hypolipidemic and hypotensive (Khan et al., 2007; Papoti and Tsimidou, 2009).

Amabeoku and Bamuamba (2010) also reported on the possibility of oleuropein being present in the leaves of the South African species of *O. europaea* subsp. *africana*. However, this active compound was not present in this sample. This finding partly conforms to the studies of Somova et al. (2003) who reported that oleuropein occurs only in trace amounts in the leaves. This absence may therefore be due to the observation of Gholivand et al. (2012) who reported that the composition of essential oil vary from place to place, as it depends on variables such as soil biology, location, climatic conditions and habitat. Radulović et al. (2012) also noted that these variables regulate the plant internal physiology leading to the various array of chemicals produced within the same plant species.

3.4.3 *In vitro* antioxidant activity

The results also revealed that the *O. europaea* extracts effectively scavenged free radicals and the percentage inhibition values were noted to increase as *O. europaea* extracts concentration increased in the assays. A standard curve was plotted by using the different concentrations of extracts and vitamin C against the percentage of inhibition obtained and this was further used to calculate the IC₅₀ (mg/ml). From the ABTS results presented in Figure 3.2 - 3.4, it was observed that the ethanol leaf extract and ethyl acetate stem extract had the highest antioxidant activity amongst the extracts used, although none of the extracts could match the vitamin C in its antioxidant activity. This therefore indicates that at the lowest concentration, *O. europaea* ethanol leaf extract and ethyl acetate stem extract could serve as inhibitors of ABTS free radical.

These findings also imply that compounds that contain a high quantity of polar solvent have the ability to inhibit ABTS radicals compared to compounds containing non-polar solvents (Sultana et al., 2009; Wintola and Afolayan, 2011).

Lipid peroxidation assay was carried out *in vitro* by inducing egg-yolk homogenates using ferrous sulphate. The scavenging activity of the lipid peroxides by the *O. europaea* leaf and stem extracts, compared with vitamin C is presented in Figures 3.5 and 3.6. The n-hexane leaf extract of the plant had a lower IC₅₀ value of 1.17 mg/ml suggesting it had overall higher lipid peroxidation inhibition than other extracts of *O. europaea* tested. The high percentage inhibition exhibited therefore implies that the n-hexane leaf extract might contain the strong lipid peroxidation inhibitory compounds which have better lipid peroxidation scavenging activity than the other extracts (Figure 3.10). Meanwhile, the relatively low inhibition of lipid peroxidation at 0.125-0.03125 mg/ml may be attributed to high lipid content of the egg homogenate (Badmus et al., 2011).

The extracts revealed their hydrogen donating ability when added to the DPPH radical, resulting in decreased absorbance and the production of a yellow colored diphenylpicryl hydrazine compound. Figures 3.7 to 3.9 illustrate the DPPH radical scavenging activity of *O.europaea* leaf and stem extracts compared with vitamin C as regards the inhibition percentage. Both *O.europaea* ethanol and ethyl acetate leaf extracts demonstrated stronger DPPH radicals scavenging activity with percentage inhibition values ranging from 67% to 36% than the n-hexane extracts which at 0.5mg/ml had 25%. At 0.25 mg/ml, it was noted that the ethanol leaf extract inhibited at a percentage of 60.04% with IC₅₀ value of 1.99 mg/ml which was commensurate to vitamin C at 0.3125 mg/ml (Figure 3.10), although at 0.25 mg/ml, the ethyl acetate leaf extracts had a scavenging activity of 64.59%.

For the stem, at 0.5mg/ml concentration the highest percentage inhibition of DPPH was exhibited by the ethanol extract, with 78% and an IC₅₀ value of 0.027mg/ml, subsequently followed by ethyl acetate extract with 76.49% and an IC₅₀ value of 0.7 mg/ml, with n-hexane extract showing 66.24 % with an IC₅₀ value of 1.28mg/ml. At this concentration, the ethanol stem extract was comparable to the standard, vitamin C at 0.125 mg/ml, which had 77.64% (Figure 3.10). While the ethyl acetate stem extract was close to the range with 76.49. These

comparisons imply that at a higher concentration, the ethanol leaf and stem extracts, as well as the ethyl acetate leaf and stem extracts have better DPPH scavenging activity.

The result of DPPH assay also suggests that these extracts of *O. europaea* contains antioxidant compounds that have the ability to donate hydrogen to the DPPH free radical in order eliminate the unpaired electron which is responsible for radical's reactivity. This finding is similar to Marwah et al. (2007) who reported that when the antioxidant activities of *O. europaea* extracts was evaluated using DPPH radicals as reference, the ethanol extracts of the plant was observed to have good antioxidant properties. The vitamin C demonstrated a very strong antioxidant activity and this could be owing to the fact that it was a pure compound. Unlike the *O. europaea* extracts which were crude in nature, consisting of various compounds with the active compound possibly present in low quantities. From the antioxidant results presented in figure 3.2-3.9, it was observed that different range of scavenging activities was exhibited by *O. europaea* extracts when tested against the different free radical systems and this may be ascribable to the various channels used by the reactions between the radicals and antioxidant in the different assays (Goswami and Chatterjee, 2014).

3.4.4 Antimicrobial Assay

The antimicrobial results presented in Table 3.3 and 3.4 demonstrates that the ethanol and ethyl acetate extracts of *O. europaea* exhibited activity against the growth of the pathogens tested while the test pathogens did not show any sensitivity to the n-hexane extracts. The ethyl acetate and ethanol extracts in particular displayed strong inhibitory activity against *S. marcescens* with MIC of 0.625mg/ml and 1.25 mg/ml respectively, while only ethanol had a lower MIC value of 1.25 mg/ml against *K. pneumoniae*. This antimicrobial property of ethanol and ethyl acetate extracts of *O. europaea* could be owing to the presence of some of the biochemical compounds identified during the GC-MS analysis. The lack of sensitivity observed against the n-hexane extracts could be due to the absence of antimicrobial compounds in the n-hexane extracts, considering that inhibition of *K. pneumoniae*, *P. aeruginosa*, *S. marcescens* and the other pathogens by ethanol and ethyl acetate extracts.

Hussain et al. (2012) gave a similar report that the ethanol and methanol crude leaves extracts of *O. europaea* plant strongly inhibited the growth of *K. pneumoniae*, *P. vulgaris*, *P. aeruginosa*, which confirmed the results of this study. The *O. europaea* leaf extracts have also been reported

to strongly inhibit the growth of *S. typhimurium*, *E. coli*, *S. aureus*, *B. cereus*, *L. monocytogenes* including *P. aeruginosa* which was also tested in this study (Ko et al., 2009). Polar solvents have been established to be good solvents capable of extracting bioactive substances (Njume et al., 2011). It is therefore thought that the solvents, ethyl acetate and ethanol permitted the extraction of the phenolic constituent contained in the *O. europaea* since the ethyl acetate and ethanol extracts were effective against the tested microorganisms. Thus, indicating that the *O. europaea* is a good antibacterial source while confirming its traditional usage in the study area against infections associated with diabetes.

3.5 Conclusion

In view of the serious health threats the continuous microbial resistance to antibiotic poses, the salient pharmacological properties of medicinal plants imply a good alternative to failing antibiotics. *O. europaea* extracts showed significant activity indicating that it is a good source of antioxidant and antimicrobial agent. The results of the present investigation complement the ethnobotanical usage of the *O. europaea*, as the plant possessed several chemical constituents when elucidated by the GC–MS. The chemical constituents contained in the *O. europaea* are in the family of diterpenes, monoterpenes and sesquiterpenes with biological activity which inhibits microorganisms. Based on the present investigation, it is concluded that *O. europaea* is a potential source of bioactive compounds with great pharmaceutical value.

3.6 References

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Chapter 4

***In vitro* Free-Radical-Scavenging and Antimicrobial Activities of *Euryops brevipapposus* Essential Oils**

Abstract

Euryops brevipapposus (Asteraceae), an endemic plant found in Cala community in the Eastern Cape Province, South Africa, serves as a traditional herb to the community members due to its locally acclaimed effectiveness in managing asthma, as a bronchodilator and easy accessibility to it. *Euryops brevipapposus* essential oil was investigated *in vitro* for its antimicrobial as well as scavenging free radicals ability using scavenging assays such as 2,2-diphenyl-2-picryl-hydrazyl free radical (DPPH), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging and lipid peroxidation. The radicals were strongly scavenged by oil with IC₅₀ value of 0.0000000671 mg/ml for DPPH, 1.05 mg/ml, ABTS and 1.170 mg/ml for lipid peroxidation. In the antimicrobial investigations, seven microorganism strains namely, *Listeria ivanovii* (ATCC 19119), *Staphylococcus aureus* (ATCC 29213), *Streptococcus uberis* (ATCC 700407), *Mycobacterium smegmatis* (ATCC 19420), *E. cloacae* (ATCC 13047), *Escherichia coli* and *Vibrio* sp. were used. The oil had antimicrobial effects on the microorganisms; the most sensitive microorganism was *E. coli* followed by *Vibrio* sp. Gas chromatography–mass spectroscopy studies on the *E. brevipapposus* oil resulted in the identification of 95 compounds which constituted 99.84% of the oil. α -pinene, α -Phellandrene, germacrene D, β -pinene, trans- β -Ocimene, Bicyclogermacrene and β -Phellandrene were the major compounds making up 59.84% of the oil. The strong scavenging and high antimicrobial activities of *E. brevipapposus* oil holds great potential as a source of bioactive compounds with good antioxidant and antimicrobial activities, thus complementing the traditional usage of the plant.

Keywords: *Euryops brevipapposus*; essential oil; Cala; free radical scavenging; antimicrobial; gas chromatography-mass spectroscopy.

4.1 Introduction

Chronic respiratory disease, an important component of NCDs, is rated among the major causes of mortality globally, with asthma being the most common chronic disease affecting children (IUATLD, 2011). Asthma is an acute inflammatory lung condition causing the reversible airflow obstruction and is denoted by wheezing, coughing and breathlessness (Wood et al., 2015). Globally, 300 million people are affected by asthma, thus, attracting much attention of recent (Vasconcelos et al., 2008; Wu, 2011). In sub-Saharan Africa, asthma contributes immensely to their burden of diseases, and around 50 million children under the age of 15 are affected, with the major proportion of these children residing in South Africa (Adeloye et al., 2013). It has been established that oxidative damage may lead to human diseases such as cardiovascular disorders, diabetes mellitus, myocardial infarction, immunologic, atherosclerosis, neurodegenerative diseases and asthma (Sugiura and Ichinose, 2008; Riedl and Nel, 2008; Skarbez et al., 2010).

Numerous researches have established that reactive oxygen species (ROS) do not only act as determinants of asthma severity but also play a major part in the airway inflammation (Sahiner et al., 2011). They are known to elevate lipid peroxide production as well as protein carbonyls in plasma (Nadeem et al., 2015), by reacting with lipids to produce ethane in addition to isoprostane, which is raised in the breath of those affected with asthma (Wedes et al., 2009).

During the occurrence of these disease-aforementioned conditions, microorganisms invade host body due to the compromised state of the host making it vulnerable to infections by microorganisms (Casqueiro et al., 2012). Some opportunistic microorganisms that have been associated with asthma include *Haemophilus influenza*, *Pseudomonas aeruginosa*, *Moraxella catarrhalis* and *Staphylococcus aureus* (Wood et al., 2009).

Oxidative stress is a root cause of many diseases that plague humans; hence the employment of antioxidants in pharmacology is studied extensively (Bjelakovic et al., 2007). The diverse levels of antioxidant defense in a healthy human, keeps the production of pro-oxidants in check. The endogenous antioxidant defense mechanisms include the release of enzymatic antioxidants such as catalase (CAT) and non-enzymatic antioxidants (ascorbic acid, and tocopherol) (Poljsak et al., 2013). However, whenever antioxidant defense is subjected to harmful environmental, pathological or physicochemical agents like toxic chemicals, ultraviolet rays, atmospheric pollutants and radiation; this maintained balance shifts in favor of pro-oxidants thereby resulting

in ‘oxidative stress’ (Sultan, 2014). As a result, endogenous antioxidants perform a critical role maintaining cell functions optimally, systemic health and general wellbeing. Although, when undergoing conditions that encourages oxidative stress, these antioxidants may not be enough and therefore supplementation with dietary antioxidants to ensure the cellular functions are maintained optimally becomes very imperative (Ozben, 2015).

Medicinal plants act as reducers or neutralizers of free radicals or as antimicrobial agents and they are known to lessen the harmful conditions generated during pathophysiology of diseases (Mittal et al., 2014). Apart from being effective in the inhibition and reduction of free radicals, these natural antioxidants have been established as safe (Morten et al., 2012; Camila et al., 2013), as their synthetic counterparts cannot be re-used neither can they be recycled once their electron has been donated thereby becoming toxic metabolic by-products that elevates the entire load of oxidative stress (Wang et al., 2008). Medicinal plants are usually used in folkloric medicine (Agbabiaka et al., 2010), as it is well known that they proffer sources of bioactive chemical compounds which can serve as antimicrobial and antioxidant agents (Edris, 2007; Ramawat et al., 2009; Verpoorte, 2009).

Till date, there is no known cure for asthma; the medications that exist (e.g. corticosteroids) are used for the management of symptoms only. Besides this limitation, the side effect that arises from their usage in children and glucocorticoids-resistant individuals brings more cause for concern (Li et al., 2013). Considering the chronic nature of asthma, the side effects associated with western medications and the serious health and death threat (250,000 deaths) posed to humans particularly those in low and middle income countries (LMIC) yearly (Bousquet et al., 2010), drugs from other origins such as plants are of high need and thus are being researched. Despite the huge potentials of medicinal plants as sources of pharmacological agents, there are not enough adequate scientific data evaluating their efficacy. Hence, this study was undertaken to screen for the *in vitro* activity of *E. brevipapposus*.

The genus *Euryops* (Cass.) Cass. belongs to the *Senecioneae* tribe of the family Asteraceae and is made up of 97 completely described species plus another five awaiting description or recently described (Nordenstam et al., 2009). *Euryops* genus is the third in Asteraceae, in terms of species numbers and size in Southern Africa (Koekemoer, 1996). It is distributed all over Africa with the exception of *E. arabicus* Steud. which is found in locations like the Arab nations (Anderberg et

al., 2007; Devos et al., 2010). *E. brevipapposus* is a small, erect aromatic plant of about 1m height.

Since there is very little published information regarding, *Euroyys brevipapposus*, a wild plant found growing on the mountains and utilized routinely in the treatment of NCDs in particular, asthma or as a bronchodilator by the traditional health practitioners of Cala, a community in Eastern Cape, South Africa, it has become of prime importance to evaluate the biological activities of *E. brevipapposus* oil. The research study objectives therefore were: 1) to determine the *in vitro* the efficacy of the oil as an antioxidant; 2) to assess antimicrobial efficacy of the oil; 3) to determine the chemical composition of the essential oils of *E. brevipapposus* using GC/MS.

4.2 Materials and Methods

4.2.1 Study Area Description

Cala is a small rural town in the Sakhisizwe Local Municipality which falls under the Chris Hani District Municipality (Statistics South Africa, 2012). It is situated at geographical coordinates of 31.5230° S, 27.6980° E in the northern region of Eastern Cape Province, South Africa. Sakhisizwe Local Municipality where Cala town is situated represents about 8% of Amathole District Municipality having a population size of about 63,582 people (Statistics South Africa, 2012). It is reported to have a low Human Development Index (HDI) of 0.49, high rate of unemployment and a high percentage of people living in poverty (75%) (CHDM, 2015). Figure 4.1 and 4.2 shows the map that illustrates the study area.



Figure 4.1: The map illustrating the study area of Cala Community (Source:<https://www.google.co.za/maps/place/Cala+Community>). **Figure 4.2:** Map showing local municipality in which Cala community is located (Source: <https://www.localgovernment.co.za>).

4.2.2 Ethical Clearance

This was procured from the committee of research ethics, University of Fort Hare prior to commencement of study and the collection of *E. brevipapposus* from the Cala community was carried out after consent was granted by the traditional health practitioners (THPs) in the community.

4.2.3 Plant Collection

The leaf part of plant materials that was collected depended on the information on the plant usage gotten from the THPs in the Cala community. The identification of *E. brevipapposus* took place at the Selmar Schonland herbarium, Botany Department, Rhodes University by plant taxonomist Tony Dold.

4.2.4 Sample Preparation

Upon identification, *E. brevipapposus* leaves were washed thoroughly with sterile water to remove dust and air dried for few days. After drying, the air-dried plant materials were then milled using Polymix (PX-MFC 90 D model) till finely powdered.

4.2.5 Essential Oil Extraction

Using hydrodistillation process, *E. brevipapposus* oil was obtained in Clevenger apparatus. After the oils were collected, they were treated with anhydrous sodium sulfate PA to get rid of the leftover water. The oil was stored in dark colored vials and refrigerated at -4°C pending further use. The oil yield was derived based on the relation between the plant weight and the essential oil. Before the extraction commenced, a clean bottle was weighed and after the oil was collected, the bottle was reweighed and both masses were recorded. The following was used to calculate the yield:

$$\text{Oil Mass (g)} = (B_O - B_E)$$

$$(\%) \text{ Percentage yield} = [(B_O - B_E) \div P_W] 100$$

Where the plant weight (g) = P_W ; empty bottle weight (g) = B_E ; and the weight of bottle + oil extracted (g) = B_O (Omoruyi et al., 2014).

4.2.6 Antioxidant Assay

This was evaluated *in vitro* using three different methods as previously described in literature and they are as follow-:

I. 2,2-Diphenyl-1-picryl hydrazyl radical (DPPH) Assay

This spectrophotometric assay was carried out to determine radicals scavenging activities of essential oil against DPPH radical as described by Odeyemi et al. (submitted for publication). The DPPH solution ($2.7 \times 10^{-6} \text{ M}$) was prepared using methanol solvent at 0.1 mM concentration and thereafter kept in the dark. Then 100 μl of methanol was added to the wells of microtiter plates except the second and third rows respectively. Thereafter, 150 μl of methanol was added into the third row. 50 μl of the oil prepared in methanol was then added into the third row in triplicates. Starting from the first column in the third row, a twofold serial dilution was done by mixing the well contents and transferring 100 μl into the second well of the same column and repeating this process till the 7th well of the same column with the remaining 100 μl from the 7th

well discarded. The two fold dilution method was used to prepare the 0.05- 0.5 mg/mL concentrations of the vitamin C (standard) and essential oil in the wells. Then 100 µl of the already prepared DPPH radical was added into all the wells. Afterwards, the reaction mixture was vortexed and left for half an hour at ambient temperature away from light. The absorbance of the resultant solution was then determined with the spectrophotometer at 517 nm.

The capacity of *E. brevipapposus* to clean-up DPPH was then evaluated as % inhibition using the expression below:

$$\% \text{ inhibition} = \{(\text{Control}_{\text{Abs}} - \text{Oil}_{\text{Abs}})\} / (\text{Control}_{\text{Abs}}) \times 100 \text{ ----- } (\%)$$

Where $\text{Control}_{\text{Abs}}$ = abs of the DPPH radical + methanol (control sample) and Oil_{Abs} = Abs of DPPH radical + oil or reference drug.

II. 2, 2-azinobis-(3-ethylbenzothiazolin - 6-sulfonic acid) diammonium salt (ABTS) Assay

The decolorization reaction method of Re et al. (1999) was used. Briefly, potassium persulfate solution (2.45 mM) was used to oxidize ABTS solution (7.0 mM) in equal amount forming pre-formed ABTS monocation (ABT^+). The mixture was then left for 12 h in a dark cupboard at ambient temperature for complete reaction. Thereafter, 1mL of the solution was mixed with 60 mL methanol to attain 0.705 ± 0.001 at 734 nm, which is the absorbance required in performing the assay.

Then 100 µl of methanol was added to the wells of the microtitre plates except the second and third rows respectively. Thereafter, 200 µl of the essential oil and standards prepared in methanol was then added into the third row in triplicates. Starting from the first column in the third row, a twofold serial dilution was done by mixing the well contents and transferring 100 µl into the second well of the same column and repeating this process till the 7th well of the same column with the remaining 100 µl from the 7th well discarded. The two fold dilution method was used to prepare the 0.05- 0.5 mg/mL concentrations of vitamin C and oil in the wells. Then 100 µl of the already prepared ABTS solution were added into all the wells. After seven minutes, the absorbance was recorded at 734 nm spectrophotometrically. ABTS^+ (%) clean-up by the oil, was then estimated by the expressing (%) as stated for DPPH• assay.

III. Lipid Peroxidation Test

With minor modifications (methanol was used for serial dilution of oils in place of distilled water), the protocol used was previously reported by Badmus et al. (2011). Ten percent of 0.5 mL homogenate (in distilled water) of egg yolk, known to be lipid-rich was mixed with the essential oil at varying concentrations of 0.05-0.50 mg/ mL and brought up to 1.0 mL. To induce the lipid peroxidation, 0.05 mL of FeSO₄ (0.07 M) was mixed with the solution above thereafter incubating for 30 min. Then, 10 % CH₃COOH (1.50 mL) of 3.50 pH adjusted with NaOH, 0.80 % of 2-thiobarbituric acid (1.5 mL) made in sodium dodecyl sulphate 1.1 % and 20 % trichloroacetic acid (0.05 mL) was combined in Eppendorf tubes and was then allowed to complete reaction in water bath at 65 °C for one hour. On cooling, 1-butanol (0.5 mL) was mixed with individual tubes and subjected to centrifugation for ten minutes at three thousand revolutions per minute. Finally, at 532 nm organic layer absorbance of each was then recorded. The solvent employed as blank was methanol. The lipid peroxide percentage inhibition activity was obtained using the expressing (×) stated for DPPH test. The results were in triplicates and average values computed.

4.2.7 Antimicrobial Assay

4.3.7.1 Preparation of Bacterial Suspensions (CLSI, 2014)

The antibacterial activity of *E. brevipapposus* was evaluated using *Listeria ivanovii* (ATCC 19119), *Staphylococcus aureus* (ATCC 29213), *Streptococcus uberis* (ATCC 700407), *Mycobacterium smegmatis* (ATCC 19420), *E. cloacae* (ATCC 13047), *Escherichia coli* (AEMREG) and *Vibrio* sp (AEMREG). The bacteria were inoculated into different tubes of 7 ml of Mueller Hinton Broth from their stock culture and incubation was for 24 h at 37°C. After 24 h incubation, the bacterial suspensions (inoculums) were diluted serially to McFarland 0.5 standard using normal saline solution. Dilutions matching with 0.5 McFarland standards were then used. The reference drug used was ciprofloxacin.

4.2.7.2 Minimum Inhibitory Concentration (MIC)

This was performed using the macro-broth dilution method. The following dilutions 200 µl, 150 µl, 100 µl, and 50 µl of the *E. brevipapposus* oil were added into Eppendorf tubes containing

800 µl, 850 µl, 900 µl, and 950 µl Mueller-Hinton broths respectively and tubes were inoculated with 20 µl cell culture suspension matching 0.5 McFarland of target microorganisms. The tubes were then incubated at 37°C for 24 h. The lowest concentration which was able to inhibit apparent microorganism's growth was noted as the MIC (Christofilogiannis, 2001; Bonjar, 2004). To determine the minimum bactericidal concentration (MBC), 10 µl from the MIC assays tubes that lacked turbidity after the period of incubation were subcultured and incubation was at 37°C for 24 hours. The MBC therefore was the lowest concentration with no visible growth, those agar plates with growth after the incubation period were regarded as having bacteriostatic effect.

4.2.8 Chemical Analysis of *E. brevipapposus* essential oil

Using the method of Omoruyi et al. (2014), this analysis was conducted at University of Fort Hare, Botany Department with a GC-MS system (HP 6890) coupled to a mass selective detector (HP5973). A needle with the oil was directly inserted into the inlet of a Hewlett Packard (HP 6890, USA) Gas Chromatograph. Maintaining the injection port temperature and inlet pressure at 220°C and 3.96 psi respectively, while the HP-5 MS cross-linked with 5% Phenyl Methyl Siloxane column of 30 m× 0.25 mm× 0.25 µm film thickness was scheduled with temperature ranging from 60°C to 150°C at 3°C per min after a 3 min delay. The carrier gas used was helium at a flow rate of 0.7 ml/min. The various compounds contained in the oil were identified when the spectrum obtained through the GC-MS analysis was compared with the standards available or well known components spectrum saved on the database of National Institute Standard and Technology (NIST).

4.2.9 Statistical Analysis

The data obtained were treated in replicates, averaged and expressed as Mean ± standard deviation (SD). Data was further statistically analyzed using MINITAB Release 17 statistical package to carry out one way ANOVA (analysis of variance). Results were significant at $P < 0.05$, Microsoft Office Excel was used to plot bar charts and the standard deviation was represented by the error bars.

4.3 Results

4.3.1 Yield of the leaves of *Euryops brevipapposus*

The hydro-distillation of *E. brevipapposus* resulted in the extraction of a yellow coloured minty essential oil. The total yield of the oil was 6.37 ml giving a percentage yield of 0.91% (Table 4.1).

Table 4.1: Results obtained for extraction yield and percentage yield of extraction values

| Parameter | <i>E. brevipapposus</i> |
|------------------------------------|-------------------------|
| Extraction yield (g) | 6.37 |
| Percentage yield of extraction (%) | 0.91 |
| Colour | Yellow |

4.3.2 Scavenging activity of *E. brevipapposus* essential oil

This capacity of *E. brevipapposus* essential oil was evaluated in DPPH, ABTS, and Lipid peroxidation assays.

4.3.2.1 DPPH

The DPPH radical inhibition activity of *E. brevipapposus* oil as compared with vitamin C is shown on Figure 4.3. The IC₅₀ value for the essential oil was 0.0000671 mg/ml (Table 4.2). *E. brevipapposus* demonstrated a high inhibition activity at all concentrations, with values of percentage inhibition of DPPH radicals ranging between 75-78%.

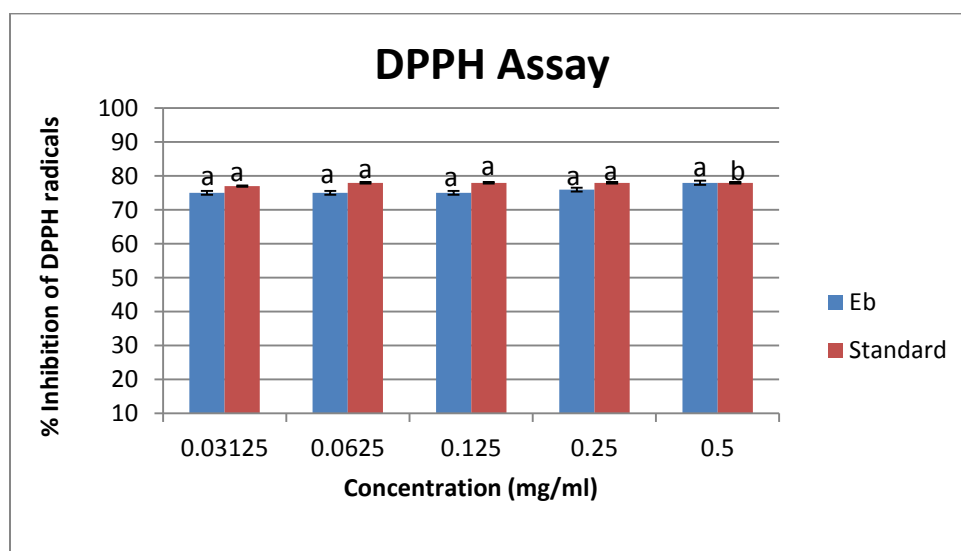


Figure 4.3 DPPH radical scavenging by *E. brevipapposus* essential oil and the standard antioxidant, vitamin C. Bar graphs with different letter superscript in the same concentration are significantly different ($P < 0.05$).

4.3.2.2 ABTS

The essential oil of *E. brevipapposus* showed significantly strong scavenging potential at 0.125 mg/ml to 0.5 mg/ml concentrations (Figure 4.4). The values of percentage inhibition of DPPH radicals ranged between 40.5% -76% while the value for the IC₅₀ of the oil was 1.06 mg/ml (Table 4.2).

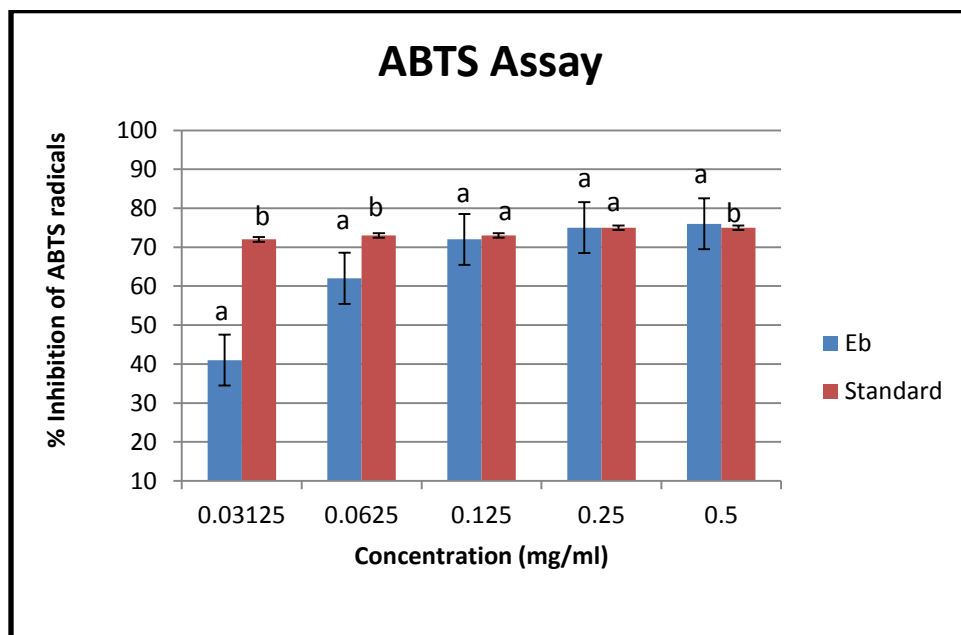


Figure 4.4 ABTS radical scavenging by *E. brevipapposus* essential oil and the standard antioxidant, vitamin C. Bar graphs with different letter superscript in the same concentration are significantly different ($P < 0.05$).

4.3.2.3 Lipid peroxidation assay

The essential oil of *E. brevipapposus* at 0.125 to 0.5 mg/ml, showed high scavenging activity, which was observed to be comparable to the standard vitamin C. The value for the IC₅₀ of the oil was 1.17 mg/ml (Table 4.2). The scavenging effect of *E. brevipapposus* is shown in Figure 4.5.

Table 4.2: Antioxidant activity of essential oils of *Euryops brevipapposus*

| S/N | Activity | Concentration(mg/ml) | %inhibition | IC50 | R ² |
|-----|----------|----------------------|-------------|--------------|----------------|
| 1. | LP | 0.03125 | 50±0.009 | 1.170 | 0.921 |
| | | 0.0625 | 53±0.003 | | |
| | | 0.125 | 61±0.006 | | |
| | | 0.25 | 67±0.003 | | |
| | | 0.5 | 68±0.002 | | |
| 2. | DPPH | 0.03125 | 75±0.0004 | 0.0000000671 | 0.517 |
| | | 0.0625 | 75±0.0004 | | |
| | | 0.125 | 75±0.0004 | | |
| | | 0.25 | 76±0.0004 | | |
| | | 0.5 | 78±0.0004 | | |
| 3. | ABTS | 0.03125 | 41±0.004 | 1.05 | 0.95 |
| | | 0.0625 | 62±0.004 | | |
| | | 0.125 | 72±0.006 | | |
| | | 0.25 | 75±0.003 | | |
| | | 0.5 | 76±0.001 | | |

LP• = lipid peroxide radical, DPPH• = 2,2-diphenylpicrylhydrazyl radicals, ABTS+• = 2,2'-azino-bis diammonium salt radicals. Values are %inhibition± SD.

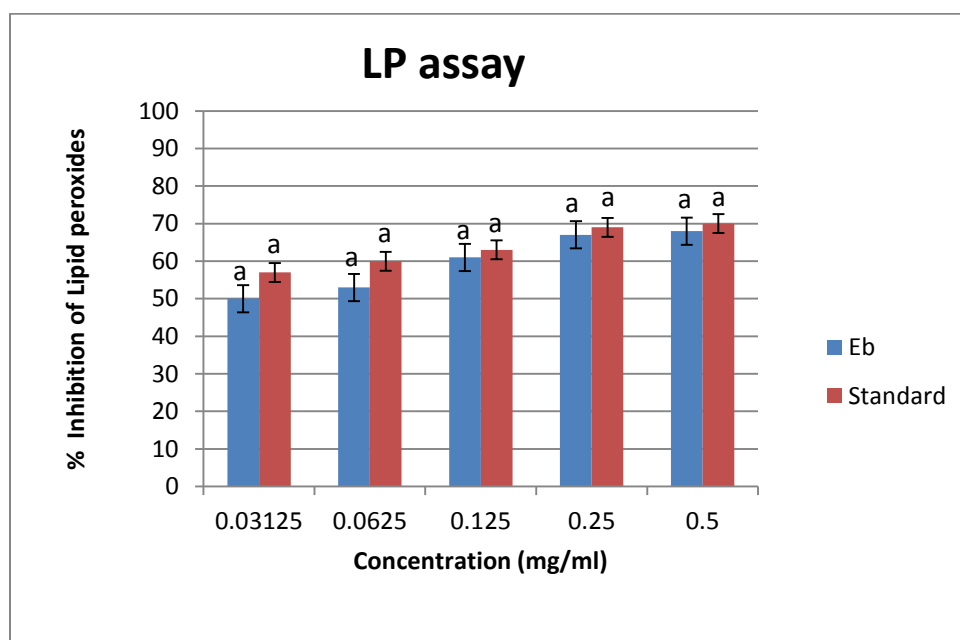


Figure 4.5 –Lipid peroxide radical scavenging by *E. brevipapposus* essential oil and the standard antioxidant, vitamin C. Bar graphs with different letter superscript in the same concentration are significantly different ($P < 0.05$).

4.3.3 Antimicrobial Activity of *E. brevipapposus* oil

The essential oil from the *E. brevipapposus* leaves exhibited high inhibitory activity against the seven test bacteria used (Table 4.3). However, ciprofloxacin had overall highest activity, when tested against the microbes compared to *E. brevipapposus*.

Table 4.3: Growth inhibition effect of *E.brevipapposus* oil

| Microorganism | Gram +/- | Inhibitory effects | Ciprofloxacin |
|---------------------|----------|--------------------|---------------|
| <i>S. aureus</i> | + | Susceptible | Susceptible |
| <i>M. smegmatis</i> | + | Susceptible | Susceptible |
| <i>L. ivanovii</i> | + | Susceptible | Susceptible |
| <i>S. uberis</i> | + | Susceptible | Susceptible |
| <i>E.coli</i> | - | Susceptible | Susceptible |
| <i>E. cloacae</i> | - | Susceptible | Susceptible |
| <i>Vibro</i> | - | Susceptible | Susceptible |

4.3.3.1 Minimum inhibitory concentrations (MIC)

The essential oil of *E. brevipapposus* showed high to moderate antimicrobial activity with MICs values that ranged between 0.055 to 0.335 mg/ml. The extent of growth inhibition of the microorganisms tested was concentration dependent. The lowest MIC value of the essential oil was 0.055 mg/ml, and it was exhibited against *E. coli* and *Vibrio* while the highest value of 0.335 mg/ml was observed when the essential oil was tested with *S. aureus*. The essential oil of *E. brevipapposus* was bacteriocidal as it had MBCs ranging from 0.125 to 0.5 mg/ml for the other test microorganisms, except for *S. uberis* which was bacteriostatic at all concentrations used. The MBC value for *S. aureus* was 0.5 mg/ml, *M. smegmatis* and *L. ivanovii* were 0.335 mg/ml, while *E. cloacae* was 0.215 mg/ml and *E.coli* and *Vibrio* were 0.125 mg/ml (Table 4.4).

Table 4.4: MIC and MBC values for *E. brevipapposus*






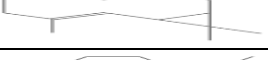



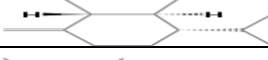


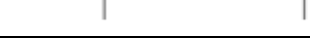

| Microorganism | Culture collection and Ref. No. | <i>E. brevipapposus</i> | | Antibiotic |
|--------------------------------|---------------------------------|-------------------------|-------|------------|
| | | MIC | MBC | |
| <i>Enterobacter cloacae</i> | (ATCC 13047) | 0.125 | 0.215 | >0.125 |
| <i>Listeria ivanovii</i> | (ATCC 19119) | 0.215 | 0.335 | >0.125 |
| <i>Staphylococcus aureus</i> | (ATCC 29213) | 0.335 | 0.5 | >0.125 |
| <i>Streptococcus uberis</i> | (ATCC 700407) | 0.5 | >0.5 | >0.125 |
| <i>Mycobacterium smegmatis</i> | (ATCC 19420) | 0.215 | 0.335 | >0.125 |
| <i>Escherichia coli</i> | AMEREG | 0.055 | 0.125 | >0.125 |
| <i>Vibro</i> sp. | AMEREG | 0.055 | 0.125 | >0.125 |







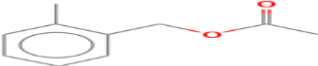


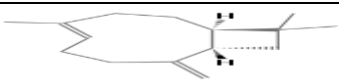


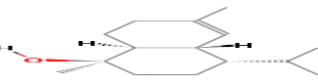
4.3.4 Chemical Compounds of *E. brevipapposus* Oil Identified by GC-MS

The *E. brevipapposus* essential oil recorded a large number of compounds, having about 95 compounds, among which 9 were the most prevailing compounds (Table 4.5). The mass spectrum of *E. brevipapposus* essential oil showed 9 prominent peaks including alpha.-Pinene, alpha.-Phellandrene, 1,6-Cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl)-, beta.-Pinene, trans-beta-Ocimene, Bicyclogermacrene, Benzene, 2-ethyl-1,3-dimethyl, 8-Isopropenyl-

1,5-dimethyl-cyclodeca-1,5-diene and α -Copaene (Figure 4.6). The less prominent peaks reveals the minor components identified at other retention times, their peak areas are given in Figure 4.6.

Table 4.5: Major composition of bioactive compounds identified using GC–MS

| S/N | Retention time (min) | Compounds | Molecular Formula | Structure | Molecular Weight | Peak Area % |
|-----|----------------------|--|---------------------------------|---|------------------|-------------|
| 1 | 3.986 | .alpha.-Pinene | C ₁₀ H ₁₆ |  | 136.23404 | 11.46 |
| 2 | 4.565 | .alpha.-Phellandrene | C ₁₀ H ₁₆ |  | 136.23404 | 12.38 |
| 3 | 4.393 | .beta.-Pinene | C ₁₀ H ₁₆ |  | 136.23404 | 7.51 |
| 4 | 4.809 | trans-.beta.-Ocimene | C ₁₀ H ₁₆ |  | 136.23404 | 6.31 |
| 5 | 8.169 | (-)-Germacrene D | C ₁₅ H ₂₄ |  | 204.3511 | 7.63 |
| 6 | 8.271 | Bicyclogermacrene | C ₁₅ H ₂₄ |  | 204.35106 | 5.39 |
| 7 | 7.428 | .alfa.-Copaene | C ₁₅ H ₂₄ |  | 204.3511 | 2.06 |
| 8 | 7.508 | 8-Isopropenyl-1,5-dimethyl-cyclodeca-1,5-diene | C ₁₅ H ₂₄ |  | 204.3511 | 2.09 |
| 9 | 8.034 | .beta.-Bisabolene | C ₁₅ H ₂₄ |  | 204.3511 | 1.67 |
| 10 | 8.084 | γ-Cadinene | C ₁₅ H ₂₄ |  | 204.3511 | 1.67 |
| 11 | 5.496 | 2,4,6-Octatriene, 2,6-dimethyl- | C ₁₀ H ₁₆ |  | 136.2340 | 1.48 |
| 12 | 8.363 | Allo-Ocimene | C ₁₅ H ₂₄ |  | 204.3511 | 1.88 |
| 13 | 4.904 | .beta.-Ocimene | C ₁₀ H ₁₆ |  | 136.2340 | 4.67 |
| 14 | 4.771 | Benzene, 2-ethyl-1,3-dimethyl- | C ₁₀ H ₁₆ |  | 136.2340 | 3.78 |

| | | | | | | |
|----|-------|--|--|---|----------|------|
| 15 | 4.698 | .alpha.-Phellandrene | C ₁₀ H ₁₆ |  | 136.2340 | 2.34 |
| 16 | 4.320 | .beta.-Phellandrene | C ₁₀ H ₁₆ |  | 136.2340 | 4.49 |
| 17 | 4.095 | 1R- α -Pinene | C ₁₀ H ₁₆ |  | 136.2340 | 1.94 |
| 18 | 3.577 | 1-Nonene | C ₉ H ₁₈ |  | 126.2392 | 1.55 |
| 19 | 3.111 | Cyclopentane, 1,1-dimethyl- | C ₇ H ₁₄ |  | 98.1861 | 1.14 |
| 20 | 6.966 | 2-Acetylcyclopentanone | C ₇ H ₁₀ O ₂ |  | 126.1531 | 0.74 |
| 21 | 6.847 | Benzenemethanol, 2-methyl-, acetate | C ₁₀ H ₁₂ O ₂ |  | 164.2011 | 0.87 |
| 22 | 7.130 | 1,5,5-Trimethyl-6-methylene-cyclohexene | C ₁₀ H ₁₆ |  | 136.2340 | 0.76 |
| 23 | 7.676 | 1H-Cycloprop[e]azulene, 1a,2,3,4,4a,5,6,7b-octahydro-1,1,4,7-tetramethyl-, [1aR-(1a.alpha.,4.alpha.,4a .beta.,7b.alpha.)]- | C ₁₅ H ₂₄ |  | 204.3511 | 0.67 |
| 24 | .757 | Caryophyllene | C ₁₅ H ₂₄ |  | 204.3511 | 0.69 |
| 25 | 7.898 | Neoisolongifolene | C ₁₅ H ₂₂ O |  | 218.3346 | 1.03 |
| 26 | 8.763 | Spatulenol | C ₁₅ H ₂₄ O |  | 220.3505 | 1.46 |
| 27 | 9.094 | .tau.-Cadinol | C ₁₅ H ₂₆ O |  | 222.3663 | 0.73 |

Abundance

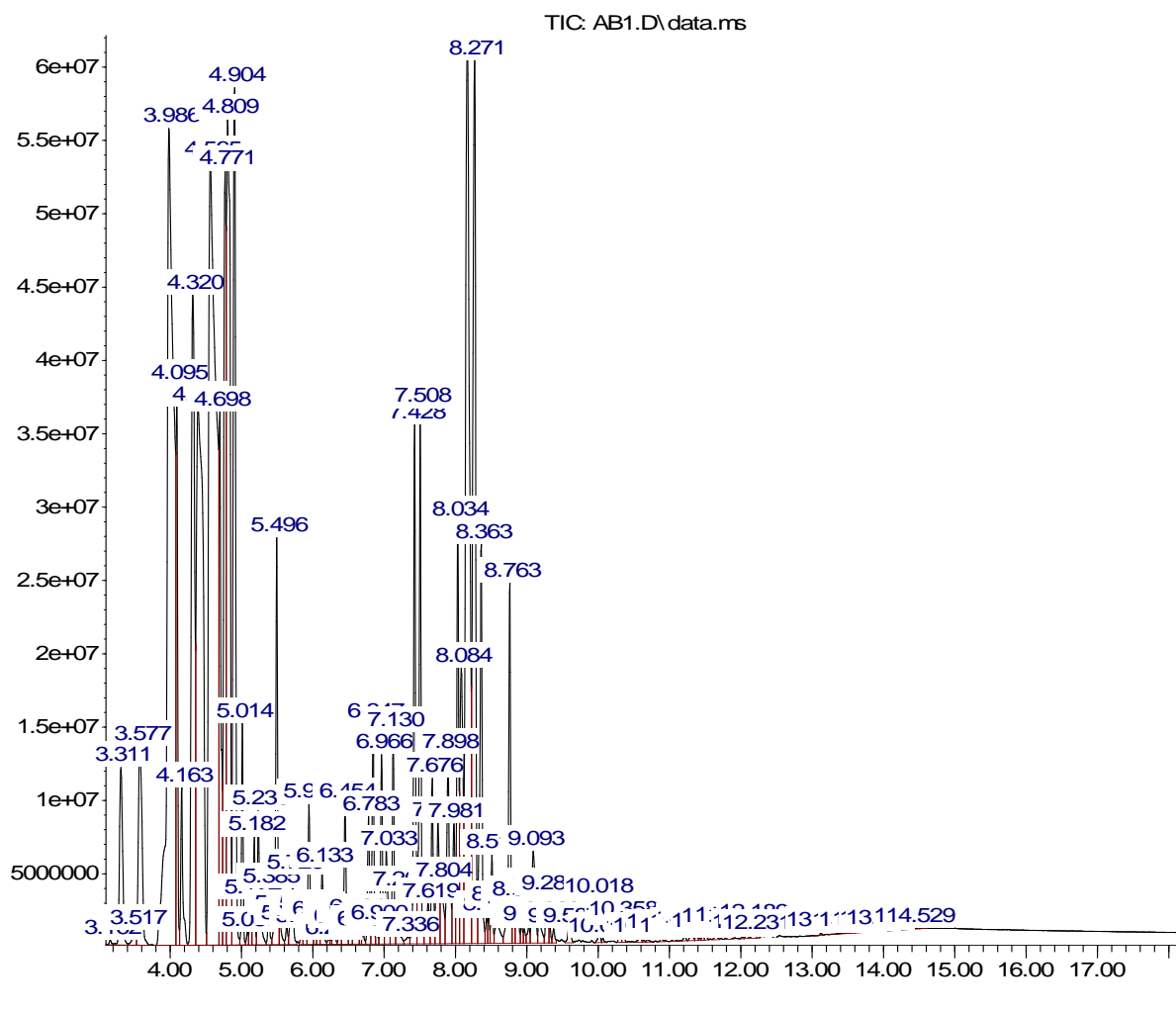


Figure 4.6: GC-MS chromatogram of *E. brevipapposus* essential oil

4.4 Discussion

The result presented in Table 4.1 shows that hydro-distillation of the *E. brevipapposus* leaves extracted a pleasant smelling yellow coloured oil with a yield of 0.91%. The obtained oil was then analyzed for its radical scavenging, antimicrobial activity, in addition to the chemical composition. From the results presented in Figures 4.3- 4.5, a lower activity was observed when the IC₅₀ was compared with the standard antioxidant used, as the essential oil had a value of 1.05mg/ml while vitamin C had 0.00013 mg/ml. Similarly in the lipid peroxidation assay, the essential oil also effectively reduced lipid peroxides radicals (IC₅₀=1.17 mg/ml), the activity was however low as vitamin C had a value of 0.5 mg/ml (Figure 4.3, Table 4.2). The DPPH assay however gave a better result for *E. brevipapposus* with IC₅₀ value of 0.0000000671 mg/ml

compared with 1.05 mg/ml, the IC₅₀ value obtained for vitamin C (Figure 4.4, Table 4.2). The high DPPH radical inhibition by the essential oil can be as a result of the oil's strong proton donating ability (Tundis et al., 2013). Findings have shown that the difference in the behaviour of essential oil in the ABTS and DPPH is due to the difference in solubility of the ABTS and DPPH reagents (Longhi et al., 2011). Thus, these comparisons indicate that *E. brevipapposus* essential oil possesses significant DPPH scavenging activity and high ABTS and lipid peroxidation activity, implying that the essential oil is a potent natural antioxidant.

Table 4.3 and 4.4 shows that the oils exhibited favorable antimicrobial activities against various microorganisms in the panel tested as it had inhibitory activities against the growth of the microorganisms (Minimum inhibition concentration values of 0.125 and 0.05 mg/ml). The microorganisms tested showed susceptibility to *E. brevipapposus* essential oil. The essential oil showed variable inhibitory activities against test microorganisms which were concentration-dependent. To the best of our knowledge, there is no previous report regarding the antimicrobial activity of the plant, although *E. arabicus*, Asteraceae species was reported to exhibit potent antibacterial activity with MIC-values of 0.13–5.25 mg/ml supporting their traditional use in wounds treatment (Mothana et al., 2011). This finding of the antimicrobial activity of the essential oil of *E. brevipapposus* seemed to confirm both its antimicrobial potential and use in traditional medicine.

Santoyo et al. (2005) described alpha-pinene, 1,8-cineole, camphor, verbenone, and borneol, present the oil they studied as being accountable for the antimicrobial activity, with the most potent being borneol, followed by camphor and verbenone. However only alpha-pinene was present in this essential oil among these compounds mentioned by Santoyo et al. (2005) report, and the quantity was high in the studied sample. The susceptibility of the microorganisms to the oil could be linked with the antimicrobial activity of the high alpha-pinene and caryophyllene content of the oil. Although, the other major and minor components of the essential oil, including those components having known antimicrobial activity such as alpha-pinene, beta-Bisabolene, beta-Ocimene, caryophyllene and their combined synergistic effect could also have enhanced the overall antimicrobial activity of essential oil. As chemical constituents of essential oils have the potential of affecting its biological activity as previously suggested by van Vuuren et al. (2007).

The GC-MS analysis of *E. brevipapposus* oil revealed the various types of compounds present in it viz., beta-Bisabolene, alpha-Pinene, beta-Pinene, beta-Ocimene and caryophyllene (Table 4.5). The major bioactive compounds in *E. brevipapposus* were alpha-Pinene (11.46), alpha-Phellandrene (12.38%), 1,6-Cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl)-, [S-(E,E

)]- (7.63%), beta-Pinene (7.51%), trans-beta-Ocimene (appearing twice 6.31 and 4.67% respectively), Bicyclogermacrene (5.39%), beta-Phellandrene (4.49%), while the other components were detected in lesser quantities (Figure 4.6).

Beta-Bisabolene has been observed to exhibit activities such as anti-rhinoviral, antiulcer, antiviral, stomachic (Duke, 1992). Alpha-pinene (α -pinene) and beta-Pinene (β -pinene), isomers of pinene, are monoterpene described to have antibacterial and antifungal activities (Leite et al., 2007, Silva et al., 2012). Alpha-Pinene (α -pinene) is also used pharmacologically as an anti-inflammatory (Zhou et al., 2004), apoptotic, and antimetastatic (Matsuo et al., 2011). α -pinene is also a well-known antioxidant that has demonstrated remarkable activities (Wang et al., 2008), and it is known to exhibit diverse effects such as antilisterial, spasmolytic, anticholinesterase and insecticidal (Sadraei et al., 2001; Lee et al., 2001; Mourey and Canillac, 2002; Savelev et al., 2003). Kelen and Tepe (2008) on the other hand showed that when tested on its own α -pinene is not able to have strong antioxidative activity in free radical assay tested.

Beta-Ocimene has been used as an expectorant and fungicide (Duke, 1992). Alpha-Phellandrene has various biological activities such as antibacterial and antistaphylococcal (Wright, 2002), fungicide, irritant, insectiphile, laxative (Duke, 1992). Caryophyllene has analgesic, anti-asthmatic, antibacterial, anti-carcinogenic, anti-endemic, anti-inflammatory, anti-tumor, anti-ulcer, anti-leishmanic, anti-proliferant properties. 1, 6-Cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl)-, [S-(E,E)]- has anti-inflammatory properties (Easa, 2003). Reviewing the available researches, no scientific report has been made on the essential oil of *E. brevipapposus*. There is therefore a dearth of information on *E. brevipapposus* as the existing current literature on it is very limited.

A previous study by Mothana et al. (2011) on the oil of *E. arabicus* led to the identification of 48 components which represented 93.5% of the oil. It was characterized by oxygen-containing sesquiterpenes (39.9%) and predominately oversesquiterpene hydrocarbons (24.1%) while the major constituents were caryophyllene oxide (8.6%), T-cadinol (7.0%), spathulenol (5.2%), (E)- β -caryophyllene (6.0%) and 2-epi-(E)- β -caryophyllene (6.0%). It is therefore assumed that there is synergism between the major and minor components of the essential oil of *E. brevipapposus* to produce the antimicrobial and antioxidant activities exhibited.

4.5 Conclusion

Owing to the numerous benefits such as availability, effectiveness, relative cheapness, little/no side effects, medicinal plants have over drugs. The study of medicinal plants has attracted much attention in recent times. In this study, *E. brevipapposus* was assessed for the first time to identify its bioactive components, followed by the investigation of its efficacy as an *in vitro* antioxidant and antimicrobial agent. The findings revealed that the essential oil has *in vitro* antioxidant and significant antimicrobial activity complementing the traditional usage of the plant for infections associated with noncommunicable diseases. Therefore, results from this study showed that the natural compounds contained in the oil could be potential sources of bioactive compounds with strong antioxidant and antimicrobial activities. However, extensive investigations need to be done to identify and better evaluate the active compounds responsible for the observed antimicrobial and antioxidant activities and to determine the *in vivo* biological efficacy as well as evaluate the cytotoxicity level of the plants, in order to explore and exploit its rich biological benefits.

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Chapter 5

General Conclusion

Two plants namely, *Olea europaea* subspecies *africana* and *Euryops brevipapposus* were investigated for chemical composition, antioxidant and general antimicrobial properties. The results also indicate that *O. europaea* and *E. brevipapposus* contained abundant phytochemical compounds which could be responsible for the biological activities observed among the plants extracts. Only the ethanol and ethyl acetate extracts of *O.europaea* had bacteriostatic effect on the microorganisms; however, the n-hexane extract including the ethanol and ethyl acetate *O. europaea* extracts exhibited significant antioxidant activities. The essential oil of *E. brevipapposus* was effective against all the microorganisms. The *E. brevipapposus* essential oil also exhibited significant *in vitro* antioxidant activities. With the results thus obtained, it is safe to conclude that these properties validate the traditional usage of both plants in the treatment/management of diseases. However, further studies should be conducted in order to evaluate the *in vivo* biological activity and to validate the cytotoxicity profile of these plants before their usage can be recommended.