Antimicrobial activity and phytochemical screening of *Olea europaea subsp. africana* (wild olive tree) against selected pathogenic microorganisms

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21888159

BSc Hons (Microbiology), 2014

BSc (Biology and Chemistry), 2013

Dissertation submitted in fulfillment of the requirements for the degree *Masters* in Biology (Microbiology) at the Mafikeng Campus of the North-West University

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Date: January, 2017

DECLARATION

I Saynab Rashid Adem declare that, the dissertation entitled "Antimicrobial activity and phytochemical screening of *Olea europaea subsp. africana* (wild olive tree) against selected pathogenic microorganisms", hereby submitted for the Degree of Master of Science in Biology (Microbiology) at the North-West University (Mafikeng Campus), has not been submitted by me for a degree at this or any other university. This is my own work in design and execution and that all material contained herein has been duly acknowledged.

Saynab Rashid Adem
(Student)

Signed at NWU on this Day of 11th October 2016

Dr R.E Gopane
(Supervisor)

Signed at NWU on this Day of 11th October 2016

DEDICATION

This work is dedicated to my family.

ACKNOWLEDGEMENTS

My thanks first and foremost to Allah almighty, who has been the source of my energy throughout my study; without Allah none of this would be possible.

I would like to thank my supervisor Dr R. E Gopane for her guidance patience and support throughout this research project. I greatly appreciate the generous financial aid from the National Research Foundation of South Africa for supporting my studies at North-West University, Mafikeng Campus.

Finally my deepest and greatest gratitude goes to my parents and family for their motivation and support.

ABSTRACT

A rich variety of medicinal plants are found in Southern Africa and nearly 80% of the populace still depend on medicinal plants to fulfil their primary health care needs. Many of these medicinal plants are used to treat illnesses such as sores, urinary tract infections, burns, colds, rheumatism, flu, gout, hypertension, cancer, diabetes, Human Immunodeficiency Virus (HIV) infections and Acquired Immunodeficiency Syndrome (AIDS). Examples of such plants are *Olea europaea subsp. africana*. The aim of this work was to investigate the phytochemical screening, antioxidant and antimicrobial activities of different crude extracts from leaves of *Olea africana*. Different organic solvents including butanol, ethanol, ethyl acetate, hexane and water were used to prepare the crude extracts. Antibacterial, antifungal, antioxidant and anti-lipid peroxidation activities were determined by agar disc diffusion, minimum inhibitory concentrations (MIC) using the broth dilution technique, antifungal screening was evaluated by disc diffusion, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and thiobarbituric acid reactive substances (TBARS) assay. Phytochemical screening for all crude extracts were tested and showed positive results for alkaloids, tannins, flavones, flavonoids, terpenoids, and steroids compounds. However, all the crude extracts did not show positive results for saponins, carotenoids, cardiac glycosides and anthraquinonoes compounds. The antioxidant activity results of the crude extracts showed that when gradually increasing the samples concentration there was an increase in absorbance. Therefore the antioxidant activity of the crude extracts as equivalent to 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was in the order of hexane > ethanol > ethyl acetate > butanol > water > methanol. This antioxidant potential corresponded with the results of DPPH spectrophotometric assay. The crude extracts of *Olea africana* were screened for antibacterial activity against the following bacteria *Bacillus cereus* (ATCC 10876), *Enterococcus faecalis* (ATCC 29212), *Moraxella catarrhalis* (ATCC 25240), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhi* (ATCC 14208) and *Staphylococcus aureus*

(ATCC 25923). All the plant extracts tested had antibacterial activity however, the plants differ in their activities against the microorganisms tested. Highest antibacterial activity was observed with methanol extract of *O. africana* against *S. aureus* (43 mm), *S. typhi* (40 mm) and *B. cereus* (34mm), respectively while minimum activity was observed with aqueous extract of *O. africana* against *Moraxella catarrhalis* and *P. aeruginosa* (7 mm). The MIC results indicated that the extracts inhibited the growth of *Bacillus cereus* (ATCC 10876), *Enterococcus faecalis* (ATCC 29212), *Moraxella catarrhalis* (ATCC 25240), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhi* (ATCC 14208) and *Staphylococcus aureus* (ATCC 25923). All the plant extracts tested showed antifungal activity, among the five solvents attempted, the methanol extract showed higher inhibitory activity (23.7 mm) followed by ethanol extract (15.5 mm) and ethyl acetate extract (12 mm) against the fungus, *Candida tropicalis*. Ethanol fraction showed the highest percentage inhibition of anti-lipid peroxidation in egg yolk homogenate (84.53%). In conclusion, all organic crude extracts could be used as potential sources of new antioxidant and antimicrobial agents.

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ACRONYMS AND ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
cfu/ml	Colony-forming units per milliliter
HIV	Human Immunodeficiency Virus
DPPH	2,2-diphenyl-1-picrylhydrazyl
$FeCl_3$	Ferric Chloride
$FeSO_4$	Ferric Sulfate
HCl	Hydrochloric acid
H_2SO_4	Sulfuric acid
IC_{50}	Concentration of an inhibitor
GC	Gas liquid chromatography
GC-MS	Gas chromatography mass spectrometry
MIC	Minimum inhibitory concentration
ml	millilitre
mm	millimetre
NaOH	Sodium hydroxide
ppm	parts per million
R_f	Retention factor
TBARS	Thiobarbituric acid-reactive species

TLC	Thin-layer chromatography
µg/ml	microgram per milliliter
WHO	World Health Organization

DEFINITION OF CONCEPTS

Antibacterial: A type of antimicrobial used in the treatment and prevention of bacterial infection. They may either kill or inhibit the growth of bacteria.

Antibiotic: A drug used to treat infections caused by bacteria and other micro-organisms.

Antifungal: An agent that inhibits or destroys fungi. Used in the treatment of various fungal problems such as *Candida.*

Anti-inflammatory: Reduces inflammation of joints, injuries etc.

Antimicrobial: is an agent that kills microorganisms or inhibits their growth.

Antioxidants: are chemicals that block the activity of other chemicals known as free radicals.

Minimum inhibitory concentration: the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation.

Lipid peroxidation: refers to the oxidative degradation of lipids. It is the process in which free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage.

Phytochemicals: are chemical compounds that occur naturally in plants.

CHAPTER 1

INTRODUCTION

1.1 Background

Infectious diseases are a major threat to human health (Ahmad *et al.*, 2008). No region in the world has been excluded from the relentless spread of increasingly drug-resistant bacteria (Cars and Nordberg, 2005). Pathogenic bacteria causing different kinds of life threatening infections have increased and is an essential cause of death in immune-compromised patients mainly in developing countries (Zembower, 2014). Even in developed nations despite the improvement made in the understanding of microbes and their control, incidence of epidemics due to drug resistant microorganisms and the emergence of yet unknown disease causing microbes generate great health concerns (Iwu *et al.*, 1999). As microbial resistance continues to change, some pathogens that were once considered harmless to treat are developing, or rather, have developed resistance to almost every antibacterial agent currently available (Wagner and Ulrich-Merzenich, 2009). This is evident in increased level of deaths from acute respiratory infections, diarrhoea diseases, measles, malaria and tuberculosis which have become chief concerns in public health (Alavijeh *et al.*, 2012). This frightening situation necessitates the need for the discovery and improvement of new drugs and new medicinal agents with novel modes of activity (Zampini *et al.*, 2012). There is a vital need to discover new antimicrobial combinations with different biochemical structures and unique mechanisms of action for new and recurring infectious diseases.

One of the most encouraging areas is herbal medicine (Chanda *et al.*, 2013). It has been documented that natural compounds play a significant role in modern medicinal care. Almost all the medicinal plants accessible in the world are likely sources for discovery as well as invention of new drugs to benefit mankind (Borde *et al.*, 2014). At present, there are many

methods to ascertaining new biologically active ingredients in medicinal plants for the preparation of safe drugs. The plant crude extracts are of specific interest because of their wide reception by consumers for their resourceful uses (Al Nomaani *et al.*, 2013). These crude extracts can be in the form of phytochemicals and other bioactive chemical compounds produced by the plants (Thomas and Shanmugasundaram, 2015). Different bioactive compounds have their own specific modes of action thereby enabling specific targeting of pathogenic bacteria by the compound extracted. In view of this there is a need to search for more potent bioactive compounds especially of natural origin to fight the effects of these pathogens.

1.2 Problem statement

Several classes of antimicrobial agents have become less effective as a result of the rise of antimicrobial resistance, probably emanating from selective pressure of antimicrobial usage (Oskay *et al.*, 2010). Presently, there is growing awareness in exploiting plants for medicinal purposes and this may stem from the fact that microorganisms are developing resistance to many drugs this has created a situation where some of the common and less expensive conventional antimicrobial agents are losing their efficacy (Montefiore *et al.*, 1989).

This study is particularly important in the present world where pathogens have developed multiple resistance against the available antimicrobial compounds and where we can explore effective antimicrobial compounds especially of natural origin to combat the effects of these pathogens.

1.3 Aim

The main aim of this research was to evaluate the efficacy of *Olea europaea subsp. africana* as a medicinal plant and to identify the main active ingredients which can be extracted from

this plant. Moreover, to clarify their possible roles in the treatment of current diseases, and how they can be used to produce more effective drugs.

1.4 Objectives

The specific objectives of the study were to:

- Evaluate the crude extract *Olea europaea subsp. africana* for antibacterial activity;
- Evaluate the crude extract *Olea europaea subsp. africana* for antifungal activity;
- Determine the minimum inhibitory concentrations (MICs) of *Olea europaea subsp. africana* crude extract;
- Evaluate the crude extract *Olea europaea subsp. africana* for antioxidant activity;
- Evaluate the lipid peroxidation content of the plant extract.
- Assess the phytochemical components of the plant extract;

CHAPTER 2

LITERATURE REVIEW

2.1 Impact of medicinal plants

The use of medicinal plants in healthcare by individuals of different nations and races across the globe is as long-standing as the history of man (Egwaikhide *et al.*, 2009). Traditionally, plant parts used as a basis of herbal preparation for the treatment of various ailments continue to play an important role in the treatment of many diseases and ailments (Mensah *et al.*, 2009) and their restorative effects have been documented since ancient times (Nweze and Eze, 2009). According to the World Health Organisation (Organization, 2004) medicinal plants are those that contain properties or compounds that can be used for therapeutic purposes or those that synthesize metabolites which act as useful drugs. For centuries, medicinal plants have been widely used by man to treat an assortment of diseases. Medicinal plants naturally contain mixtures of different chemical compounds that may act independently or additively to improve health. A single plant may contain bitter substances that stimulate digestion, anti-inflammatory compounds that reduce swelling and pain, phenolic compounds that act as antioxidants, antibacterial and antifungal compounds that act as natural antibiotics, and toxins and alkaloids that enhance mood and give a sense of well-being and euphoria (Mahomoodally, 2013). Studies have revealed that some plant-derived compounds have antiviral, antibacterial and antifungal properties (Selvamohan *et al.*, 2012).

The healing properties of medicinal plants have been traced to their phenolic content including flavonoids and phenolic acids with their potential role in the prevention of diseases related to preventing oxidative stress (Djeridane *et al.*, 2006). Oxidative stress results from an imbalance between development and removal of free radicals (Abheri *et al.*, 2010). These radicals are possibly involved in a number of illnesses including tissue injury, DNA damage,

cardiovascular malfunctions and tumour promotion among other ailments (Horton, 2003). The herbal remedies occupy distinct positions right from the original period to present day. In the last few decades, traditional knowledge on primary health care has been widely acknowledged across the world. WHO estimates that 60% of the world populace and 80% of the populace of developing countries depend on traditional medicine, mostly plant drugs for their primary health needs (Shrestha and Dhillion, 2003). WHO has acknowledged the aid of traditional health care in tribal communities (Dwivedi and Kohli, 2012). These medicines have less side effects and man can obtain the herbs without difficulty from nature. Plants have been a source of medicinal agents for thousands of years from which a large number of modern drugs have been formulated. Plants are the cheapest and safest alternative source of antimicrobials; plant parts which include the roots, leaves, branches/stems bark, flowers and fruits which are commonly rich in phenolic acids, tannins, coumarins and lignins (Cai *et al.*, 2004).

Therefore, within the traditional drug discovery process, medical plants have been found to be of importance to man (Raskin *et al.*, 2002). Very few plants can be scientifically and clinically recommended due to lack of widespread scientific research (Heinrich *et al.*, 2004). Plant-derived compounds of known structure are used as commercial drugs (Samuelsson, 2004). Drugs such as quinine were first obtained from the plant *Cinchona officinalis* which is still in use today (Butler, 2004). Credibility in terms of its effectiveness can therefore be provided as a result of human experience.

2.2 Antimicrobial agents in plants

Many plants possess antimicrobial properties and provide effective remedies for the management of infectious diseases. The antimicrobial effectiveness of medicinal plants are a desirable feature for controlling and treating infections. Bacterial and fungal infections may be easily diagnosed by traditional healers and community members, as such there is more chance of finding a successful traditional remedy from plant material used in treatment of such

infections. Numerous antimicrobial agents, derived from traditional medicinal plants, are used to treat various diseases caused by microorganisms. The growing occurrence of multidrug resistant strains of bacteria and the presence of strains with reduced susceptibility to antibiotics, increases the threat of untreatable bacterial infections and justifies the need to search for new infection-fighting strategies (Janovska *et al.*, 2003). The various classes of antimicrobial agent each have a unique mode of action against a particular microorganism, which is normally associated to the organism's cell structure. As an example, membrane arrangements of gram negative and gram positive bacteria are altered, which totally upsets their antimicrobial resistance mechanisms (Holley and Patel, 2005).

2.3 Modes of Antimicrobial Action

Antimicrobial agents use different antimicrobial actions in which they may affect cell wall separation, inhibit nucleic acid production, inhibit protein production or block metabolic pathways to inhibit growth of microorganisms or eradicate them (Jorgensen and Ferraro, 1998). Antimicrobial agents can thwart cell wall synthesis, simply by blocking the synthesis of peptidoglycan layer which covers the outer surface of the cytoplasmic membrane.(Jorgensen and Ferraro, 1998). A number of antibacterial agents act by inhibiting ribosome function (Neu and Gootz, 1996) . Bacterial ribosomes contain two subunits, a 50S and a 30S subunits, binding to these sites may cause protein chain synthesis to be terminated and inhibit protein production. This type of antimicrobial agent plays a role in disruption and destabilization of the cytoplasmic membrane. A large number of agents interfere with purine and pyrimidine synthesis or with the deployment of nucleotides. Other agents act as nucleotide analogs that are incorporated into polynucleotides. Antimicrobial agents may also bind to the enzyme gyrase to block DNA replication.

2.4 Medicinal Plants with Antioxidant Properties

Free radicals are highly volatile chemical units normally produced but often overproduced in all higher organisms. When free radicals are produced in excess, they can damage biomolecules and can be among the many reasons for the early incidence of worsening illnesses. Free radicals, including the superoxide radical (O_2^-), hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2) , and lipid peroxide radicals have been implicated in a number of disease processes, including asthma, cancer, cardiovascular disease, cataracts, diabetes, gastrointestinal inflammatory diseases, liver disease, macular degeneration, periodontal disease, and other inflammatory processes(Miller, 1996). These reactive oxygen species (ROS) are produced as a normal result of biochemical processes in the body (Pourmorad *et al.*, 2006).

Free radicals due to environmental pollutants, radiation, chemicals, toxins, deep fried and spicy foods as well as physical stress, cause a reduction of the immune system and antioxidants, alteration in gene expression and give rise to abnormal proteins. The oxidation process is one of the most important paths for producing free radicals in food, drugs and even living systems.

Free radicals or oxidative injury appears to be the fundamental mechanism underlying a number of human disorders(Atawodi, 2005, Sabu and Kuttan, 2002). Hence, therapy using free-radical scavengers (antioxidants) has the potential to stop and delay many of these disorders (Delanty and Dichter, 2000). There has been an increase in the in the therapeutic potentials of medicinal plants as antioxidants in reducing free radical induced tissue injury. Well known and traditionally used natural antioxidants from tea, wine, fruits, vegetables and spices, as well as some natural antioxidants (e.g. rosemary and sage) are already exploited commercially either as antioxidant additives or as nutritional supplements. Also, many other plant species have been investigated in the search for novel antioxidants (Chu *et al.*, 2000, Oke

and Hamburger, 2002) but generally there is still a demand to find more information concerning the antioxidant potential of plant species. The antioxidant activity of plants is mainly due to their phenolic compounds (Pourmorad *et al.*, 2006). Many medicinal plants of African origin have been reported to possess antioxidant properties.(Mahomoodally, 2013).

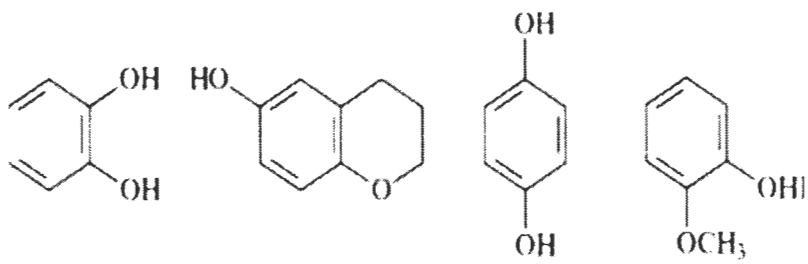


Figure 1: Basic chemical structure of antioxidants (Pokorný, 2007)

2.5 Phytochemicals found in Medicinal Plants

Phytochemicals are bioactive compounds found in plants and have beneficial effects on human health. The bioactive constituents of plants are mainly alkaloids, saponins, tannins, flavonoids and phenolic compounds (Ayoola, 2008). They are responsible for protecting the body against cardiovascular diseases, some forms of cancer and other degenerative diseases (Ayoola, 2008). They function as antioxidants which react with free radicals in the body (Liu, 2003). Attention has been mainly focussed on edible plants, especially those that are rich in phytochemicals, possessing antimicrobial activities. Still, there is an increasing interest in the antioxidant activity of phytochemicals present in the diet (Kubo *et al.*, 2004). In addition to vitamins; nutritional phytochemicals have protective effects and protect the body against several biochemical, physiological and metabolic disorders. Intake of these phytochemicals should be from dietary sources and therefore, fruits and vegetables should be consumed at the maximum in order to benefit from the nutrients and phytochemicals they contain (Liu, 2003).

2.5.1 Alkaloids

Alkaloids are a group of nitrogen-containing compounds resulting from a variety of sources, including microorganisms, aquatic organisms and plants, via complex biosynthetic pathways. These nitrogenous compounds function in the defence of plants against herbivores and pathogens, and are widely exploited as drugs and stimulants, due to their potent biological activities. In nature, the alkaloids exist in large proportions in the seeds and roots of plants, and have many pharmacological applications (Madziga *et al.*, 2010). More than 12,000-alkaloids are known to exist in about 20% of the plant species and only a few have been researched for medicinal purposes. These compounds show promise as they are novel inhibitors of the multidrug resistance phenotype to cancer cells (Kinghom *et al.*, 2003). Alkaloids are pharmaceutically significant, e.g. morphine as a narcotic analgesic, codeine in the treatment of coughs, colchicine in the treatment of gout, quinine as anti-malarial, and quinidine as an anti-arrhythmic (Gurib-Fakim, 2006).

2.5.2 Saponins

Saponins include a vast group of glycosides, which occur in many plants. They are characterized by their chemical agent properties; they dissolve in water and when shaken and produce a foamy solution (Gurib-Fakim, 2006). Saponins exhibit various pharmacological activities such as anti-inflammatory and analgesic activities (Sarker and Nahar, 2007).

2.5.3 Tannin

Tannin is termed as any phenolic compound of sufficiently high molecular weight containing sufficient hydroxyls and other suitable groups (i.e. carboxyls) to form effectively strong complexes with protein and other macromolecules under the particular environmental conditions being studied (Kumari and Jain, 2012). Tannins can be classified into two broad groups: hydrolysable tannins and condensed tannins/proanthocyanidins (PA). Tannin

compounds which are presented in many species of plants play a role in the defence from predation, pesticides, and also in plant growth regulation (Katie and Thorington, 2006). Tannins causes the dryness and creases inside the mouth when it is consumed in red wines (McGee, 2004). Hydrolysable tannins are present only in trace amounts in commonly consumed foods. The more predominant condensed tannins are of more concern due to their anti-nutritional effects. The implication of food tannins on human health is a public concern, but it has preventive benefits to health also, as some tannins are antioxidants. They have been considered to be cardio-protective, anti-inflammatory and also active against pathogenic bacteria (Kennedy and Wightman, 2011). The antimicrobial mode of action is related to their ability to inactivate microbial adhesion enzymes (Coiocan and Bara, 2007). These protective effects are related to their capacity to act as free radical scavengers as well as to activate antioxidant enzymes (Kumari and Jain, 2012).

2.5.4 Flavonoids

Flavonoids are multiple groups of polyphenolic compounds widely distributed in the plant kingdom and over 4000 structurally unique flavonoids have been identified in plant sources (Patel, 2008). Abundant reports support their use as antioxidants or free radical scavengers (Kar, 2007). These are known as the pigments responsible for the many shades of yellow, orange, and red in flowers, fruit and leaves (Patel, 2008). These natural products were known for their positive effects on health long before they were isolated as the effective compounds. Flavonoids are the subject of medical research (Cushine and Lamb, 2005). In vitro studies shows that flavonoids have many useful properties, including anti-inflammatory, oestrogenic, enzyme inhibition, antimicrobial, anti-allergic, and anti-diarrheal (Spencer, 2008, Cushine and Lamb, 2005, De Sousa *et al.*, 2007, Schuier *et al.*, 2005). Further research suggests a protective role of dietary flavonoids against coronary heart disease and also indicate that flavonoid intake is inversely linked with mortality due to coronary heart disease (Skibola and Smith, 2000).

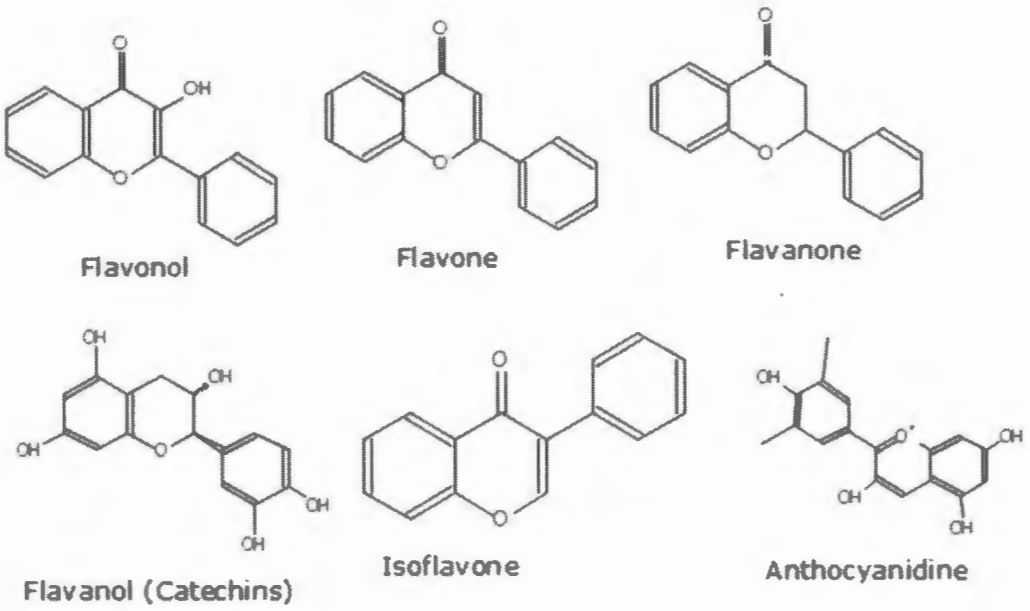


Figure 2: Chemical structures of flavonoids(Lakhanpal and Rai, 2007)

2.5.5 Phenolics

Phenolic compounds are the most ample secondary metabolites of plants. Chemically they are defined as substances possessing an aromatic ring with one or more hydroxyl substituents Phenols are chemical components that occur universally as natural colour pigments responsible for the colour of fruits and plants. Phenol in plants is mostly produced from phenylalanine via the action of phenylalanine ammonia lyase (PAL) (Doughari, 2012). They are very important to plants and have multiple functions (Doughari, 2012). The most important role may be in plant defence against pathogens and thus are applied in the control of human pathogenic infections (Puupponen-pimia *et al.*, 2008). They form one of the simplest groups of bioactive phytochemicals, containing a single substituted phenolic ring (Das, 2010). This group seems to be toxic to microorganisms because of the sites and number of hydroxyl groups present on the phenolic ring; increasing the hydroxylation results an increase of toxicity (Coiocan and Bara, 2007). Phenolics fundamentally represent a host of natural antioxidants, used as nutraceuticals with vast ability to combat cancer and are also thought to prevent heart ailments

to an appreciable degree and sometimes are anti-inflammatory agents (Doughari, 2012). Phenolic compounds are among the natural antioxidants being studied by the scientific community due to their biological properties, e.g., antioxidant and antimicrobial activities (Zhu *et al.*, 2004, Pereira *et al.*, 2006, Malheiro *et al.*, 2011).

2.6 Olea europaea subsp. africana (wild olive tree)

Figure 3: *Olea europaea subsp. africana* (wild olive tree)

2.6.1 Description

Olea europaea subsp. africana also known as the wild olive is a species belonging to the family *Oleaceae* (Laurentis *et al.*, 1997).The wild olive is mainly located in Africa. In history, the wild olive is one of the most quoted in literature. The olive fruit is of major agricultural importance in the Mediterranean region as a source of olive oil. In Southern Africa, the wild olive is one of the most popular plants used by Sotho, Xhosa and Zulu tribes (Van Wyk and

Gericke, 2000). Of 120 species, wild olive was designated the most important plant in traditional medicine.

The wild olive is a neatly shaped evergreen tree with a dense spreading crown (9 x 12 m) of glossy grey-green to dark-green foliage. Leaves are grey-green to dark-green above and greyish below. The tiny, lightly scented white to greenish flowers spray from October to February, followed from March to July with small, spherical, fleshy fruits, either sweet or sour, which ripen purple-black. Common names of the wild olive is named umNquma (Xhosa), Isadlulambazo (Zulu), Motholoari (Sotho) (Van Wyk *et al.,* 2000), Mutlhwari (Venda), Motlhware (Tswana) and Swartolienhout (Afrikaans). *Olea africana* is rich in bioactive phenolic compounds. Oleuropein is one of the utmost important phenolic constituents of *Olea africana*, which has broad pharmacological activities including antibacterial, antifungal, antiviral, antihypertensive, and anti-diabetic properties.

2.6.2 Distribution

This tree is found in a variety of habitats, often near water on rocky hillsides, on stream banks and in woodland. It is widespread in Africa, Mascarene Islands, Arabia, India and China. The olive tree grows in a wide variety of soils with marked preference for calcareous (containing calcium carbonate, calcium, limestone and chalky) soil and coastal climate conditions. The tree is very tolerant to drought and high soil pH (Arabshahi-D *et al.*, 2007).

2.6.3 Cultural uses

Tea can be made from the leaves. The hard, heavy and beautiful golden-brown wood is used for furniture, ornaments, spoons and durable fence posts. An ink is made from the juice of the fruit.(Boudhrioua *et al.*, 2009)

2.6.4 Medicinal uses

Infusions prepared from the leaves of *Olea africana* are used to treat eye infections or as a gargle to relieve sore throat (Agrawal *et al.*, 2010) . The infusion is also taken internally as a remedy for colic or urinary tract infection and to improve kidney function (Masoko and Makgapeetja, 2015). The powdered leaf is used as a styptic (Long *et al.*, 2010) . The fruit is used to treat diarrhoea (Bisi-Johnson *et al.*, 2010) . Traditional remedies prepared from the leaves, roots or stem bark are used to lower blood pressure and to treat related cardiovascular diseases (Sudjana *et al.*, 2009). Customarily, olive tree leaves have been used as a folk remedy for fevers and other diseases such as malaria (Benavente-Garcıa *et al.*, 2000). The antimicrobial activity of olive polyphenols has also been widely investigated by scientists, and in-vitro tests discovered that oleuropein and its hydrolysis products could inhibit the growth of a broad spectrum of pathogenic microorganisms, including bacteria (*Staphylococcus aureus , Salmonella enteritis, Bacillus cereus, Escherichia coli*), fungi, viruses and parasitic protozoan, but have no effect against yeast (Markin *et al.*, 2003). The precise mechanism of antimicrobial action remains uncertain, but it appears that oleuropein could interfere with the synthesis of amino acids necessary for the survival of micro-organisms (Aziz *et al.*, 1997). Nevertheless, in-vivo studies are still required to confirm whether this broad antimicrobial activity of oleuropein can occur in the human body (Lee and Lee, 2010). Olive leaf extract also has great potential as a food ingredient, dietary supplement and source of natural antioxidants (Benavente-Garcıa *et al.*, 2000).

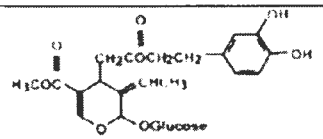
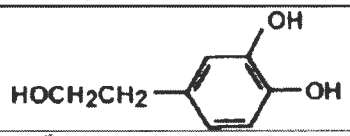
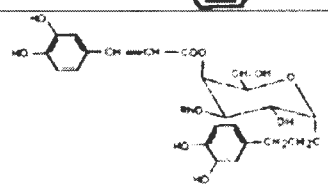
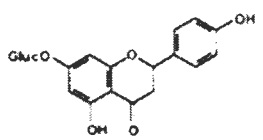
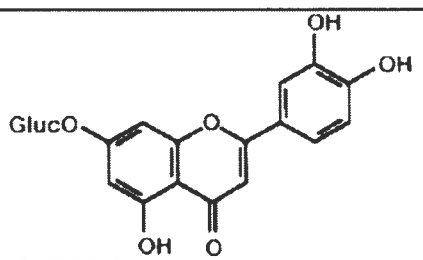
Recent studies have shown that olive leaf extract had the capacity to lower blood pressure vasodilator activity, increase blood flow in the coronary arteries and relieve arrhythmia and prevent intestinal muscle spasms (Benavente-Garcıa *et al.*, 2000). Olive leaf extract and especially oleuropein has been found to have strong antimicrobial activity against fungi, bacteria, viruses and other parasites (Tassou *et al.*, 1991, Koutsoumanis *et al.*, 1998). In

particular, olive leaf extract was found to be effective against many microorganisms, such as *Staphylococcus aureus* (at low concentrations it reduces the growth rate, at higher concentrations inhibits growth, and inhibits the production of enterotoxin B, regardless of concentration), *Vibrio cholerae, Vibrio parahaemolyticus, Haemophylus influenza, Salmonella* spp., *Bacillus cereus* (inhibits spore germination) (Aziz *et al.*, 1997). Studies of six major phenolic compounds found in ethyl acetate extracts of green olives have shown that these have antimicrobial properties (Fleming *et al.*, 1969). Moreover, *Lactobacillus plantarum, Staphylococcus carnosus, Enterococcus faecalis, Salmonella enteridis, Pseudomonas fragi,* and some fungi were inhibited by oleuropein (Aziz *et al.*, 1997).

2.7 Chemical composition

2.7.1 Major Phenolic Compounds in Olive Leaf

Table 1: Chemicals Structures of the Most Abundant Phenolics in Olive Leaf Extract

Phenolic Compounds	Chemical Formula	Chemical Class
Oleuropein		Secoirdoids
Hydroxytyrosol		Phenolic alcohol
Verbascoside		Flavonoids
Apigenin-7-glucoside		Flavonoids
Luteolin-7-glucoside		Flavonoids

(MacDonald-Wicks *et al.*, 2006)

Oleuropein is the main active phenolic compound found in olive leaf extract. Oleuropein is a bitter, secoiridoid glycoside that can be found in fruit, bark and leaves of olive tree (Soni *et al.*, 2006, Benavente-Garcıa *et al.*, 2000), which are produced from the secondary metabolism of terpenes as signs of indole alkaloids, and are usually derived from the oleoside type of glucosides, which is a combination of elenoic acid and a glucosidic residue. This type of compound is exclusive to the *Olea* family (Silva *et al.*, 2006).

Oleuropein has various pharmacological and health promoting properties including, antiarrhythmic, spasmolytic, immune-stimulant, cardioprotective (by inhibiting low-density

lipoprotein oxidation), hypotensive and anti-inflamatory (responsible for inhibition of 5-lipoxygenase enxyme), hypoglycemic, antiviral (even against HIV), cytostatic (against McCoy cells), molluscicidal endocrinal and an enzyme modulator effects due to its antioxidative properties (Lee-Huang *et al.*, 2003, Al-Azzawie and Alhamdani, 2006). Oleuropein and its metabolite hydroxytyrosol both have a catechol group which is required for optimum antioxidant and scavenging activity. Both oleuropein and hydroxytyrosol have been reported to be scavengers of superoxide anions and inhibitors of the respiratory burst of neutrophils and hypochlorous acid-derived radicals(Al-Azzawie and Alhamdani, 2006). They have also been proven to inhibit or delay the growth rate of several human intestinal or respiratory track pathogens such as *Heamophilus influenza*, *Moraxella catarrhalis*, *Salmonella typhimurium*, *Vibrio parahaemplyticus*, *Staphylococcus aureus*, *Vibrio cholerae* and *Vibrio alginolyticus* mainly due to their protective action provided by their phenolic structures (Pereira *et al.*, 2007).

2.8 Extraction of Phenolic Compounds from Olive Leaves

Fresh olive leaf usually needs drying and grinding before extraction. As a preservation method, drying is carried out to remove the water from the leaves consequently protecting the leaves against spoilage by enzyme action. Grinding the dried leaves can reduce particle size and facilitate solvents entering into the cells of the leaves. It also improves extraction efficiency. Many different drying approaches have been explored by researchers, but air drying, microwave drying and freezing drying have been mostly reported in the literatures. Air drying can be carried out at room temperature or high temperatures for different time periods (Savournin *et al.*, 2001). Generally longer times are required for lower temperatures to achieve the same extent of dryness of leaves. Careful temperature control is needed during the drying since it may cause degradation of polyphenols. It was reported that air drying at 25°C or elevated temperature such as 30°C and 40°C in the oven results in good recovery of oleuropein, however, drying at 60°C would result in substantial losses of polyphenols possibly due to

degradation of oleuropein and other polyphenols (Malik and Bradford, 2008). Freeze-drying is another way to effectively avoid thermal degradation while removing the water from leaves which was used. The leaves are immediately frozen in liquid nitrogen, and lyophilized before extraction (Briante *et al.*, 2002, Goulas *et al.*, 2010). The elimination of water through lyophilization generally does not disturb the phenolic compounds extremely, and allows samples to be kept for longer periods (Bravo and Mateos, 2008). The temperature of -80°C is commonly used to store biological samples; it is also used for storage of plant tissues for the purpose of keeping bioactive compounds in the plant.

There is little literature reporting extraction of phenolics from fresh olive leaves, and it was reported that higher levels of oleuropein were found in dried leaves than in fresh leaves probably due to the conversion of oleuropein glucoside into oleuropein by β-glucosidase present in fresh leaves (Silva *et al.*, 2006).

2.9 The Methods of Extraction

Solvent extraction was the main method adopted in this research to extract phenolics from olive leaves. This procedure is designed to separate soluble compounds by diffusion from a solid medium using a liquid medium. This process takes place in two steps, which are the adsorption of solvent into the solid phase by osmotic forces, by capillary and by solvation of the ions in the cells, then followed by diffusion from the solid phase (Bravo and Mateos, 2008). The purpose of the extraction is to concentrate antioxidant constituents; the extraction process involves a forceful agitation of the ground raw materials with extraction solvent at ambient or elevated temperatures and subsequent separation of the residue by filtration. Repeated extraction steps may be accomplished to increase the extract yield. After extraction, suspensions were filtered using filter paper. The solvent was removed by various methods such as rotary evaporators and freezer drier, and then a dry or a concentrated extract was obtained (Savournin *et al.*, 2001, Bouaziz and Sayadi, 2005, Lee *et al.*, 2009).

There are a number of important parameters which affect extraction yield of antimicrobials. They are the type of solvent, concentration of solvent, particle size, extraction temperature and time, the ratio of liquid-to-solid as well as pH of solvent. Before extraction, pre-treatment may be needed for various purposes such as removal of lipid or chlorophyll. Purification of the crude extracts may be essential, especially for the alcoholic extracts, in order to remove the undesirable co-extracts and improve the antioxidant properties and to create a product with a light colour, odour and taste. (Tzia and Liadakis, 2003).

2.10 The Effect of the Extracting Solvent

Both the extraction yield and antimicrobial capacity of extracts are strongly influenced by the solvent, due to the different polarities and different antimicrobial potential of compounds extracted. Therefore, organic solvents of higher polarity are more effective in quantitative recovery of phenolic compounds than non-polar solvents and methanol was reported in many studies as a good solvent for extraction of phenolics from the plants including olive leaves. Ethanol and water are the most commonly employed solvents for safety reasons (Moure *et al.*, 2001). Ethanol alone exists as a solvent for extraction of phenolic compounds from olive leaves, and water has an important role in the extraction process by increasing the diffusion of extractable polyphenols through the plant tissues (Altıok *et al.*, 2008). Ethanol, methanol, ethyl acetate, boiling water, hexane, diethyl ether, chloroform and butanol were the main solvents used by researchers for olive leaf. Of these solvents, aqueous methanol or ethanol were the most commonly used and the concentration of solvent varied between 40% and 80% (v/v). Extraction with 80% methanol (v/v) was reported as the utmost effective method for olive leaf polyphenols (Malik and Bradford, 2008). Boiling of dried leaves was also a very effective method for extracting oleuropein and verbascoside resulting in 96% and 94% recoveries of these compounds, respectively (Malik and Bradford, 2008).

Interestingly, the polyphenol extracts with ethyl acetate from natural materials were reported to have strong antioxidant capacity. Less polar solvents such as ethyl acetate delivered more active extracts than ethanol or methanol, although ethanol and methanol extracts also presented high lipid peroxidation-inhibiting capacity (Moure *et al.*, 2001).

2.11 The Effect of Extraction Temperature, Time and Solvent to Solid Ratio

The removal of the extract is a function of how fast the compound is dissolved and the equilibrium is achieved by liquid solvents (Pinelo *et al.*, 2006). The temperature has impact on solubility, diffusion coefficient and the stability of phenolics compounds (Luthria, 2008). Rise in temperature and a decrease of viscosity significantly increase the diffusion rate. However, high temperature may degrade the phenolics. The driving force for the extraction is the concentration gradient within the particles, which is related to solvent: solid ratio. The rate of extraction increases with a larger concentration gradient (Cacace and Mazza, 2003). A range of extraction temperatures and time were employed by researchers in the extraction of phenolic compounds from olive leaves (Bouaziz and Sayadi, 2005). Generally less extraction time is required with the increasing temperature. When ethanol was used as a solvent, the extraction processes took place either at room temperature (most frequently used) or elevated temperatures of 40°C for 24-48 hours with agitation (Bouaziz and Sayadi, 2005, Jemai *et al.*, 2008, Kiritsakis *et al.*, 2010). A water bath was used to achieve required temperatures. With boiling water, 10 to 30 minutes extraction time was used by researchers (Pereira *et al.*, 2007, Malik and Bradford, 2008). This kind of extraction is purely a static process and easy to operate. The solvent to solid ratio is normally expressed as the ratio of the volume of solvent (millilitre) to the weight of extraction sample (gram). The solvent to solid ratio employed by the researchers for olive leaf extraction varied hugely from 4 to 100, but a ratio between 10 and 50 was mostly reported in the literature (Savournin *et al.*, 2001).

2.12 Microorganisms under investigation for this study

2.12.1 *Bacillus cereus*

Bacillus cereus is a Gram-positive, rod-shaped bacterium found in soil and food. *B. cereus* is a type of bacteria that produces toxins. These toxins can cause two types of illness characterized by diarrhoea and the other by nausea and vomiting.

2.12.2 *Enterococcus faecalis*

Enterococcus faecalis is a Gram-positive bacterium inhabiting the gastrointestinal tracts of humans and other mammals. *E. faecalis* can cause septicemia, urinary tract infections, meningitis, and other infections in humans.

2.12.3 *Moraxella catarrhalis*

Moraxella catarrhalis, non-motile, Gram-negative, aerobic, oxidase-positive diplococcus that can cause infections of the respiratory system, middle ear, eye, central nervous system, and joints of humans.

2.12.4 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, rod-shaped bacterium that causes diseases in plants and animals, plus humans. It is the most common pathogen isolated from patients who have been hospitalized longer than one week, and it is a frequent cause of nosocomial infections. These infections are complicated and can be life-threatening.

2.12.5 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive coccal bacterium and is frequently found in the nose, respiratory tract, and skin. It is a common cause of skin infections such as abscesses, respiratory infections such as sinusitis, and food poisoning.

2.12.6 *Aspergillus niger*

Aspergillus niger is a fungus and one of the most common species of the genus *Aspergillus*. *A. niger* is less likely to cause human disease than some other *Aspergillus*. *A. niger* is one of the most common causes of otomycosis (fungal ear infections), which can cause pain, temporary hearing loss, and, in severe cases, damage to the ear canal and tympanic membrane.

2.12.7 *Candida tropicalis*

Candida tropicalis is a species of yeast in the genus Candida. It is a common pathogen which causes bloodstream infection (candidaemia) and less commonly tissue invasive candidiasis.

2.12.8 *Candida albicans*

Candida albicans is a dimorphic fungus that grows both as yeast and filamentous cells and one of the few species of the Candida genus that cause the infection candidiasis in humans. *C. albicans* biofilms may form on the surface of implantable medical devices. In addition, hospital-acquired infections by *C. albicans* have become a cause of major health concerns. About 85-95% of vaginal infections cases are responsible for physician office visits every year.

2.12.9 *Cryptococcus neoformans*

Cryptococcus neoformans is an encapsulated yeast and an obligate aerobe that can live in both plants and animals. Most infections with *C. neoformans* occur in the lungs. However, fungal meningitis and encephalitis, is a secondary infection acquired by AIDS patients,

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant Materials

The leaves of *O. africana* used in this research were chosen according to the availability of fresh plant material from the botanical gardens in Mafikeng, a region in the North West Province of South Africa, during the 2015, 2016 period. The plants were identified by a taxonomist.

3.1.2 Microorganisms under investigation for this study

The bacterial and fungal isolates used in this work were obtained from Davies Diagnostics (Pty) Ltd, South Africa (Table 2)

Table 2: Microorganisms under investigation for this study

Bacteria species	**Fungal species**
Bacillus cereus (ATCC 10878)	*Aspergillus niger*
Enterococcus faecalis (ATCC 29212)	*Candida albicans*
Moraxella catarrhalis (ATCC 25240)	*Candida tropicalis (ATCC 13803)*
Pseudomonas aeruginosa (ATCC 27853)	*Cryptococcus neoformans*
Salmonella typhi (ATCC 14208)	
Staphylococcus aureus (ATCC 25923)	

3.2 Methods

3.2.1 Extraction of plant samples

The leaves of *O. africana* were washed, cut and air dried. The plant parts were ground to a fine powder and stored in plastic bags. 100g of the powder were weighed and placed into conical flasks and extracted with various solvents of varying polarity (butanol, ethanol, ethyl acetate, hexane, methanol and water), plugged with cotton wool and kept in a rotary shaker at 190-

220rpm for 72 hours at 35°C. After 72 hours the plant extracts were filtered through Whatman filter paper. The filtrate collected was concentrated in a vacuum tube (rotary evaporator) to remove the organic solvent and this left the aqueous part. The aqueous filtrate was then freeze-dried in a lyophilizer to collect the crude extracts. The crude extracts were stored in bottles and kept in the freezer for further use.

Figure 4: Rotary evaporator used to remove organic solvents

Figure 5: Lyophilizer (Freeze Dryer) used to remove water from crude extract.

3.2.2 Antimicrobial Tests

3.2.2.1 Antibacterial Activity

Sensitivity testing of the crude extracts from the plants was determined using agar-well diffusion method as described by (Irobi, 1994). Bacterial isolates were sub-cultured into pre-sterilized nutrient broth and incubated at 37°C in an incubator for 18-24 hours. 0.2 ml of the standardized test isolates (106 cfu/ml of 0.5 McFarland standards) were then sub-cultured on to Mueller-Hinton agar. The medium was allowed to set and wells were then bored into the agar medium using a 6mm Cork borer. The wells were then filled up with the prepared solution of each extract; sterile distilled water was used as negative control and ampicillin as positive control. Care was taken not to allow the extracts to overflow onto the surface of the medium. The plates were then allowed to stand for about an hour to allow diffusion of the solution into the medium. The plates were then incubated in an upright position at 37°C for 24 hours and later observed for zones of inhibition.

3.2.2.2 Antifungal Activity

Antifungal activity was investigated by the disc diffusion method (Alzoreky and Nakahara, 2003). A fungal suspension ($2x10^5$ µl) was streaked on the potato dextrose agar (PDA) medium containing petri plates. Then, sterile discs (made from Whatman filter paper) each about 5mm in diameter were impregnated with the $1x10^5$ µl leaf extracts and placed on the inoculated plates. Each plate was placed with a sterile disc, Ampicillin, as positive control. All the plates were incubated at 28°C for 48 hours. The zones of growth inhibition around the disc were measured after 48 hours. The sensitivity of the fungal species to the plant extracts was determined by measuring the sizes of inhibition zones (diameter of the zone) on the agar surface around the disc.

3.2.2.3 Antioxidant Activity

The estimation of free radical scavenging activity of different dry crude extracts of *Olea africana* was carried out as described by Blois (Blois, 1958) with minor modifications. The dry crude extracts of *Olea africana* were prepared at different concentrations (equivalent to 12.5, 25, 50, 100 and 200 µg/ml) using distilled water in separate test tubes. One milliliter of DPPH (2,2-diphenyl-1-picrylhydrazyl) solution (0.1 Mm) was dissolved in methanol and added to each test tube and shaken vigorously and allowed to stand at 27°C in a dark place for 45 min. The blank and positive controls were prepared in the same way without any extract. Methanol with DPPH was used as a standard at the concentration of 50 ppm. The absorbance of the prepared samples were measured at a wavelength of 517 nm. Radical scavenging activity of the tested crude extract samples were estimated as an inhibition percentage and was calculated by using the following formula, (Alabri *et al.*, 2014) shown below.

$$\%\ Inhibition = \frac{A_{control} - A_{extract}}{A_{control}} \times 100$$

3.2.2.4 Minimum Inhibitory concentration (MIC)

The minimum inhibitory concentrations (MICs) of the plant extracts were determined using the standard method of the European Committee for Antimicrobial Susceptibility Testing (EUCA ST, 2000). Dilutions of the plant extract ranging from 0.05 - 25 mg/ml were prepared in water and incorporated into molten nutrient agar (Bio-lab) at 50°C and poured into sterile plates. The plates were allowed to set and then streaked with standardized inocula of the test bacteria. Plates were incubated at 37°C for 18 hours under aerobic conditions. The MIC was defined as the lowest concentration of the plant extracts that completely inhibited visible growth of the test organism.

3.2.2.5 Anti-Lipid peroxidation assay (TBARS)

A modified thiobarbituric acid-reactive species (TBARS) assay (Singh *et al.*, 2009) was used to measure the lipid peroxide formed using egg yolk homogenate as the lipid rich medium. Egg homogenate (0.5ml of 10% v/v) and 0.1ml of extract were added to a test tube and made up to 1ml with distilled water. About 0.005ml of $FeSO_4$ [Iron (II) sulphate] (0.07M) was added to induce lipid peroxidation and incubated for 30 min at 37°C. Then 1.5ml of 20% acetic acid (pH adjusted to 3.5 with NaOH) and 1.5ml of 0.8% (w/v) TBA in 1.1% sodium dodecyl sulphate and 0.5ml of 20% TCA were added and the resulting mixture vortexed and heated to 95°C for 60 min. To eliminate non-MDA interference by high levels of anthocyanin, another set of samples were as above except no TBA was added cooling, 5.0ml of butanol were added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532nm. Inhibition of lipid peroxidation (%) by the extract was calculated using the formula

$$1 - {}^{E}/_{C} \times 100 \qquad 3.2$$

where C is the absorbance value of the fully oxidized control and E is (Abs_{532}+TBA – Abs_{532} –TBA)].

3.2.3 Phytochemical Analysis

The phytochemical analysis of the extract was determined using the method described by (Evans, 2002, Harborne, 1998). The tests were based on the visual observation of a colour change or formation of a precipitate after the addition of specific reagents.

3.2.3.1 Test for alkaloid

0.5 g of the extract was stirred with 5 ml of 1% HCl solution on a steam bath for 10 minutes 1 ml of the filtrate was tested separately with 2 drops of Wagner, Picric acid and Dragendorff

T.S. reagents. Turbidity or precipitation with at least both Mayer and Dragendorff T.S reagents was taken as preliminary evidence for the presence of alkaloids.

3.2.3.2 Test for saponins

Frothing test

Five millilitre of distilled water was added to 0.2 g of the extract in a clean test tube and the mixture was shaken vigorously. The production of the honeycomb like frothing which persists on warming for up to 15 minutes is indicative of saponins.

3.2.3.3 Test for tannins

The presence of tannins were tested for by mixing 0.5 g of the extract with 20 mL of water in a test-tube or a beaker. 2 mL of 0.1M $FeCl_3$ [Iron(III) chloride] was added. Formation of a blue black colouration indicated the presence of tannins.

3.2.3.4 Test for flavones

The presence of flavones were tested for by adding 5 mL of ammonium solution to 1 mL of aqueous filtrate of the plant extract followed by addition of 2 mL sulphuric acid. A yellow colouration indicated the presence of flavones.

3.2.3.5 Test for flavonoids

Five millilitres of diluted ammonium solution was added to a portion of the aqueous filtrate of each of the extracts followed by the addition of concentrated sulphuric acid. A yellow colouration observed in each of the extracts indicated the presence of flavonoids.

3.2.3.6 Test for carotenoids

The leaf extracts were subjected to a test for carotenoids whereby 1 g of each extract was boiled in 20 mL water. The filtrate was then treated with concentrated sulphuric acid. A blue colour indicated the presence of carotenoids.

3.2.3.7 Test for terpenoids

Test for presence of terpenoids was carried out by adding 1 ml of chloroform extract and then an equal volume of concentrated sulphuric acid was added. Formation of a bluish red colouration indicated presence of terpenoids.

3.2.3.8 Test for steroids

The Salkowski Test

0.5 g of the extract was dissolved in 2 ml of $CHCl_3$ and filtered. Concentrated H_2SO_4 was added to the filtrate to form a lower layer. A reddish brown colour at the interface was taken as a positive test for steroids.

3.2.3.9 Test for cardiac glycosides

The Keller-Killiani test

0.5 g of the extract was dissolved in 2 ml of glacial acetic acid containing 1 drop of ferric chloride solution (1%). This was under-laid with 1 ml of concentrated H_2SO_4 (sulphuric acid). A brown ring obtained at the interface indicated the presence of a desoxy sugar, characteristic of cardiac glycosides. A violet ring may appear below the brown ring while in the acetic acid layer; a greenish ring may form just above the brown ring and gradually spread throughout this layer.

3.2.3.10 Test for anthraquinonoes

Combined anthraquinone test

1 g of the extract was boiled with 10 ml of 1% HCl and filtered. The filtrate was shaken with 5 ml of benzene and the benzene layer removed. To this was added 10% ammonia solution (equal to half the volume of benzene). A pink, red or violet colour in ammoniacal layer indicated presence of anthraquinone derivatives.

3.2.4 Thin layer chromatography (TLC) for phytochemical analysis

Thin layer chromatography (TLC) was used to separate the different parts of *Olea africana* extract into different spots on the chromatographic plate spotted on silica gel precoated TLC plate and allowed to rise in different solvent systems in a saturated TLC chamber. The chromatograms developed on the microscope slide were dried and observed under UV light for the various different spots of plant extract. The developing solvent that was used was ethyl acetate and methanol in ratios of 3:2, respectively. Each of the extracts was co-chromatographed with standard samples of flavonoid (Kaempherol) as markers. Their R_f (retention factor) values were recorded as the ratio of distance travelled by the solute to the distance travelled by solvent front on the TLC plate as described by Kajaria et al., (2011):

$$R_f = \frac{Distance\ travelled\ by\ the\ solute\ (mm)}{Distance\ travelled\ by\ the\ solvent\ front\ TLC\ plate\ (mm)}$$

3.2.5 Gas chromatography-mass spectrometry (GC-MS) for phytochemical analysis

The six (butanol, methanol, ethanol, ethyl acetate, hexane and water) plant extracts were analysed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). GC was carried out on a BP-20 column (60 m × 0.25 mm × 0.25 μm). The temperature was 50°C (5 min) to 220°C (15 min) at 3°C/min with helium as the carrier gas. The temperature of the injector and detector were both set at 220°C. Two columns were used for GC-MS: (a) a BP-20 column (30 m × 0.35 mm × 0.25 μm, injector temperature 220 °C) programmed from 35°C to 220°C at 3°C/min and (b) a BP column (30 m × 0.25 mm × 0.25 μm), injector temperature 250°C), programmed from 35°C (5 min) to 250°C (15 min) at 5°C/min or 80°C (5 min) to 300°C (30 min) at 10°C/min, both with helium as the carrier gas. Mass spectra were recorded in

electron impact (EI) mode at 70eV,scanning from41 to 450 m/z. Compounds were identified by their identical GC retention times and retention indices relative to n-alkanes and by comparison of their mass spectra with either pure standards or published spectra in the NISTGC-MS library and those in the literature. (Sparkman, 2005).

CHAPTER 4

RESULTS

4.1 Yield of plant extract

Table 3 shows yields in milligrams of extracts from all extracted plant materials. Differences in yield between polar and non-polar organic solvents were not big. Methanol extracts of all plants had the highest yields.

Table 3: Yield in milligrams of each plant extract per 100 g of plant material

Plant name	Butanol	Ethanol	Ethyl acetate	Hexane	Methanol	Water
			mg			
Olea europaea subsp. africana	135	142	156	120	184	110

4.2 Antimicrobial tests

4.2.1 Antibacterial activity of *O. africana* extract by agar-well diffusion

The sensitivity of six pathogenic bacteria to the leaf extracts of *O. africana* were tested and compared to that of antibacterial antibiotic Ampicillin. The results shown Table 4 are the average zones of inhibition for each extract. All the plant extracts tested showed antibacterial activity; however, the plant extracts differ in their activities against the microorganisms tested. Highest antibacterial activity was observed with methanol extract of *O. africana* against *S. aureus* (43 mm), *S. typhi* (40 mm) and *B. cereus* (34 mm), respectively while minimum activity was observed with aqueous extract of *O. africana* against *Moraxella catarrhalis* and *P. aeruginosa* (7 mm) (Table 4).

Table 4: Antibacterial activity of various extracts of *O.africana*. Tabulated values are the mean ± standard deviation of 3 replicates.

Microorganisms	Zone of inhibition (mm)						
	Crude Extracts						
	B	E	EA	H	M	W	Ampicillin
***Bacillus cereus* (ATCC 10876)**	8±0.2	23±0.2	15±0.4	16±0.3	30±0.2	6±0.2	34±0.2
***Enterococcus faecalis* (ATCC 29212)**	15±0.2	26±0.4	12±0.2	19±0.4	20±0.6	0.0±0.2	27±0.5
***Moraxella catarrhalis* (ATCC 25240)**	11±0.4	14±0.3	16±0.4	15±0.6	15±0.3	10±0.4	17± 0.6
***Pseudomonas aeruginosa* (ATCC 27853)**	17±0.3	19±0.6	13±0.6	15±0.8	16±0.5	7±0.3	25± 0.3
***Salmonella typhi* (ATCC 14208)**	19±0.1	24±0.5	14±0.4	18±0.7	15±0.4	17.0±0.1	40±0.3
***Staphylococcus aureus* (ATCC 25923)**	10±0.05	19±0.3	13±0.7	19±0.5	28±0.6	00±0.05	43±0.5

Key: Ampicillin was used as a positive control (B: Butanol, E: Ethanol, EA: Ethyl Acetate, H: Hexane, M: Methanol, W: Water)

4.2.2 Antifungal activity of *O. africana* extract by disc diffusion

The sensitivity of a pathogenic fungus to the leaf extracts of *O. africana* was tested and compared to the antibiotic Ampicillin. The results shown in Table 5 are the average zones of inhibition for each extract. All the plant extracts tested showed antifungal activity, among the five solvents used. The methanol extract showed the highest inhibitory activity (23.7 mm) followed by ethanol extract (15.5 mm) and ethyl acetate extract (12mm) against the fungus, *Candida tropicalis*.

Table 5: Antifungal activity of various extracts of *O.africana*. Tabulated values are the mean ± standard deviation of 3 replicates.

Microorganism	Zone of inhibition (mm)						
	Crude Extracts						
	B	**E**	**EA**	**H**	**M**	**Water**	**Ampicillin**
Aspergillus niger	15.3±0.4	16±0.8	13.6±0.6	9.3±0.5	26.5±0.4	10.8±0.5	33.4±0.4
Candida tropicalis (ATCC 13803)	13.4±0.3	15±0.4	12.2±0.6	8.3±0.5	23.7±0.2	9.8±0.3	31.3±0.6
Candida albicans	11.8±0.3	16±0.3	10.6±0.6	9.4±0.5	24.9±0.2	12.3±0.5	33.6±0.6
Cryptococcus neoformans	14.5±0.3	17±0.5	11.3±0.6	8.6±0.9	27.6±0.2	13.4±0.3	34.7±0.6

Key: Ampicillin was used as a positive control (B: Butanol, E: Ethanol, EA: Ethyl Acetate, H: Hexane, M: Methanol, W: Water)

4.2.3 Determination of the minimum inhibitory concentrations (MICs)

The minimum inhibitory concentrations (MIC) of the *O. africana* extracts against selected bacterial isolates are shown in Table 6. The minimum inhibitory concentration (MIC) of all crude extracts ranged from 62.5ug/ml to 250ug/ml. The lower the MIC of the extract the greater the chances of it destroying the microorganism. And the higher the MIC of the extract the less chances of it destroying the microorganism. Despite this, the ability of the extract to destroy the bacterial species also depends directly on the resistance profile of the test organism against the particular extract.

Table 6: The results of MIC for antibacterial activity of *O.africana* extract at various doses on bacterial strains.

		Serial dilution (ug/ml)					
Microorganisms	**Crude Extracts**	**250**	**125**	**62.5**	**31.2**	**15.6**	**7.8**
***Bacillus cereus* (ATCC 10876)**	**Butanol**	–	–	–	+	+	+
	Ethanol	–	–	–	+	+	+
	Ethyl Acetate	–	–	–	+	+	+
	Hexane	–	–	–	+	+	+
	Methanol	–	–	–	+	+	+
	Water	–	–	–	+	+	+
***Enterococcus faecalis* (ATCC 29212)**	**Butanol**	–	–	–	+	+	+
	Ethanol	–	–	–	+	+	+
	Ethyl Acetate	–	–	–	+	+	+
	Hexane	–	–	–	+	+	+
	Methanol	–	–	–	+	+	+
	Water	–	–	–	+	+	+
***Moraxella catarrhalis* (ATCC 25240)**	**Butanol**	–	–	–	+	+	+
	Ethanol	–	–	–	+	+	+
	Ethyl Acetate	–	–	–	+	+	+
	Hexane	–	–	–	+	+	+
	Methanol	–	–	–	+	+	+
	Water	–	–	–	+	+	+
***Pseudomonas aeruginosa* (ATCC 27853)**	**Butanol**	–	–	–	+	+	+
	Ethanol	–	–	–	+	+	+
	Ethyl Acetate	–	–	–	+	+	+
	Hexane	–	–	–	+	+	+
	Methanol	–	–	–	+	+	+
	Water	–	–	–	+	+	+
***Salmonella typhi* (ATCC 14208)**	**Butanol**	–	–	–	+	+	+
	Ethanol	–	–	–	+	+	+

	Ethyl Acetate	–	–	–	+	+	+
	Hexane	–	–	–	+	+	+
	Methanol	–	–	–	+	+	+
	Water	–	–	–	+	+	+
***Staphylococcus aureus* (ATCC 25923)**	**Butanol**	–	–	–	+	+	+
	Ethanol	–	–	–	+	+	+
	Ethyl Acetate	–	–	–	+	+	+
	Hexane	–	–	–	+	+	+
	Methanol	–	–	–	+	+	+
	Water	–	–	–	+	+	+

Key: resistant (+) sensitive (–)

4.3 Antioxidant activity assessment

In this test the antioxidant activity is based on an electron transfer and involves reduction of a coloured oxidant. A simple method that has been developed to determine the antioxidant activity of food utilizes the stable 2, 2- diphenyl-1-picrylhydrazyl (DPPH) radical. The structure of DPPH and its reduction by an antioxidant are shown below (figure 6). The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm and is purple in colour as DPPH is reduced. The colour turns from purple to yellow as DPPH is reduced. The DPPH reagent reacts with the crude extracts and changes the colour from that of α, αα-diphenyl-β-picrylhydrazyl to that of α,αα-diphenyl-β-picrylhydrazine. The amount of change of colour decrease indicates the scavenging potentials of the sample. In our experiments, the six different solvents of various polarities from the leaves of *Olea africana* of all crude extracts were able to change the colour of DPPH solution due to the presence of different phenolic compounds. The antioxidant activity of plant extracts is strongly dependent on the nature of the extracting solvent due to the presence of several antioxidant compounds of diverse chemical characteristics and polarities. Polar solvents are most frequently employed for the recovery of polyphenols, which are some of the main groups of compounds responsible for antioxidant activity (Peschel *et al.,* 2006). In addition, during the sample processing and extraction by different solvents, some volatile active compounds have been destroyed or evaporated from the samples. In this regard, the antioxidant activity was different among the six crude leaf extracts of *Olea africana.* The crude extracts from the leaves of *Olea africana* contain very high contents of total phenols and flavonoids. Also all six crude extracts from this plant showed very high percentage of antioxidant activity.

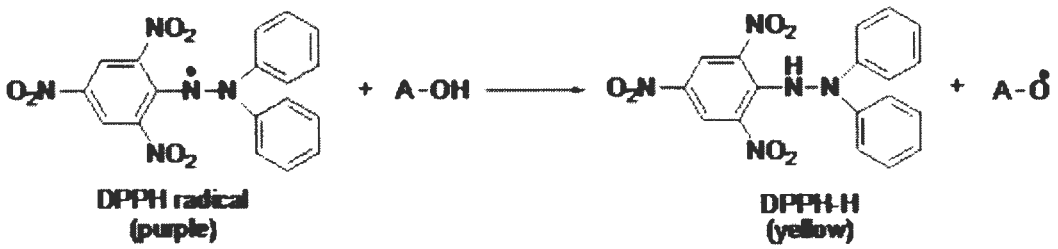


Figure 6: The structure of DPPH and its reduction by an antioxidant

The antioxidant activity of butanol, ethanol, ethyl acetate, hexane, methanol and water crude extracts from the leaf samples of *Olea africana* at different concentrations (12.5, 25, 50, 100 and 200 ppm) showed activity ranging from 18.85-83.87 % (Table 7). The absorbance gradually increased with increasing concentrations of crude extracts from the leaf samples (Figure 7) indicates a concentration dependence of it. Antioxidant activity which is depended on the amount of total polyphenolic compounds present in the leaf extract.

The IC_{50}, which is concentration required to reduce 50% of the DPPH radical, was computed to be 0.34 mM. The smaller the IC_{50}, means the higher the antioxidant activity. High slopes mean that the antioxidant is potent (obtained by linear regression Appendix 1), and the percent inhibition increases as the concentration of the antioxidant increases.

Table 7: Antioxidant potential of Butanol, Ethanol, Ethyl Acetate, Hexane, Methanol and Water crude extracts from leaves of *Olea africana.*

Concentration µg/ml	% Inhibition					
	B	**E**	**E A**	**H**	**M**	**W**
12.5	18.85	19.92	18.87	26.01	17.78	20.08
25	29.88	32.26	31.28	35.06	28.15	28.23
50	37.86	40.66	37.04	42.47	35.14	36.05
100	62.55	64.44	60.74	51.19	61.73	47.57
200	83.87	84.53	75.88	76.71	82.63	75.88
IC_{50}	0.34	0.33	0.29	0.25	0.34	0.28

Key: B: Butanol, E: Ethanol, EA: Ethyl Acetate, H: Hexane, M: Methanol, W: Water

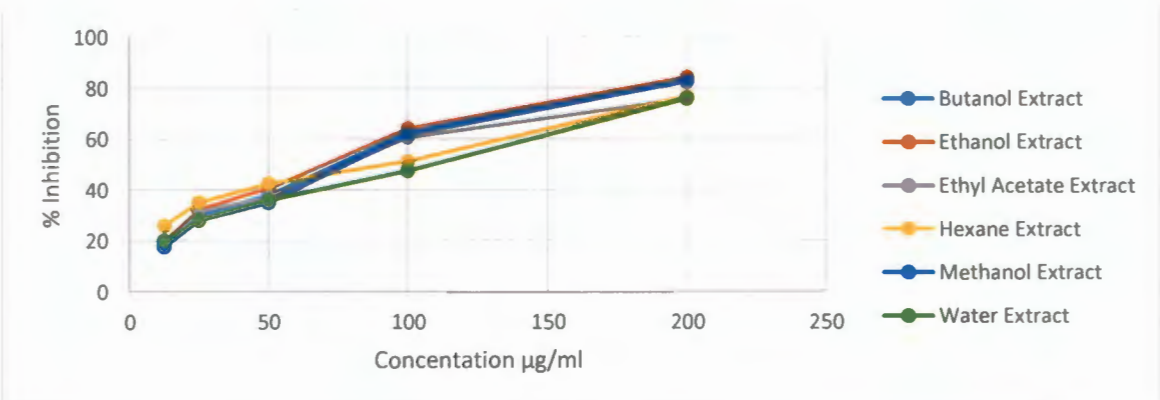


Figure 7: DPPH scavenging activity of Butanol, Ethanol, Ethyl Acetate, Hexane, Methanol and Water crude extracts from leaves of *Olea africana.*

4.4 Anti-Lipid peroxidation assay (TBARS)

To confirm the effects of *Olea africana* plant extract against lipid peroxidation as a direct antioxidant, thiobarbituric acid-reactive substance (TBARS) assay was used. The extent of lipid peroxidation was estimated by the levels of MDA measured using the thiobarbituric acid. $FeSO_4$ was added to egg yolk homogenates, as lipid rich media, to induce lipid peroxidation.

MDA a secondary end product of polyunsaturated fatty acid oxidation, reacts with two molecules of thiobarbituric acid (TBA) yielding a pinkish red chromogen with an absorbance maximum at 532 nm. As presented in Table 8, the ethanol extract significantly showed higher lipid peroxidation inhibition compared with the hexane extract, while no significant difference was observed when compared with methanol, butanol and ethyl acetate fractions as shown in Figure 8.

Table 8: Percentage inhibition of lipid peroxidation at different concentrations of leaf extracts of *Olea africana*. Tabulated values are the mean ± standard deviation of 3 replicates.

Concentration of crude extract (ug/ml)	**% Inhibition**					
	B	**E**	**EA**	**H**	**M**	**W**
200	77.44±0.41	88.44±0.81	76.41±0.15	69.95±0.07	79.26±0.23	89.44
100	63.43±0.32	67.33±0.21	86.20±0.18	53.60±0.11	77.60±0.17	67.20
50	65.44±0.41	70.06±0.51	64.10±0.22	50.08±0.44	48.90±0.41	49.90
25	52.08±0.56	30.09±0.43	36.10±0.39	43.70±0.13	41.20±0.29	41.00
12.5	41.09±0.51	43.87±0.54	41.60±0.31	39.30±0.19	40.50±0.54	39.20

Key: B: Butanol, E: Ethanol, EA: Ethyl Acetate, H: Hexane, M: Methanol, W: Water

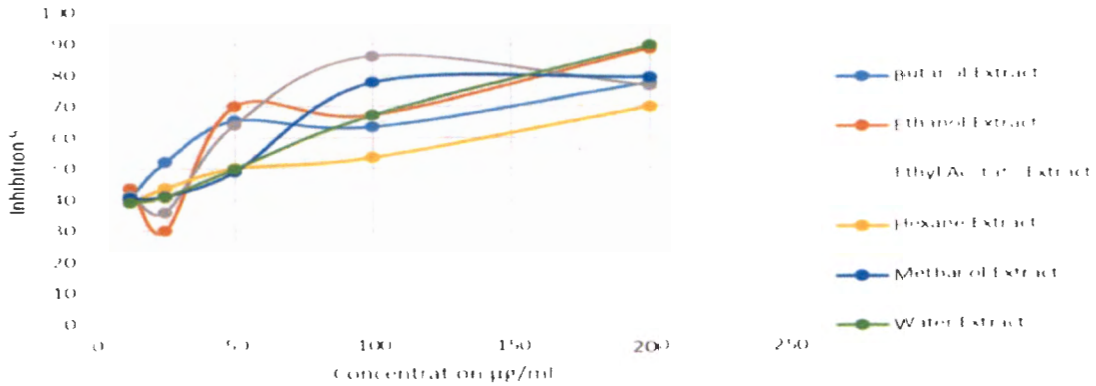


Figure 8: Anti-lipid peroxidation potential of Butanol, Ethanol, Ethyl Acetate, Hexane, Methanol and Water crude extracts from leaves of *Olea africana*

4.5 Phytochemical analysis

4.5.1 Preliminary phytochemical screening

The present study revealed the presence of medicinally active constituents. The phytochemically active compounds of *Olea africana* were analysed and the results are presented in Table 9. Among these phytochemical were alkaloids, saponins, tannins, flavones, flavonoids, terpenoids and steroids which were present in all solvent extracts whereas cardiac glycosides and anthraquinonoes were absent in all the solvent extracts.

Table 9: Phytochemical potential of Butanol, Ethanol, Ethyl Acetate, Hexane, Methanol and Water crude extracts from leaves of *Olea africana*.

Phytochemicals	Tests	B	E	EA	H	M	W
Alkaloids	Dragendorff's test	+	+	+	+	+	+
Saponins	Frothing test	–	–	–	–	–	–
Tannins	Ferric chloride test	+	+	+	+	+	+
Flavones	Alkaline test	+	+	+	+	+	+
Flavonoids	Alkaline test	+	+	+	+	+	+
Carotenoids		–	–	–	–	–	–
Terpenoids	Salkowski test	+	+	+	+	+	+
Steroids	Liebermann-Burchardt test	+	+	+	+	+	+
Cardiac glycosides	Keller-Killiani test	–	–	–	–	–	–
Anthraquinonoes	Combined anthraquinone test	–	–	–	–	–	–

Key: + Denotes present, – Denotes absent, B: Butanol, E: Ethanol, EA: Ethyl Acetate, H: Hexane, M: Methanol, W: Water

4.5.2 Thin layer chromatography (TLC) for phytochemical analysis

Hexane and ethyl acetate were used as a solvent system to achieve good resolution. TLC analysis of all the fractions revealed the presence of promising spots as shown in figure 12, 13, 14. The green spot that appears is the Kaempferol standard, which is a natural flavonol, found in a variety of plants and plant-derived foods. Kaempferol acts as an antioxidant by reducing oxidative stress. Many studies suggest that consuming kaempferol may reduce the risk of various cancers. All six solvent extracts revealed the presence of flavonoids which is an important phytochemical. The variation in R_f values provides a very important clue in

understanding the polarity and selection of appropriate solvent systems for the separation of pure compounds incorporated in different fractions by column chromatography (Table 10).

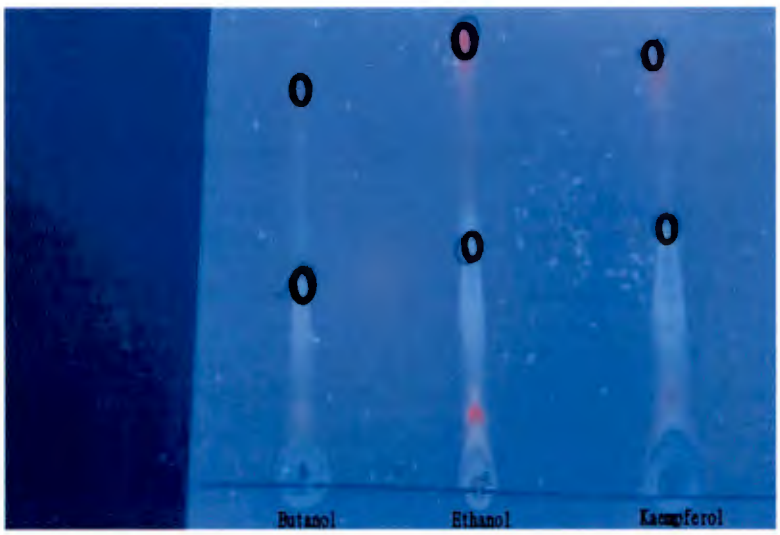


Figure 9: TLC plate of butanol and ethanol extract developed with hexane, ethyl acetate as solvent carriers.

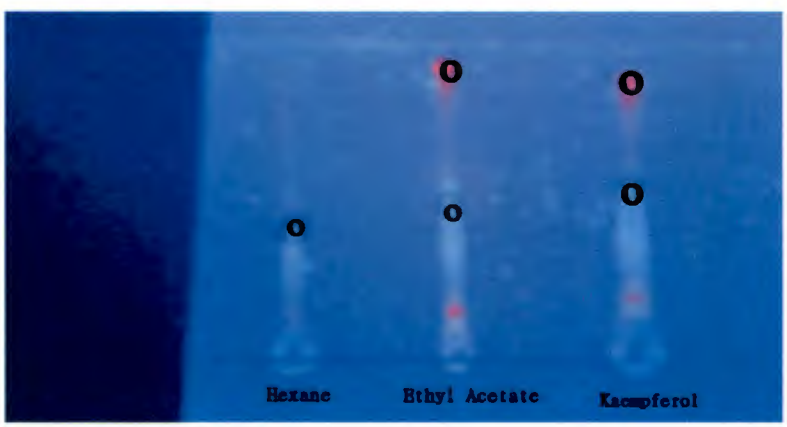


Figure 10: TLC plate of hexane and ethyl acetate extract developed with hexane, ethyl acetate as solvent carriers.

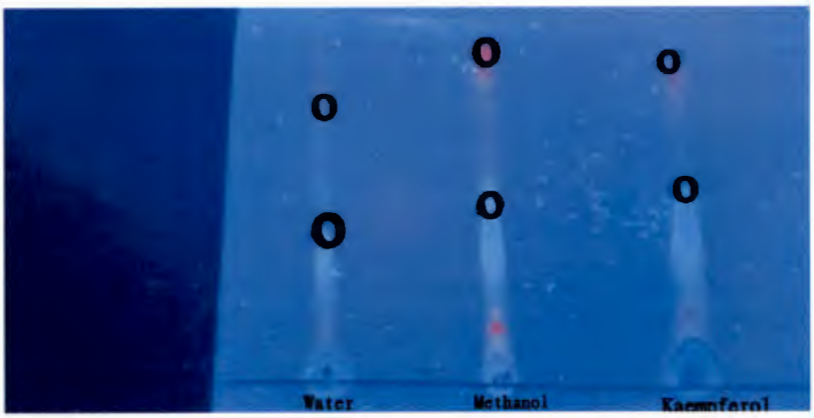


Figure 11: TLC plate of water and methanol extract developed with hexane, ethyl acetate as solvent carriers.

Table 10: R_f values of TLC solvent system for different fractions of *Olea africana*

Plant extracts	Solvent system Hexane: Ethyl acetate(1:1)	
	No of spots	R_f values (mm)
Butanol	2	0.42
		0.9
Ethanol	2	0.44
		0.93
Ethyl acetate	2	0.46
		0.94
Hexane	1	0.43
Methanol	2	0.471
		0.91
Water	2	0.47
		0.89
Kaempferol	2	0.48
		0.93

4.5.3 Gas chromatography-mass spectrometry (GC-MS) for phytochemical analysis

4.5.3.1 GC and GC-MS analysis of all six solvent extracts *Olea. africana*

The six solvent extracts (butanol, ethanol, ethyl acetate, hexane, methanol and water) of *Olea africana* were analysed by GC-MS. The mass spectrum figures are presented in figures 15 and

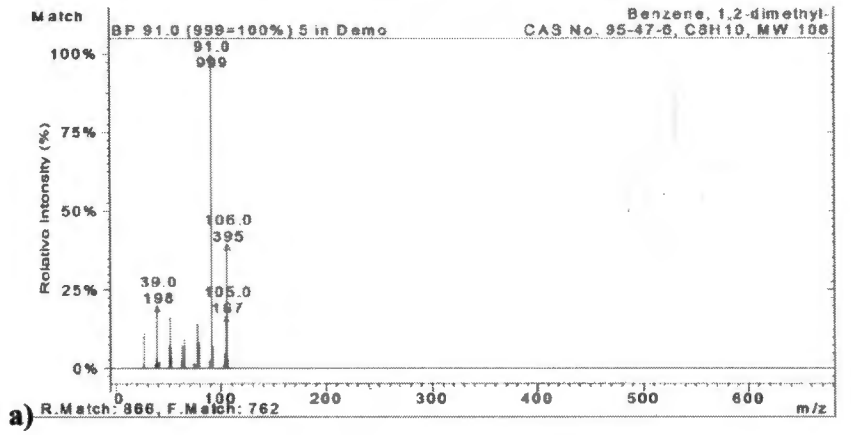
16. The chromatograms are presented in figure 17, 18 and 19. The GC-MS analysis revealed presence of five compounds. The identified compounds with their molecular weight, retention times, peak areas and molecular formula are shown in Table 11.

Two of the identified compounds camphor, and oleuropein, have antioxidant and antimicrobial properties.

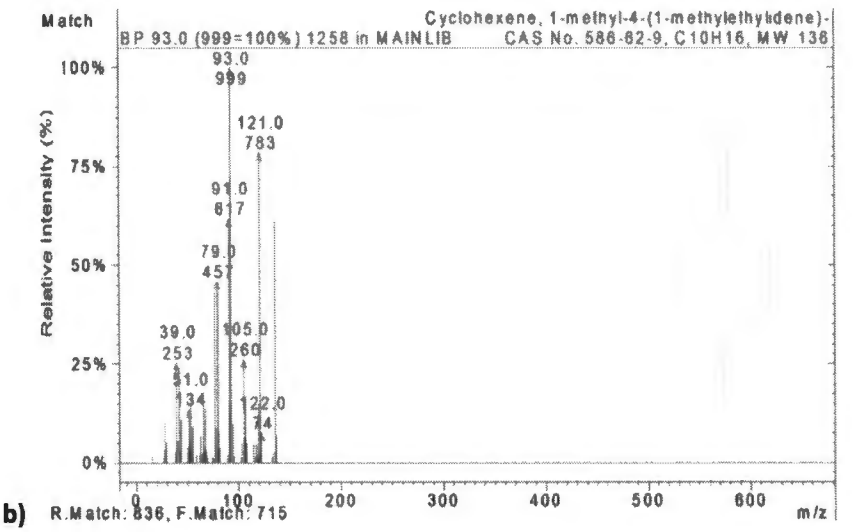
Table 11: GC-MS analysis of six crude extracts of *Olea africana*

Crude extract	Peak number	Retention time	Peak area	Molecular weight (g/mol)	Molecular formula	Identified compound
Butanol	1	3.188	58255	106.168	C_8H_{10}	Benzene1,2-dimethyl
	2	4.343	65432	540.518	$C_{25}H_{32}O_{13}$	Oleuropein
Ethanol	1	3.175	58279	136.328	$C_{10}H_{16}$	Cyclohexene,1-methyl-4-(1-methylethylidene)
	2	5.143	65412	540.518	$C_{25}H_{32}O_{13}$	Oleuropein
Ethyl acetate	1	6.159	749911	136.328	$C_{10}H_{16}$	Bicyclo[4.1.0]hept-2-ene,3,7,7-trimethyl
	2	7.243	844321	540.518	$C_{25}H_{32}O_{13}$	Oleuropein
Hexane	1	5.999	217661	152.237	$C_{10}H_{16}O$	Camphor
	2	6.338	234321	540.518	$C_{25}H_{32}O_{13}$	Oleuropein
Methanol	1	5.904	206471	152.237	$C_{10}H_{16}O$	Camphor
	2	6.345	24432	540.518	$C_{25}H_{32}O_{13}$	Oleuropein
Water	1	8.533	208711	152.237	$C_{10}H_{16}O$	Camphor
	2	9.343	22432	540.518	$C_{25}H_{32}O_{13}$	Oleuropein

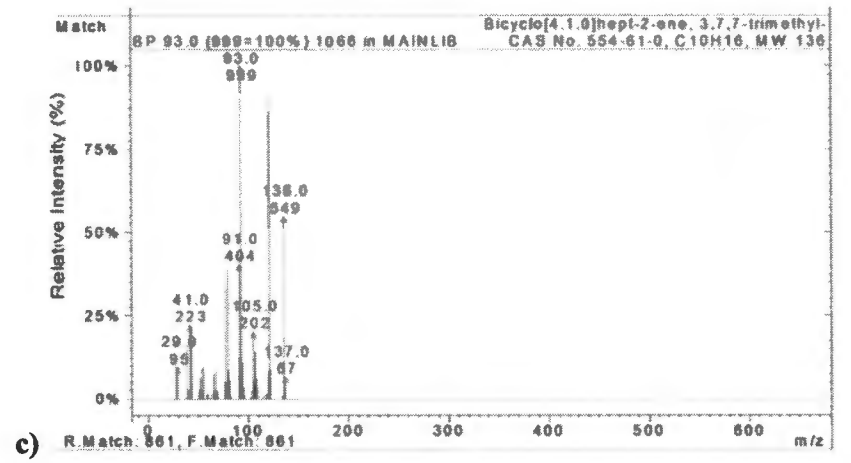
Figure 12: The mass spectrums of **a)** butanol extract, **b)** ethanol extract and **c)** ethyl acetate extract



a)

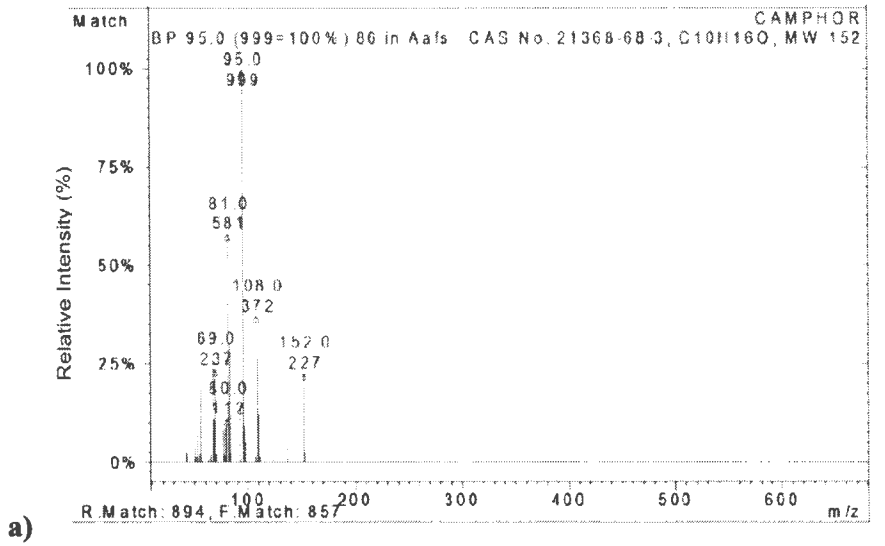


b)

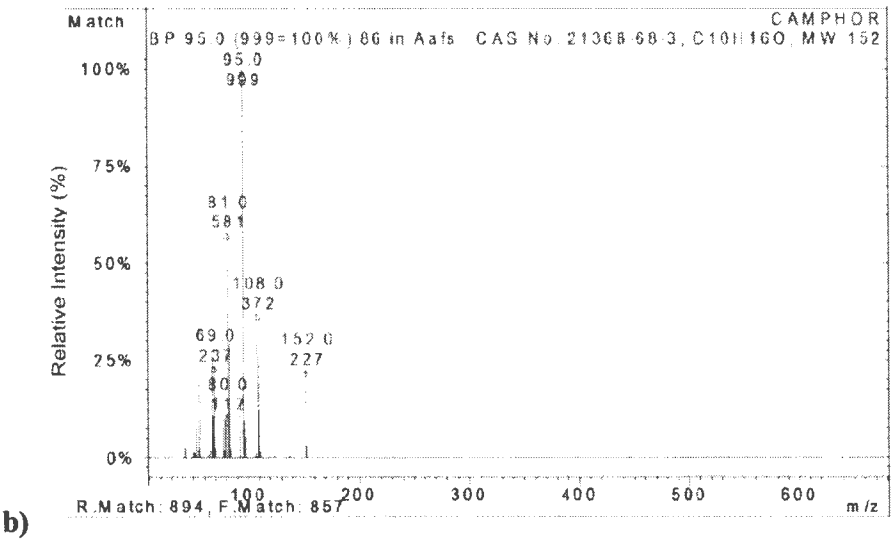


c)

Figure 13: The mass spectrums of **a)** hexane extract **b)** methanol extract and **c)** water extract



a)



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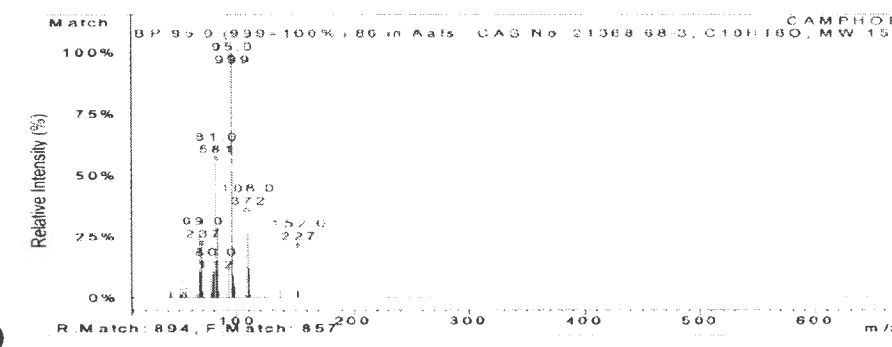
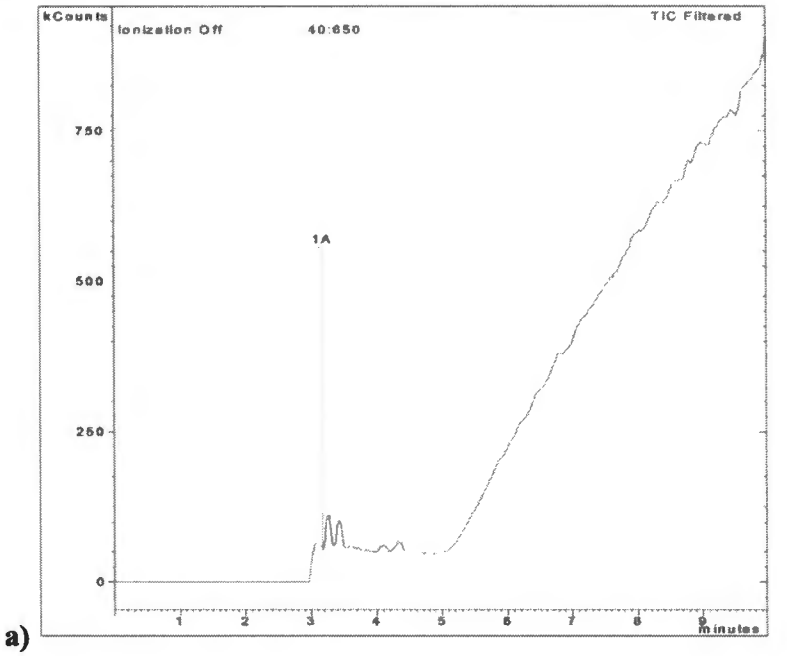
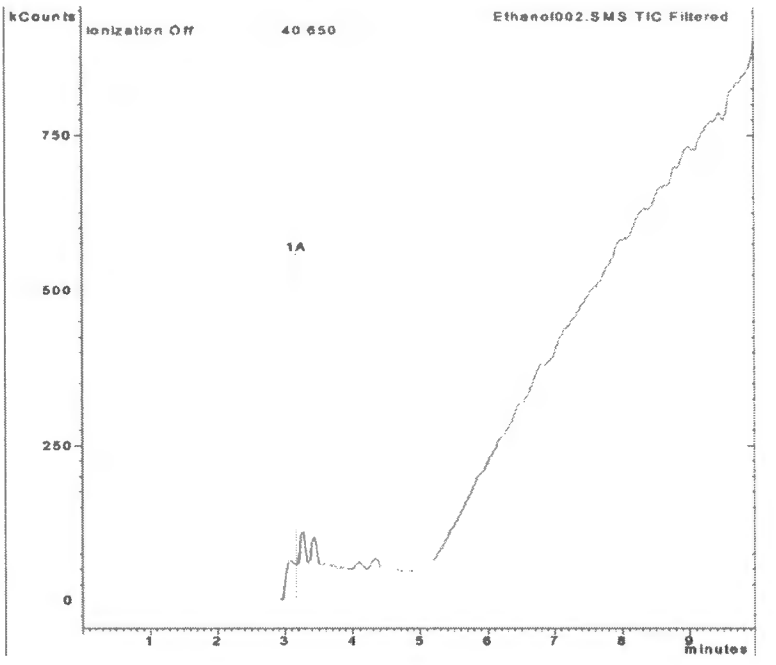


c)

Figure 14: Chromatograms of **a)** butanol extract **b)** ethanol extract

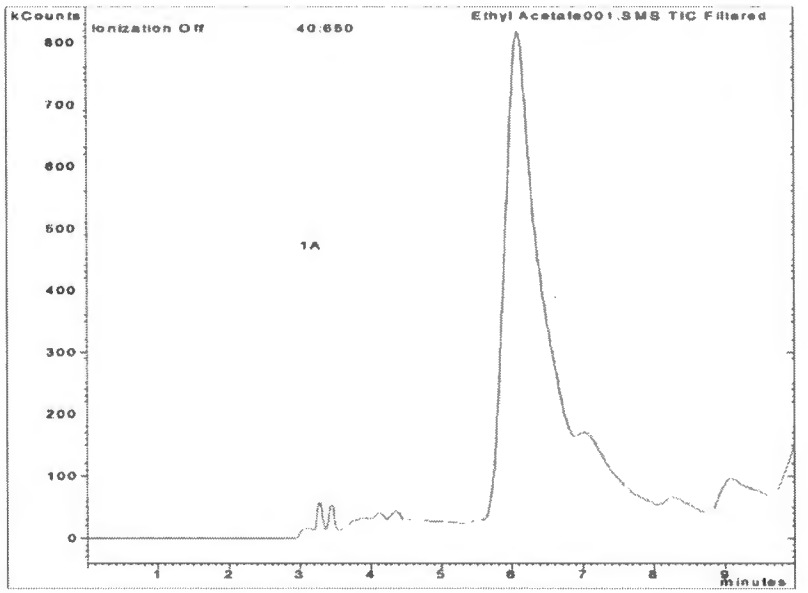


a)

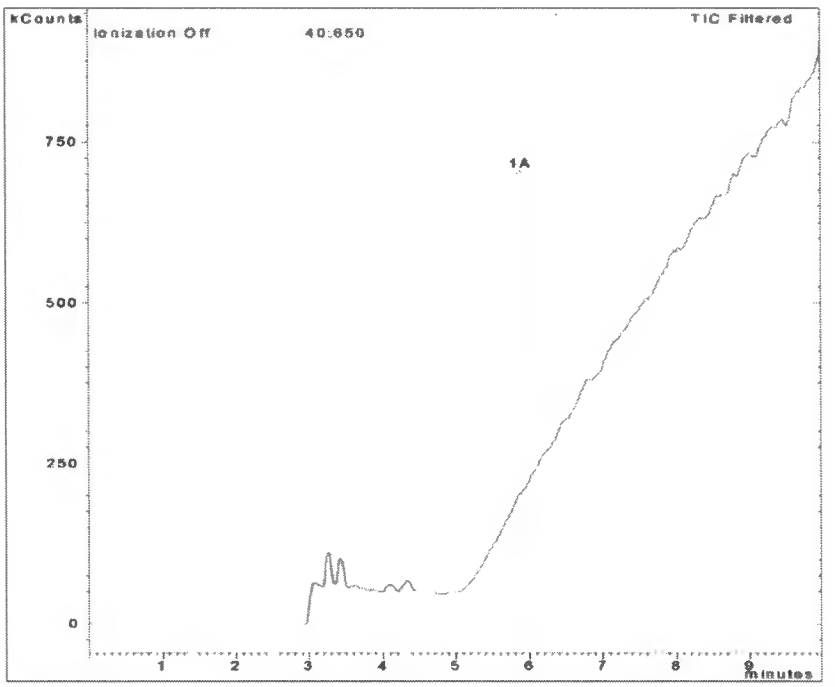


b)

Figure 15: Chromatograms of **a)** ethyl acetate extract **b)** hexane extract

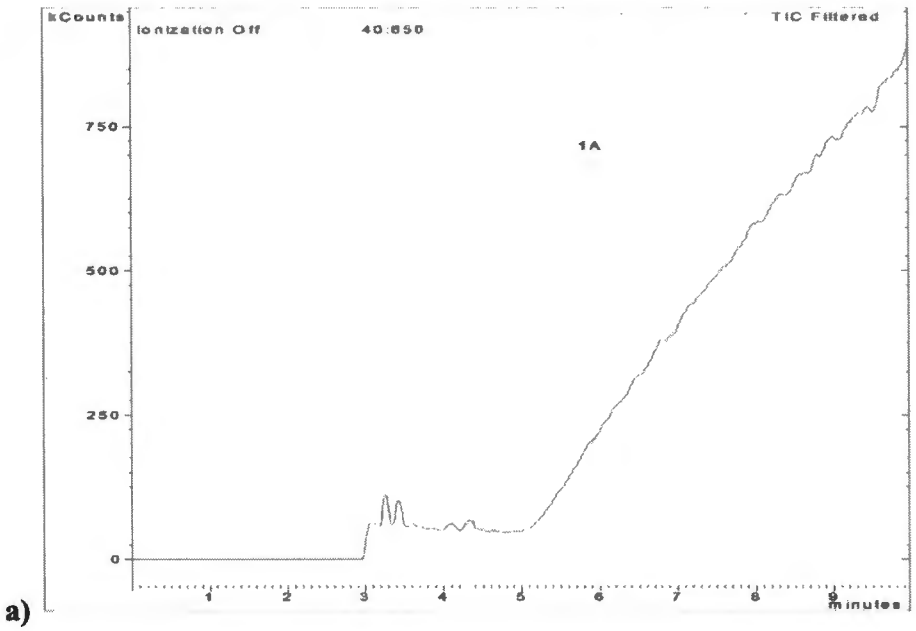


a)

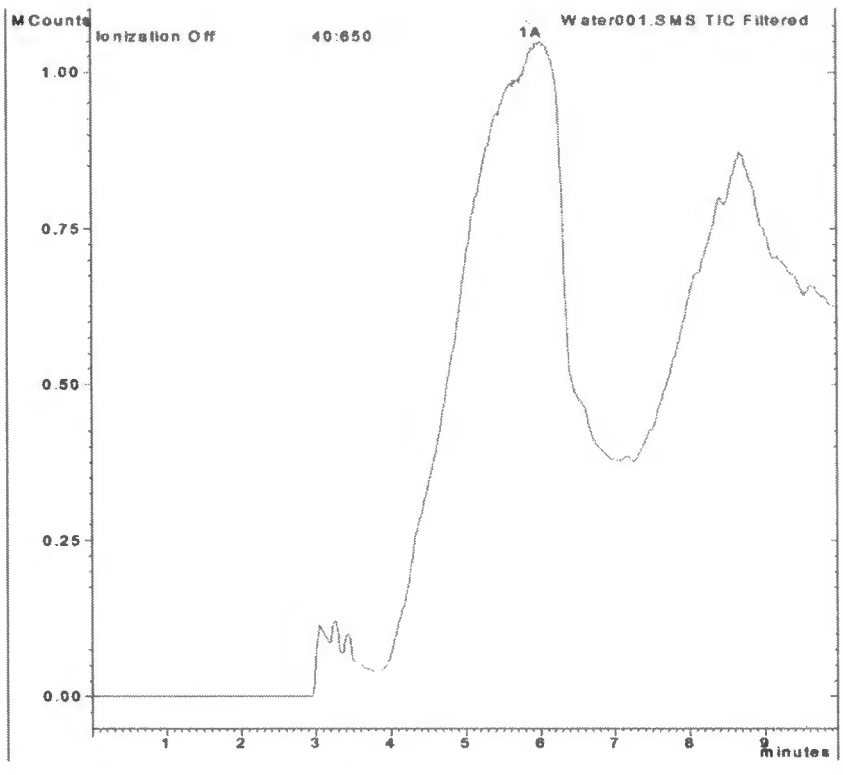


b)

Figure 16: Chromatograms of **a)** methanol extract **b)** water extract



a)



b)

CHAPTER 5

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

Pathogenic microorganisms have developed resistance to antibiotics in response to the indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. This situation and the undesirable side effects of certain antibiotics, and the emergence of previously uncommon infections, has forced scientists to look for new antimicrobial substances from various sources, such as medicinal plants. The use of higher plants and preparations made from them to treat infections is a longstanding practice in a large part of the population, especially in the developing countries, where there is dependence on traditional medicine for a variety of ailments (Ahmad *et al.*, 1998). Recently, the antimicrobial effects of various plant extracts against certain pathogens have been reported by a number of researchers (Bhalodia and Shukla, 2011) .

This research was carried out with an aim to validate *Olea africana* as an antimicrobial agent in order to harness its resources for the benefit of mankind. The results of these investigations showed that the six extracts obtained from the leaves of *Olea africana* exhibited *in vitro* antibacterial activities against selected test pathogens (Table 4). The extracts of *Olea africana* at the concentration of 20 mg/ml inhibited the growth of *Bacillus cereus* (ATCC 10876), *Enterococcus faecalis* (ATCC 29212), *Moraxella catarrhalis* (ATCC 25240), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhi* (ATCC 14208) and *Staphylococcus aureus* (ATCC 25923). This indicates that the plant contains active principles that can inhibit growth of some microorganisms. The results of the study showed that the extracts exhibited a broad spectrum of antibacterial activity. The extraction with water did not show activity against *Staphylococcus aureus* (ATCC 25923) and *Enterococcus faecalis* (ATCC 29212). Several

studies support that the olive leaves in the aqueous extracts have antimicrobial activity against pathogenic bacteria (Aliabadi *et al.*, 2012, Keskın *et al.*, 2012). Maybe, if some conditions were different, for example if the extraction time was superior, the results would have been different for these extracts. This can be attributed to the permeability of the Gram positive cell wall which is more susceptible and for that reason does not restrict the penetration of antimicrobials. The activity of the methanol extract was just slightly better than that of the water extract; this may be as a result of the active components of the plant being soluble in methanol.

Besides the different plant characteristics, extraction solvents played a major role in the concentration of plant material obtained from the medicinal plants. Previous studies have reported on the influence extraction solvents have on the concentration of plant material extracted and subsequent antimicrobial testing. A difference was observed in the amount of plant material extracted with the different extraction solvents and medicinal plants Table 3. The characteristics of extraction solvents and the specific chemical composition of medicinal plants may have influenced the amount of plant material extracted.

The results of the antifungal screening reported in (Table 5) confirmed that the *Olea africana* exhibited inhibitory effects on some of the fungal isolates. Results obtained from the present investigation revealed that the highest antifungal activity was exhibited by the ethanol extract and the least by water extracts. The basis of the varying degree of sensitivity of the different fungi may be due to the intrinsic tolerance of the various fungi and the nature and combinations of phytochemicals present in the crude extracts.

The results of minimum inhibitory concentration (MIC) determination reported in Table 6 confirmed that the *Olea africana* exhibited inhibitory effects on some of the bacterial isolates. The MIC of the methanol extract against bacterial strains was found to be 125 µg/ml for *B. cereus* (ATCC 10878), *S. aureus* (ATCC 25923) and *E. faecalis* (ATCC 29212), and 250

μg/ml for *S. typhii*, compared to that of the aqueous extract which was 250 μg/ml for all four susceptible bacteria. *Olea africana* extracts presented moderate activity against the susceptible bacteria against the control. Further purification of the active ingredients in these extracts would provide possible identification of the mechanisms of action and possible lead compounds for new development. It would also validate the use of *Olea africana* in the treatment of bacterial infection.

The antioxidant activity of *Olea africana* leaves was investigated by the DPPH method. During the DPPH free radical reaction, the degree of discolouration (decrease in absorbance) of the DPPH solution indicates the scavenging potentials of the sample antioxidant. The magnitude of the antioxidant power depends on the extraction solvent used. Ethanol and methanol were the solvents that yielded the highest antioxidant activity as assessed by the DPPH method (Table 7), which is probably due to their ability to extract phenolic compounds. The crude extracts of *Olea africana* contain plant secondary metabolites such as alkaloids, saponins, tannins, flavones, flavonoids, terpenoids and steroids. All these bioactive compounds have the ability to discolour DPPH solution by their hydrogen donating ability. The results of antioxidant activity of the extracts expressed in percentage DPPH activities are presented in this study.

The electron-donating properties of flavonoids and many phenolic acids have been repeatedly emphasized as the basis of their antioxidant action (Rice-Evans, 2001). The scavenging effects of different amounts of crude plant extracts and tincture extracts on the free radical DPPH increased with increasing amounts of extract. Antioxidants are believed to intercept the free radical chain of oxidation and to donate hydrogen from the phenolic hydroxyl groups, thereby forming stable radicals which do not initiate nor propagate further oxidation of lipids. As DPPH is known to abstract the labile hydrogen atom of chemical compounds(Ratty *et al.*, 1988), the antioxidant that can scavenge the DPPH radical is expected to depress lipid peroxidation

(Aniya *et al.*, 1999). In this study, DPPH scavenging activity was found in all the extracts tested and it was confirmed that these extracts would also inhibit lipid peroxidation. Thus, the action of the plant extracts on DPPH would suggest that they can act as free radical inhibitors and primary antioxidants. The individual phenolic compounds in olive leaf extract showed strong in vitro activities, the antioxidant and antimicrobial activities of the combined phenolics showed similar or better effects than the individual phenolics. Therefore, these results suggest that olive leaf extract has great potential as a functional food ingredient, particularly as a source of phenolic compounds. In addition, if olive leaf is to be utilized as a functional food material, a more cost-effective and safer extraction method may be to prepare crude olive leaf extract.

Lipid peroxide assays are often used to establish the antioxidant potential of plant extracts and individual compounds. Although it is not possible to elucidate exact mechanisms of action, the observed antioxidant activity on lipid peroxidation is the result of all oxidation and reduction processes which might occur *in vivo* (van Acker *et al.*, 1998). Comparison of results between authors is generally difficult, due to differences in method and experimental conditions. As Ethanol significantly showed higher lipid peroxidation inhibition compared with hexane extract in egg yolk (Table 8), while no significant difference was observed when compared with methanol, butanol and ethyl acetate fractions.

The phytochemical analysis of *Olea africana* extract showed that it contains phytochemicals that are of medicinal importance such as, flavonoids, tannins, and alkaloids (Table 9). These classes of compounds are known to have curative activity against several pathogens and therefore could validate the traditional use of *Olea. africana* for the treatment of various illnesses (Usman and Osuji, 2008, Hassan *et al.*, 2004). Flavonoids increase values of therapeutic activities and are known to be biologically active, aiding the antimicrobial activities of the extracts. These biological functions of flavonoids include protection against allergies, inflammation, platelets aggregation, microbes, ulcer, viruses and tumor (Okwu, 2004). Apart

from its antimicrobial property, flavonoids are anti-inflammatory, antiangionic, analgesic, anti-allergic, cytostatic and antioxidant (Hodek, 2002). Flavonoids represent the most common and widely distributed groups of plant phytochemicals (Singh, 2012). Phytochemical compounds have been extensively used in disinfection's and remain a standard with which bactericides are compared (Okwu, 2001). Plants have been extensively used to heal diseases, including infectious ones, with the presence of phytochemical compounds like flavonoids being the active ingredient of their antibacterial action against pathogenic bacteria (Ayo, 2010). Doss and co-workers compared (Doss *et al.*, 2009), the sensitivity of some pathogenic bacteria to the tannins of the leaves of *Solanum trilobatum* to that of the antibiotic Streptomycin. The results revealed out that tannins possess the highest relative antibacterial activity against the bacteria *S. typhii*, *P. aeruginosa* and *P. vulgaris* and active against the remaining bacteria, when compared to Streptomycin. Several plants which are rich in tannins have been shown to possess antimicrobial activities against a number of microorganisms. The antimicrobial activity of *Olea. africana* extracts may therefore be due to the combination of alkaloids, tannins, and flavonoids present in the various extracts (Table 9).

TLC profiling of all six extracts revealed the presence of several phytochemicals. Various phytochemicals gives different R_f values in different solvent system. This variation in R_f values of the phytochemicals provides a very important clue in understanding of their polarity and also helps in selection of appropriate solvent system for separation of pure compounds by column chromatography.

GC and the interpretation of the MS spectra were carried out using gas chromatography machine. The molecular weight of the unknown compound was obtained from the mass spectrum. The leaves extract upon quantification by colorimetric methods were found to be rich in phenolic compounds.

5.2 CONCLUSION AND RECOMMENDATION

The antimicrobial activity exhibited by *Olea africana* extracts makes it a potential source for the production of drugs to combat by infections pathogenic organisms. It has been observed that many of these pathogens are now becoming resistant to the available antimicrobial compounds, so drugs formulated from plants like this one, will serve better in health care. Thus, the crude extract from *Olea africana* used in this study appears to be a potential source of antibacterial compounds that could be relevant in the treatment of infections caused by the susceptible test pathogens in this study. Based on the results, it can also be concluded that *Olea africana* leaves could be used as a natural source of antioxidants and its regular consumption in the diet could provide health benefits to humans by the protection against oxidative stress. Further detailed in vitro and in vivo correlation studies along with isolation of active constituents are needed to unravel novel treatment strategies for free radical induced diseases.

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APPENDICES

Appendix 1: Determination of DPPH. Radical Scavenging Activity of Olive Leaf Extract

Reagent

1 ml of DPPH (2, 2-diphenyl-1-picryhydrazyl radical) was completely dissolved in 500 ml of absolute methanol, stored the DPPH radical solution at 27°C in the dark.

Calculation of Result

$$\% \, Inhibition = \frac{A_{control} - A_{extract}}{A_{control}} \times 100$$

The percentage of DPPH radical scavenging activity was plotted against the plant extract concentration (µg/ ml) to determine the amount of extract necessary to decrease the DPPH radical concentration by 50% (called IC_{50}).

Table 1: The DPPH scavenging activity of butanol extract at different concentrations

Extract	Concentration ug/ml	Absorbance at 517nm	% Radical Scavenging Activity
	Control	1.215	0
Butanol	12.5	0.986	18.85
	25	0.852	29.88
	50	0.755	37.86
	100	0.453	62.96
	200	0.196	83.87

% DPPH Radical scavenging activity = (1.215-0.986)/1.215x100%=18.85%

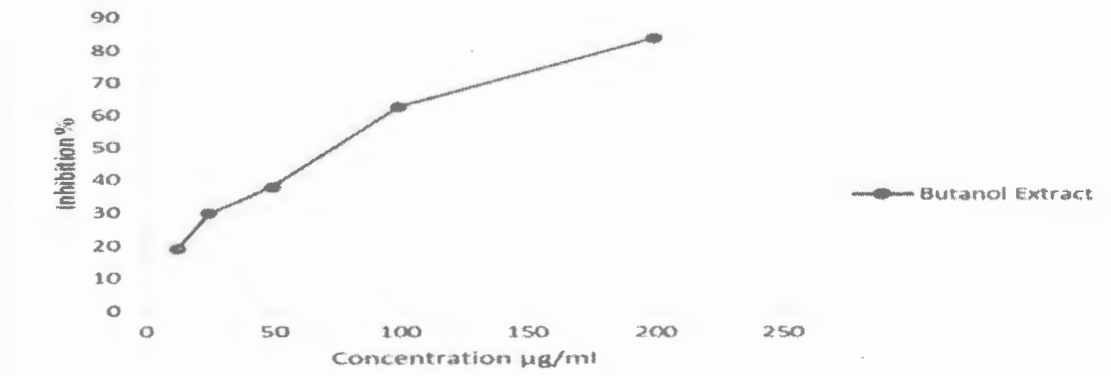

Figure 1: DPPH scavenging activity of butanol extract from leaves of *Olea africana*.

The IC_{50}, which is concentration required to quench 50% of the DPPH radical was computed to be 0.34ug/ml for the butanol extract, using the equations of the line in Figure 2 obtained by linear regression. The smaller the IC50, means the higher the antioxidant activity.

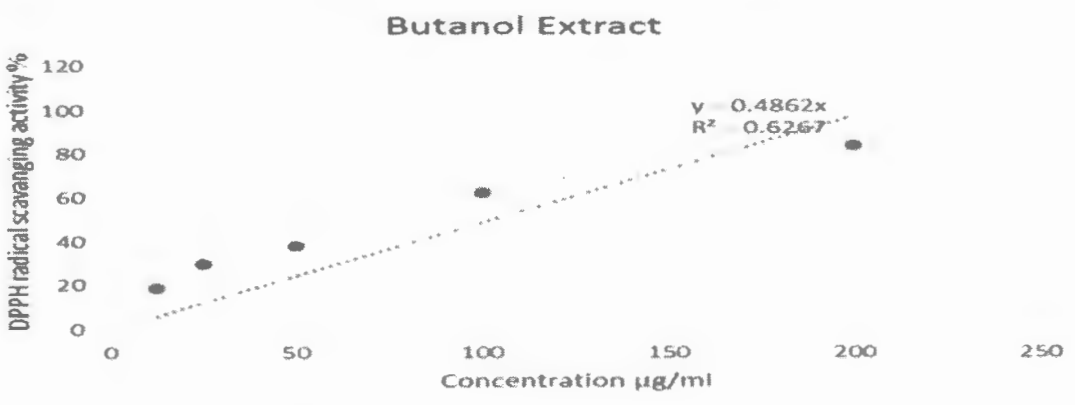

Figure 2: Antioxidant activity of butanol extract

Table 2: The DPPH scavenging activity of ethanol extract at different concentrations

Extract	Concentration ug/ml	Absorbance at 517nm	% Radical Scavenging Activity
	Control	1.215	0
Ethanol	12.5	0.973	19.92
	25	0.823	32.26
	50	0.721	40.66
	100	0.432	64.44
	200	0.188	84.53

% DPPH Radical scavenging activity = (1.215-0.973)/1.215x100%=19.92%

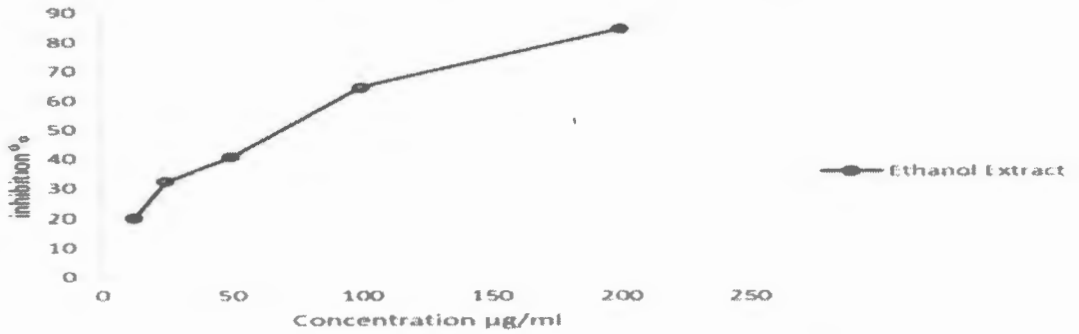

Figure 3: DPPH scavenging activity of ethanol extract from leaves of *Olea africana*.

The IC_{50}, which is concentration required to quench 50% of the DPPH radical was computed to be 0.33ug/ml for the ethanol extract, using the equations of the line in Figure 4 obtained by linear regression. The smaller the IC50, means the higher the antioxidant activity.

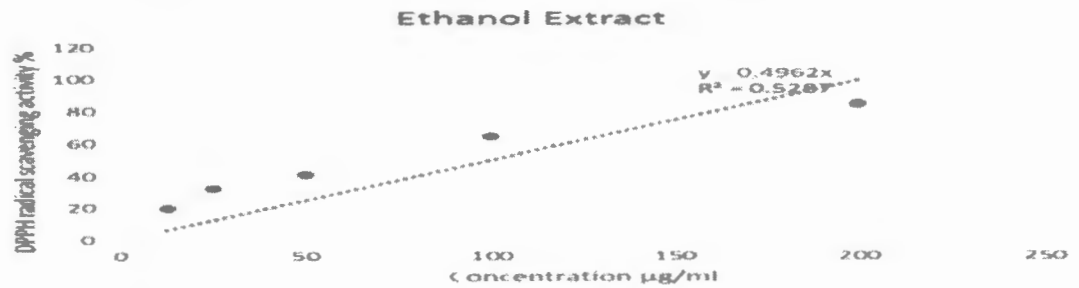

Figure 4: Antioxidant activity of ethanol extract

Table 3: The DPPH scavenging activity of ethyl acetate extract at different concentrations

Extract	Concentration ug/ml	Absorbance at 517nm	% Radical Scavenging Activity
	Control	1.215	0
Ethyl acetate	12.5	0.987	18.87
	25	0.835	31.28
	50	0.765	37.04
	100	0.477	60.74
	200	0.293	75.88

% DPPH Radical scavenging activity = (1.215-0.987)/1.215x100%=18.87%

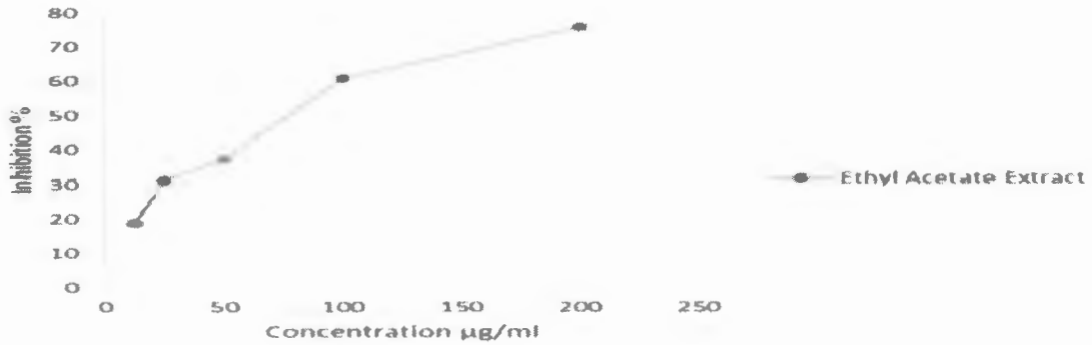

Figure 5: DPPH scavenging activity of ethyl acetate extract from leaves of *Olea africana*.

The IC_{50}, which is concentration required to quench 50% of the DPPH radical was computed to be 0.29ug/ml for the ethyl acetate extract, using the equations of the line in Figure 6 obtained by linear regression. The smaller the IC50, means the higher the antioxidant activity.

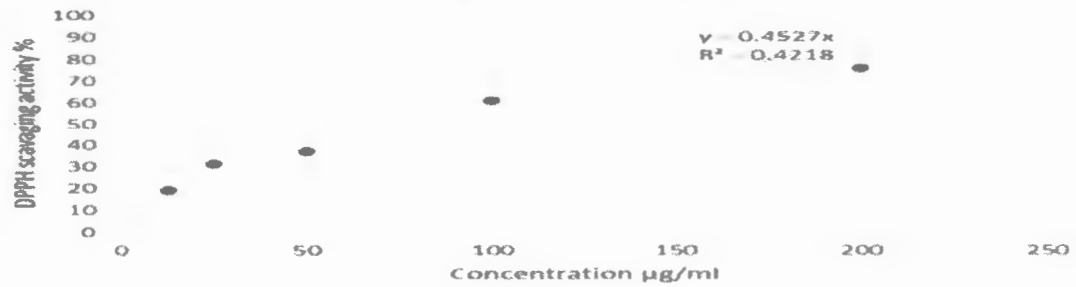

Figure 6: Antioxidant activity of ethyl acetate extract

Table 4: The DPPH scavenging activity of hexane extract at different concentrations

Extract	Concentration ug/ml	Absorbance at 517nm	% Radical Scavenging Activity
	Control	1.215	0
Hexane	12.5	0.899	26.01
	25	0.789	35.06
	50	0.699	42.47
	100	0.593	51.19
	200	0.283	76.71

% DPPH Radical scavenging activity = (1.215-0.899)/1.215x100%=26.01%

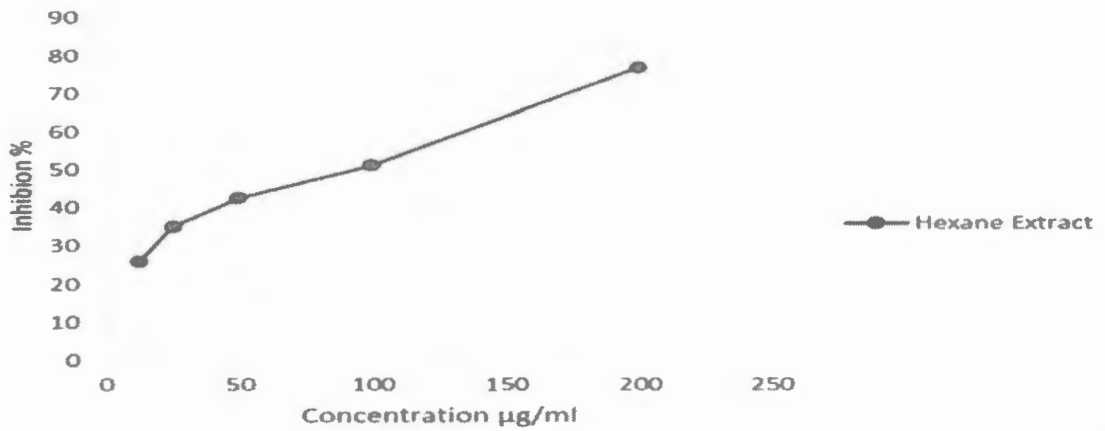

Figure 7: DPPH scavenging activity of hexane extract from leaves of *Olea africana*.

The IC_{50}, which is concentration required to quench 50% of the DPPH radical was computed to be 0.25ug/ml for the hexane extract, using the equations of the line in Figure 8 obtained by linear regression. The smaller the IC50, means the higher the antioxidant activity.

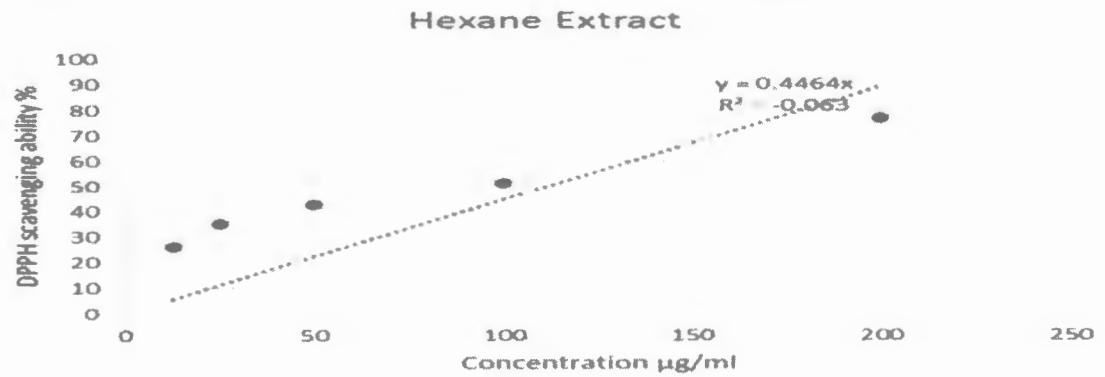

Figure 8: Antioxidant activity of hexane extract

Table 5: The DPPH scavenging activity of methanol extract at different concentrations

Extract	Concentration ug/ml	Absorbance at 517nm	% Radical Scavenging Activity
	Control	1.215	0
Methanol	12.5	0.999	17.78
	25	0.873	28.15
	50	0.788	35.14
	100	0.465	61.73
	200	0.211	82.63

% DPPH Radical scavenging activity = (1.215-0.999)/1.215x100%=17.78%

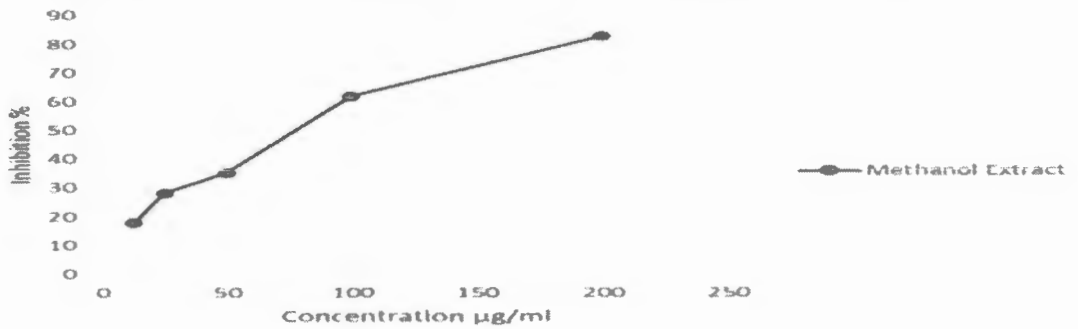

Figure 9: DPPH scavenging activity of methanol extract from leaves of *Olea africana*.

The IC_{50}, which is concentration required to quench 50% of the DPPH radical was computed to be 0.34ug/ml for the methanol extract, using the equations of the line in Figure 10 obtained by linear regression. The smaller the IC50, means the higher the antioxidant activity.

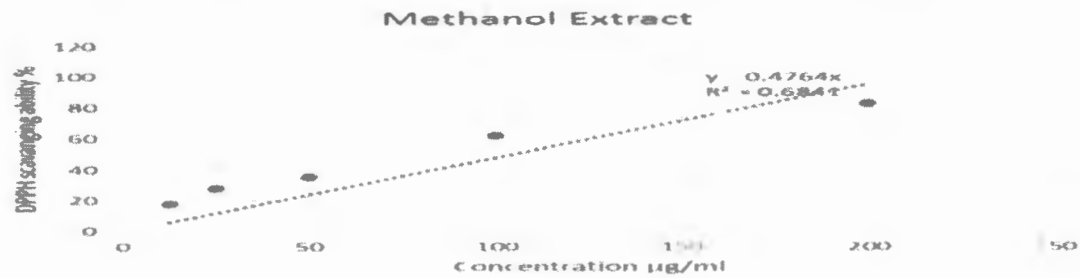

Figure 10: Antioxidant activity of methanol extract

Table 6: The DPPH scavenging activity of water extract at different concentrations

Extract	Concentration ug/ml	Absorbance at 517nm	% Radical Scavenging Activity
	Control	1.215	0
Water	12.5	0.971	20.08
	25	0.872	28.23
	50	0.777	36.05
	100	0.637	47.57
	200	0.293	75.88

% DPPH Radical scavenging activity = (1.215-0.971)/1.215x100%=20.08%

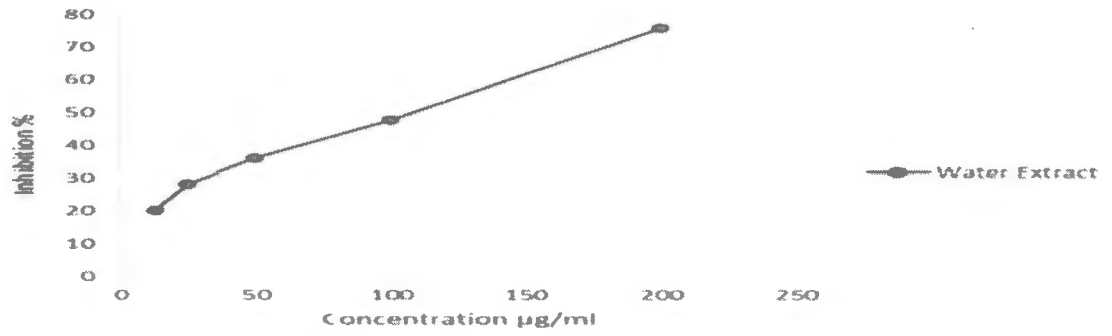

Figure 11: DPPH scavenging activity of water extract from leaves of *Olea africana*.

The IC_{50}, which is concentration required to quench 50% of the DPPH radical was computed to be 0.34ug/ml for the methanol extract, using the equations of the line in Figure 12 obtained by linear regression. The smaller the IC50, means the higher the antioxidant activity.

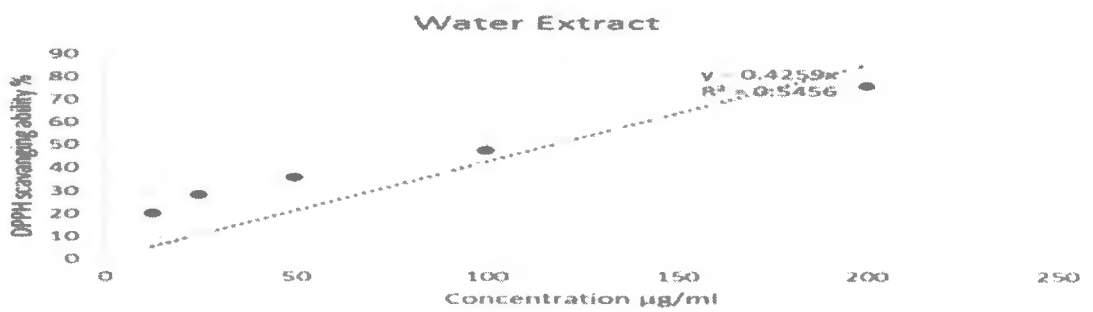

Figure 12: Antioxidant activity of water extract