

**SCREENING, ISOLATION AND CHARACTERISATION OF ANTIMICROBIAL AND  
ANTIOXIDANT COMPOUNDS FROM *OLEA EUROPAEA* SUBSPECIES  
*AFRICANA* LEAVES**

By

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DISSERTATION

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## DECLARATION

I **Kholofelo Sarah Mamabolo**, declare that the dissertation titled “**Screening, isolation and characterisation of antimicrobial and antioxidant compounds from *Olea europaea* subspecies *africana* leaves**”, submitted to the University of Limpopo, for the degree of Master of Science in Biochemistry has not been previously submitted by me for a degree at this or any other university. This is my work in design and execution, and all the material contained herein has been duly acknowledged.

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Signature

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Date

## **DEDICATION**

I dedicate this work to my son Kgoshi Hlomphe Mamabolo, my mother Makgoshi Herminah Mamabolo and my brothers, Mamadimo Joseph and Mathata Alfred Mamabolo.

## CONFERENCE PRESENTATIONS AND PUBLICATIONS

### Oral paper

1. **Kholofelo S. Mamabolo, Peter Masoko.** “Preliminary screening of antimicrobial and antioxidant bioactive compounds from *Olea europaea* subspecies *africana*”. 20<sup>th</sup> annual Indigenous Plant Use Forum (IPUF) conference held at Batter Boys Village, Pretoria, Gauteng, South Africa, July, 2017.
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### Manuscripts

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## LIST OF ABBREVIATIONS

|                  |                                      |
|------------------|--------------------------------------|
| $\cdot\text{OH}$ | Hydroxyl radical                     |
| $^{13}\text{C}$  | Carbon-13                            |
| $^1\text{H}$     | Hydrogen-1                           |
| $^1\text{O}_2$   | Singlet oxygen                       |
| A                | Acetone                              |
| ATCC             | American type culture collection     |
| B                | Butanol                              |
| BEA              | Benzene/Ethanol/Ammonia hydroxide    |
| C                | Chloroform                           |
| CAT              | Catalase                             |
| CEF              | Chloroform/Ethyl acetate/Formic acid |
| COSY             | Correlated Spectroscopy              |
| D                | Dichloromethane                      |
| Da               | Dalton                               |
| DCF              | 2, 7-dichlorofluorescein             |
| DMEM             | Dubleco's modified essential medium  |
| DMSO             | Dimethyl sulfoxide                   |
| DNA              | Deoxyribonucleic acid                |
| DPPH             | 2, 2-diphenyl-1-picrylhydrazyl       |
| E                | Ethanol                              |
| EA               | Ethyl Acetate                        |
| EMW              | Ethyl acetate/methanol/water         |
| ESBL             | Extended-spectrum $\beta$ -lactamase |

|                                |  |
|--------------------------------|--|
| FRAP                           | Ferric reducing antioxidant potential              |
| GAE/g                          | Gallic acid equivalence per gram                   |
| H                              | Hexane   |
| H <sub>2</sub> O <sub>2</sub>  | Hydrogen peroxide                                  |
| H <sub>2</sub> SO <sub>4</sub> | Sulphuric Acid                                     |
| HIV                            | Human immunodeficiency virus                       |
| HMQC                           | Heteronuclear Multiple Quantum Correlation         |
| HNO <sub>2</sub>               | Nitrous acid                                       |
| HPLC                           | High Performance Liquid Chromatography             |
| HSV                            | Herpes simplex virus                               |
| IC <sub>50</sub>               | Half maximal inhibitory concentration              |
| INT                            | ρ-iodonitrotetrazolium violet                      |
| IR                             | Infrared   |
| IUPAC                          | International Union of Pure and Applied Chemistry  |
| KHz                            | Kilohertz  |
| LC/MS                          | Liquid Chromatography/ Mass spectroscopy           |
| LC <sub>50</sub>               | Lethal concentration for 50% of the cells          |
| LC-NMR                         | Liquid Chromatography-Nuclear Magnetic Resonance   |
| M                              | Methanol   |
| MEM                            | Minimum Essential Medium                           |
| MFP                            | Membrane Fusion Protein                            |
| MIC                            | Minimum inhibitory concentration                   |
| MRSA                           | Methicillin-resistant <i>Staphylococcus aureus</i> |
| MS                             | Mass spectrometry                                  |

|                              |  |
|------------------------------|--|
| MTT                          | 3-(4, 5-dimethylthiazol-2-yl)-2, 4-diphenyltetrazolium bromide |
| NaCl                         | Sodium chloride  |
| NMR                          | Nuclear magnetic resonance spectroscopy                        |
| NO <sup>•</sup>              | Nitric oxide radical   |
| NO <sub>2</sub> <sup>•</sup> | Nitrogen dioxide radical                                       |
| NOESY                        | Nuclear Overhauser Enhancement Spectroscopy                    |
| O <sup>2-</sup>              | Superoxide radicals  |
| OMF                          | Outer Membrane Factor  |
| ORAC                         | Oxygen Radical Absorbance Capacity                             |
| PBP                          | Penicillin Binding Proteins                                    |
| PBS                          | Phosphate Buffered Saline                                      |
| QE/g                         | Quercetin equivalence per gram                                 |
| R <sub>f</sub> value         | Retardation factor Value                                       |
| RNA                          | Ribonucleic acid   |
| RND                          | Resistance Nodulation Division                                 |
| RNS                          | Reactive Nitrogen Species                                      |
| ROS                          | Reactive oxygen species  |
| TEAC                         | Trolox equivalent antioxidant capacity                         |
| TLC                          | Thin layer chromatography                                      |
| TNF-α                        | Tumor necrosis factor-alpha                                    |
| USA                          | United States of America                                       |
| UTI                          | Urinary Tract Infection  |
| UV                           | Ultraviolet light  |
| W                            | Water  |

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## ABSTRACT

Medicinal plants have been used as a key source for medication and they remain to provide new therapeutic remedies to date. Extracts of *Olea europaea* subspecies *africana* leaves are used extensively in South Africa to treat various diseases traditionally. The diseases have been noted to be associated with free radicals, bacterial infections, and inflammation. However, there is little information about the antioxidant, antibacterial, and anti-inflammatory activities of the leaves of this plant in literature and the cytotoxicity of the leaf extracts is still a concern. The information about the isolated compounds is also minimal hence this study was aimed at filling in those gaps in relation to the traditional use of the leaves in southern Africa and subsequently isolating and identifying the active compounds using bioassay-guided fractionation.

Preliminary screening of the crude extracts for antioxidant, antibacterial and anti-inflammatory activities indicated that the extracts possessed all biological activities. The presence of major phytochemicals in the crude extracts was determined through the use of standard chemical methods and TLC analysis. The colorimetric methods (Folin-Ciocalteu and Aluminum chloride) were used for quantification purposes. TLC-DPPH assay was used to screen antioxidant activities of the crude extracts. The observed activity was quantified using the spectrophotometric method of DPPH and reducing power. The antibacterial properties of the leaf extracts were determined by direct bioautography and the serial broth microdilution assay using *E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus* as test bacteria. Screening of the acetone crude extract for anti-inflammatory activities was done using the LPS-stimulated RAW 264.7, cells where the inhibition of ROS generation was studied. MTT assay was used to determine the cytotoxicity effects of the leaves. Isolation of bioactive compounds started with serial exhaustive extraction, followed by column chromatography packed with silica gel. NMR analysis was conducted to identify the isolated compound.

The results revealed the presence of tannins, terpenoids, steroids and flavonoids with the total phenolic ( $99.67 \pm 2.52$  mg of GAE/g) and tannin content ( $114.33 \pm 9.02$  mg of GAE/g) found in high amounts. All crude extracts exhibited antioxidant activities and the antioxidant activity quantified via the DPPH assay demonstrated to

have EC<sub>50</sub> value of 1.05 ± 0.0071 mg/mL. The reducing capacity was found to be dose-dependent and great significance was seen at concentration 0.5 mg/mL to 1 mg/mL that was about 2/3 of that of L-ascorbic acid (standard) at a similar concentration. Screening of the crude extracts for antibacterial activity revealed that all crude extracts except n-hexane and water extracts, inhibited the growth of the tested bacteria on the previously developed TLC plates. The activity was seen as clear zones on the bioautograms. Serial broth microdilution assay indicated that dichloromethane, acetone and ethanol had average MIC values of 0.30, 0.32 and 0.35 mg/mL against all tested bacteria, respectively.

Good anti-inflammatory activity of the crude extract was demonstrated at the highest concentration of 0.90 mg/mL. MTT assay indicated that the crude extract had no adverse cytotoxic effects. This was demonstrated by the LC<sub>50</sub> values greater than 20 µg/mL and considered non-cytotoxic according to the National Cancer Institute (NCI). Isolation following the bioassay-guided-fractionation resulted in the selection of acetone extract to isolate the bioactive compounds from as it demonstrated good antioxidant and antibacterial activities. Fractionation of the compound by column chromatography yielded three combinations (pools) of fractions and of the three from which only pool 1 was considered for further fractionation. NMR spectra information identified the isolated compound as a mixture of ursolic acid (minor) and oleanolic acid (major). This compound had antibacterial and anti-inflammatory activities and no cytotoxic effects. The leaves of *Olea europaea* subspecies *africana* have been proven to possess antioxidant, antibacterial and anti-inflammatory properties. Evaluation of the biological activities of the crude extracts was to validate the use of the leaves traditionally to treat free radical and bacterial-related diseases and potential drug that are safe and has less side effects may be produced from the leaves.

## CHAPTER 1: GENERAL INTRODUCTION

Over the years, humans have developed a broad knowledge of useful plants through continuous contact with the environment. This intrigued the researchers to conduct scientific research in vegetables, fruits, medicinal plants and spices. The interest was based on the presence of phytochemicals with antioxidant and antimicrobial properties in plants. The use of medicinal plants, both in the simplest forms and in the advanced manufacturing industry is motivated by the plant's properties to generate beneficial reactions to the human body (Nitu *et al.*, 2017).

Medicinal plants are indispensable for human well-being and provide all or significant number of the remedies required in the health care (de Lima *et al.*, 2017). They are widely distributed throughout the world and have the ability to combat infections and diseases such as eye infection, sore throat, urinary tract infections, kidney problems and backaches or headaches (Somova *et al.*, 2003). They have also demonstrated their contribution to the treatment of symptoms related to HIV/AIDS, malaria, diabetes, sickle-cell anaemia, mental disorders and microbial infections. Medicinal uses of plants range from the administration of the roots, barks, stems, leaves and seeds to the use of extracts and decoctions from the plants (Borokini and Omotayo, 2012).

Various parts of the plant contain different bioactive compounds, these include saponins, tannins, essential oils, flavonoids, alkaloids and other chemical compounds found as secondary metabolites (Masoko and Makgapeetja, 2015). These secondary metabolites are produced from primary metabolism where basic molecules such as carbohydrates, amino acids, nucleic acids, proteins and lipids are produced through subsequent chemical reactions. They are defined as substances that are produced and used by the plants for protection in relation to abiotic stress. The stress can be a result of changes in temperature, water status, light levels, UV exposure and mineral nutrients (Borokini and Omotayo, 2012).

The secondary metabolites produced by plants are largely regarded as a potential source of novel antibiotics, insecticides and herbicides (Maaroufi *et al.*, 2017). Furthermore, they offer health benefits such as antioxidant, anti-aging, anti-

atherosclerotic, antimicrobial and anti-inflammatory activities (Mulaudzi *et al.*, 2012). It has also been reported that using plant derived medicines is much safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment (Borokini and Omotayo, 2012).

Natural products, either as pure compounds or as standardised plant extracts have been reported to have multi-antimicrobial properties which provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity. Therefore, researchers are increasingly turning their attention towards medicinal plants, looking for new leads to develop better drugs against microbial infections. In the last three decades, scientists have achieved enormous new antibiotics due to advances in technology and science to combat different types of infectious diseases caused by pathogens. However, due to increased and indiscriminate use of antibiotics for treatment of humans, microorganisms have developed resistance against antibiotics and multidrug, leading to an emergence of new bacterial strains “superbugs”, which are multidrug-resistant (Rahman *et al.*, 2016).

In general, the bacteria that cause infectious diseases have the genetic ability to transmit and acquire resistance to drugs that are used as therapeutic agents. These organisms are the major causes of morbidity and mortality, functional disability, extended hospitalisation, greater use of antibiotics and economic burden among patients. Due to the increase of antibiotic resistance by microorganisms, there is an urgent need to discover new antimicrobial agents with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases (Mulu *et al.*, 2012; Rahman *et al.*, 2016).

Besides the pandemic caused by the antimicrobial resistant microorganisms, there is also an alarming issue involving oxidative stress, which has become increasingly recognised to the point that it is now considered a component of almost every disease (Santo *et al.*, 2016). The imbalance between the formation of reactive oxygen species (ROS) or free radicals and the antioxidant defence mechanism causes cellular oxidative stress (Cabello-Verrugio *et al.*, 2016). Free radicals may be defined as molecules or molecular fragments, containing one or more unpaired



electrons in the outermost electron orbital and are capable of independent existence. They are dangerous substances produced by the body along with toxins and wastes, formed during normal metabolic processes, and can be generated by chemicals in our external environment (Sen *et al.*, 2010).

Oxidative stress has consequences that can be very subtle to very serious, depending on the balance between reactive species generation and the antioxidant defence. At low to moderate concentrations, free radicals regulate cell-signalling cascades and damage all biomolecules at high concentrations by inducing DNA damage, lipid peroxidation, and protein modification, causing disruption of signal transduction, mutations and eventually cell death. Oxidative damages due to free radicals have been implicated in the pathogenesis of a number of conditions, such as atherosclerosis, aging, ischemic heart disease, cancer, and Alzheimer's disease (Biswas, 2016; Santo *et al.*, 2016).

Antioxidants play an important role as health protecting factors. They help reduce the number of free radicals that form in the body, lower the energy levels of existing free radicals, and stop oxidation chain reactions to lower the amount of damage caused by free radicals. They are defined as any substance that when present at low concentrations compared to those of an oxidisable substrate delays or prevents oxidation of that substrate. Scientific evidence suggests that antioxidants derived from medicinal plants reduce the risk of developing chronic diseases. Therefore, an increased intake of antioxidants can prevent the development of diseases and therefore lower the health problems. Plant sourced antioxidants like vitamin C, vitamin E, carotenes, phenolic acids etc. have been recognised as having the potential to reduce disease risk (Halliwell, 1996; Tailor and Goyal, 2014; Rahman *et al.*, 2016). This suggests that natural products, mainly obtained from dietary sources provide a large number of antioxidants, which are capable to terminate the free radical chain reactions (Hashmi *et al.*, 2015).

It has been demonstrated by recent researchers that antioxidants from plant origin with free-radical scavenging properties, could have great importance as therapeutic agents in several diseases caused by oxidative stress. Antioxidants of plant origin are preferred over synthetic ones because, synthetic antioxidants have shown toxic

and/or mutagenic effects, which have stimulated the interest of many investigators to search for natural antioxidants (Sen *et al.*, 2010).

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## CHAPTER 2: LITERATURE REVIEW

### 2.1. Background of the study

From time memorable to humans, plants have been a source of medicinal agents used to treat and prevent various ailments. A large number of drugs have been produced from isolated phytochemicals, based on their use in traditional medicine (Chah *et al.*, 2006). These phytochemicals have disease preventative properties. Phytochemicals like flavonoids, carotenoids and polyphenols have been reported to have antimicrobial activities and serve as source of antimicrobial agents against pathogens (Minakshi *et al.*, 2016). Similarly, phytochemicals such as phenolic compounds have shown to have antioxidant activities (Duthie *et al.*, 2000).

Pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococci faecalis* and *Staphylococcus aureus*, have been reported to be associated with infections such as urinary tract, eye, skin, kidney infections, diarrhoea and intestinal infections and inflammation-related diseases (Marrs *et al.*, 2005; Wiedenmann *et al.*, 2006; Mittal *et al.*, 2009; Miller *et al.*, 2009; Arthur *et al.*, 2012). These infections have been reported to be traditionally treated using the different parts (leaf, bark and root) of *Olea europaea* subspecies *africana*. Diseases such as rheumatism and arthritis have also been reported to be treated using the leaves of this plant (Msomi and Simelane, 2017). The use of this plant for therapeutic applications has been demonstrated since the primordial age. The health benefits of the leaf extracts have been reported to be due to the presence of additive and/or synergistic effects of their phytochemicals (Ghanbari *et al.*, 2012). The leaves have also been reported to be used in the treatment of diseases associated with free radicals such as diabetes and hypertension (Somova *et al.*, 2003).

In literature, there is little information about the antioxidant, antibacterial and anti-inflammatory activities of the leaves of this plant. Similarly, the information about isolated bioactive compounds from this plant is minimal; hence, this study was aimed at filling in those gaps in literature in relation to the traditional use of the leaves in Africa. Cytotoxicity studies will also be done to determine the safety of the leaves on human cells.

## **2.2. Plant secondary metabolites of therapeutic relevance**

Plants are considered great source of many important drugs because of their ability to produce different entities and bioactive molecules through the process of metabolism. This process comprises of both primary and secondary metabolism. Primary metabolism deals with the production of biomolecules such as nucleic acids, sugars, proteins and lipids, whereas secondary metabolism is activated at certain stages of the plants growth, particularly when the plant is under environmental stress due to limitation of nutrients, or under the attack by microbes (Yazaki *et al.*, 2008). The stress may also be due of pollution and various other abiotic stresses (Oz and Kafkas, 2017).

Secondary metabolites are complex and synthesised from primary metabolites through processes like modifications, such as methylation, hydroxylation and glycosylation. They are categorised based on their chemical structure (e.g. aromatic rings, sugars), composition (containing nitrogen or not), solubility in solvents and the pathways they undergo to be synthesised (Sharma *et al.*, 2012). Phytochemicals have many health benefits on humans; these include protecting our bodies against the build-up of free radicals and fighting against infections caused by bacteria, fungi, and viruses. They also possess activities like lowering cholesterol, antithrombotic and anti-inflammatory responses (Oz and Kafkas, 2017). Secondary metabolites derived from plants are mostly phenols or oxygen-substituted derivatives. Structural variations and chemical composition of these compounds result in differences in their therapeutic relevance (Asfour, 2017).

### **2.2.1. Phenolics**

This is considered the largest class of secondary metabolites produced widely in plants. Structurally, various phenolic compounds have one or more hydroxyl groups (OH) attached directly to an aromatic hydrocarbon chain. Phenolic compounds are classified according to parameters such as number of hydroxyl groups, chemical composition and substitutes present in the carbon skeleton. They can also be classified based on the number of aromatic rings and carbon atoms located on the side chain (Singh, 2017).

Gallic acid is the most common phenolic acid. These can be obtained through the intake of fruits and vegetables like citrus fruits, pomegranate, nuts, berries, onions and processed food and beverages like red wine, cocoa and black tea. These foods may contain phenolic transformation products that are best described as derived polyphenols (Del Rio *et al.*, 2013).

Polyphenols have been reported to have potential health properties to human such antioxidants, anti-allergic, anti-inflammatory, anticancer, anti-hypertensive and antimicrobial activities (Daglia, 2012). The major types of plant phenolic compounds are phenols, coumarins, curcuminoids, lignans, stilbenes tannins and flavonoids, quinones and phenolic acid (Khoddami *et al.*, 2013).

### **2.2.3. Tannins**

They are described as water soluble, polyphenolic compounds with high molecular weight ranging from 500 Da to more than 3000 Da. They have the properties enabling them to bind to proteins and form insoluble or soluble tannin-protein complexes. They are capable of forming complexes with polysaccharides (cellulose, hemicellulose, pectin, etc.), alkaloids, nucleic acids and minerals. They also have protective role against mammalian herbivores and insects. They are divided into two groups; these are hydrolysable and condensed tannins. Their classification on based on their chemical structure and properties. The chemical structures of casuarictin (hydrolysable tannin) and proanthocyanidins (non-hydrolysable or condensed tannins) are shown in figure 2.2A and figure 2.2B, respectively. They are located in the root, bark, stem and outer layers of the plant tissue. Tannins are considered to be used as antiseptic and this is due to the presence of the phenolic group. Medicinal plants rich in tannins have been traditionally used as healing agents in a number of diseases. In Ayurveda, a primary healthcare system in India, preparation of medicine from tannin-rich plants has been used to treat diseases like leucorrhoea, rhinorrhoea and diarrhoea (Singh, 2017).

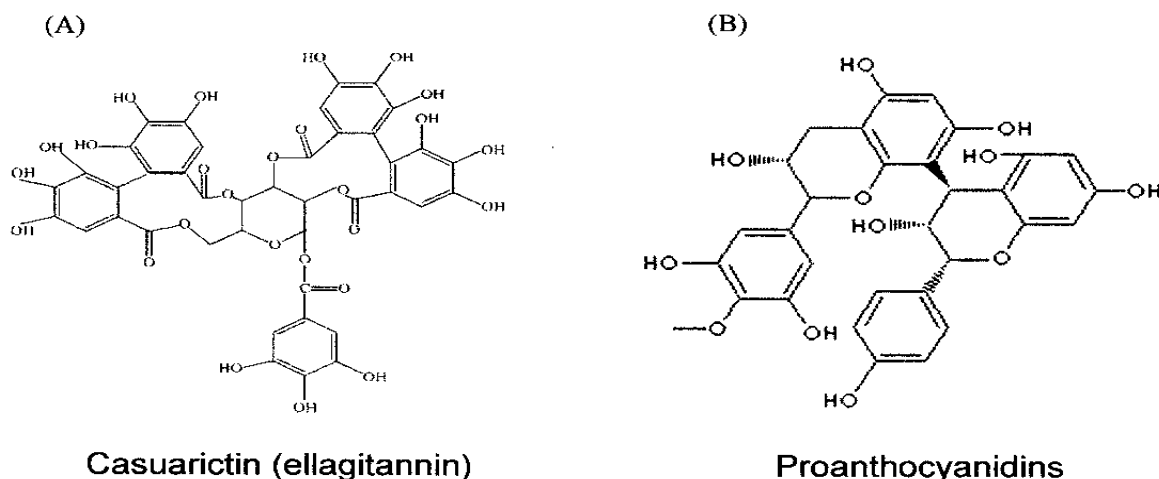


Figure 2.2: Chemical structures of hydrolysable tannin (A) and non-hydrolysable or condensed tannins (B) (Giada, 2013).

They are considered toxic to fungi, bacteria and yeast. They are of value to the food science, wood science, soil science, plant pathology, pharmacology and human and animal nutrition. Mechanisms used by tannin to inhibit microbial growth include astringency by enzyme inhibition and substrate deprivation. This was observed when enzymes such as cellulases and pectinases were found to be inhibited when raw culture filtrates or purified enzymes were mixed with tannins (Scalbert, 1991).

### 2.2.2. Flavonoids

These are water-soluble polyphenols, with more than one hydroxyl group substituted into the aromatic ring. The basic chemical structures of the main classes of flavonoids are presented in figure 2.1. They comprise of 15 carbons with two aromatic rings connected by a three-carbon bridge.

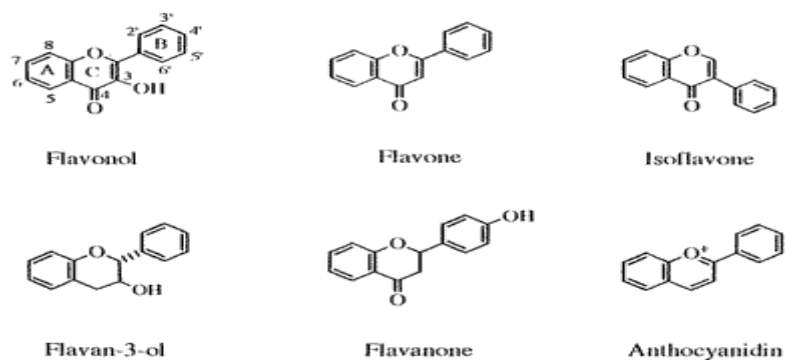


Figure 2.1: Chemical structures of the main classes of flavonoids (Giada, 2013).

They are widely distributed in plants, are associated with the production of pigment, protection of plants against ultraviolet radiation, diseases, and play a major role in symbiotic nitrogen fixing. The main subclasses of flavonoids are flavones, flavonols, flavan-3-ols, isoflavones, flavanones and anthocyanidins; other flavonoid groups (Figure 2.1) which comparatively are minor components of the diet quantitatively (Singh, 2017).

They have various functions in plants including protection from herbivores and microbial infections, as attractants for pollinators and seed-dispersing animals, as allelopathic agents, UV protectants, and signal molecules in the formation of nitrogen-fixing root nodules. In human, there has been an increasing evidence that long term intake of food/herbs containing flavonoids have favourable effects on incidence of cancers and chronic diseases, including cardiovascular disease, type II diabetes, and impaired cognitive function. They also possess properties like anti-inflammatory activity, oestrogenic activity, enzyme inhibition, antimicrobial activity and antioxidant activity. The main subclasses are flavones, flavonols, flavan-3-ols, isoflavones, flavanones, and anthocyanidins (Figure 2.3). The majority of flavonoids occur naturally as glycosides rather than aglycones (Del Rio *et al.*, 2013; Oz and Kafkas, 2017).

#### **2.2.4. Terpenoids**

Terpenoids or isoprenoids are the largest and most diverse class of chemicals among myriad compounds produced by plants. For years, humans have used terpenoids in the food, pharmaceutical, agriculture and chemical industries, and more recently, they have exploited in the development of biofuel products (Tholl, 2015). In an experiment done by Prabuseenivasan and colleagues (2006), cinnamon oil has demonstrated broad-spectrum activity against *Pseudomonas aeruginosa* and this proved that plant oils, which contained terpenes, have shown increasing promises in *in vivo*, inhibiting multiple species of bacteria.

They are classified based on the number of isoprene units (C<sub>5</sub>) they contain. Terpenoids are terpenes which contain some extra elements, usually oxygen in their molecular structure as illustrated in figure 2.3 (Singh, 2017). They are the building blocks of metabolites like plant hormones, sterols, carotenoids, rubber, the phytol tail of chloroform and turpentine. The isoprene unit has the ability to build upon it in



various ways. An isoprene unit bonded to a second isoprene unit is called a monoterpene (C<sub>10</sub>) and three isoprene units bonded together are called sesquiterpenes (C<sub>15</sub>), diterpenes (C<sub>20</sub>) and triterpenes (C<sub>30</sub>) contain two and three terpene units, respectively while polyterpenes contain more than four terpene units (Zwenger and Basu, 2008).

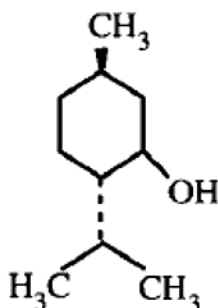


Figure 2.3: Molecular structure of menthol, an example of a terpenoid (Singh, 2017).

The characteristics of these compounds include volatility, flavour/aroma and toxicity. Due to the possession of these characteristics, terpenoids are able to play an important role in plant defence, plant-to-plant communication, and pollinator attraction (Pichersky and Gershenzon, 2002). Besides their ecological importance, they also exhibit pharmacological activities such as anti-viral, antibacterial, anti-malarial, anti-inflammatory, inhibition of cholesterol synthesis and anti-cancer activities (Nassar *et al.*, 2010; Mahato and Sen, 1997).

### 2.2.5. Alkaloids

These secondary metabolites contain nitrogen atom and are of basic nature. Besides carbon, hydrogen and nitrogen, some of the alkaloids may also contain oxygen, sulphur and rarely some other elements like chlorine, bromine and phosphorus. There is no clear difference between alkaloids and other nitrogen containing natural substances such as amino acids, nucleotides and amines. A well-known example of alkaloids is morphine. Unlike other secondary metabolites, alkaloids have molecular structures that are more diverse and therefore, there is no any uniform classification for them. However, they have been classified under three groups, i.e. true alkaloids, pseudoalkaloids and protoalkaloids. The true alkaloids are derived from amino acids and contain nitrogen in the heterocyclic ring; best examples are cocaine and nicotine. The pseudoalkaloids are not synthesised from amino acids while the

protoalkaloids are derived from amino acids, but the nitrogen is not present in a heterocyclic ring (Singh, 2017).

### **2.2.6. Steroids**

They are a group of cholesterol derived lipophilic and the compounds have low molecular weight. They can be derived from marine, terrestrial and synthetic sources. Some hydrocarbons such as a number of hormones (gonadal and adrenal cortex), sterols and bile salts are fall within the steroid family. Unlike other secondary metabolites, all steroid classes and their metabolites play a major role in the physiology and biochemistry of living organisms in which these are found. Synthetic steroids are being extensively used as anti-hormones (Jovanovic-Santa *et al.*, 2015), Contraceptive drugs (Lopez *et al.*, 2006), anti-cancer agents (Thao *et al.*, 2015), cardiovascular agents (Rattanasopa *et al.*, 2015), antibiotics, anaesthetic, anti-inflammatory and anti-asthmatic agents (Aav *et al.*, 2005). All sterols share the same basic perhydro-1, 2-cyclopentenophenanthrene skeleton but differ because of variations in this skeleton or the introduction of functional groups. These variations result in various classes of steroids. The most common steroids are usually known by their trivial names for example cortisol, testosterone, etc. however, it is recommended to use systematic International Union of Pure and Applied Chemistry (IUPAC) steroidal nomenclature, as trivial names cause confusions (Sultan and Rauf Raza, 2015).

## **2.3. Biological activities of plants**

### **2.3.1. Antioxidant activity**

Reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) result from redox cellular processes and are excessively produced during cellular metabolism. Excessive production of ROS/RNS or decrease in antioxidant level, leads to oxidative stress, which may lead to progressive cell damage and ultimate cell death. The equilibrium between free radical production and scavenging determines whether the free radicals would serve as signalling molecules or could cause oxidative damage to the tissues. Besides being signalling molecules, they also play a major role in the regulation of transcription factors that regulate the expression

of various genes encoding proteins that are responsible for tissue injury (Sharma *et al.*, 2012). They are also important for the maturation process of cellular structures and forms part of the host defence system. This is demonstrated by the body's ability to signal phagocytes (neutrophils, macrophages, monocytes) to release free radicals to destroy pathogenic microbes (Pham-Huy *et al.*, 2008).

ROS and RNS are terms that describe free radicals and other non-radical reactive derivatives called oxidants. They have been defined as molecules with one or more unpaired electrons in their outer shells. Besides being produced through cellular metabolism, they also result from breakage of chemical bonds of molecules such that each fragment keeps one electron. Free radicals include molecules such as superoxide anion ( $O_2^{\cdot-}$ ), hydroxyl radical ( $\cdot OH$ ), nitric oxide ( $NO^{\cdot}$ ), nitrogen dioxide ( $NO_2^{\cdot}$ ), as well as nonradical molecules (oxidants) like hydrogen peroxide ( $H_2O_2$ ), nitrous acid ( $HNO_2$ ), singlet oxygen ( $^1O_2$ ), and so forth. Oxidants are not free radicals but can easily lead to free radical reactions in living organisms. This demonstrates that free radicals are highly unstable molecules, which can react with various organic substances such as lipids, proteins and DNA. This potency is made possible by the availability of unpaired electrons (Pham-Huy *et al.*, 2008).

At high concentrations, ROS are considered extremely harmful to the cell and the cell is said to be in an 'oxidative stress'. At this state, cellular functions become deregulated in a series of events, leading to various pathological conditions. Conditions like cardiovascular dysfunction, neurodegenerative diseases, gastroduodenal pathogenesis, and metabolic dysfunction of almost all the vital organs, cancer and premature aging arise due to oxidative stress (Bandyopadhyay *et al.*, 1999). The cells may also undergo processes such as peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death (PCD) pathway and ultimately leading to cell death (Meriga *et al.*, 2004).

Excess free radicals are scavenged or detoxified through an efficient oxidative system, comprising of enzymatic and nonenzymatic antioxidants. The examples of enzymatic antioxidants include superoxide dismutase, glutathione reductase and catalase. Non-enzymatic antioxidants include ascorbate, vitamin C, vitamin E

glutathione, carotenoids, tocopherols, and phenolics (Sharma *et al.*, 2012). The super peroxide dismutase is the first line of defence against free radicals, it catalyses the dismutation of superoxide anion radical ( $O_2^{\cdot-}$ ), into hydrogen peroxide ( $H_2O_2$ ) by reduction, then transformed to water and oxygen ( $O_2$ ) by the enzyme catalase (CAT). The human body has the ability to counteract oxidative stress by producing antioxidants, which are either naturally synthesised *in situ* (endogenous antioxidants) or externally supplied through food and/or supplements (exogenous antioxidants). The presence of antioxidants which are regarded as 'free radical scavengers', helps in preventing and repairing damages caused by ROS and RNS, and therefore boosting the immune defence and lowering the risk of development of cancer and other diseases (Pham-Huy *et al.*, 2008).

The antioxidants from plants taken as supplements have the ability to terminate the free radical reactions thus preventing oxidative damage to cells. Phytoconstituents and herbal products, which are safer than synthetic medicine may be beneficial in the treatment of the diseases, caused by free radicals and most importantly, this protects the body by inhibiting free radicals from causing tissue injury. Antioxidants are defined as substances that even in small amounts can prevent oxidation of easily oxidisable materials. They may also be defined as substances capable of inhibiting a specific oxidising enzyme or a substance that reacts with oxidising agents prior to causing damage to other molecules (Bhatt *et al.*, 2013). Natural antioxidants offer an advantage because they confer fewer side effects and are compatible to the body physiology (Sen *et al.*, 2010). Antioxidants have various mechanisms of action; these include direct trapping of reactive oxygen species, inhibition of enzymes responsible for producing superoxide anions, chelating transitional metals involved in processes forming radicals, and prevention of the peroxidation process by reducing alkoxy and peroxy radicals (Bridi and Montenegro, 2017).

To assess the total number of antioxidants present in dietary plants, a number of methods can be used. These assays are used to generally assess the plant's functions as reducing agents, hydrogen donors, singlet oxygen quenchers or metal chelators (Kasote, 2013). This include the 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox) equivalence antioxidant capacity (TEAC) assay, the ferric reducing antioxidant potential (FRAP) assay and the oxygen radical absorbance

capacity (ORAC) assay (DeLange and Glazer, 1989; Benzie and Strain, 1996; Miller *et al.*, 1996).

The TEAC and the ORAC assays are based on the ability of an antioxidant to react with or neutralise free radicals generated in the assay system. While FRAP assay is based on measuring the reduction of Fe<sup>3+</sup> (ferric iron) to Fe<sup>2+</sup> (ferrous iron) in the presence of antioxidants. FRAP assay has an advantage in that it directly measures antioxidants or reductants in a sample, while other assays are indirect and measure the inhibition of reactive species (free radicals). The results obtained, depend on which reactive species are to be assayed. Another commonly used assay that is simple and inexpensive, is the 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assays. It is based on the ability of compounds to act as free radical scavengers or hydrogen donors and to evaluate antioxidant activity (Halvorsen *et al.*, 2002).

### **2.3.2. Antimicrobial activity**

Since times immemorial, plants have been resisting the continuous attacks of microorganisms (Parasites, fungi, Bacteria and Viruses) by producing endless secondary metabolites (Gilani, 2005). Different types of antimicrobials have been developed from the metabolites, to fight off pathogens responsible for causing diseases. Antimicrobials are substances that kill or inhibit the growth of microorganisms. These could be in the form of antibiotics, which are products of microorganisms or synthesised derivatives, or antimicrobial peptides produced by complex organisms (Jenssen *et al.*, 2006; Cowan 1999).

There are different types of antimicrobials, these include: antibiotics, anti-viral, anti-fungal and antiprotozoan. The treatment of antibacterial infection can be through the use of antibiotics produced from natural or synthetic sources. The examples of antibiotics from natural sources are phenyl propanoids (chloramphenicol), polyketides (tetracycline), aminoglycosides (streptomycin, gentamycin), macrolides (erythromycin), glycopeptides (vancomycin) and second-generation  $\beta$ -lactams (cephalosporin). Plant-derived antimicrobials inhibit microbial growth through the disintegration of cytoplasmic membrane, destabilisation of the proton motive force, electron flow, active transport and coagulation of the cell content (Silva and Fernandes, 2010). The antibiotics produced from synthetic sources are

sulphonamides, quinolones and oxazolidinones. Most of them exert their actions either by inhibition of the bacterial cell wall or protein synthesis. However, there are some that mainly inhibits DNA synthesis such as quinolones and others that inhibit the synthesis of metabolites used for the synthesis of deoxyribonucleic acid (DNA) (Singh and Barrett 2006).

With the world facing the re-occurring resistance of pathogenic microorganisms to antibiotics, investigation of other sources of antimicrobials, such as medicinal plants, for their antimicrobial properties is gaining ground. This investigation is also influenced by the adverse side effects presented by the synthetic antibiotics. The phytochemicals produced by plants have been reported to possess antimicrobial activities when used alone and as synergists or potentiators of other antibacterial agents. These phytochemicals usually act through different mechanisms than conventional antibiotics and could therefore be of use in the treatment of resistant bacteria (Abreu *et al.*, 2012).

Several methods are employed in medical research laboratories to test for bioactive compounds with antimicrobial activity, which includes bioautography and micro-broth dilution methods (Renisheya *et al.*, 2011). TLC-bioautography methods combine chromatographic separation and *In-situ* activity determination. This facilitates the localisation and target-directed isolation of active constituents in a mixture. In this method, the microbial growth inhibition is the main component used to detect antimicrobial components of extracts chromatographed on a TLC layer. This methodology has been considered as the most efficacious assay for the detection of anti-microbial compounds (Sasidharan *et al.*, 2011).

### **2.3.3. Anti-inflammatory activity**

Inflammation may be defined as a process that protects our bodies against harmful stimuli and hastens the recovery process (Krishnaraju *et al.*, 2009). A pathogenic bacterium, virus, wound or an endogenous trigger may act as a stimulus. Inflammation is characterised by redness, pain, swelling, immobility and heat (Hsu *et al.*, 2013). The inflammatory response is complex and involves many reactions such as enzyme activation, innate immune cell activation and migration, release of reactive oxygen species and tissue repair (Vadivu and Lakshmi, 2011).

The process of inflammation may be categorised into two distinct types, acute and chronic inflammation. Acute inflammation is the state which therapeutically allows the body to heal after an injury. At this stage, the immune cells, enzymes and reactive oxygen species all congregate at the site of injury leading to the propagation of biochemical cascades that act to remove the pathogen or seal the wound (Spite and Serhan, 2010). Chronic inflammation has many features of acute inflammation but is usually of low grade and persistent, resulting in responses that lead to tissue degeneration (Franceschi and Campisi, 2014).

Inflammation has been implicated in a broad range of diseases, including diabetes, cancer, hypertension and atherosclerosis (Ramírez-Cisneros, 2012). The effects of chronic inflammation are thought to be due to the excessive amounts of free radicals and depletion of antioxidant mechanisms. Chronic inflammation accompanied by oxidative stress has been linked to various steps involved in tumorigenesis, such as cellular transformation, promotion, proliferation, invasion, angiogenesis, and metastasis (Lai *et al.*, 2011).

There are many components involved in inflammation; these include inflammatory mediators, free radical activity and oxidative stress. The mediators are the chemokines which include tumor necrosis factor (TNF)- $\alpha$ , nuclear factor kappa beta (NF $\kappa$ B), interferon (IFN)- $\gamma$ , nitric oxide and interleukins (Hong *et al.*, 2009). Bacteria activate NF- $\kappa$ B through receptors found on macrophages via several signaling pathways. The activated macrophages can release TNF- $\alpha$  which is responsible for the up-regulation of the production of other inflammatory mediators that include prostaglandins and nitric oxide (Franceschelli *et al.*, 2011).

Overproduction of reactive nitrogen species has been associated with diseases such as rheumatoid arthritis, diabetes and hypertension (Park *et al.*, 2013). Damage at the site of inflammation may result if the over production of free radicals is not eliminated (Miguel, 2010). Free radicals that are produced during an inflammatory response called the respiratory burst. This is characterised by the release of immune cells such as phagocytes and macrophages to kill harmful bacteria and viruses (Biller-Takahashi *et al.*, 2013).

Currently, the treatment of inflammatory diseases involves mainly the interruption of the synthesis or action of critical mediators that drive the host's response to injury (Tung *et al.*, 2008). Narcotic, steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) are the current treatment options against inflammation. However, the steroidal drugs have been reported to have reduced efficacy due to adverse effects and relatively high potency. They have side effects such as hypertension, gastrointestinal bleeding and improper clotting of blood. Non-steroidal drugs, on the other hand, have relatively fewer and less adverse effects (Burk *et al.*, 2010).

The ideal anti-inflammatory drugs should be effective with minimal to no side effects when administered over a long-term period. The agents should also prevent the development of chronic and degenerative diseases. Plants have been considered to be sources of the new anti-inflammatory agents due to their extensive use in folk medicine globally to treat various ailments (Boukhatem *et al.*, 2013). Plants have advantages over synthetic drugs in that they are non-narcotic in nature, are biodegradable, have minimal environmental hazards and adverse effects, and are relatively affordable (Umapathy *et al.*, 2010).

Medicinal plants have been used extensively in traditional medicine in the treatment of inflammatory disorders (Matthew *et al.*, 2013). Phytochemicals such as phenols, vitamins, carotenoids, phytoestrogens and terpenoids are some of the have been found to possess anti-inflammatory activity which could justify their use in folk medicines. Phenolic compounds and terpenoids have been reported to have an effect in the increase of activity of inflammatory mediators such as the NO radical instead of inhibiting it (Gouveia *et al.*, 2011). Some of plants that have been used in the treatment of inflammation include *Olea europaea* (Suntar *et al.*, 2010), *Agathosma betulina*, *Aloe ferox* and *Harpagophytum procumbens* (Street and Prinsloo, 2012).

## **2.4. *Olea europaea* subspecies *africana***

### **2.4.1. Morphology**

The genus *Olea* was derived from the Greek word “*elaia*” and the Latin word “*oleum*,” but it is known by nearly 80 different names with the common one being the



olive tree (M'édail *et al.*, 2001). The genus *Olea* comprises 30 species (Bracci *et al.*, 2011) with *Olea europaea* L. being the most popular member of the genus *Olea*. *Olea europaea* subspecies *africana* is an evergreen tree, belonging to the Oleaceae family and was previously known as *Olea europaea* subspecies *africana* (Mill) P.S. Green (Heenan *et al.*, 1999). It is commonly known as the wild olive and its vernacular names include mohlware (Sotho), olienhout (Afrikaans) and umquma (Xhosa and Sulu). Its alternative name at the subspecies rank is subspecies *cupidata* (Hamman-Khalifa *et al.*, 2007). The wild olive tree is a shrub which grows to 5–10 m in height and irregularly reaching 18 m. The trees mature into a wild, rounded pattern with a solid upper layer and twisted trunk when exposed to dry conditions (Figure 2.4A). Flowers are greenish white in colour, 6–10 mm long, with a sweet aroma and held insecurely in axillary or occasionally terminal heads (Figure 2.4). The ovoid fruit are thinly fleshy, about 7–10 mm in dimension, and upon maturation it turns black or dark brown (Figure 2.4C). The bark is grey to brownish and flaky once it matures (Figure 2.4D) (Msomi and Simelane, 2017).

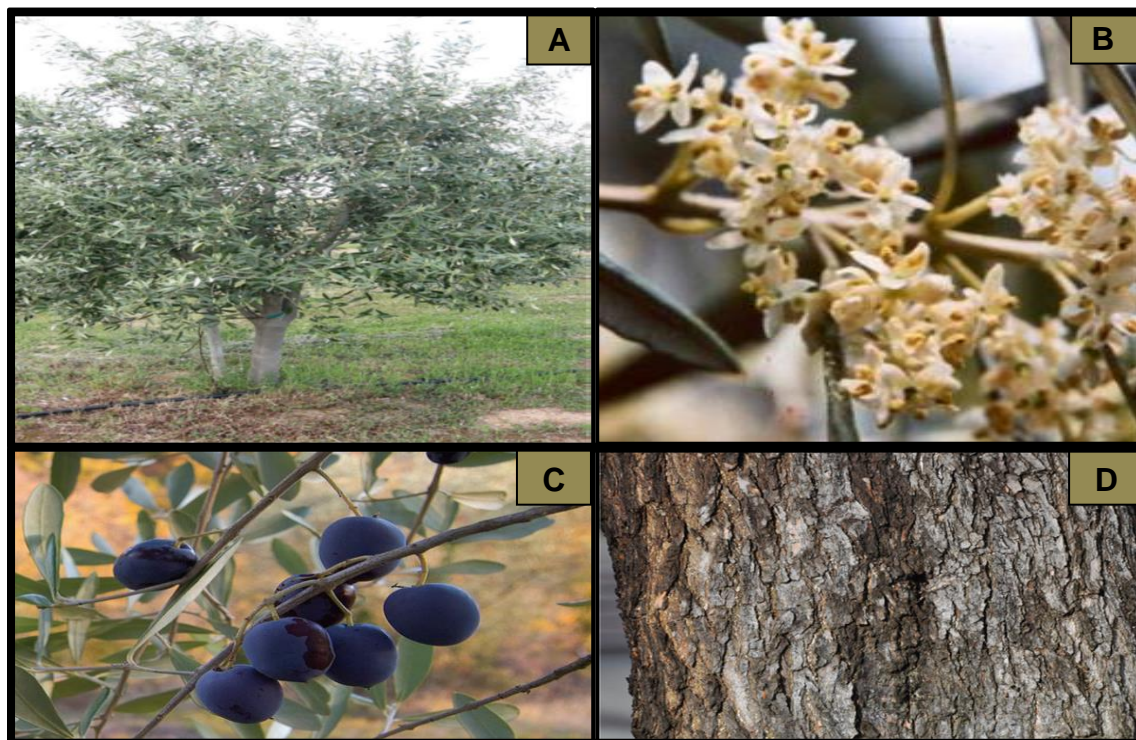


Figure 2.4: *Olea europaea* subspecies *africana*: (A) tree; (B) leaves; (C) flowers; (D) ripe fruits; (E) stem bark (Hashmi *et al.*, 2015).

#### 2.4.2. Traditional use of *Olea europaea* subspecies *africana*

*Olea europaea* subspecies *africana* has been stated to be “the most important plant” from 120 plants being used in traditional medicine (Somova *et al.*, 2003). It has a number of traditional uses medically that are summarised in table 2.1. The bark, leaves, roots and fruits are used in different forms, alone or sometimes in combination (Long *et al.*, 2010).

Table 2.1: Ethnobotanical uses of *Olea europaea* subspecies *africana* in southern Africa and the rest of Africa (Msomi and Simelane, 2017).

| Traditional uses in southern Africa   | Reference                          |
|---|------------------------------------|
| Dried and powdered leaf is applied on fresh wounds as a styptic.  | Pappe, 1857.                       |
| Powered leaf stops nosebleeds by using it as a snuff.   | Smith, 1966.                       |
| Leaves are used as a treatment for malaria, urinary tract infections, backaches and kidney problems.                  | Masoko and Makgapeetja, 2015.      |
| Leaf infusions are used as lotion for the treatment of eye infections or to relieve sore throats.                     | Mkize <i>et al.</i> , 2008.        |
| Bark, which is dried, pound and powdered, is applied for eye illnesses. Boiled bark is administered for itchy rashes. | Kipkore <i>et al.</i> , 2010.      |
| Decoction of stem bark is used to treat helminthiasis, asthma, rheumatism and lumbago in Samburu district, Kenya.     | Nanyingi <i>et al.</i> , 2008.     |
| Bark, root and leaf infusions are taken to relieve colic, leaf infusion taken to treat sore throats and diphtheria.   | Reida <i>et al.</i> , 2006.        |
| Fruit infusion treats bloody stool and diarrhoea.   | Bisi-Johnson <i>et al.</i> , 2010. |
| Decoction of the fruits and leaves is used in treating blood pressure in the Transkei region.                         | Bhat, 2014.                        |

#### 2.4.3. Research done on *Olea europaea* subspecies *africana*

Phytochemicals that have been isolated from *Olea europaea* subspecies *africana* include phenolic compounds like flavonoids (Amabeoku and Bamuamba, 2010),

triterpenoids (oleanolic acid and ursolic acid) (Somova *et al.*, 2003), (erythrodiol and uvaol) (Douglas *et al.*, 2016) and coumarin glycosides (esculin and scopolin) (Nishibe, 1982). The isolated triterpenoids were extracted with dichloromethane, ethyl acetate and methanol (van Wyk *et al.*, 2008).

A study conducted by Douglas and colleagues (2016), investigated the antibacterial activity of the subspecies *africana* leaf extracts (methanol, ethyl acetate and dichloromethane) against *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus*, by means of micro-broth diffusion method. Another study by Abdel-Sattar and colleagues (2013) reported on the antioxidant properties of *Olea europaea* subspecies *africana* methanol leaf extract. The antioxidant activity was studied by exploring scavenging activity of 1, 1-diphenyl-2-picrylhydrazyl free radical (DPPH). The leaf extract reduced the radical to a yellow-coloured diphenylpicrylhydrazine, confirming its antioxidant property.

Toxicological studies done by Somova and colleagues (2003) investigated the toxicity effects of the methanolic leaf extract on Sprague-Dawley rats using the doses 20, 40, 60 and 80 mg. It was observed using the brine shrimp test that the leaf extract had toxicity with LC<sub>50</sub> of 1.25 at a dosage of 60 mg/kg which was relatively low. The reference substances, ursolic and oleanolic acid, showed low toxicity with LC<sub>50</sub> of 0.95 and 0.10 mg/mL, respectively. In this study, it was therefore concluded that *Olea europaea* subspecies *africana* was non-toxic and could be used as medical drug.

## **2.5. Separation and purification of compounds**

The study of medicinal plants begins with pre-extraction and the extraction procedures. Pre-extraction procedures involve drying and grinding of plant materials while extraction involves maceration, infusion, percolation and decoction (Azwanida, 2015). Bioactive compounds usually occur as a mixture of compounds of various types and polarities. Identification of the phytochemicals is done through phytochemical screening, which involves procedures that are simple, quick, and inexpensive to perform (Sasidharan *et al.*, 2011).

Application of different separation techniques may be done, to obtain pure compounds. Such techniques include the use of thin layer chromatography (TLC), flash chromatography, sephadex chromatography, high performance liquid chromatography (HPLC) and column chromatography. Firstly, the components in the crude extracts may be separated by preparative thin layer chromatography then followed by purification using column chromatography. TLC has been regarded as the most used method because it gives a quick answer as to how many components are there in a mixture. It can also support the identity of a compound in a mixture when the retention factor ( $R_f$ ) of a compound is compared with the  $R_f$  of a known compound (Sasidharan *et al.*, 2011).

In chromatography, the separation is based on polarity and one or two solvents may be combined and used to further purify the compounds. To confirm the purity on an isolated compound, thin layer chromatography is employed (Visht and Chaturvedi, 2012). It is important to isolate the bioactive compounds from medicinal plants because they represent a major source of standards for screening and drug analysis. After the isolation and purification of compounds, pure compounds are analysed using NMR ( $^1\text{H}$  and  $^{13}\text{C}$ ) to elucidate their structures. The molecular weights of the compounds are determined by Mass Spectrometry (MS) (Patra *et al.*, 2012).

## **2.6. Structure elucidation of compounds**

No single method is sufficient to study the bioactivity of phytochemicals from a given plant. An appropriate assay is required to first screen for the presence of the source material then purify and subsequently identify the compounds therein (Doughari, 2012). HPLC can be used to identify compounds by the identification of peaks, which will be achieved by comparison to the known standard. The identified peak should have a reasonable retention time and be separated from the extraneous peaks. Ultraviolet (UV) detection is applied because the majority of naturally occurring compounds have UV absorbance at low wavelengths (190-210 nm). Liquid chromatography coupled with mass spectrometry (LC/MS) can also be used to analyse complex botanical extracts. It provides enough information to elucidate structures when combined with tandem mass spectrometry. The other techniques that are employed to separate and determine structures of antifungal and

antibacterial plant compounds involves the application HPLC or it can be coupled with UV photodiode array detection, Liquid Chromatography-Ultraviolet (LC-UV), Liquid Chromatography-Mass Spectrophotometry (LCMS) and Liquid Chromatography-Nuclear Magnetic Resonance (LC-NMR) (Oleszek and Marston, 2000).

Libraries of spectra can be searched for comparison with complete or partial chemical structures. Hyphenated chromatographic and spectroscopic techniques are powerful analytical tools that are combined with high throughput biological screening in order to avoid re-isolation of known compounds as well as for structure determination of novel compounds (Doughari, 2012).

## **2.7. Pathogens**

Practically any microbe has the potential to cause an infection in hospitalised patients. About 90 percent of the nosocomial infections are of bacterial origin, whereas mycobacterial, viral, fungal or protozoal agents are less commonly involved. The most frequently reported nosocomial pathogens have been *Escherichia coli*, *Staphylococcus aureus*, *Enterococci* species and *Pseudomonas aeruginosa*. (Sanglard, 2016). These pathogens have been selected as the test bacteria in this study because they are among the bacterial species that are antibiotic resistant. They are also causative agents of many diseases that are traditionally treated using the plant investigated in this study.

### **2.7.1. *Escherichia coli***

*Escherichia coli* are Gram-negative facultative anaerobes, which oxidises negatively. It is one of the common organisms, which have the ability to cause Gram-negative sepsis and endotoxin-induced shock. Infections caused by *E. coli* include urinary tract and wound infections, diarrheal disease or gastroenteritis infections, pneumonia in immunocompromised hospitalised patients and meningitis in neonates. These microorganisms possess a wide range of virulence factors, which members of the Enterobacteriaceae family share. For example, characteristics such as the production of endotoxins and capsules, antigenic phase variations, sequestration of growth factors and the ability to become resistant to serum killing and antimicrobials

(Bereket *et al.*, 2012). The strain ATCC 25922 is commonly used as a quality control strain, particularly in antibody sensitivity assays. It is of serotype O6 and biotype 1. It is used in susceptibility disc testing of neomycin, kanamycin, cephalixin, cephalothin, tetracycline, cephaloglycin and chloramphenicol (Minogue *et al.*, 2014).

### **2.7.2. *Pseudomonas aeruginosa***

*Pseudomonas aeruginosa* is a Gram-negative bacterium, which has a potential to cause diseases in both humans and animals. It is located in soil, water, skin flora and most man-made environments. Its versatility enables the organism to invade damaged tissues or tissues with reduced immunity, which causes inflammation and sepsis. It is identified by its colonial characteristics and mucoid polysaccharide capsule. This can be done through performing biochemical tests. It has the ability to infect the respiratory and gastrointestinal tracts of hospitalised patients, particularly those treated with broad-spectrum antibiotics, exposed to respiratory therapy equipment, or hospitalised for extended period. Pathogenesis by this organism begins when a normal defence mechanism of a body is compromised. The effects caused by *P. aeruginosa* include blue-green pus producing wound infections, meningitis, urinary tract infection, and necrotising pneumonia (Bereket *et al.*, 2012). *Pseudomonas aeruginosa* ATCC 27853 is usually used for the analysis of antimicrobial activity (Fang *et al.*, 2012).

### **2.7.3. *Enterococcus faecalis***

The microorganisms composed in this genus are facultative anaerobic organisms that have the ability to survive in high concentrations of salt. *Enterococcus* species are Gram-positive cocci and are arranged in pairs and short chains. They are facultative anaerobic and achieve optimum growth at 35 °C on complex media with a supplement of vitamin B, nucleic acid base and carbon source as glucose. They form part of the normal flora of the gastrointestinal and genitourinary tracts of humans. These organisms are responsible for the urinary tract infections, followed by intra-abdominal and pelvic infections. They are also responsible for surgical wound infection, bacteraemia, endocarditis, neonatal sepsis, and rarely meningitis. *E. faecalis* is the most common cause of infections (80-90 percent) (Bereket *et al.*, 2012).

This species is often resistant to multiple antibiotics, displaying both inherent and acquired traits. *E. faecalis* ATCC 29212 was first isolated from a human urine sample collected in Portland, Oregon, United States, and it is common in laboratories for research (Minogue *et al.*, 2014). It is often used as a control strain to assess the quality of commercially prepared microbiological culture media, susceptibility of pathogens to antibiotics, biochemical identification of bacterial species commercially and microbiological quality testing of food and animal feeding stuffs and are tolerant to heat (Kim *et al.*, 2012).

#### **2.7.4. *Staphylococcus aureus***

The genus *Staphylococcus* comprises of several species with *S. aureus* being one of them and thus far the most important nosocomial pathogen. The bacteria in this genus are non-motile, non-spore forming, catalase positive, Gram-positive cocci and facultative anaerobe. *Staphylococcus aureus* can be both commensal and pathogenic. It is the primary causative agent of lower respiratory tract infections and the surgical site infections and it is considered the second leading cause of nosocomial bacteraemia, necrotising pneumonia and cardiovascular infections. The manner in which the virulence factors of *S. aureus* are assembled is extensive, where both its structural and secreted products play a role in the infections (Bereket *et al.*, 2012). Its numerous virulence factors are responsible for its resistance to a multitude of antibiotics, which narrows the options available for its treatment. *Staphylococcus aureus* subspecies *aureus* ATCC 29213 is a clinical isolate that is utilised as a standard quality-control strain in laboratory testing. It is sensitive to a wide range of antimicrobials, including methicillin (Soni *et al.*, 2015).

### **2.8. Antimicrobial resistance of pathogens**

The increasing antibiotic resistance in bacterial pathogens has been observed for many years; it was initially discovered in Gram-negative bacteria then later in Gram-positive bacteria (Witte, 1999). Historically, the emergence of antibiotic resistance initiated soon after the discovery and use of penicillin G and sulfonamides in the 1940s. The most commonly high profile nosocomial organisms are multidrug resistant, either with acquired resistance e.g. methicillin-resistant *Staphylococcus*

*aureus* (MRSA) and extended-spectrum  $\beta$ -lactamase (ESBL) producers, or natural resistance (Marothi *et al.*, 2005).

### 2.8.1. Gram-negative bacteria

Gram-negative pathogens have features that enable them to efficiently up-regulate or acquire particular genes that code for mechanisms of antibiotic/ drug resistance. This usually occurs when the organism is under the selection pressure of antibiotics. Furthermore, they have plenty of resistance mechanisms, often executing multiple mechanisms against the same antibiotic or executing a single mechanism to affect multiple antibiotics (Figure 2.5).

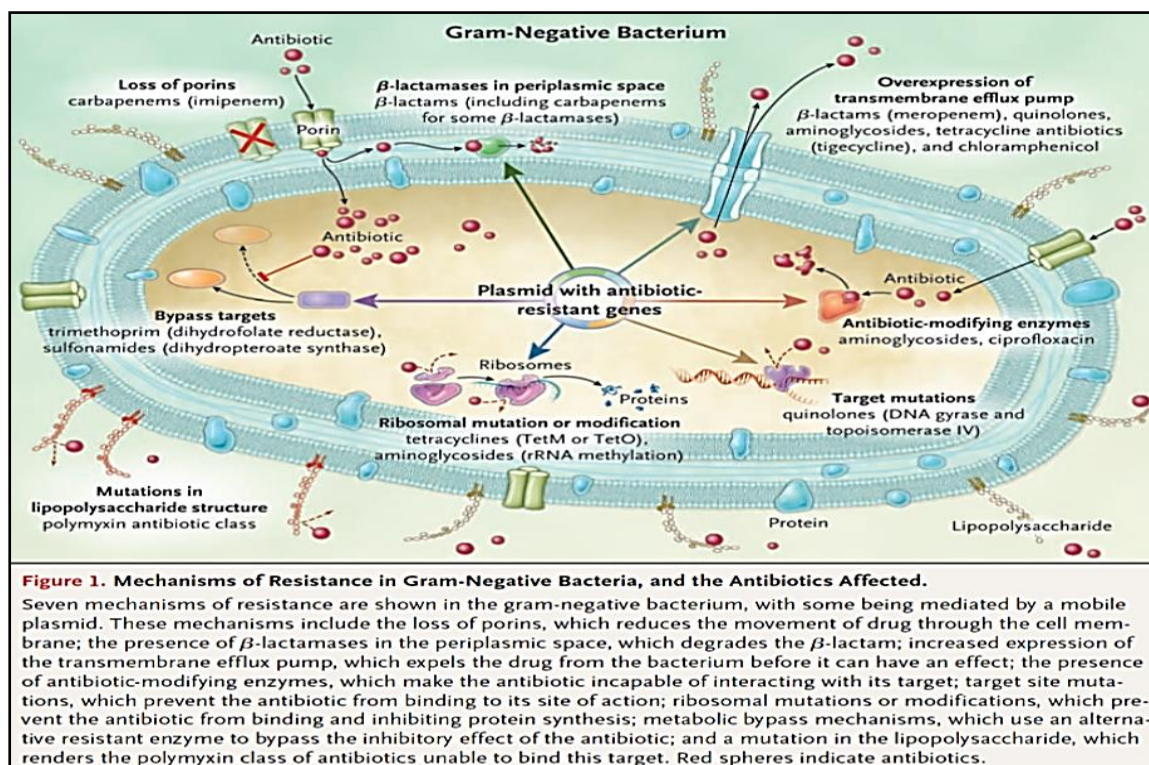


Figure 2.5: Mechanism of resistance in Gram-negative bacteria, and antibiotics affected (Peleg *et al.*, 2010).

Gram-negative microorganisms are more resistant to antimicrobials than Gram-positive microorganisms. This is due to the presence of the outer membrane permeability barrier, which has the ability to minimise the accessibility of antimicrobial agents to their targets in the bacterial cell. In many cases, the



resistance is exhibited to be intrinsic and occurs independently of mutation or acquired foreign resistance determinants (Poole, 2001).

The multidrug efflux systems are broadly specific systems used by bacteria, to promote drug excretion and entry into the cells. In that way, drug accumulation is reduced, thus protecting the cells from deleterious effects of drugs. The main systems responsible for the antibiotic effluxes include resistance-nodulation-division (RND), membrane fusion protein (MFP) and the outer membrane factor (OMF). These systems play a significant role in resistance to clinically relevant antimicrobials and are widely distributed amongst Gram-negative bacteria such as *E. coli* and *P. aeruginosa*. These systems work in conjunction to bring about the efflux of antibiotics across both membranes of a typical Gram-negative organism (Poole, 2001).

The intrinsic resistance of Gram-negative bacteria, acquired resistance via multiple mechanisms have also been observed in *P. aeruginosa*. The mechanisms used to promote resistance include the production of  $\beta$ -lactamases and carbapenemases, up regulation of multidrug efflux pumps and finally cell wall mutations leading to reduction in porin channels. This hinders the small antibiotics like  $\beta$ -lactams and quinolones to enter the cell of *P. aeruginosa* since they require the aqueous porin channels to penetrate the membrane. In addition, the mutation of genes encoding antibacterial targets such as DNA gyrase for fluoroquinolones contributes to the resistance of antibiotics (Slama, 2008).

### **2.8.2. Gram-positive bacteria**

Gram-positive pathogens such as *Enterococci* species have the ability to survive in an environment full of heavy antibiotics. Like Gram-negative bacteria, the antibiotic resistance of *Enterococci* is of two types: intrinsic and acquired resistance. Intrinsic resistance is species specific and chromosomally mediated, while acquired resistance occurs when the pathogen's DNA gets mutated or new DNA is acquisitioned into the cell (Marothi *et al.*, 2005). For vancomycin resistance to occur, an expression of several genes has to take place, and consequently, genes from another organism have to be acquired into the cell (Bereket *et al.*, 2012).

The mechanism of antibiotic resistance varies between pathogens; this is dictated by the pathogen's type of cellular structure (Marothi *et al.*, 2005). *Enterococci* are resistant to commonly used antimicrobial agents such as aminoglycosides, cephalosporin, vancomycin and semisynthetic penicillin. The low affinity of penicillin binding proteins (PBP) to B-lactam antibiotics enables *Enterococcus* species to synthesise cell wall components even in the presence of modest concentration of most B-lactam antibiotics. The pathogens are inhibited in the presence of B-lactam antibiotics and not killed by these agents. The B-lactamase enzyme produced by *E. faecalis* has high level of resistance to ampicillin and penicillin. Its production is plasmid mediated and the enzyme is constitutively produced. This property demonstrates acquired characteristics. The tolerance of antibiotics by *Enterococci* after an exposure of few doses of an antibiotic develops very quickly (Marothi *et al.*, 2005).

Microorganisms resistant to penicillin and newer narrow spectrum B-lactamase-resistant penicillin antimicrobial drugs, (e.g., methicillin, oxacillin) emerged soon after being introduced into clinical practice in the 1940s and 1960s, respectively. The methicillin-resistant *Staphylococcus* spp. emergence was soon reported within a year after the introduction of methicillin in 1959-1960. This was achieved by the modification of the penicillin binding proteins encoded by the chromosomal *mecA* gene carried on a mobile genetic element. This modification in the target site enables bacteria to be resistant to all B-lactams and their derivatives (Bereket *et al.*, 2012). The PBP2a is an additional penicillin binding protein with low affinity for all B-lactam antibiotics (Witte, 1999).

## 2.9. Aim and Objectives

### 2.9.1. Aim

The aim of this study was to investigate and isolate compounds exhibiting antimicrobial, antioxidant and anti-inflammatory activities from the leaves of *Olea europaea* subspecies *africana* and study their cytotoxic effects.

### 2.9.2. Objectives

The objectives of the study are:

- i. To collect, dry, grind and perform extraction on the *Olea europaea* subspecies *africana* leaves with n-hexane, chloroform, dichloromethane, ethyl acetate, acetone, ethanol, methanol, n-butanol, and water.
- ii. To determine the chemical profile of the extracts using TLC analysis.
- iii. To determine the antioxidant potential of the extracts using DPPH assay and the reducing power assay.
- iv. To assess the extracts for possible antimicrobial activities using serial broth microdilution assay and direct bioautography assay.
- v. To determine the anti-inflammatory activities of the leaves using the Lipopolysaccharide-stimulated RAW 264.7 cells.
- vi. To determine the presence of major phytochemicals using the standard chemical methods.
- vii. To quantify the major phytochemicals using the standard chemical methods.
- viii. To determine the cytotoxicity of the plant extract against normal cells using Vero monkey cell line.
- ix. To isolate and identify bioactive compounds showing antimicrobial and antioxidant activities through open column chromatography.
- x. To determine the chemical structure of the identified compounds using NMR and Mass spectroscopy.

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## CHAPTER 3: EXTRACTION AND PRELIMINARY PHYTOCHEMICAL ANALYSIS

### 3.1 INTRODUCTION

Medicinal plants serve as reservoirs of potentially useful chemical compounds, which could serve as newer leads and clues for modern drug design (Majekodunmi, 2015). It is of significance to know the correlation between the phytochemicals and the bioactivity of a plant, in order to synthesise compounds with specific activities, to treat various health ailments and chronic diseases (Pandey *et al.*, 2013). Preliminary screening of phytochemicals is a valuable step, which assists in the detection of bioactive compounds present in medicinal plants and may subsequently lead to isolation of the compounds. If required, extracts resulting from extraction, may be directly used as medicinal agents in the form of tinctures or fluid extracts or further processed to be incorporated in any dosage form such as tablets and capsules. This is important in the discovery and development of novel therapeutic agents with improved efficacy (Ncube *et al.*, 2008; Yadav *et al.*, 2014).

Extraction of bioactive compounds from medicinal plants mainly focuses on separating medicinally active portions of plant tissues, by using selective solvents through standard chemical procedures. Final products obtained after extraction are of complex mixtures of metabolites, either in liquid or semisolid state or (after removing the solvent) in dry powder form (Tiwari *et al.*, 2011). The basic principle of grinding the plant material (dry or wet) finer, is to increase the surface area for extraction, thereby increasing the rate of extraction. To obtain compounds of great quality and quantity, it is ideal to use the solvent to dry sample ratio of 10:1 (v/w) (Das *et al.*, 2010).

The choice of solvent is influenced by what is intended with the extract. Since the product will contain traces of residual solvent, the solvent should be non-toxic and should not interfere with the bioassays. Water is commonly used by traditional healers for extraction purposes. It is known to have abilities of extracting compounds with antimicrobial activity. However, organic solvents have been found to give extracts with more consistent antimicrobial activity compared to water extracts. Organic solvents that are often used are n-hexane, chloroform, dichloromethane, ethyl acetate, acetone, ethanol, methanol and n-butanol. These solvents extract

different phytochemicals (Table 3.1) which vary in polarity strength i.e. Non-polar, intermediate, and polar. In a sample, different solvents can be mixed for extraction or they can be used in a sequence in the same sample material (Majekodunmi, 2015).

Table 3.1: Solvents used for bioactive component extraction (Pandey and Tripathi, 2013).

| <b>Water</b>  | <b>Ethanol</b> | <b>Methanol</b> | <b>Chloroform</b> | <b>Acetone</b> |
|---------------|----------------|-----------------|-------------------|----------------|
| Anthrocyanins | Tannins        | Anthrocyanins   | Terpenoids        | Phenol         |
| Starches      | Polyphenols    | Terpenoids      | Flavonoids        | Flavonols      |
| Tannins       | Polyacetylenes | Saponins        |                   |                |
| Saponins      | Flavonols      | Tannins         |                   |                |
| Terpenoids    | Terpenoids     | Flavones        |                   |                |
| Polypeptides  | Sterols        | Phenones        |                   |                |
| Lectines      | Alkaloids      | Polyphenols     |                   |                |

In this study, extraction was done using maceration and serial exhaustive extraction methods. These were selected due to their simplicity and straightforwardness. In maceration, a whole or coarsely powdered plant is kept in contact with the solvent in a closed container for a defined period, with frequent agitation until soluble matter is dissolved. The mixture is then strained and the liquid is clarified by filtration or decantation after standing (Pandey and Tripathi, 2013). In serial exhaustive extraction, successive extraction with solvents of increasing polarity from non-polar (n-hexane) to more polar (methanol) solvent takes place. This is to ensure that wide ranges of compounds of varying polarity are extracted. These two methods are best suited for extraction of thermolabile compounds (Das *et al.*, 2010).

After extraction, the extract is concentrated by leaving it open at room temperature to evaporate the solvent. When reconstituting the extract, a measured volume of solvent is used to dissolve the extract to required working concentrations. The extract is then used for metabolic profiling and tested for different biological activities or any other analysis as required (Majekodunmi, 2015).

Thin layer chromatography (TLC) is a chromatography technique used to separate mixtures. It can be employed in various purposes, these include monitoring the



progress of a reaction, identification of compounds present in a given substance and determination of the purity of a substance. It can be performed on a sheet of glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide, or cellulose (blotter paper). The thin layer is referred to as the stationary phase. In this technique, the test sample is applied at the bottom of the plate and a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action. Separation then occurs because different analytes ascend the TLC plate at different rates. Separation of compounds is based on the solubility of compounds to the eluent system (Bele and Khale, 2011).

The presence of major phytochemicals in crude extracts is detected by the performance of standard chemical tests. These include; the Wagner's and Hager's test for alkaloids, Borntrager's test for anthraquinones, Keller killiani test for cardiac glycosides, Ferric chloride test for tannins, Frothing and Haemolysis test for saponins and the Salkowski's test for steroids (Patil and Khan, 2016). Quantification of the major phytochemicals such as the total phenolic and tannin compounds is done using the Folin-Ciocalteu method. This involves a reaction between the Folin-Ciocalteu reagent and phenolic compounds present in the extracts and result in the formation of a blue color complex that absorbs radiation and allows quantification. The total flavonoid content can be determined using the aluminum chloride. In the presence of flavonoid compounds, aluminum chloride forms a complex with the carbonyl and hydroxyl groups of the compounds and produces a yellow color (Pontis *et al.*, 2014). The aim of this chapter was to carry out a preliminary phytochemical screening of major classes of phytochemicals using TLC and the standard chemical tests, and quantify them using the Folin-Ciocalteu and aluminium chloride method.

## **3.2. METHODOLOGY**

### **3.2.1. Plant collection**

The *Olea europaea* subspecies *africana* medicinal herb was collected in February 2016, from the University of Limpopo, South Africa. The voucher specimen was deposited at the University of Limpopo Larry Leach Herbarium to be verified by Dr Bronwyn Egan. The herb was verified as *Olea europaea* subspecies *africana* and allocated a specimen number UNIN 11938. The leaves were removed from the

branches and air dried for few days under shade. Air-drying was ideal, as it does not force plant materials to dry using high temperature; hence, heat-labile compounds were preserved prior extraction. The leaves were ground into fine powder using an electric grinder (Sundy hamercrusher SDHC 150) and stored in an airtight black polythene bag for later use. The bags provided an environment that was light and air free.

### **3.2.2. Preliminary extraction procedure**

The dried finely grounded leaves of *Olea europaea* subspecies *africana* (1g) were extracted with 10 mL of different organic solvents of varying polarities (n-hexane, dichloromethane, ethyl acetate, acetone, ethanol, methanol, n-butanol, chloroform and water) in 50 ml centrifuge tubes. The mixtures were shaken separately for 10 minutes at high speed (200 rpm) in a shaking incubator machine (New Brunswick Scientific Co., Inc.). Upon completion of extraction, the extracts were filtered using Whatman filter papers into labelled pre-weighed glass vials and the solvents were evaporated using a fan. The quantity of the plant material extracted was determined by subtracting the mass of the empty pre-weighed glass vials from the mass of the dried crude extracts in the vials. The dried crude extracts were stored at room temperature in the dark until use. The final extracts were reconstituted in acetone to a concentration of 10 mg/ml for phytochemical analysis.

### **3.2.3. Preliminary Phytochemical Screening**

Ground powdered leaves (1 g) were extracted with 10 mL of 95% ethanol and distilled water separately; these were treated as stock solutions for the testing of the presence of alkaloids, flavonoids, steroids, tannins, terpenoids, anthraquinones, tannins, saponins, phlobatannins and cardiac glycosides.

#### **3.2.3.1. Test for alkaloids (Dragendroff's Test)**

Three drops of Dragendroff's reagent (Sigma ®) were added to the 2 mL of the ethanol aqueous filtrate. Formation of a red precipitate indicated the presence of alkaloids (Harborne, 1973).

### **3.2.3.2. Flavonoids (Alkaline Reagent Test)**

Three drops of diluted ammonia solution (Sigma ®) were added to the 2 mL of the ethanol aqueous filtrate, followed by an addition of 1 ml concentrated sulphuric acid (Sigma ®). A yellow colouration that disappears on standing indicated the presence of flavonoids (Borokini and Omotayo, 2012).

### **3.2.3.3. Test for steroids (Liebermann-Burchard's test)**

Two millilitres of acetic anhydride (Sigma ®) was added to the 0.5 g of ethanol extract, followed by an addition of 2 ml sulphuric acid (Sigma ®) down the side of the test tube to form a layer underneath. The test tube was observed for green colouration as indicative of steroids (Borokini and Omotayo, 2012).

### **3.2.3.4. Test for terpenoids (Salkowski test)**

Three millilitres of concentrated sulphuric acid (H<sub>2</sub>SO<sub>4</sub>) (Sigma ®) was carefully added to 0.5 g of ethanol extract. A reddish brown colouration formed at the interface indicated the presence of terpenoids (Borokini and Omotayo, 2012).

### **3.2.3.5. Test for anthraquinones**

Ten millilitres of 97% sulphuric acid (H<sub>2</sub>SO<sub>4</sub>) was added to 0.5 g of ethanol extract, boiled and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipetted into another test tube and 1 ml of dilute ammonia was added. A pink colour indicated the presence of anthraquinones in the ammonia phase (Ayoola *et al.*, 2008).

### **3.2.3.6. Test for tannins (Ferric chloride Test)**

Three drops of 0.1% ferric chloride (Sigma ®) were added to the 2 mL of the water aqueous filtrate. A Brownish green or blue-black colouration represented the presence of tannins (Trease and Evans, 1989).

### **3.2.3.7. Test for saponins (Froth Test)**

Thirty millilitres of tap water was added to 1 g of powdered leaves. The mixture was vigorously shaken and heated at 100 °C. Froth formation indicated the presence of saponins (Odebiyi and Sofowora, 1978).

### **3.2.3.8. Test for phlobatannins**

A solution of 2% HCL (Sigma ®) was added to the 2 mL of the aqueous filtrate and boiled. The positive test was indicated by the formation of a red colour (Borokini and Omotayo, 2012).

### **3.2.3.9. Test for cardiac glycosides (Keller- Killiani test)**

Five millilitres of distilled water was added to 0.5 g of the dry water extract, this was then followed by an addition of 2 mL glacial acetic acid (Sigma ®) with one drop of 0.1% ferric chloride solution. This mixture was mixed with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of deoxysugar, which is a characteristic of cardenolides (Borokini and Omotayo, 2012).

### **3.2.4. Thin Layer Chromatography (TLC) analysis**

The chemical profile of extracts was determined by TLC to confirm the presence of bioactive compounds extracted in various extracts mentioned in Section 3.2.2. The plates used were aluminium-backed TLC plates (Merck, silica gel 60 F<sub>254</sub>) at a size of 10 × 10 cm. The extracts were reconstituted to a concentration of 10 mg/mL and volumes of 10 µl were spotted on TLC plates, 1 cm from the bottom of the plates. The TLC plates were developed in solvent systems of varying polarity, i.e. ethyl acetate/ methanol/ water (40:5.4:5): [EMW] (polar/ neutral); chloroform/ ethyl acetate/ formic acid (5:4:1): [CEF] (intermediate polarity/acidic); benzene/ ethanol/ ammonia hydroxide (90:10:1): [BEA] (non-polar/basic) (Kotze and Eloff, 2002). At the end of the chromatographic development, the plates were removed from the chromatographic tank, air dried under a fume-hood cabinet and observed under ultraviolet (UV) light (365 nm) for fluorescing compounds. To detect the presence of the secondary metabolites in the leaf extracts which were not visible under UV light, vanillin-sulphuric acid reagent [0.1 g vanillin (Sigma ®): 28 ml methanol: 1 ml concentrated sulphuric acid] was sprayed on the TLC plates. The plates were subsequently heated at 110 °C for 1 to 2 minutes for optimal colour development. The plates were scanned and analysed.

### **3.2.5. Quantification of major phytochemicals**

#### **3.2.5.1. Total phenolic content determination**

A solvent mixture of 70% aqueous acetone was used to extract the powdered leaves. This was followed by the determination of the total phenolic content using spectrophotometric method described by Singleton *et al.*, 1999 with minor modifications. The Folin-Ciocalteu method was used, where 0.1 mL of extract and 0.9 mL of distilled water were mixed in a 25 mL volumetric flask. To this mixture 0.1 mL of Folin-Ciocalteu phenol reagent (Sigma ®) was added and the mixture was shaken well. One milliliter of 7% Sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) solution (Sigma ®) was added to the mixture after 5 minutes. The volume was made up to 2.5 mL with distilled water. A set of standard solutions of gallic acid (0.0625, 0.125, 0.25, 0.5, and 1 mg/mL) were prepared in the same manner. The mixtures were incubated for 90 minutes at room temperature and the absorbance for test and standard solutions were determined against the reagent blank at 550 nm with an Ultraviolet (UV)/visible spectrophotometer. The formula obtained from the standard curve of gallic acid was used for the determination of total phenolic content. Total phenolic content was expressed as mg of GAE/g of extract (Tambe and Bhambar, 2014).

#### **3.2.5.2. Total tannin content determination**

The tannin content was determined using the Folin-Ciocalteu reagent method. About 0.1 mL of the 70% aqueous acetone extract was added to a 10 mL volumetric flask containing 5 mL of distilled water. To this mixture 0.2 mL of 2 M Folin-Ciocalteu phenol reagent and 1 mL of 35%  $\text{Na}_2\text{CO}_3$  solution was added and this was made up to 10 mL with distilled water. The mixture was shaken well and kept at room temperature for 30 minutes. A set of standard solutions of gallic acid (0.0625, 0.125, 0.25, 0.5, and 1 mg/mL) were prepared in the same manner. Absorbance for test samples and standard solutions were measured against the blank at 725 nm with a UV/Visible spectrophotometer. The formula obtained from the standard curve of gallic acid was used for the determination of total tannin content. The tannin content was expressed as mg of GAE/g of extract (Tambe and Bhambar, 2014).

### **3.2.5.3. Total flavonoid content determination**

Total flavonoid content was determined by the aluminum chloride colorimetric assay. One milliliter of 70% aqueous acetone extract was mixed with 4 mL of distilled water in a 10 mL volumetric flask; this was followed by an addition of 0.30 mL of 5% sodium nitrite. About 0.3 mL of 10 % aluminum chloride (Sigma ®) was added to the mixture after 5 minutes. After 5 minutes, 2 mL of 1 M Sodium hydroxide (Sigma ®) was added and this was made up to 10 mL with distilled water. A set of reference standard solutions of quercetin (0.0313, 0.0625, 0.125, 0.25, 0.5 mg/mL) were prepared in the same manner. The absorbance for test and standard solutions were determined against the reagent blank at 510 nm with a UV/Visible spectrophotometer . The formula obtained from the standard curve of quercetin was used for the determination of total flavonoid content. The total flavonoid content was expressed as mg of QE/g of extract (Tambe and Bhambar, 2014).

### **3.2.5.4. Statistical analysis**

All the experiments were carried out in triplicates and the data was presented as mean  $\pm$  standard deviation. Calculations were carried out using MS Office Excel 2007. All data was calculated using a linear regression formula ( $y=mx+c$ ) obtained from each standard curve.

### 3.3. RESULTS

#### 3.3.1. The quantity of plant material extracted

Active compounds from *Olea europaea* subspecies *africana* leaves were extracted using nine solvents of increasing polarity. The quantity of the extracts was measured in mg as shown in figure 3.1. Water was the best extractant, extracting (158.2 mg) of plant material. Methanol was the second best extractant (109.1 mg), followed by acetone (66.9 mg), ethanol (59.9 mg) and ethyl acetate (57.2 mg) and n-hexane (12.8 mg) extracted the least plant material.

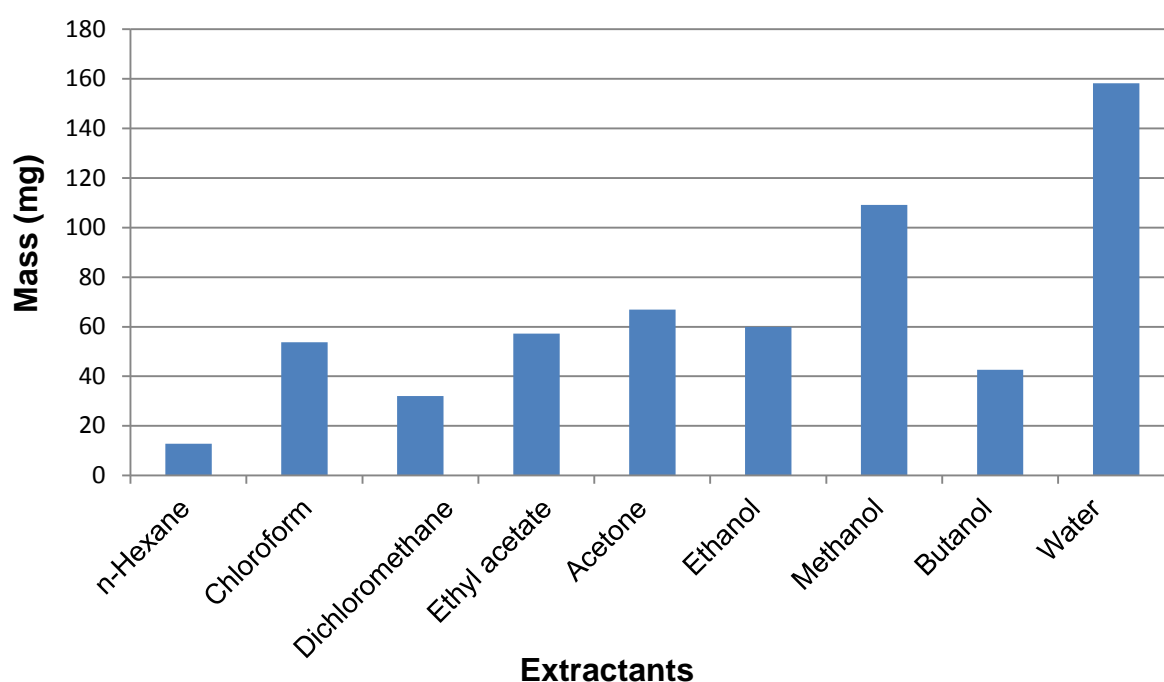


Figure 3.1: Mass of *Olea europaea* subspecies *africana* extracts, extracted using solvents of increasing polarity.

### 3.3.2. Preliminary phytochemical screening

#### 3.3.2.1. Phytochemical screening through standard chemical methods

Preliminary phytochemical screening of *Olea europaea* subspecies *africana* leaves showed the presence of flavonoids, steroids, tannins and terpenoids (Table 3.2), while the rest of the tested phytochemicals were absent in the extracts.

Table 3.2: Qualitative phytochemical screening of *Olea europaea* subspecies *africana* leaves.

| Phytochemicals     | Occurrence |
|--------------------|------------|
| Alkaloids          | -          |
| Anthraquinones     | -          |
| Cardiac glycosides | -          |
| Flavonoids         | +          |
| Phlobatannin       | -          |
| Saponins           | -          |
| Steroids           | +          |
| Tannins            | +          |
| Terpenoids         | +          |

Keys: + = Present, - = Absent



### 3.3.3.2. Phytochemical screening by Thin Layer Chromatography

Thin layer chromatography was performed to separate phytochemicals present in the leaves of *Olea europaea* subspecies *africana*. Phytochemicals represented by bands were detected by UV light (365 nm) (Figure 3.2A) and vanillin-sulphuric acid reagent (Figure 3.2B). Most bands were detected on the TLC plates developed in BEA followed by EMW then CEF solvent system.

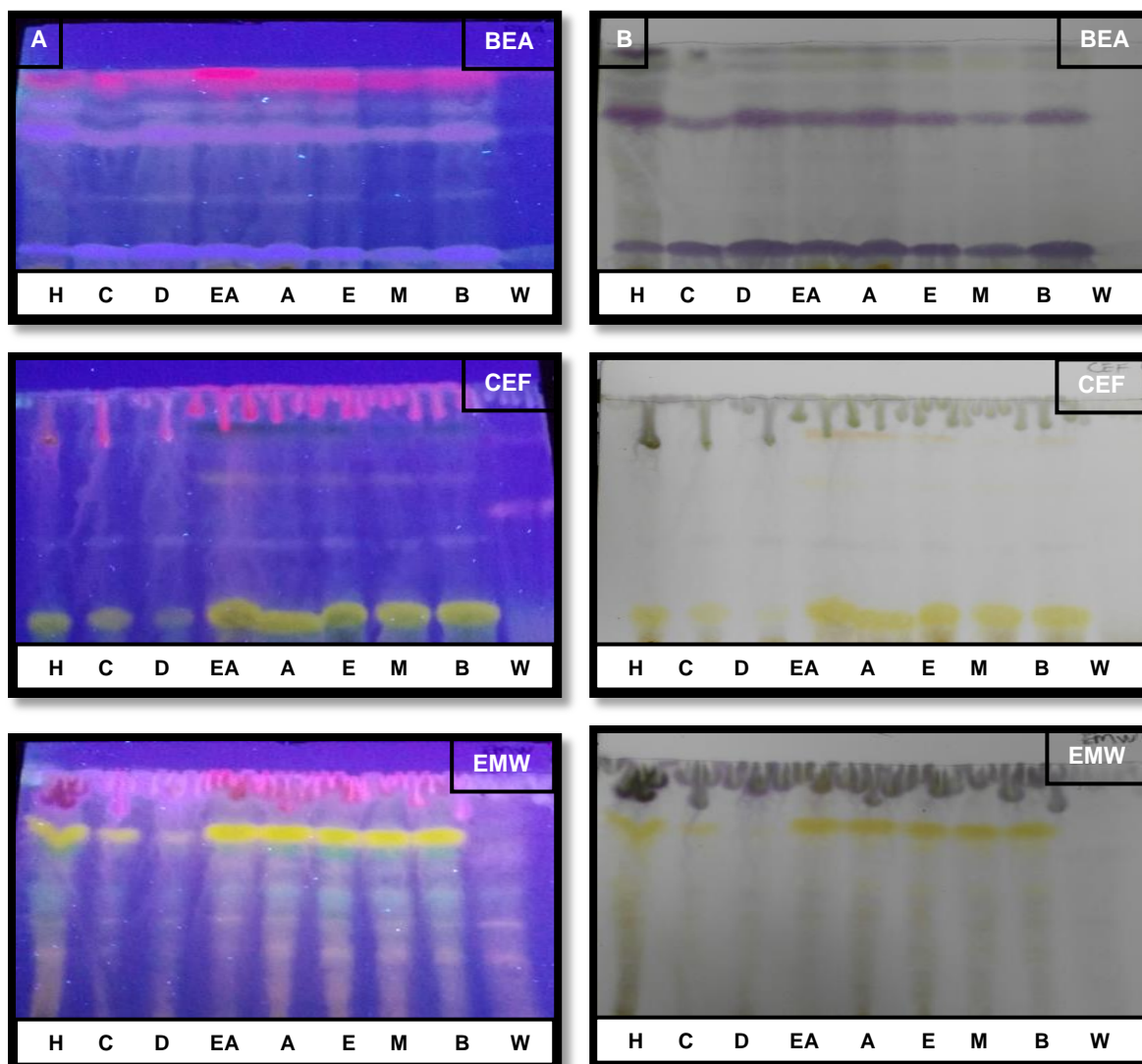


Figure 3.2: TLC plates visualised under UV light at 365 nm (A) and sprayed with vanillin-sulphuric acid reagent (B). The compounds separated on the TLC plates were extracted with n-hexane (H), chloroform (C), dichloromethane (D), ethyl acetate (EA), acetone (A), ethanol (E), methanol (M), butanol (B) and water (W).

### 3.3.3.3. Quantification of major phytochemicals

The total concentrations of phenolic, tannin and flavonoid contents were determined using the equations obtained from the calibration curves of gallic acid (Figure 3.3 and 3.4) and quercetin (Figure 3.5) at different concentrations. The curves were linear, indicating a direct relationship between absorbance and concentration of the phytochemicals.

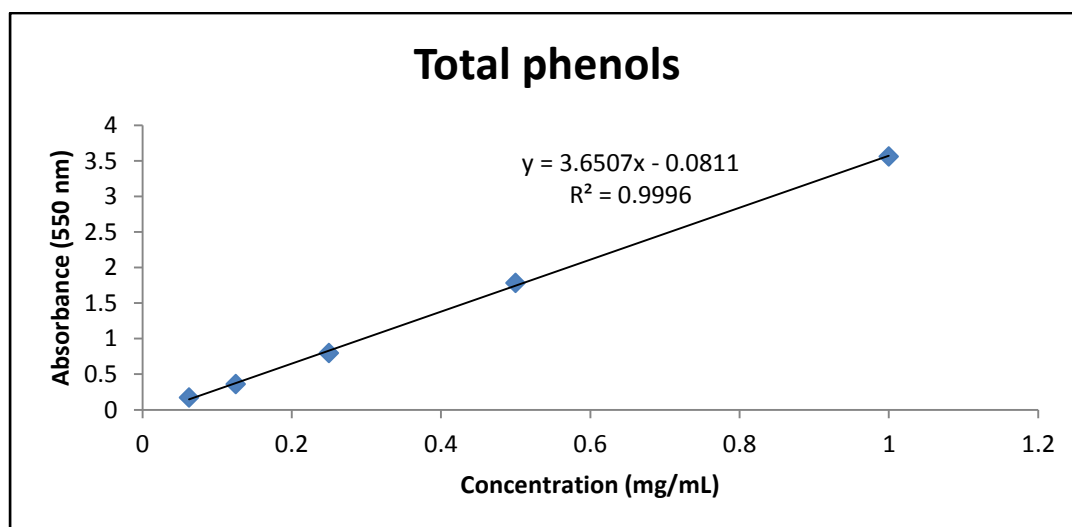


Figure 3.3: Standard curve of gallic acid in mg/mL used as a standard to quantify total phenolic content in *Olea europaea* subspecies *africana* at 550 nm.

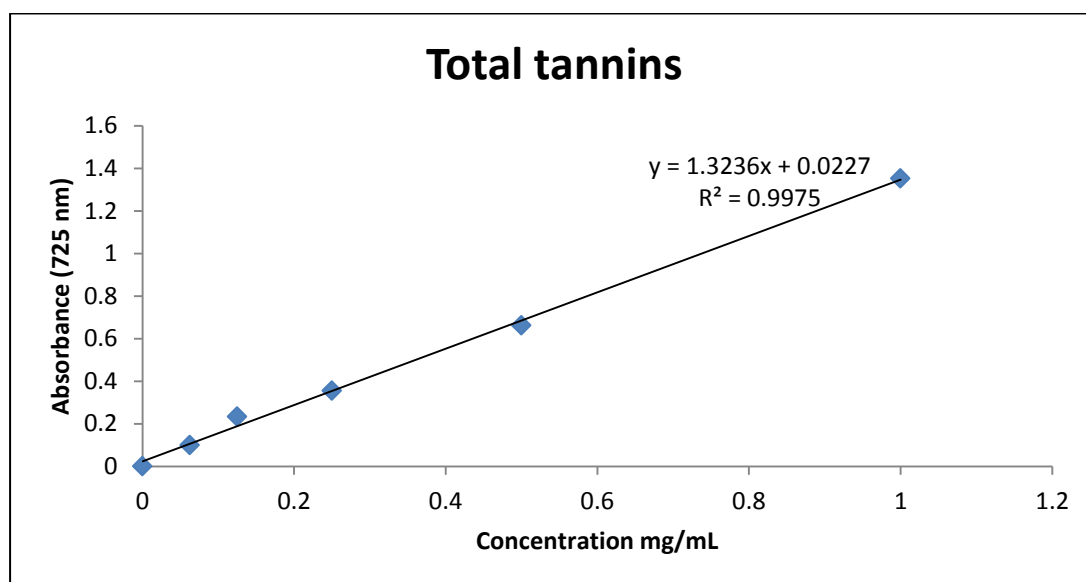


Figure 3.4: Standard curve of gallic acid in mg/mL used as a standard to quantify total tannin content in *Olea europaea* subspecies *africana* at 725 nm.

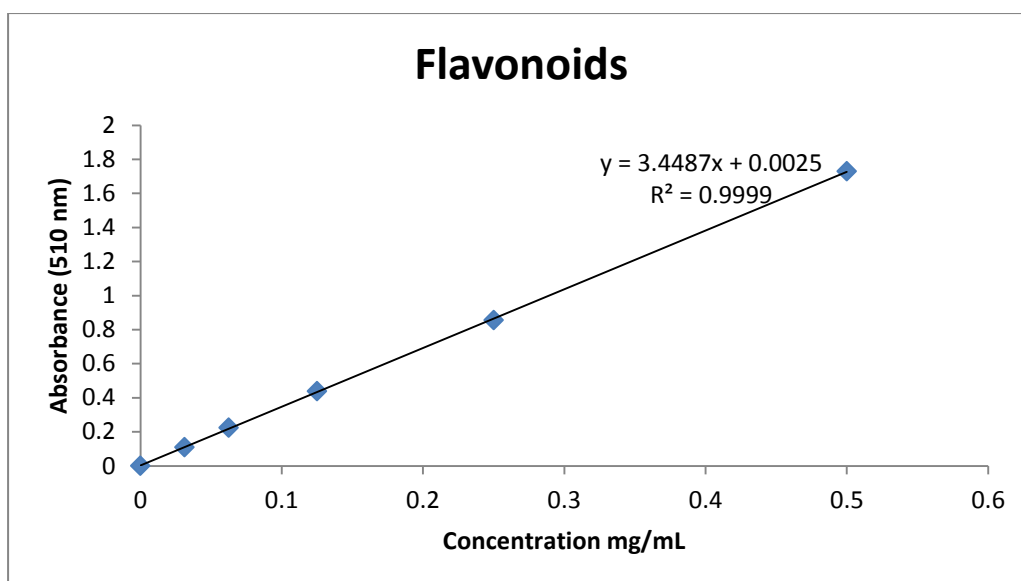


Figure 3.5: Standard curve of quercetin in mg/mL used as a standard to quantify total flavonoid content in *Olea europaea* subspecies *africana* at 510 nm.

An extract prepared with 70% aqueous acetone was used for the analyses. The concentrations of the assayed phytochemicals ranged from  $23.97 \pm 1.15$  mg of QE/g to  $114.33 \pm 9.02$  mg of GAE/g. Tannin content was the highest among the assayed phytochemicals with a value of  $114.33 \pm 9.02$  mg of GAE/g and flavonoids content was low with a value of  $23.97 \pm 1.15$  mg of QE/g (table 3.3).

Table 3.3: Quantification of total phenolic, tannins and flavonoid content in the leaves of *Olea europaea* subspecies *africana*.

| Phytochemicals          | Quantity          |
|-------------------------|-------------------|
| Phenols(mg of GAE/g)    | $99.67 \pm 2.52$  |
| Tannins (mg of GAE/g)   | $114.33 \pm 9.02$ |
| Flavonoids (mg of QE/g) | $23.97 \pm 1.15$  |

Results were represented as  $\pm$  standard deviation; GAE: Gallic acid equivalence; QE: quercetin equivalence.

### 3.4. DISCUSSION

*Olea europaea* subspecies *africana* was selected in this study based on its use by traditional healers in southern Africa and around the world for combating bacterial, free radical and inflammation related diseases (Long *et al.*, 2010). Before extraction, the leaves of *Olea europaea* subspecies *africana* were ground to coarse smaller particles, to increase the surface area and ensure efficient extraction. It was reported that particle sizes smaller than 0.5 mm were ideal for efficient extraction, hence grinding of plant material prior extraction is paramount (Nollet *et al.*, 2016).

Throughout the study, the ground leaves were stored in an air tight, black plastic bag. This was to avoid air and direct sunlight. Both air and sunlight have oxidation abilities and may result in chemical degradation/reactions of bioactive compounds in the leaves. Most scientists prefer to use dried samples over fresh samples because fresh samples are fragile and tend to deteriorate faster than dried samples (Azwanida, 2015).

Solvents of increasing polarities were used in this study to extract bioactive compounds of varying polarities. The phytochemical groups of interest ranged from non-polar terpenoids extracted with n-hexane to polar saponins extracted with methanol and water. For phytochemical analysis, acetone was used to reconstitute the plant extracts. The use of acetone to reconstitute has been reported by Eloff (1998a) and is confirmed to be non-toxic towards bacteria and fungi (Masoko *et al.*, 2007) and it is able to dissolve both hydrophilic and hydrophobic components in an extract.

The extraction yields represented in figure 3.1, showed that water extracted most of the plant material (158.2 mg), followed by methanol (109.1 mg). Despite water extracting most of the plant material, it was not considered the best extractant to further the study with. The reasons being that it only extracts polar compounds, it partially solubilises phytochemicals with antioxidant activity, its extracts have been found to possess less potent antimicrobial activities and it does not evaporate quickly from the extracts (Lin *et al.*, 1995; Das *et al.*, 2010; Xu *et al.*, 2012). Besides the mentioned down falls, it is still conventionally used by the traditional healers to prepare their concoctions. This is because it is easily accessible and non-toxic to

human and animals; however, it is not recommended for phytochemical analysis in a laboratory environment (Eloff, 1998a).

The ability of methanol to extract most leaf material (109.1 mg) could be because the plant consisted of many polar compounds. This was also observed by Paulsamy and Jeeshna (2011), where in their study, methanol also extracted a great quantity of plant material and they concluded that this may be due to methanol's high polarity which can extract a variety of plant constituents. The low extraction yield demonstrated by n-hexane could be because it had a zero index of polarity and was only able to extract lipophilic compounds (Abarca-Vargas *et al.*, 2016).

Preliminary phytochemical analysis is important in determining the chemical constituents present in plant materials. It is also useful in locating the source of pharmacologically active chemical compounds. That is why it was firstly done through the use of standard chemical methods and the results indicated the presence of flavonoids, tannins, steroids and terpenoids were present in the leaf extracts (Table 3.2). These phytochemicals serve as defence mechanisms in plants against microorganisms, insects and other herbivores (Mehni *et al.*, 2004). They were also reported to be responsible for scavenging free radicals in plants (Ikhwan Rizki *et al.*, 2017).

The presence of the phytochemicals was further strengthened by TLC analysis, to demonstrate various constituents in the leaf extracts (Figure 3.2). The use of TLC analysis indicated how many constituents are there in the crude extracts and it validated the presence of different phytochemicals. The phytochemicals were indicated by different colour developments, which appeared as bands when viewed under UV light (365 nm) and after spraying with vanillin-sulphuric acid reagent (Sasidharan *et al.*, 2011).

After spraying the chromatograms with vanillin-sulphuric acid reagent, it was revealed that BEA separated the phytochemicals better than EMW. However, more bands were also observed under UV light on the TLC plate developed in EMW. The results suggested that the plant contained more non-polar and polar compounds. Both the solvent systems (EMW and BEA) could be used in the isolation of pure compounds by column chromatography to obtain pure compounds.

The major phytochemicals proven present in the extracts were quantified to determine their concentration in the crude extracts (Table 3.3). Tannin content was the highest among the assayed phytochemicals, with a value of  $114.33 \pm 9.02$  mg of GAE/g, followed by total phenolics ( $99.67 \pm 2.52$  mg of GAE/g) then flavonoids with a value of  $23.97 \pm 1.15$  (mg of QE/g). It was reported that the concentration of phenolic content is directly proportional to its antioxidant activity and that phenolic compounds have antimicrobial activities (Berkada, 1978; Stankovic, 2011).

Since phenolic phytochemicals include flavonoids and tannins, then in this study it was evident that the tannin content dominated as phenolic compounds, whilst flavonoids were only constituted in low quantities. These findings suggested that, *Olea europaea* subspecies *africana* could have strong antioxidant and antimicrobial activities.

### **3.5. CONCLUSION**

Extraction of phytochemicals resulted in isolation of a wide range of isolates of varying polarities. This enabled efficient screening for the presence of major phytochemicals and their quantities in the leaves of *Olea europaea* subspecies *africana*. The major phytochemicals assayed and found to be present, are known to possess both antioxidant and antibacterial activities. Further investigations to determine whether the plant indeed contained the compounds responsible for the mentioned activities will be evaluated in the next chapter.

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## CHAPTER 4: ANTIOXIDANT ASSAYS

### 4.1. INTRODUCTION

Antioxidants are gaining so much popularity, more especially in the health sector and in the food industry. They are defined as substances that even in small amounts can prevent oxidation of easily oxidisable materials (Bhatt *et al.*, 2013). In the ancient times, people used to obtain their antioxidants from traditional herbal medicines and dietary foods and these protected them from the damage caused by free radicals (Sen and Chakraborty, 2011). Phytochemicals that have been reported to have the most powerful antioxidant properties include the phenolic compounds and flavonoids (Halliwell, 2007). The hydroxyl groups in these phytochemical's structures are the ones responsible for their antioxidant properties (Saggu *et al.*, 2014).

There are various methods that could be used to investigate the antioxidant property of samples. Several *in vitro* assays have been reported and can be grouped into two main groups: those that evaluate lipid peroxidation and those that measure free radical scavenging ability (Miguel, 2010). A single method is not sufficient to fully describe the antioxidant activity of a sample since it is a complex procedure that is usually influenced by many factors and occur through several mechanisms. Therefore, it is of importance to perform more than one type of assay to measure the antioxidant capacity of a sample, to take into account the various mechanisms of antioxidant action (El Jemli *et al.*, 2016).

In this study, two complementary tests were used to assess the antioxidant activity of the leaves of *Olea europaea* subspecies *africana*. The assay 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging activity and the reducing power assay were employed. These assays were selected based on their simplicity, rapidness, accuracy and they are inexpensive to perform. DPPH assay is the most widely used assay, to evaluate the free radical scavenging activity of plant extracts (Pavithra and

Vadivukkarasi, 2015). In this assay, plant extracts are tested for their ability to reduce the violet 2, 2-diphenyl-1-picrylhydrazyl to a yellow colored 1,1-diphenyl-2-picrylhydrazine radical. The scavenging potential of the extracts with antioxidant activity is indicated by the degree of discoloration which is measured spectrophotometrically at 517 nm (Dehpour *et al.*, 2009).

The reducing power assay is generally based on the reduction of potassium ferricyanide ( $\text{Fe}^{3+}$ ) to form potassium ferrocyanide ( $\text{Fe}^{2+}$ ) by the antioxidants; this is then followed by the reaction of potassium ferrocyanide ( $\text{Fe}^{2+}$ ) with ferric chloride to form a ferric-ferrous complex that has a maximum absorption at 700 nm. In this assay, an increase in the absorbance of the reaction mixture indicates the degree of the reducing power of the sample. The concentration of the  $\text{Fe}^{2+}$  produced is monitored by measuring the formation of Perl's Prussian blue spectrophotometrically at 700 nm (Jayaprakasha *et al.*, 2001; Lee *et al.*, 2012; Loganayaki *et al.*, 2013).

Other *in vitro* assays include the hydrogen peroxide scavenging ( $\text{H}_2\text{O}_2$ ) assay, nitric oxide scavenging activity assay, trolox equivalent antioxidant capacity (TEAC) method/ABTS radical cation decolorization assay, ferric reducing-antioxidant power (FRAP) assay, hydroxyl radical scavenging activity assay, oxygen radical absorbance capacity (ORAC) assay, Cupric Reducing Antioxidant Power (CUPRAC) assay, organic radical scavenging (2,2-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid, ABTS) assay and metal chelating activity assay to mention a few (Pérez-Jiménez and Saura-Calixto, 2008; Alam *et al.*, 2013).

The aim of this chapter was to screen for the antioxidant capacity of the leaves of *Olea europaea* subspecies *africana* qualitatively through the use of the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay using Thin Layer Chromatography (TLC), as well as quantitatively through the use of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and the reducing power assays. The latter assays were performed spectrophotometrically.

## **4.2. METHODS AND MATERIALS**

### **4.2.1. DPPH free radical scavenging assay on TLC**

The crude extracts prepared as mentioned in Section 3.2.2 were used for qualitative antioxidant assay on TLC plates. The chromatograms prepared as mentioned in Section 3.2.4 were sprayed with 0.2% (w/v) of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma®) in methanol as an indicator. The development of yellow spots against a purple background on TLC plates indicated the antioxidising abilities of the extracts (Deby and Margotteaux, 1970).

### **4.2.2. DPPH free radical scavenging assay**

A concentration of 1 mg/mL of 70% aqueous acetone extract was prepared and used in the analysis of free radical scavenging activity. The assay was carried out according to the method described by Brand-Williams *et al.* (1995) with some modifications. A set of concentration ranging from 0.031 mg/mL to 1 mg/mL of the acetone aqueous extract were prepared serially. In each test tube, 1 mL of 0.2% DPPH was mixed with 1 mL of the extract at different concentrations followed by an addition of 10 mL methanol (Sigma ®) to dilute the solution and give a final volume of 12 mL. L-ascorbic acid was prepared using the same manner as the extracts. Methanol was used as blank while the DPPH solution was used as a standard control. The test tubes were shaken well and incubated in the dark for 20 minutes. The absorbance reading was measured at 517 nm using a spectrophotometer. The results were represented as EC<sub>50</sub> obtained from the linear regression formulas of the sample and standard control.

### **4.2.3. Reducing power assay**

This assay was used to measure the ability of an extract to reduce the potassium ferricyanide (Fe<sup>3+</sup>) to form potassium ferrocyanide (Fe<sup>2+</sup>) which will react with ferric chloride to form a ferric-ferrous complex that is monitored spectrophotometrically as described by Oyaizu (1986). Concentrations ranging from 0.0625 mg/mL to 1 mg/mL of aqueous acetone extract were prepared. A volume of 2 mL of the plant extract was mixed with 2 mL sodium phosphate buffer (1M, pH 6.6) and 2 mL potassium ferricyanide (1% w/v in distilled water), mixed well and incubated in a water bath for 20 minutes at 50 °C. To stop the reaction, 2.5 mL trichloroacetic acid (10% w/v in

distilled water) was added and the mixture was centrifuged at 650 rpm for 10 min. After centrifugation, 3 mL of the supernatant was added into a test tube followed by an addition of 10 mL distilled water and 1 mL ferric chloride (Sigma ®) (0.1% w/v in distilled water) solution. The solution was mixed well and the absorbance reading was measured at 700 nm. Blank was prepared similar to the plant extracts, however, an addition of the plant extract was replaced with acetone.

## 4.3. RESULTS

### 4.3.1. TLC-DPPH assay

The antioxidant activity of *Olea europaea* subspecies *africana* was determined qualitatively through TLC-DPPH assay, where crude extracts were screened for their ability to scavenge free radicals upon spraying the chromatograms with 0.2% DPPH in methanol solution. The results in figure 4.1, demonstrated the reduction of a violet colour of DPPH to yellow on all chromatograms. The results also indicate that most compounds exhibiting the antioxidant activities are intermediate to polar as many bands were observed in plates developed in CEF and EMW solvent system.

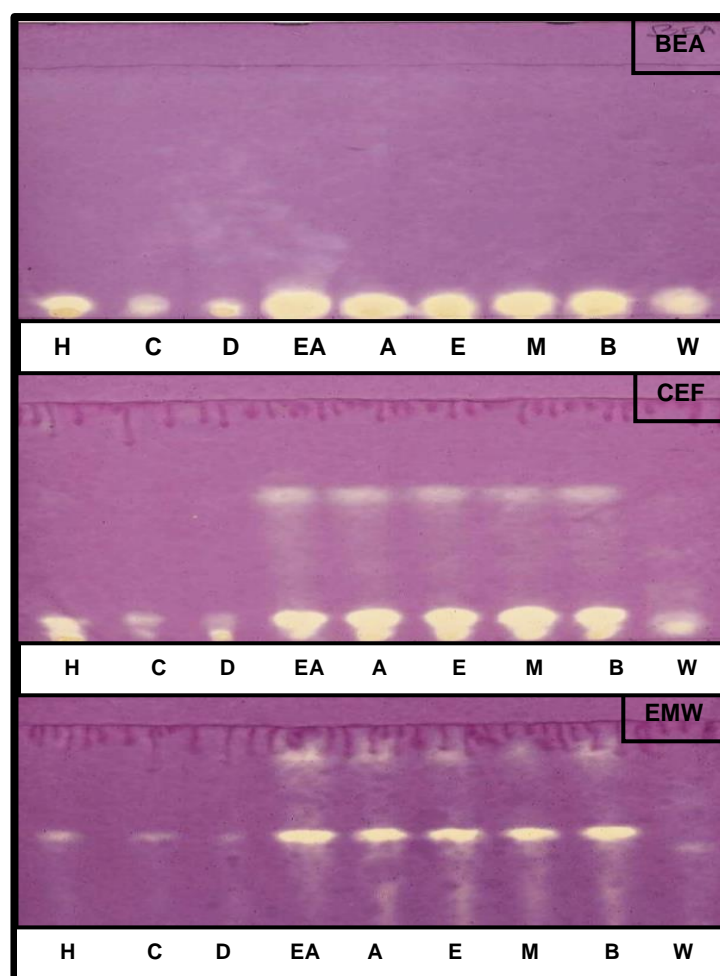


Figure 4.1: Chromatograms of *Olea europaea* subspecies *africana* developed in BEA, CEF and EMW solvent systems and sprayed with 0.2% DPPH. The yellow spots indicate the extracts containing compounds with antioxidant activity. The compounds separated on the TLC plates were extracted with n-hexane (H), chloroform (C), dichloromethane (D) ethyl acetate (EA), acetone (A), ethanol (E), methanol (M), butanol (B) and water (W).

### 4.3.2. DPPH assay

The 70% aqueous acetone extract was used to analyse the antioxidant potential of the plant quantitatively through DPPH assay. The EC<sub>50</sub> value was expressed as the concentration of antioxidant required to scavenge 50% of the DPPH radical. The smaller values indicated high antioxidant activity of the plant extract. The results demonstrated good free radical scavenging capacity (EC<sub>50</sub> of 1.05 ± 0.0071) (Table 4.1). This value was slightly higher than that of the standard control, however it is still regarded to be low and indicated good antioxidant activity.

Table 4.1: The EC<sub>50</sub> of the aqueous acetone extract and L-ascorbic acid. L-ascorbic acid was used as a standard control.

| Sample  | DPPH scavenging potential EC <sub>50</sub> |
|---|--|
| <i>Olea europaea</i> subspecies <i>Africana</i> | 1,05 ± 0,0071                              |
| L-Ascorbic acid (Standard)                      | 0,10 ± 0,0014                              |

Data is expressed as mean ± standard deviation of three independent experiments.

### 4.3.3. Reducing power assay

Antioxidant activity of the 70% aqueous acetone extract was also quantified using the reducing power assay. This was based on the ability of extracts with antioxidant activities to reduce ferric ions to a ferrous complex. An increase in the absorbance of the reaction mixture indicated the degree of the reducing power of the extract. The reducing power of ascorbic acid (standard) was higher than that of the extract at all concentrations. Nonetheless, the extract as well showed good reducing power and the activities in both the ascorbic acid and the extract are concentration dependent (Figure 4.2).

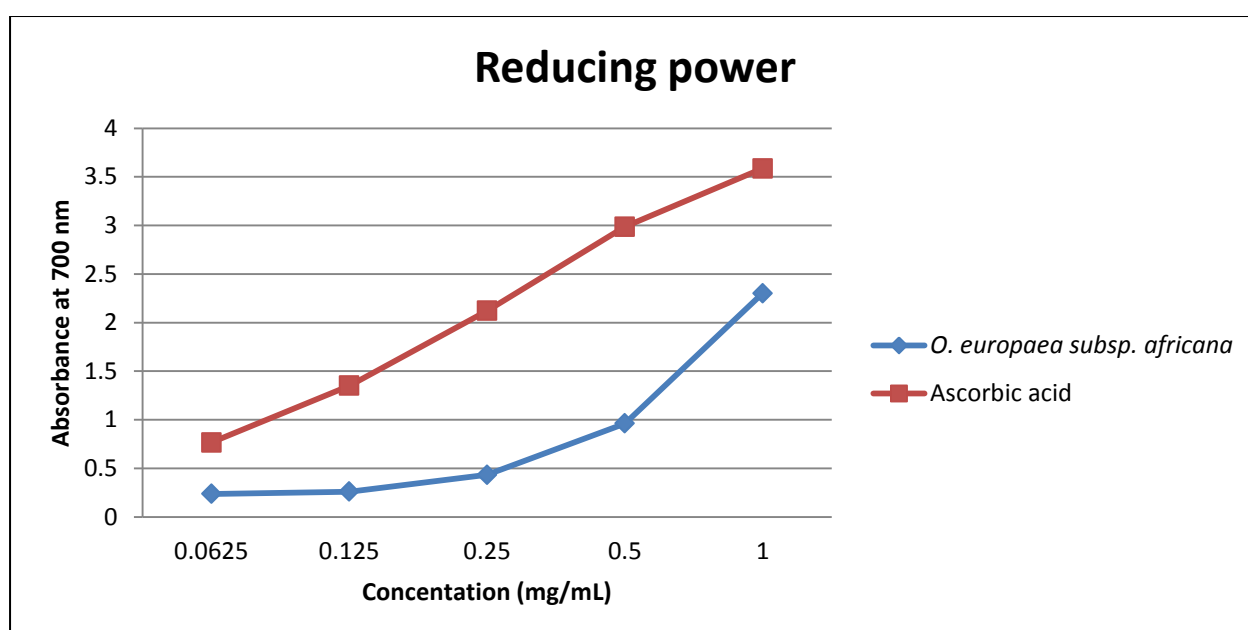


Figure 4.2: The reducing power of the aqueous acetone extract at an absorbance of 700 nm with respect to increasing concentrations. L-ascorbic acid was used as a reference standard.



#### 4.4. DISCUSSION

Screening of the leaf extracts qualitatively, using TLC-DPPH assay revealed that all extracts possessed antioxidant activities (Figure 4.1). This was seen as yellow spots on the TLC plate against a violet background after spraying the plates with DPPH reagent. On chromatogram developed in BEA, the extracts failed to separate as compared to the extracts separated in CEF and EMW. These demonstrated that the active compounds were polar, given the fact that the solvent systems used, i.e. CEF and EMW are polar.

Best separation of the extracts was seen on the TLC plates developed EMW, wherein all extracts separated into different compounds. On chromatogram developed in CEF, n-hexane, chloroform, dichloromethane, and water extracts failed to separate. The results demonstrated the nature of the active compounds in terms of polarity; this gave an indication that the active compounds need a solvent system that is polar to isolate them in column chromatography. The results also indicated that the extracts to consider for isolation were ethyl acetate, acetone, ethanol, methanol and n-butanol. This was guided by the intensity of the yellow color of the spots on the TLC plates. Extracts of chloroform, dichloromethane and water showed low antioxidant activities, indicated as by the low yellow intensity. Most antioxidants have been reported to be polar (Kotze and Eloff, 2002).

The extracts demonstrating good activity were noted to be extracted with intermediate to polar solvents. Polarity plays a huge role in extraction of compounds with antioxidant activities. In most cases, polyphenols from fruits and vegetables were reported to be recovered from the plant matrix using polar solvents (Peschel *et al.*, 2006). The solvents frequently used to extract such compounds include ethyl acetate, acetone, ethanol and methanol. Solvent mixtures such as acetone-water were also found to be effective in extracting compounds with antioxidant activities and are therefore used in the extraction of polar antioxidants (Zlotek *et al.*, 2016).

Prior the quantification analyses, the powdered leaves were extracted with 70% acetone: 30% water. The observed antioxidant activity was quantified spectrophotometrically through the DPPH free radical scavenging activity assay. One huge advantage of using this method is that it allows quantification of both lipophilic and hydrophobic compounds and not restricted by the nature of the

antioxidants as compared to other available methods (Apak *et al.*, 2016), hence it was selected to be used in this study.

The activity was expressed as EC<sub>50</sub>, which is a parameter that is typically employed to express the antioxidant capacity as well as to compare the activity of different compounds (Chen *et al.*, 2013). EC<sub>50</sub> was defined as the concentration of the antioxidants required to scavenge 50% of DPPH, and the smaller the value, the higher the antioxidant activity of the plant extract (Maisuthisakul *et al.*, 2007). The results obtained indicated that the aqueous acetone extract was found to have an EC<sub>50</sub> of 1.05 ± 0.0071 mg/mL while L-ascorbic acid had a value of 0.10 ± 0.0014 mg/mL (Table 4.1); despite that, the results indicated that *Olea europaea* subspecies *africana* was a potent source of antioxidant compounds.

The reducing power of the extract was represented in figure 4.2 and an increase in the absorbance of the reaction mixture indicated that the sample had greater reducing power (Irshad *et al.*, 2012). The results indicated that, the reducing power of the extract increased with an increase in the amount of extract. At the lowest concentration of 0.0625 mg/mL, the reducing power was low, with an absorbance of 0.236 nm when compared to the reducing power of the standard (L-ascorbic acid) which had absorption of 0.765 nm at a similar concentration. However, the reducing power of the leaves increased gradually at concentration 0.125 mg/ml, followed by a two-fold increase from concentration 0.5 mg/mL to 1 mg/mL. At the highest concentration, *Olea europaea* subspecies *africana* leaves showed a reducing power of about 2/3 of that of L-ascorbic acid, indicating good reducing power ability.

The results obtained in the two assays employed are supported by the results demonstrated in the previous chapter (Table 3.3), of the high amount of total phenolic content (99.67 ± 2.52 mg of GAE/g) that the plant contained. It has been reported that plants demonstrating high amounts of phenolic contents, have high antioxidant capacity. This is because phenolic compounds are the main antioxidant components and their total contents are directly proportional to their antioxidant activity (Razali *et al.*, 2008; Liu *et al.*, 2009). It has also been reported that there is a direct correlation between antioxidant activity and the reducing power of plants (Mohamed *et al.*, 2009). The high phenolic content was in correlation with the reducing power of the leaves as well, since this assay was based on the ability of an

antioxidant to donate an electron, which is a very important mechanism of phenolic antioxidant action (Benzie *et al.*, 1999; Koleva *et al.*, 2012).

#### **4.5. CONCLUSION**

The antioxidant activity observed qualitatively and quantitatively was found to be directly related to the phenolic content contained in the leaves. The solvent systems and the extracts that separated best, guided isolation in terms of which solvent system to use and which extracts to consider for isolation.

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## CHAPTER 5: ANTIBACTERIAL ASSAYS

### 5.1. INTRODUCTION

Traditional healers have been using plants for a very long time to prevent and cure illnesses. The biologically active substances contained in the plants i.e. phytochemicals, are responsible for medicinal activities of the plant. The medicines prepared from plants are used to combat infections caused by pathogens such as bacteria. These pathogens are responsible for the prevalence of human diseases in most immunocompromised patients (Suleiman, 2010; Savithramma *et al.*, 2011; Choma and Jesionek, 2015). With the world facing the emergence of antibiotic resistant bacteria and the evolution of new diseases, there is a need to develop new antibiotics (Demain, 1999).

Knowledge of the chemical constituents of plants is desirable because such information will be of value for the synthesis of complex chemical substances. For this reason, phytochemical screening of plants is essential. To detect these chemical constituents, bioassays were developed with the purpose of screening for compounds with therapeutic relevance (Hostettmann *et al.*, 2001). The biological activity of a plant such as antimicrobial activity can be detected by various methods, like diffusion, dilution and bioautography (Patil *et al.*, 2013).

The dilution method has been regarded as the most appropriate method to use for the determination of minimum inhibitory concentration (MIC) values of the plant extracts. This is because the concentration of the tested antimicrobial agent in the broth medium can be easily estimated. The MIC value obtained is defined as the lowest concentration of the assayed antimicrobial agent that completely inhibits the visible growth of the microorganism tested. The value can be expressed as mg/mL or mg/L. Test tubes may be used in macrodilution and for smaller volumes, 96-well microtiter plate (microdilution) may be used. For the determination of MIC values, a number of colorimetric methods based on the use of dye reagents have been developed. These include *p*-iodonitrotetrazolium chloride (INT), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2,3-bis {2-methoxy-4-nitro-5-[(sulfenylamino) carbonyl]-2H-tetrazolium-hydroxide} (XTT) (Balouiri *et al.*, 2016). The results may be read using a microplate-reader or visual reading in the



absence of a spectrophotometer. This biological assay is regarded of simple, reproducible, sensitive and inexpensive to perform (Elisha *et al.*, 2017).

Another method that can be used to assay for antimicrobial activity is bioautography. This is a sensitive method that can detect antimicrobial compounds even in small amounts. It can also allow localisation of activity in complex plant extracts; this therefore, aids the target-direct isolation of the active constituents (Rahalison *et al.*, 1991; Patil *et al.*, 2013). This technique is simple, rapid, effective and inexpensive, which means it can be performed in sophisticated laboratories as well as small research laboratories. In most cases, bioautography is applied to fast screen a large number of samples for bioactivity such as antibacterial, antifungal, antioxidant and antitumor enzyme inhibition (Dewanjee *et al.*, 2015).

TLC analysis is often combined with bioautography, its open layer enables solvent evaporation and allow comparison of the bioactivity of samples of various origins in parallel (Choma and Jesionek, 2015). Bioautography can be approached in three techniques to localise antimicrobial activity on TLC chromatogram. The three techniques include agar diffusion or contact bioautography, immersion or agar-overlay bioautography and direct bioautography. All these assays are based on a quick screening of new antimicrobial compounds through bioassay-guided isolation (Cos *et al.*, 2006). In this study, microbroth microdilution assay and TLC-direct bioautography were employed to screen the leaf extracts for antimicrobial activity against the selected bacterial strains.

## **5.2. METHODS AND MATERIALS**

### **5.2.1. Test microorganisms**

The test microorganisms were supplied by the Department of Biochemistry, Microbiology and Biotechnology section at the University of Limpopo. Two Gram-positive bacteria (*Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212) and two Gram-negative bacteria (*Escherichia coli* ATCC 28922 and *Pseudomonas aeruginosa* ATCC 27853) were used. The microorganisms were maintained on nutrient agar (Oxoid) at 4 °C as stock cultures. Prior screening tests, the microorganisms were cultured in a nutrient broth (Fluka analytical) and incubated overnight at 37 °C.

### **5.2.2. Bioautography assay**

Bioautography described by Begue and Kline (1972) was used to test for antimicrobial activity of crude extracts against test microorganisms. The crude extracts were reconstituted in acetone as mentioned in section 3.2.2. Thin layer chromatography plates were separated as described in section 3.2.4, with the only difference being 20 µL of extracts loaded on TLC plate instead of 10 µL. After the development of the chromatograms, the plates were dried at room temperature under a stream of air for 3-5 days to completely evaporate the solvents. The plates were sprayed with the test microorganisms, until wet and incubated at 37 °C in 100% relative humidity for 24 hours. For detection of microbial growth, 2 mg/mL of *p*-iodonitrotetrazolium chloride (INT) (Sigma®) was sprayed on chromatograms followed by 1 hour incubation. The appearance of clear zones on chromatograms against the purple-red background indicated inhibition of bacterial growth. The plates were scanned for later retrieval for data analysis.

### **5.2.3. Serial broth microdilution assay**

Minimum inhibitory concentration (MIC) values were determined using the serial broth microdilution method developed by Eloff (1998a). MIC was described as the lowest concentration that the assayed antimicrobial agent completely inhibited the visible growth of the microorganism. Crude extracts were reconstituted in acetone as mentioned in section 3.2.2. The microorganisms were serially diluted to 50% with

water in 96 well microtiter plates. Hundred microliters of each of the plant extracts was added into the first well and serially diluted with water. Bacterial cultures (100  $\mu$ L) were added to each well. Ampicillin (Sigma®) in  $\mu$ g/mL was used as a positive control and acetone was used as a solvent control. For negative control, only water and the tested bacteria were added into the wells. The microtiter plates were covered with plastic wrap (Glad) and incubated at 37 °C overnight to allow bacterial growth. To indicate the bacterial growth, 40  $\mu$ L of p-iodonitrotetrazohium violet [INT] (Sigma) dissolved in water was added to each microtiter well and incubated at 37 °C for 30 minutes. All determinations were carried out in triplicates and the results are the mean of the triplicates. The MIC was determined visually, where positive results were indicated by clear wells and negative results were indicated by a purple-red colour.

To determine which plant extracts contain potent antimicrobial activities and can be further used for isolation purposes, total antibacterial activity was calculated. This was determined by dividing the quantity extracted in milligrams from one gram of powdered leaves with the MIC value in milligrams per millilitre. The significance of total antibacterial activity is for determining the volume to which 1 g of plant material can be diluted and still inhibit the growth of the test organism (Eloff, 1999).

### 5.3. RESULTS

#### 5.3.1. Bioautography assay

Bioautograms of *E. coli* (Figure 5.1A), *P. aeruginosa* (Figure 5.1B), *E. faecalis* (Figure 5.2A) and *S. aureus* (Figure 5.2B). Clear bands on the chromatograms indicated the antibacterial activities of the leaf extracts, which inhibited the growth of the tested bacteria. Only the chromatograms developed in BEA demonstrated significant antibacterial activity against all tested bacteria and not much activity was observed on the bioautograms developed in CEF and EMW.

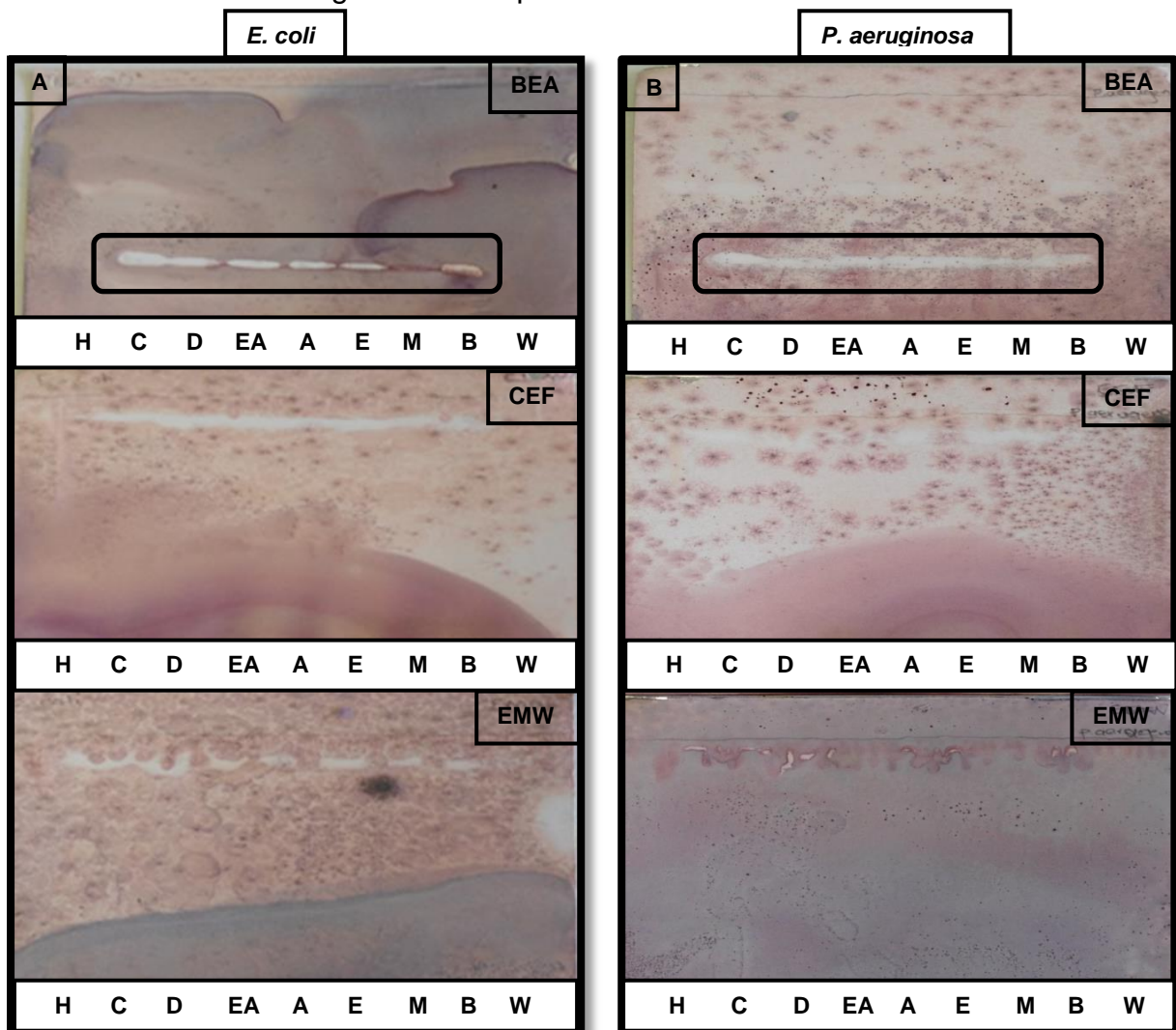


Figure 5.1: Bioautograms of *Olea europaea* subspecies *africana* leaf extracts extracted separated in BEA, CEF and EMW solvent systems and sprayed with *E. coli* (A) and *P. aeruginosa* (B).

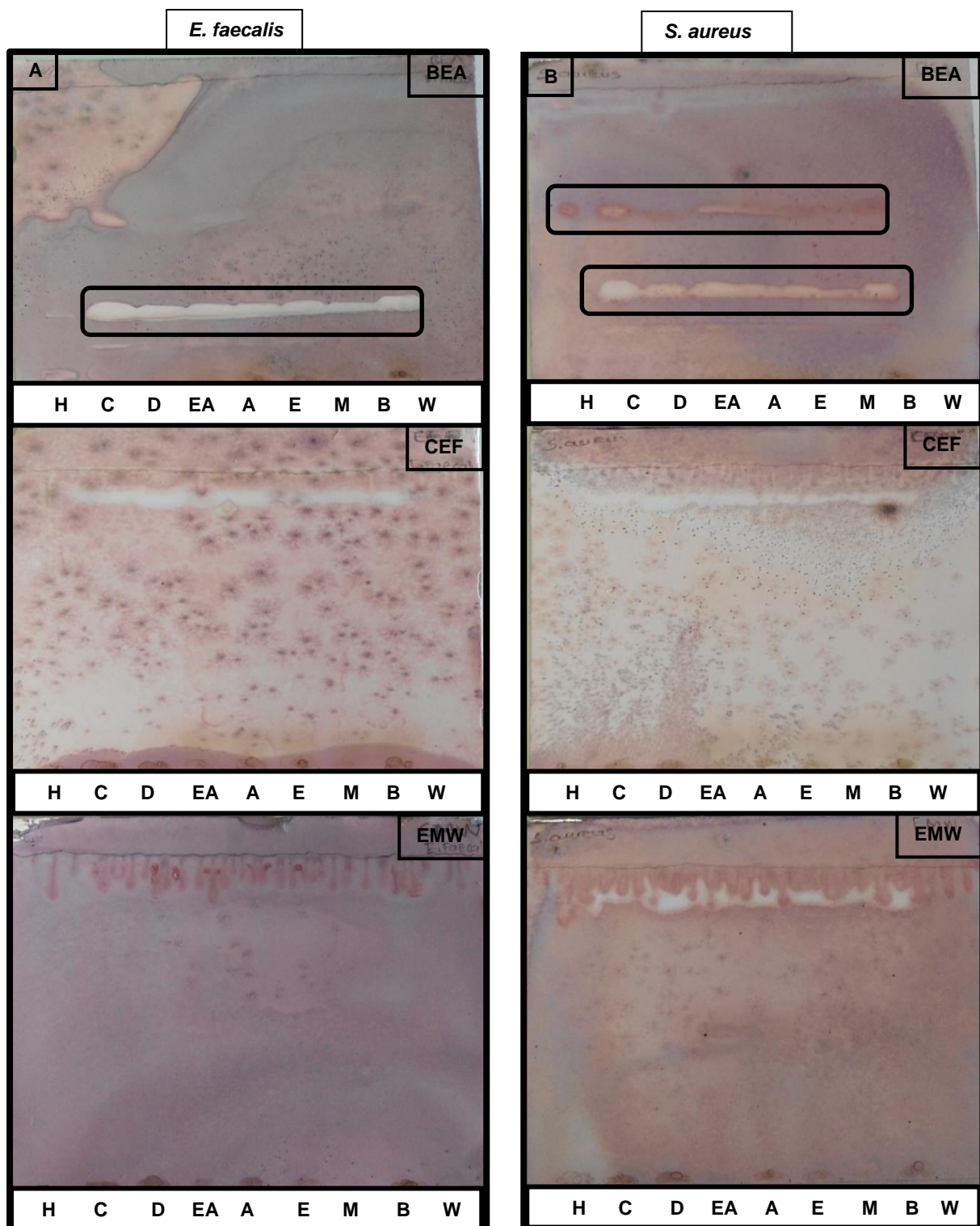


Figure 5.2: Bioautograms of *Olea europaea* subspecies *africana* leaf extracts extracted separated in BEA, CEF and EMW solvent systems and sprayed with *E. faecalis* (A) and *S. aureus* (B).

### **5.3.2. Serial broth microdilution assay**

The MIC values obtained following broth microdilution assay were recorded in table 5.1. The results showing bacterial sensitivity to the different extracts ranged from 0.16 to 2.50 mg/mL. The most sensitive bacterium to all the extracts was found to be *E. faecalis* with an average MIC value of 0.31 mg/mL. Ethyl acetate and acetone extracts had the lowest MIC values of 0.30 and 0.32 mg/mL, respectively. Methanol extract had the highest total antibacterial activity (Table 5.2), with a value of 1058 mL/g against *E. faecalis* and was an overall great antibacterial compound extractant.

Table 5.1: Minimum inhibitory concentration (MIC) values of various leaf extracts against four tested bacterial species and ampicillin (positive control).

| Microorganism        | MIC values (mg/mL) |             |             |             |             |             |             |             |             |             | AMP<br>(µg/mL) |
|----------------------|--------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|----------------|
|                      | H                  | C           | D           | EA          | A           | E           | M           | B           | W           | AVG         |                |
| <i>E. coli</i>       | 0.63               | 0.31        | 0.31        | 0.31        | 0.31        | 0.31        | 0.31        | 0.31        | >2.5        | <b>0.35</b> | 0.03           |
| <i>P. aeruginosa</i> | 1.66               | 0.52        | 1.04        | 0.47        | 0.42        | 0.52        | 0.63        | 0.73        | 2.08        | <b>0.9</b>  | 0.02           |
| <i>E. faecalis</i>   | 0.31               | 0.47        | 0.16        | 0.16        | 0.24        | 0.31        | 0.24        | 0.24        | 0.63        | <b>0.31</b> | 0.03           |
| <i>S. aureus</i>     | 1.66               | 0.26        | 0.37        | 0.26        | 0.31        | 0.26        | 0.31        | 0.26        | 2.5         | <b>0.67</b> | 0.08           |
| <b>Average</b>       | <b>1.07</b>        | <b>0.39</b> | <b>0.47</b> | <b>0.30</b> | <b>0.32</b> | <b>0.35</b> | <b>0.37</b> | <b>0.39</b> | <b>1.74</b> |             |                |

Key: n-hexane (H), chloroform (C), dichloromethane (D), ethyl acetate (EA), acetone (A), ethanol (E), methanol (M), butanol (B), water (W), (AVG) average, (AMP) ampicillin.

Table 5.2: Total antibacterial activity of the leaf extracts in mL/g.

| Microorganism        | Total antibacterial activity (mL/g) |            |            |            |            |            |            |            |            |            |
|----------------------|-------------------------------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
|                      | H                                   | C          | D          | EA         | A          | E          | M          | B          | W          | AVG        |
| <i>E. coli</i>       | 67                                  | 284        | 216        | 428        | 511        | 696        | 827        | 710        | NA         | <b>467</b> |
| <i>P. aeruginosa</i> | 25                                  | 169        | 64         | 282        | 377        | 415        | 407        | 301        | 121        | <b>240</b> |
| <i>E. faecalis</i>   | 136                                 | 187        | 419        | 829        | 660        | 696        | 1068       | 917        | 401        | <b>590</b> |
| <i>S. aureus</i>     | 25                                  | 338        | 181        | 510        | 511        | 830        | 827        | 846        | 101        | <b>463</b> |
| <b>Average</b>       | <b>63</b>                           | <b>245</b> | <b>220</b> | <b>512</b> | <b>515</b> | <b>659</b> | <b>789</b> | <b>694</b> | <b>208</b> |            |

Key: n-hexane (H), chloroform (C), dichloromethane (D), ethyl acetate (EA), acetone (A), ethanol (E), methanol (M), butanol (B), water (W), (AVG) average, (NA) not applicable.

## 5.4. DISCUSSION

The aim was to evaluate the antimicrobial activity of the leaves of *Olea europaea* subspecies *africana* by employing bioautography and serial broth microdilution assays. The bacterial growth indicator used in this study was  $\rho$ -iodonitrotetrazolium (INT). It acts as an electron acceptor and is reduced to a purple-red coloured formazan product by biologically active organisms (Eloff, 1998b). Its reduction is due to the activity of the mitochondria enzyme in the active bacterial cells (Berrington, 2013).

Screening the leaf extracts qualitatively by bioautography (Figure 5.1 A and B) and (Figure 5.2A and B), revealed that the extracts possessed compounds with good antibacterial activities. The appearance of clear zones indicated that at that particular band, INT reduction did not take place because the bacterial growth was inhibited. Of all solvent systems, BEA effectively separated most compounds with antibacterial activities, followed by CEF, then lastly EMW, which showed the least zones of inhibition. Antimicrobial activity was only observed on the bioautograms separated in the non-polar solvent system (BEA) and it was therefore concluded that the antimicrobial compounds were non-polar in nature. This gave a guidance that, upon isolation of active compounds, a non-polar solvent system would be best suited for further analysis.

All leaf extracts exhibited inhibitory characteristics towards the tested bacteria, except n-hexane and water extracts. The bioautograms sprayed with *E. coli* and *P. aeruginosa* showed similar profiles of clear zones (Figure 5.1A and B). However, n-hexane extract indicated a bit of antibacterial activities against *E. faecalis* and *S. aureus* as represented in figure 5.2A and B, respectively. The antibacterial activities demonstrated by the leaf extracts explains the use of the plant in treating infections such as urinary and respiratory tract infections as mentioned in section 2.4.2.

The inhibitory characteristics of the leaf extracts agree with the results obtained by Pereira and colleagues (2007), where the extracts of *Olea europaea* subspecies *europaea*, a close relative of *Olea europaea* subspecies *africana*, were evaluated for their antibacterial activities against the Gram-positive bacteria (*Bacillus*



*cereus*, *Bacillus subtilis*, and *Staphylococcus aureus*) and Gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumonia*). The results indicated good antibacterial activities.

Water extracts did not show any antibacterial activity against all tested bacteria. This observation was also done by Korukluoglu and colleagues (2010), where the aqueous extract of the olive leaves showed no antibacterial effect against *Salmonella enteritidis*, *Klebsiella pneumonia*, *Bacillus cereus*, *Escherichia coli*, *Streptococcus thermophiles*, *Enterococcus faecalis* and *Lactobacillus bulgaricus*. The effectiveness of *Olea europaea* subspecies *africana* against *S. aureus* also validates the plant's uses to treat wound infection, throat infections, and serious inflammations.

Quantification of the observed antibacterial activity done by serial broth microdilution assay, which indicated that the MIC values of the tested bacteria ranged from 0.16 to 2.50 mg/mL (Table 5.1). Ampicillin was used as a positive control as it is regarded as the commonly used broad-spectrum antibiotic in most laboratory environments. Having positive and negative controls in the experiment help in providing reference points to quantify the inhibitory effects of the extract dilutions (Frey and Meyers, 2010). In this study, the MIC values of 0.10 mg/mL or less were considered good while values up to 0.32 mg/mL were considered reasonable and the MIC values above 0.64 mg/mL were considered as having poor activity as described by Adamu *et al.* (2014).

The results indicated that *E. faecalis* (0.31 mg/mL) was the most susceptible bacterium, followed by *E. coli* (0.35 mg/mL). These microorganisms belong to the Gram-positive and Gram-negative groups, respectively. The results obtained indicated that the mechanism of bacterial inhibition might not be cell membrane targeted; hence, the extracts were effective against both Gram-positive and Gram-negative bacteria. It was reported that plant based-antimicrobials have different target sites in bacterial cells such as the disintegration of cytoplasmic membrane and coagulation of the cell content, unlike the conventional antibiotics, which act either by inhibiting cell wall, protein wall, DNA, RNA synthesis and other mechanisms (Singh and Barrett 2006; Silva and Fernandes, 2010). *Pseudomonas aeruginosa* and

*Streptococcus aureus* were the least sensitive microorganisms with the overall average MIC values of 0.90 and 0.67 mg/mL respectively.

The extracts that had the lowest average MIC values were dichloromethane and ethyl acetate; they both had MIC values of 0.16 mg/mL against *E. faecalis*. This demonstrated the effectiveness of the extracts. The extracts also exhibited stronger activity and a much broad-spectrum with an overall average MIC values of 0.30 and 0.32 mg/mL, respectively. Water extract had the highest MIC value of 2.50 mg/mL against *S. aureus*, which signified poor antimicrobial activity. This extract was also the least active extract against all four test bacteria with an average MIC value of 1.47 mg/mL, followed by n-hexane (1.07 mg/mL).

The MIC and total antibacterial activity values are useful pharmacological tools in determining the activity of extracts in mg/mL (potency) and the (efficacy) in mL/g, which is useful for the selection of plant species (Eloff, 2004). The results of total antibacterial activity of *Olea europaea* subspecies *africana* extracts against *E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus* were represented in table 5.2. For instance, methanol extract, which had the highest total activity (1068 mL/g) against *E. faecalis*, could be diluted to 1068 mL/g and it would still inhibit the growth of *E. faecalis*. n-Hexane extract displayed the lowest total activity with the values of 25 mL/g against both *P. aeruginosa* and *S. aureus*. The high total activity of methanol extract may be due to the presence of tannins, flavonoids and terpenoids and the fact that it extracted much plant material.

These secondary metabolites were found to be present in the leaves. They have been reported to have different mechanisms of exerting antimicrobial activities. Tannins inhibit cell wall synthesis by forming irreversible complexes with prolene rich proteins and terpenoid destroy the cell wall of microorganisms by weakening the membranous tissues. Steroids exert their antimicrobial activity by associating with membrane lipids and cause leakage from liposomes, thereby killing the microorganism (Mujeeb *et al.*, 2014). In both the direct bioautography assay and the serial broth microdilution assay, n-hexane and water extracts showed less to no clear zones on bioautography and high average MIC values of 1.07 and 1.74 mg/mL, respectively. These gave an indication that for isolation of active compounds, these

extracts should not be considered and only consider ethyl acetate or acetone extracts, which showed good antibacterial activity.

## **5.5. CONCLUSION**

The leaf extracts of *Olea europaea* subspecies *africana*, exhibited good antibacterial activities, this explains the traditional uses of this plant. BEA solvent system demonstrated good resolution of extracts; its polarity gave an indication that antibacterial compounds contained in the leaves are non-polar in nature. The MIC and the total antibacterial activity results showed that extracts with high antibacterial activity were species and not cell wall dependent. Ethyl acetate and acetone extracts had good average MIC values and indicating that these solvents have the potential to extract compounds with antibacterial activity.

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## CHAPTER 6: ANTI-INFLAMMATION ACTIVITY AND CYTOTOXICITY

### 6.1. INTRODUCTION

Oxidative stress is one of the most critical factors implicated in disease conditions. It may be caused by over production of ROS in response to inflammation and can cause major damage to the cells and their components such as DNA. It is also associated with several human degenerative diseases like cancer, neurodegenerative disorders and inflammation. These species are released by the neutrophils and activated macrophages in response to inflammation (Conforti *et al.*, 2008). Bacterial infections caused by *E. coli* which contains a lipopolysaccharide (LPS) has also been reported to cause excessive production of ROS as well as nitric oxide (Lonkar and Dedon, 2011; Sekhar *et al.*, 2015)

As such the LPS-treated RAW 264.7 cells have been widely used to study inflammatory responses (MacMicking *et al.*, 1997). Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria and has often been used in inflammatory response because it promotes the secretion of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF- $\alpha$ ) in many cell types, especially in macrophages and mediators (Debnath *et al.*, 2013). The RAW 264.7 cell line was used in the study as a model system for human macrophages. The macrophage cell line is used as a model system to screen for anti-inflammatory agents. The enzymes, effector free radicals and signaling pathways can be investigated using various detection techniques. The enzymes that are frequently measured in activated RAW cells are COX-1 and 2 and the iNOS due to their importance in the search for new anti-inflammatory agents (Criddle *et al.*, 2006).

Due to the probability of finding novel anti-inflammatory agents from medicinal plants that are already in use by herbalists, this study sought to evaluate the anti-inflammatory activity of the leaves of *Olea europaea* subspecies *africana*, which are used to relieve pain. The herbal remedies are used based on the anthropological knowledge of a community that is passed from one generation to the other. These communication methods can be prone to manipulation and misinformation (Li *et al.*, 2004). Hence, there is a need to validate some of the claims of the herbalists and

custodians of ethnomedicinal uses of plants and to document the use the medicinal plants with inflammatory activity.

Medicinal plants are assumed to be non-toxic and regarded safe due to their natural origin and long use in traditional medicine to treat various forms of diseases (Fennell *et al.*, 2004). However, there have been some indications of cytotoxic, genotoxic, and carcinogenic effects of many phytochemicals reported in scientific studies on efficacy and safety of some medicinal plants (Ernst, 2004). It should be noted that because traditional healers prepare their extracts using water, therefore, if a different extractant is used then the safety ascribed to traditional use is no longer relevant. The adverse effects of medicinal plant use arise due to organ toxicity, adulteration, contamination, contents of heavy metals, herb-drug interactions, poor quality control and inherent poisonous phytochemicals. The toxicity of some phytochemicals have been associated with diseases of the heart, liver, blood, kidney, central nervous system, gastrointestinal disorder such as diarrhea, and less frequently carcinogenesis (Tabasum and Khare, 2016).

The most commonly used assays for measuring cytotoxicity or cell viability following exposure to toxic substances are lactate dehydrogenase (LDH) leakage, protein quantification, neutral red and mitochondrial reduction (MTT) assays (Mosmann, 1983; Fotakis and Timbrell, 2006). The MTT assay is a rapid colorimetric test that was designed to measure only living cells. The assay is based on the ability of mitochondrial succinate dehydrogenase enzymes of metabolically active cells to reduce [3-(4,5-dimethylthiazol-2-yl)-2- 5diphenyltetrazolium bromide] MTT to a water insoluble purple formazan (Mosmann, 1983).

Due to an increased interest in research and development of medicinal plants worldwide, it is empirical to know the toxicity potential and efficacy of plants utilised ethnobotanically to treat ailments. Before embarking on studying the biological activity of medicinal plants, information about the toxicity properties of the plant should be searched in literature. In the absence of such information, the plant should be assayed for cytotoxicity to detect any potential toxicity. There is a clinical need to identify new compounds that are safe, for the prevention and treatment of inflammatory diseases and so far medicinal plants have been proven to be viable



alternatives to the discovery of new safer bioactive compounds (Gautam and Jachak, 2009; Hur *et al.*, 2012).

## **6.2. METHODS AND MATERIALS**

### **6.2.1. Anti-inflammatory assay**

Anti-inflammatory assay was carried out according to the method described by Sekhar *et al.* (2015). The cells were plated (2000 cells/well) in a 96-well plate and once they reached confluence (approximately 200,000 cells/well as visualised under inverted microscope), they were treated with the acetone extract at concentrations 0.32, 0.16 and 0.90 mg/mL. LPS (10 mg/ml) was added to each well to stimulate ROS production in the cells and then incubated the plate for 24 hours. The same treatment was done for curcumin (50  $\mu$ M) which was used as a positive control. Fluorescent dye, CM-H<sub>2</sub>DCFDA (100  $\mu$ l of 20 $\mu$ M) was added to cells and incubated for 30 min in a CO<sub>2</sub> incubator. After incubation, the cells were washed with cold-phosphate buffered saline (phosphate buffer saline [PBS]: 50 mM PBS [pH 7.2] containing 0.8% NaCl) and the fluorescence intensity of the stained cells was measured at an excitation and emission wavelength of 480 nm. The experiment was done in duplicates in three independent experiments.

### **6.2.2. Cell viability assay**

The tetrazolium-based colorimetric (MTT) assay described by Mosmann (1983) was used to determine the viable cell growth after incubation of African green monkey kidney (Vero) cells when tested with the acetone extract [0.025-1 mg/mL]. The cells of a subconfluent culture were harvested and centrifuged at 200 x g for 5 min. The cells were resuspended in growth medium to 5 x 10<sup>4</sup> cells/mL. Minimal Essential Medium (MEM, Whitehead Scientific) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Highveld Biological) was the growth medium used. A total of 200  $\mu$ l of the cell suspension was pipetted into each well of a sterile 96-well microtitre plate. Incubation was done for 24 hours at 37 °C in a 5% CO<sub>2</sub> incubator, until the cells were in the exponential phase of growth. The cells were washed with 150  $\mu$ L phosphate buffered saline (PBS, Whitehead Scientific). A volume of 200  $\mu$ L of crude extract with concentrations ranging from 10 to 200  $\mu$ g/mL was added into

the wells and the experiment was performed in quadruplicates. The serial dilutions of the test extracts and compounds were prepared in MEM. The microtitre plates were incubated at 37 °C in a 5% CO<sub>2</sub> incubator for 48 hours with test compound and extract. Untreated cells and positive control (doxorubicin chloride, Pfizer Laboratories) were also included. Detection of viable cells was done after incubation by adding 30 µL MTT (Sigma, stock solution of 5 mg/mL in PBS) into each well and incubated for 4 hours at 37 °C. The medium together with MTT (190 µL) were aspirated off the wells, DMSO (50 µL) was added and the plates shaken until the solution dissolved. The absorbance for each well was measured at 540 nm in a micro-titre plate reader (BioTek Synergy) at 570 nm. The wells containing medium and MTT but no cells, were used to blank the plate reader. The IC<sub>50</sub> values were calculated as the concentration of test compound resulting in a 50% reduction of absorbance compared to untreated cells.

### **6.2.3. Selectivity index**

The selectivity index indicates the cytotoxic selectivity (i.e. safety) of the crude extract against normal (Vero) cells versus bacterial cells. This was determined by dividing the LC<sub>50</sub> (in µg/mL) of the extract on Vero kidney cells by the MIC (µg/mL) values of the extracts on the tested pathogens (Elisha *et al.*, 2017).

## 6.3. RESULTS

### 6.3.1. Anti-inflammatory assay

Anti-inflammatory assay (Figure 6.1) revealed that the production of ROS in RAW 264.7 cells upon stimulation with LPS and treated with the acetone extract was inhibited in a dose dependent manner. The highest ROS inhibition was observed at the highest concentration tested (0.9 mg/mL).

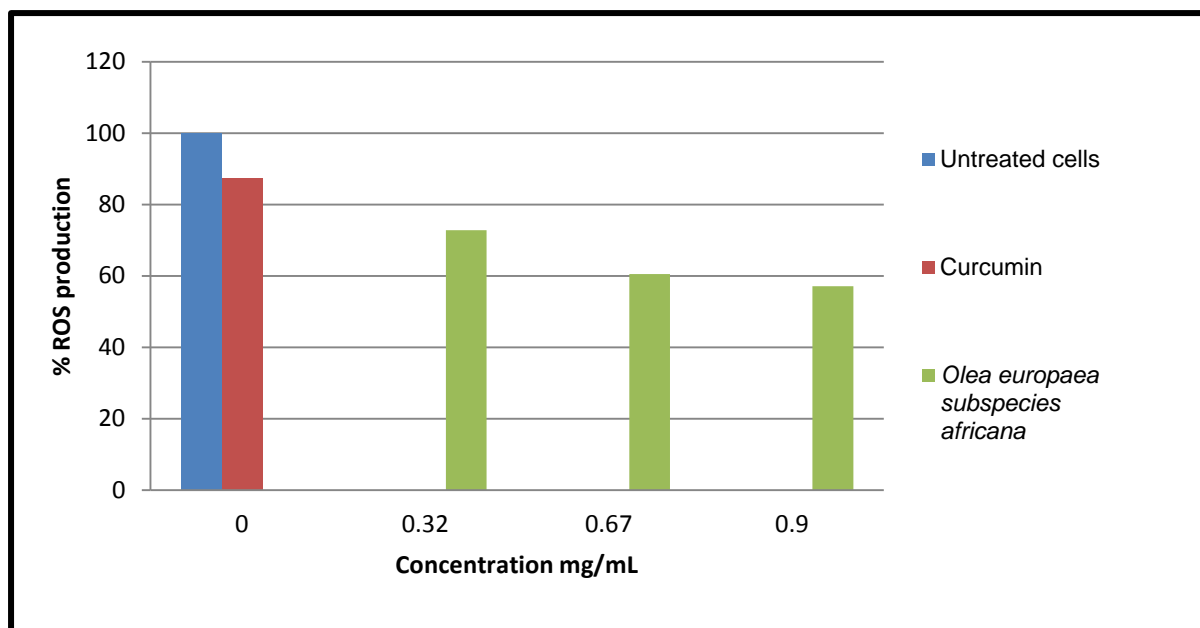


Figure 6.1: Anti-inflammatory activity of acetone crude extract determined by measuring the production of reactive oxygen species (ROS) in percentages in lipopolysaccharide-stimulated RAW 264.7 cells. Curcumin was used as a positive control.

### 6.3.2. MTT Cell Proliferation assay

The acetone extract was found to be non-cytotoxic on the Vero kidney cells; this was demonstrated by the LC<sub>50</sub> of 282.4 µg/mL. This LC<sub>50</sub> value was higher than that of Doxorubicin (control), indicating that it is safe to use on mammalian cells.

Table 6.1: LC<sub>50</sub> values of the acetone extract and the doxorubicin in µg/mL.

| Sample          | LC <sub>50</sub> |
|-----------------|------------------|
| Acetone extract | 282.4            |
| Doxorubicin     | 2.29             |

### 6.3.3. Selectivity index

The selectivity indices ranged from (1.23-2.77 µg/mL) against the four tested bacteria (Table 6.1). All the selectivity indices were low, however, they were above 1 indicating that they are still safe to use on animal cells.

Table 6.2: The MIC and selectivity indices of the acetone extract against the four tested bacteria.

| Microorganisms       | MIC values (µg/mL) | SI values |
|----------------------|--------------------|-----------|
| <i>E. coli</i>       | 230                | 1.23      |
| <i>P. aeruginosa</i> | 160                | 1.77      |
| <i>E. faecalis</i>   | 130                | 2.77      |
| <i>S. aureus</i>     | 160                | 1.77      |

## 6.4. DISCUSSION

The aim of this chapter was to investigate the anti-inflammatory activity of the leaves of *Olea europaea subspecies africana* by evaluating the inhibition of ROS production in LPS-stimulated RAW-264.7 cells. The leaves were also evaluated for cytotoxic effects by determining the LC<sub>50</sub> of the acetone extract against the Vero kidney cells and calculating the selectivity index of the extract against the tested bacteria (*E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus*).

The detection of the ROS production was facilitated by an exposure of the cells to CM-H<sub>2</sub>DCFDA, a fluorescent dye widely used to label ROS in macrophages. This dye enters the cells and reacts with ROS to form 2', 7'-dichlorofluorescein and is trapped within the cells (Shekar *et al.*, 2015). Treatment of the RAW 264.7 cells with LPS only, resulted in generation of intracellular ROS and the ROS produced was assumed to be 100%. This was used as a negative control to determine how much ROS was produced in the treated cells. At the lowest concentration of 0.32 mg/mL (Figure 6.1), ROS production in treated cells was not significantly different from cells treated with LPS only. However, at concentration 0.67 mg/mL, ROS production was inhibited to 60% and at the highest concentration (0.90 mg/mL), the percentage of ROS production was inhibited to 58 percent. It was noted that as the concentration of the extract was increased, the ROS production decreased.

Phytochemicals such as tannins have been reported to have anti-inflammatory activities. They affect the inflammatory response via their radical scavenging activities (Lee *at al.*, 2003). Terpenoids, flavonoids and saponins were also reported to have anti-inflammatory activities. These phytochemicals found in the leaves of *Olea europaea subspecies africana*, may have been responsible for the anti-inflammatory effects of the leaves (Hosseinzadeh and Younesi, 2002; Hortelano, 2009).

This study is the first to demonstrate the anti-inflammatory activities of the leaves of *Olea europaea subspecies africana* in *in vitro* studies using LPS-stimulated RAW 264.7 murine macrophage cells. The therapeutic potential of these leaves has been credited to its capacity to neutralise ROS produced under *in vitro* conditions when RAW 264.7 cells were stimulated with bacterial LPS. The long term use of this plant to treat anti-inflammatory related ailments (eye injuries, enlarged tonsils,

rheumatism and arthritis) indicated the plant's potential to have anti-inflammatory properties. Therefore, the potential of the plant extract to inhibit ROS production validates the traditional use of the leaves and the leaves could be potential sources of leads for novel anti-inflammatory agents.

Performing the *in vitro* cytotoxicity analysis is important; this is done to define the basal cytotoxicity of the extract such as the intrinsic ability of an extract to cause cell death as a result of damage to several cellular functions. The acetone extract was found to have an LC<sub>50</sub> of 282.492 µg/mL as shown in table 6.1. At this level, extracts are considered safe, considering that the value is greater than 100 µg/ml, which is considered safe for plant extracts (Patel *et al.*, 2010). Doxorubicin which was used as a positive control and had an LC<sub>50</sub> of 2.29 µg/mL, this value is much lower than that of the extract. It indicated that the leaf extract was less toxic to Vero cells than the positive control, doxorubicin. This validated the safeness of the extract and showed that it is much more safer to use on mammal cells.

The selectivity indices (SI) determined, were represented in table 6.2, along with the MIC values of the acetone extract against *E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus*. The best SI value was seen against *E. faecalis* (2.77) and the lowest was demonstrated against *E. coli* (1.23). A high selectivity index is an indication of a large safety margin, while a low SI value is an indication of cytotoxicity. The SI values were low but not cytotoxic to animal cells since their SI values were greater than 1. According to Makhafole *et al.* (2014), the *in vitro* toxicity does not equate to *in vivo* toxicity because of a difference in physiological microenvironment in live animal and tissue culture. Moreover, other factors relating to chemical kinetics which may include absorption, biotransformation, distribution and excretion, which influence the exposure at the level of target cells *in vivo*, cannot be adequately simulated *in vitro*. The toxic components in the crude extracts may be eliminated by manipulation of the extract and more suitable antibacterial extracts may be yielded (Dzoyem *et al.*, 2014).

## **6.5. CONCLUSION**

Since acetone extract has demonstrated to have anti-inflammatory properties then it may be used in the treatment of inflammatory-related diseases. Anti-inflammatory drugs of high potency and of natural origin may be isolated from it. The anti-inflammatory properties documented in this study, validate the use of the leaves. The cytotoxicity analysis proved that the leaves were non-cytotoxic to the normal cells.

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## CHAPTER 7: ISOLATION AND PURIFICATION OF ANTIOXIDANT AND ANTIBACTERIAL COMPOUNDS

### 7.1. INTRODUCTION

Plants are known to contain several classes of compounds with markedly different structures. The classes contained therein are made up of different kinds of compounds which are closely related in structure. Separation and isolation of single pure compounds from related compounds is termed natural product chemistry. Compounds that are usually targeted in the natural product chemistry are the secondary metabolites. Isolation of these metabolites from the target plant begins with extraction using solvents (Sticher, 2008).

There are many methods developed for preparing extracts depending on the type of substance to be isolated, these include soxhlex, tissue homogenisation, serial exhaustive extraction and maceration. Serial exhaustive extraction was selected to be used in this study to extract bioactive compounds in the powdered leaves. This technique is widely used for extraction of plant antioxidants and antimicrobial compounds. It involves the use of solvents to extract, starting with a non-polar (n-hexane) to medium polar (dichloromethane) then to a more polar solvents (methanol) to ensure extraction of a wide range of secondary metabolites (Ncube *et al.*, 2008; Sultana *et al.*, 2009).

Isolation of compounds with desired biological activities is achieved through the use of bioassays. Bioassays are employed to evaluate large numbers of initial samples in order to determine whether or not they have any bioactivity of the desired type. They also play an important role of guiding fractionation of a crude material towards isolation of pure bioactive compounds and this is referred to as bioassay guided fractionation. As such, bioassay tests must be simple, rapid, reliable, reproducible, sensitive, meaningful and most importantly, predictive (Pieckova *et.al.*, 1999). Upon determination of a biological activity, the complex mixture should be purified to isolate the bioactive compound(s) and an integration of various separation methods are usually required (Brusotti *et al.*, 2014).

Isolation of pure, pharmacologically active constituents from plants is a long tedious process. As such, unnecessary separation procedures should be eliminated. In order

to achieve that, chemical screening is performed to locate and target new or useful types of compound with potential activities (Marston and Hostettmann, 1999).

Isolation of the various types of bioactive compounds still remains a challenge, more especially their identification and characterisation. These compounds have been isolated using various chromatographic methods such as TLC, High-Performance Thin-Layer Chromatography, paper chromatography, column chromatography, Gas chromatography and High-performance liquid chromatography (HPLC) to obtain pure compounds. The structure and biological activity of the pure compounds is then determined thereafter (Ingle *et al.*, 2017). Non-chromatographic techniques such as immunoassay, which use monoclonal antibodies (MAbs), phytochemical screening assay, Fourier-transform infrared spectroscopy (FTIR), can also be used to obtain and facilitate the identification of the bioactive compounds (Sasidharan *et al.*, 2011).

Column chromatography and thin layer chromatography are the two chromatographic techniques that are simple, flexible and inexpensive to perform. These are most suited for laboratories that do not have expensive equipments. Subsequent chromatographic steps may be performed using HPLC for further purification depending on the nature of the research (Bajpai, 2016). TLC and open column chromatography were opted for, in this study. Isolation using column chromatography involves fractionation of solutes in a mixture due to differential migration through a closed tube of stationary phase. The mobile phase in this technique is liquid and the stationary phase can be either solid or liquid supported by an inert solid. The stationary phases that could be used are selected based on the polarity of the test sample. Silica gel and sephadex are the examples of stationary phases. The length and diameter of the column determines the amount of sample to be loaded, the separation mode to be used, and the degree of resolution required. Longer and narrower columns usually enhance resolution and separation. The aim was to extract a large scale of leaf material using serial exhaustive extraction and perform bioassay-guided fractionation to guide the isolation of compounds with antioxidant and antibacterial activities through TLC and open column chromatography.

## **7.2. METHODS AND MATERIALS**

### **7.2.1. Serial exhaustive extraction**

Mass of 1.2 kg of leaf powder was extracted three times with 5 litres of each solvent (n-hexane, dichloromethane, acetone and methanol) in increasing polarities. Shaking was done at 200 rpm and in 3 hours intervals. The first batch was shaken overnight at room temperature. The supernatant was filtered out and concentrated using a rotary evaporator (Buchi B-490) and transferred to pre-weighed beakers (250 mL). The remaining solvents were evaporated from the extracts under a stream of cold air at room temperature, followed by the determination of total mass extracted as described in section 3.2.2.

### **7.2.2. Phytochemical analysis by Thin Layer Chromatography**

The chemical profile of crude extracts was analysed using aluminium-backed thin layer chromatography (TLC) plates (Fluka, silica gel F254) as described in section 3.2.4.

### **7.2.3. TLC- DPPH assay**

Qualitative DPPH assay described by Deby and Margotteaux (1970) was done using thin layer chromatography as described in section 4.2.1.

### **7.2.4. Bioautography assay**

Bioautography was done according to Begue and Kline (1972) as described in section 5.2.2, using only *E. faecalis*. Only this bacterium was used to determine the antibacterial activities of the extracts because it demonstrated good bacterial susceptibility during the preliminary screening.

### **7.2.5. Serial broth microdilution assay**

Serial broth microdilution method was employed as described by Eloff (1998a) to determine the minimum inhibitory concentration (MIC) values of crude extracts against *E. faecalis* as described in section 5.2.3.

## 7.2.6. Isolation of active compounds

### 7.2.6.1. First open column chromatography

Acetone extract resulting from serial exhaustive extraction was selected to be subjected to the open column chromatography, as it exhibited high antioxidant and antibacterial activities. An open column (35 x 4 cm) was packed with silica gel 60 (particles size 0.063-0.200 mm) (Fluka) using 100% n-hexane. Wet packing method was used to pack the column with silica gel. Cotton wool was put above the slurry extract to ensure no disturbance of the surface occurred when the solvent was introduced. Fifteen elution systems of 2 litres each were carefully added into the column in the order illustrated in table 7.1. A vacuum pump (Vacutec) was used to collect the fractions. The fractions were concentrated using a rotary evaporator (Buchi B-490) and the remaining solvents were completely evaporated under a stream of cold air in pre-weighed beakers. Masses of the resulting fractions were calculated as described in section 3.2.2. As this is an assay guided fractionation, the resulting fractions were tested for antioxidant and antibacterial activity using TLC-DPPH (Section 7.2.3), bioautography (Section 7.2.4) and broth micro-dilution (Section 7.2.5).

Table 7.1: Solvent systems used in the first open column chromatography.

| <b>Solvent System</b>   | <b>Percentages (%)</b> |
|-------------------------|------------------------|
| n-Hexane                | 100                    |
| n-Hexane: Ethyl acetate | 90                     |
|                         | 80                     |
|                         | 70                     |
|                         | 50                     |
|                         | 30                     |
|                         | 10                     |
| Ethyl acetate           | 100                    |
| Ethyl acetate: Methanol | 90                     |
|                         | 80                     |
|                         | 70                     |
|                         | 60                     |
|                         | 50                     |
|                         | 40                     |
| Methanol                | 100                    |

### **7.2.6.2. Combination of similar fractions**

After running each column chromatography and subsequently performing all biological assays, the fractions with similar chromatographic characteristics and activities were combined and their mass was determined as described in section 3.2.2.

### **7.2.6.3. Second open column chromatography**

The first combination (Pool 1) of fractions with similar profiles, was subjected to the second open column chromatography (35 x 5 cm) which was packed as described in section 7.2.6.1 however, this time; 100% chloroform was used as a packing solvent. The column was first eluted with 3 litres of 100% chloroform followed by an introduction of 20% and 30% ethyl acetate. The fractions were collected in test tubes and evaporated under a stream of cold air to concentrate the fractions. After evaporating the fractions to half their original volume, the chemical profile of the fractions were analysed by TLC as described in section 3.2.4. The biological activities fractions were assayed for, using TLC-DPPH (Section 7.2.3), bioautography (Section 7.2.4) and serial broth micro-dilution assays (Section 7.2.5).

## 7.3. RESULTS

### 7.3.1. Serial exhaustive extraction

#### 7.3.1.1. The quantity of plant material extracted

Serial exhaustive extraction resulted in extraction of a total mass of 358.95 grams (Table 7.2). Methanol was the best extractant, extracting a total mass of 246.08 grams while n-hexane extracted the least leaf material (14.56 grams).

Table 7.2: The masses of extracts obtained after performing serial exhaustive extraction using four solvents of increasing polarity.

| Extracts        | Mass Residue (g) |               |
|-----------------|------------------|---------------|
|                 | Mass             | Total         |
| n-Hexane        | H1               | 9.44          |
|                 | H2               | 3.29          |
|                 | H3               | 1.83          |
| Dichloromethane | D1               | 22.21         |
|                 | D2               | 12.47         |
|                 | D3               | 4.88          |
| Acetone         | A1               | 43.48         |
|                 | A2               | 11.23         |
|                 | A3               | 4.01          |
| Methanol        | M1               | 164.15        |
|                 | M2               | 50.96         |
|                 | M3               | 30.97         |
| <b>Total</b>    |                  | <b>358.95</b> |



### 7.3.1.2. Phytochemical analysis by Thin Layer Chromatography

Crude extracts of *Olea europaea* subspecies *africana* were separated in three solvent systems of varying polarities i.e. BEA, CEF and EMW. The chromatograms were visualised under UV light (365 nm) (Figure 7.1A) and sprayed with vanillin-sulphuric acid reagent (Figure 7.1B). Chromatograms developed in BEA demonstrated a greater number of compounds when visualised under both UV light and spraying with vanillin-sulphuric acid reagent.

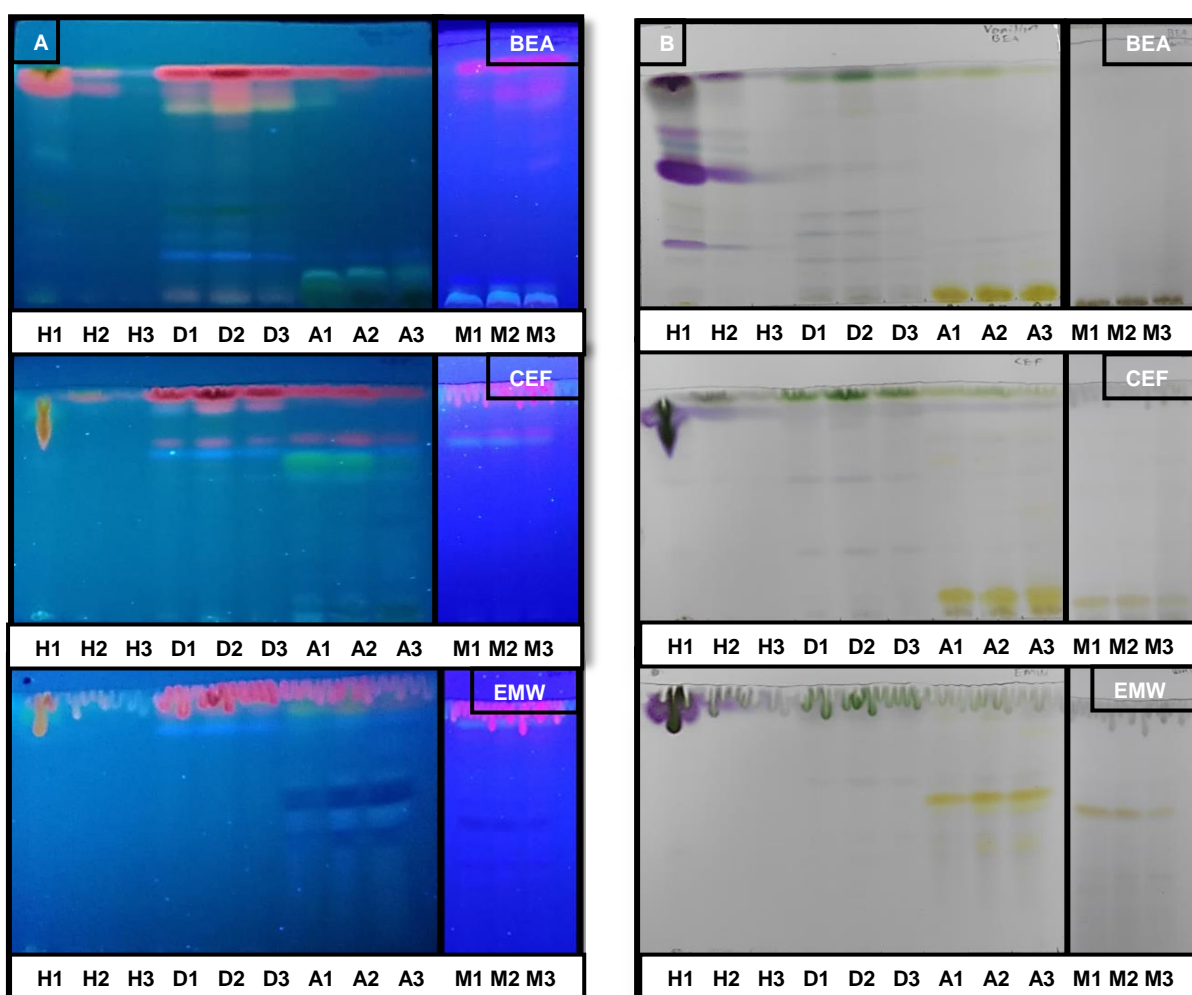


Figure 7.1: Chromatograms of leaf extracts extracted with n-hexane (H), Dichloromethane (D), acetone (A) and methanol (M). These were separated in three solvent systems; BEA, CEF, EMW and visualised under UV light (365 nm) (A) and sprayed with vanillin-sulphuric acid reagent (B).

### 6.3.1.3. TLC-DPPH assay

The antioxidant activities of the crude extracts determined by spraying the TLC plates with 0.2% DPPH in methanol. Acetone and methanol extracts had a prominent antioxidant activity across all chromatograms. A good resolution of the extracts was observed on a chromatogram developed in EMW solvent system (Figure 7.2). n-Hexane and dichloromethane extracts did not show any antioxidant activity.

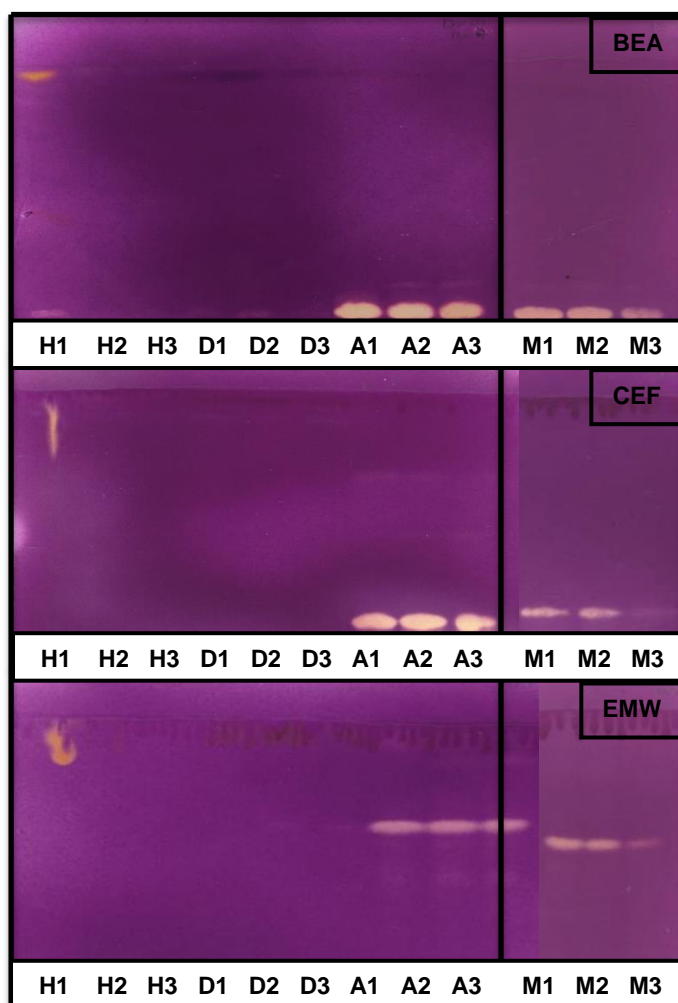


Figure 7.2: Chromatograms of leaf extracts extracted with n-hexane (H), dichloromethane (D), acetone (A) and methanol. Development of the plates was done in BEA, CEF, EMW solvent systems and sprayed with 0.2% DPPH in methanol to detect the antioxidant activities of the extracts.

#### 7.3.1.4. Bioautography assay

Antibacterial activity of the crude extracts was analysed through direct bioautography, where *E. faecalis* (Figure 7.3) was sprayed on previously developed chromatograms and incubated in a humid atmosphere. Only *E. faecalis* was used as a test bacterium. INT reagent was used to detect the antibacterial activity of the extracts. The clear or creamy zones on the chromatograms indicated antibacterial activity of the extracts. The chromatograms were developed in BEA, CEF and EMW solvent systems. n-Hexane and dichloromethane extracts had prominent antibacterial activities against the test bacterium. Great separation of compounds was observed in the chromatogram developed in BEA.

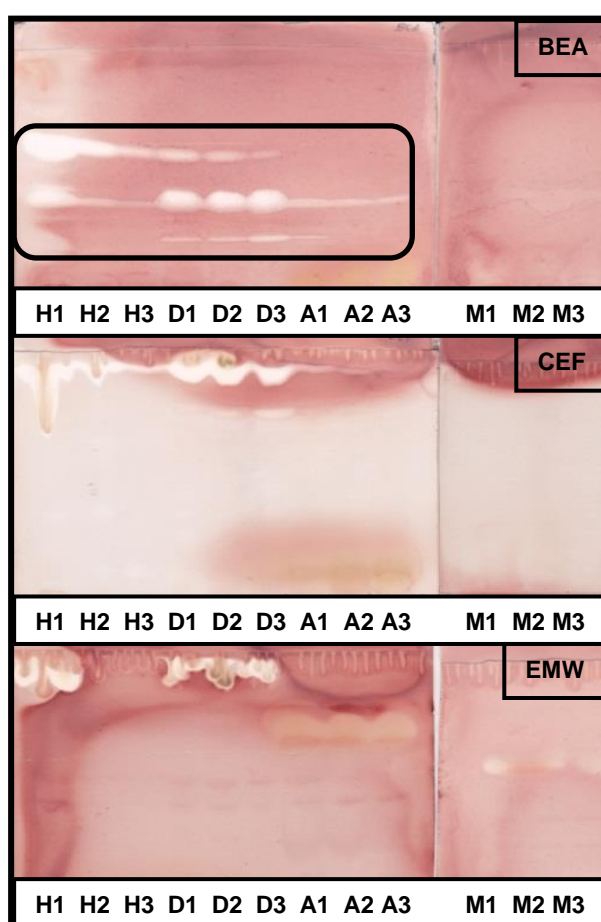


Figure 7.3: Bioautograms of *Olea europaea* subspecies *africana* leaf extracts separated with BEA, CEF, EMW and sprayed with *E. faecalis*, clear or creamy zones indicated antibacterial activity of an extract.

### 7.3.1.5. Serial broth microdilution assay

The observed antibacterial activity was quantified using the serial broth microdilution assay (Table 7.3), against *E. Faecalis* and the MIC values ranged from 0.13 mg/mL to 2.5 mg/mL. The lowest MIC value (0.13 mg/mL) was observed in all acetone extracts and the highest was observed in dichloromethane (D1) (1.8 mg/mL). Acetone extracts had the best minimal inhibitory concentrations. Ampicillin, which was used as a positive control, had an MIC value of 0.03 ug/mL.

Table 7.3: Minimum inhibitory concentration (MIC) values of various leaf extracts against *E. faecalis* and ampicillin (positive control).

| Microorganism      | Extracts       | MIC values (mg/mL) | Averages    |
|--------------------|----------------|--------------------|-------------|
| <i>E. faecalis</i> | H1             | 1.25               | <b>1.18</b> |
|                    | H2             | 1.25               |             |
|                    | H3             | 1.04               |             |
|                    | D1             | 1.67               | <b>2.02</b> |
|                    | D2             | 2.5                |             |
|                    | D3             | 1.88               |             |
|                    | A1             | 0.13               | <b>0.13</b> |
|                    | A2             | 0.13               |             |
|                    | A3             | 0.13               |             |
|                    | M1             | 0.52               | <b>1.15</b> |
|                    | M2             | 0.42               |             |
|                    | M3             | 2.5                |             |
|                    | AMP<br>(ug/mL) | 0.03               |             |

Key: n-hexane (H), dichloromethane (D), acetone (A), methanol (M), (AVG) average, (AMP) ampicillin.

## 7.3.2. Isolation of active compounds from acetone extract

### 7.3.2.1. First open column chromatography

#### 7.3.2.1.1. The masses of the collected fractions

The first open column chromatography was eluted using varying percentages of solvents listed in table 7.1. The masses for fractions collected were calculated and recorded. Elution of the column with 90% ethyl acetate in methanol yielded the highest mass (19.84 grams) of fractions collected while, 90% n-hexane in ethyl acetate yielded the lowest mass (0.11 grams) of fractions collected. The results were tabulated in Table 7.4.

Table 7.4: The masses (grams) of fractions from fractionation of the acetone extract.

| Numbering | Elution solvent         | Percentages (%) | Mass (g)     |
|-----------|-------------------------|-----------------|--------------|
| 1         | n-Hexane                | 100             | 0.71         |
| 2         | n-Hexane: Ethyl acetate | 90              | 0.11         |
| 3         |                         | 80              | 0.37         |
| 4         |                         | 70              | 1.46         |
| 5         |                         | 50              | 1.51         |
| 6         |                         | 30              | 1.41         |
| 7         |                         | 10              | 0.95         |
| 8         | Ethyl acetate           | 100             | 0.70         |
| 9         | Ethyl acetate: Methanol | 90              | 19.84        |
| 10        |                         | 80              | 2.54         |
| 11        |                         | 70              | 5.93         |
| 12        |                         | 60              | 3.66         |
| 13        |                         | 50              | 10.08        |
| 14        |                         | 40              | 1.64         |
| 15        | Methanol                | 100             | 0.48         |
|           | <b>Total</b>            |                 | <b>50.91</b> |

### 7.3.2.1.2. Phytochemical analysis by Thin Layer Chromatography

The chemical profiles of the fractions resulting from the first open column chromatography. The compounds were detected by UV light at 365 nm (Figure 7.4A) and vanillin-sulphuric acid reagent (Figure 7.4B). A wide range of phytochemicals were observed as bands in all chromatograms. The solvent systems used to develop the TLC plates were BEA, CEF and EMW. Best separation was observed on TLC plates developed in the CEF solvent system.

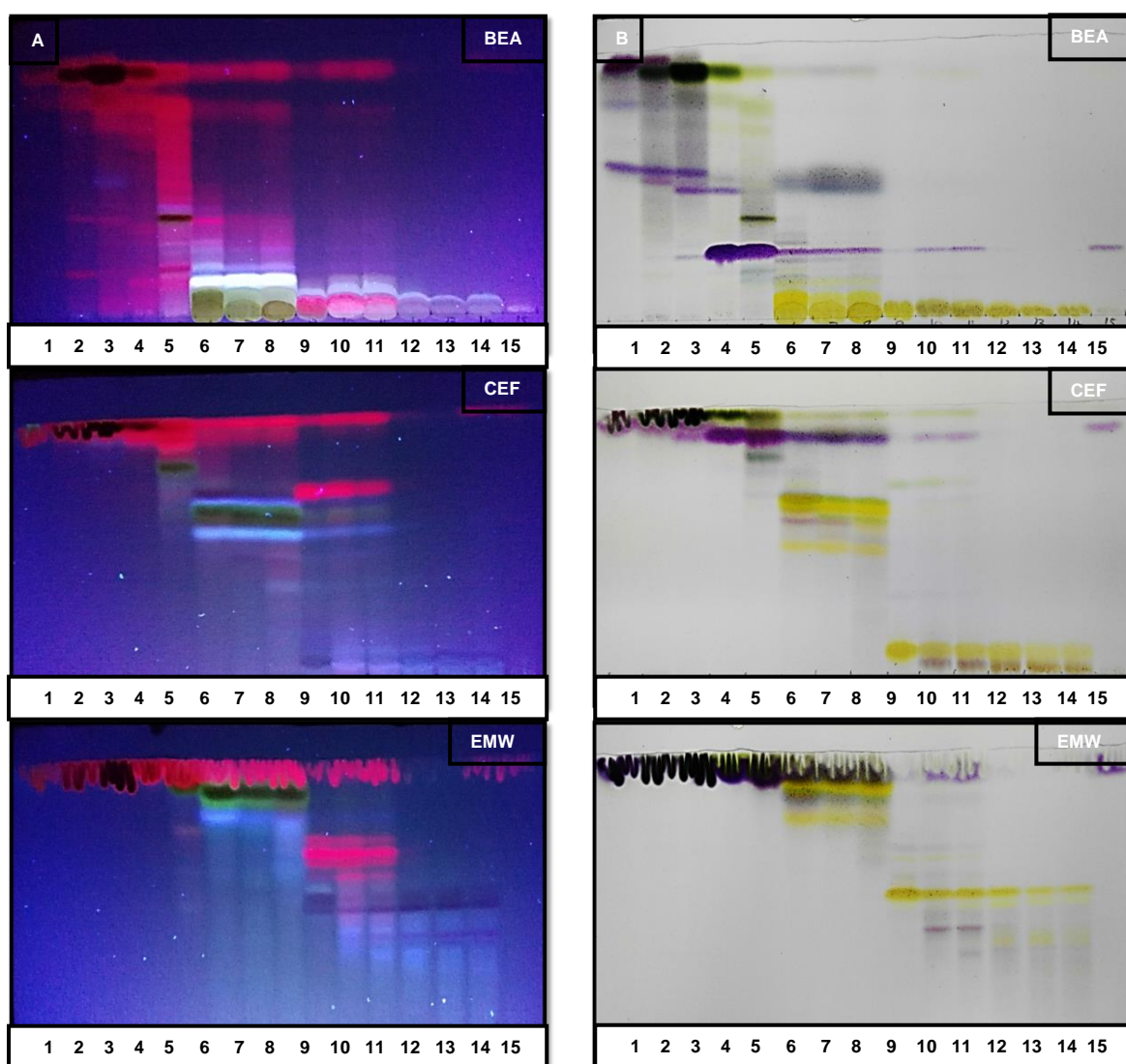


Figure 7.4: Thin layer chromatography analysis of acetone fractions separated in BEA, CEF and EMW solvent systems and visualised using UV light (A) at 365 nm and vanillin-sulphuric acid (B) to reveal the active compounds.

### 7.3.2.1.2. TLC-DPPH assay

The antioxidant activity of the fractions collected was observed on fractions 6 to 14 of the acetone extract on all chromatograms. These fractions were eluted starting with 30% n-hexane in ethyl acetate until 40% ethyl acetate in methanol (Figure 7.5). The best separation of the bioactive compounds was observed in the chromatograms separated in the solvent system CEF and EMW.

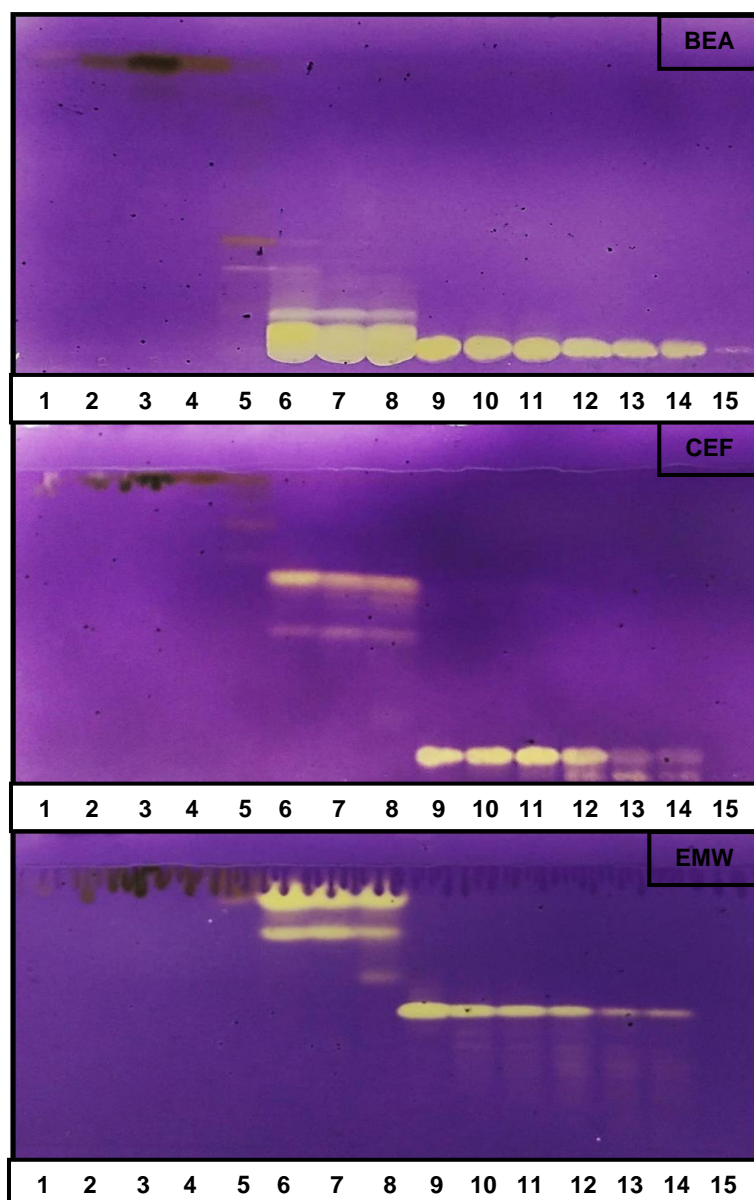


Figure 7.5: Antioxidant activities of acetone fractions collect after performing the first open column chromatography prayed with 0.2% DPPH to reveal antioxidant compounds isolated with various eluent systems.

### 7.3.2.1.3. Bioautography assay

Antibacterial activity of the acetone fractions was determined using the direct bioautography method. The solvent systems used to separate the active compounds were BEA, CEF and EMW and the tested bacteria were *E. coli* (Figure 7.6A), *P. aeruginosa* (Figure 7.6B), *S. aureus* (Figure 7.7A) and *E. faecalis* (Figure 7.7B). The clear or creamy zones indicated the antibacterial activity of the fractions. Fractions 4 and 5 demonstrated prominent activity on all chromatograms separated in BEA solvent system against all tested bacteria.

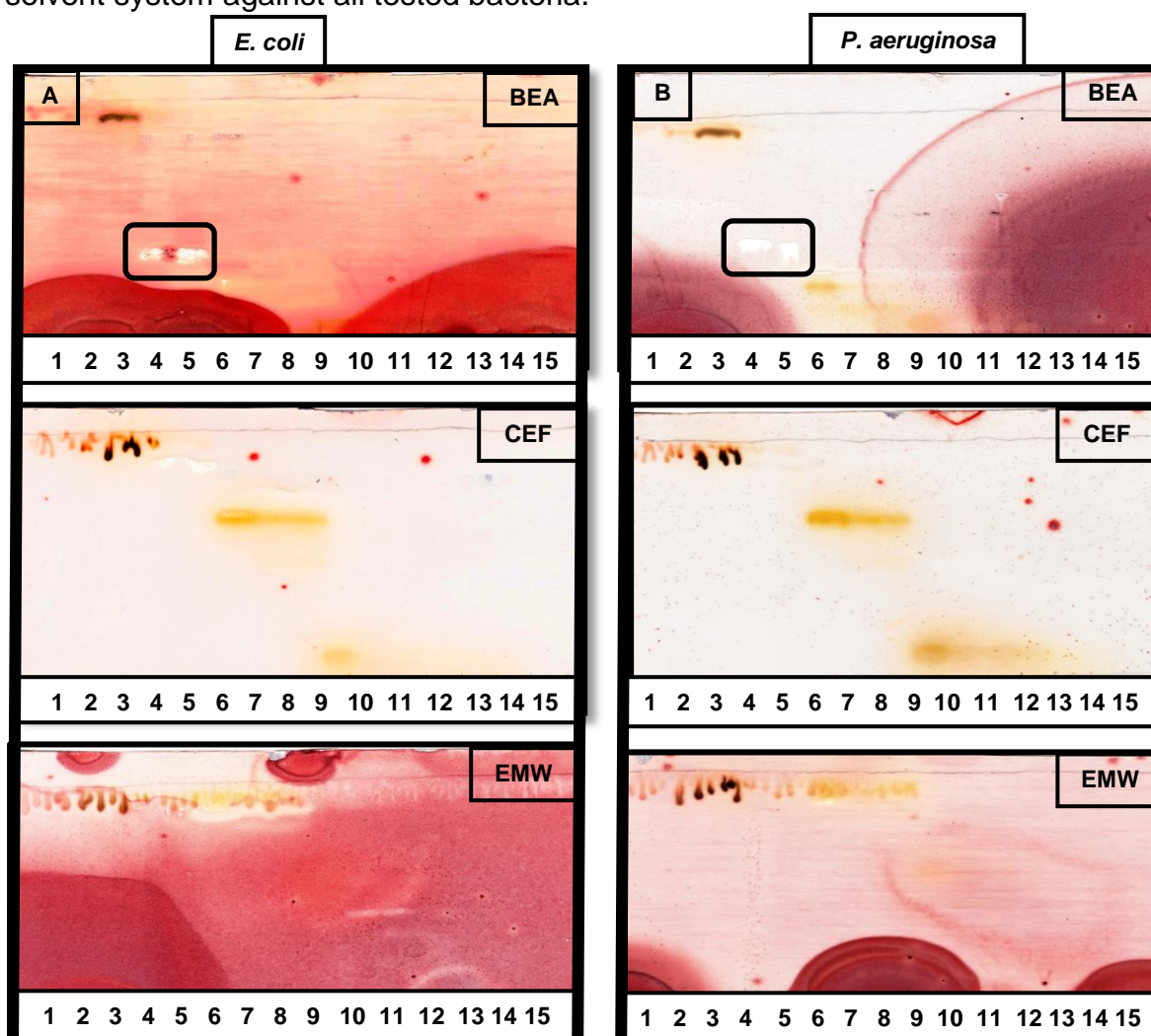


Figure 7.6: Bioautograms of fractions separated in BEA, CEF and EMW and sprayed with the Gram-negative bacteria *E. coli* (A) and *P. aeruginosa* (B). The growth of the bacteria was detected with INT reagent. The clear or creamy zones indicate active compounds that inhibited growth of tested bacterial species.



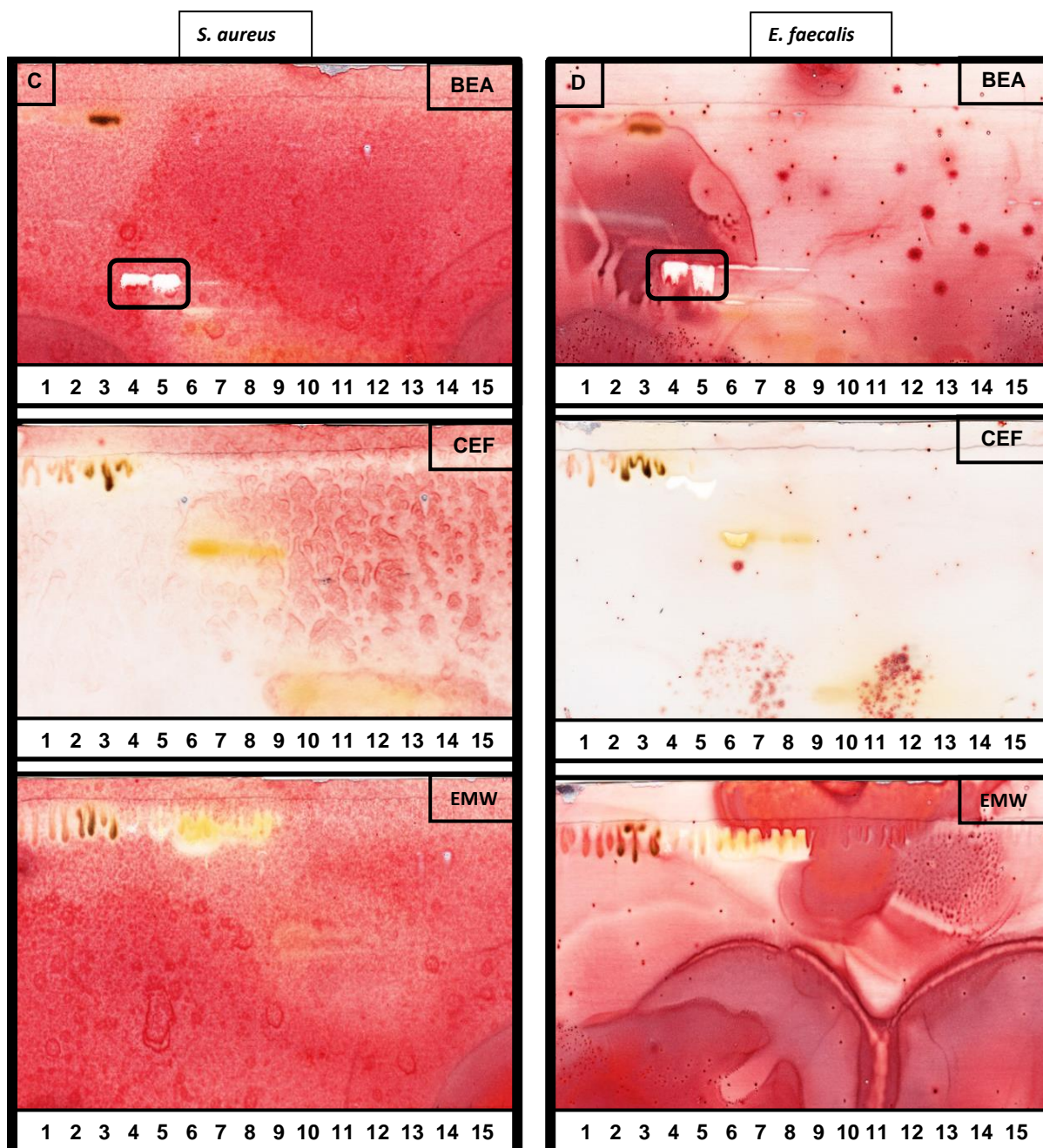


Figure 7.7: Bioautograms of fractions separated in BEA, CEF and EMW and sprayed with the Gram-positive bacteria *S. aureus* (A) and *E. faecalis* (B). The growth of the bacteria was detected with INT reagent. The clear or creamy zones indicate active compounds that inhibited growth of tested bacterial species.

#### **7.3.2.1.4. Serial broth microdilution assay**

Serial broth microdilution assay was performed to quantify the antibacterial activity of the fractions. On average, fractions 4 to 10 had the lowest MIC values indicating good antibacterial activity and the highest MIC values were seen in fractions 1 and 15 against all tested bacteria. Fraction 11 to 15 did not have any activity against *S. aureus*. The overall most susceptible bacteria was *S. aureus* with an MIC value of 0.35 mg/mL.

Table 7.5: Minimum inhibitory concentration (MIC) values of fractions resulting from acetone extract against four tested bacteria and ampicillin (Positive control).

| Microorganism        | MIC values (mg/mL) |             |             |             |            |             |             |             |             |             |             |             |              |             |             | AMP<br>(µg/mL) |      |
|----------------------|--------------------|-------------|-------------|-------------|------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|--------------|-------------|-------------|----------------|------|
|                      | 1                  | 2           | 3           | 4           | 5          | 6           | 7           | 8           | 9           | 10          | 11          | 12          | 13           | 14          | 15          |                | AVG  |
| <i>E.coli</i>        | 1.68               | 1.15        | 0.65        | 0.29        | 0.26       | 0.08        | 0.08        | 0.05        | 0.07        | 0.13        | 0.13        | 0.13        | 0.13         | 0.23        | 1.46        | <b>0.43</b>    | 0.03 |
| <i>P. aeruginosa</i> | >2.5               | NA          | 1.04        | 0.73        | 0.24       | 0.13        | 0.12        | 0.13        | 0.21        | 0.47        | 0.21        | 0.21        | 0.16         | 0.26        | 2.5         | <b>0.64</b>    | 0.02 |
| <i>E. faecalis</i>   | 0.63               | 0.84        | 0.63        | 0.31        | 0.16       | 0.08        | 0.04        | 0.05        | 0.13        | 0.31        | 2.5         | 1.67        | 1.46         | 1.04        | >2.5        | <b>0.70</b>    | 0.03 |
| <i>S. aureus</i>     | 0.63               | 1.04        | 0.63        | 0.31        | 0.26       | 0.08        | 0.04        | 0.07        | 0.16        | 0.31        | >2.5        | >2.5        | >2.5         | >2.5        | >2.5        | <b>0.35</b>    | 0.08 |
| <b>Average</b>       | <b>1.36</b>        | <b>1.01</b> | <b>0.74</b> | <b>0.41</b> | <b>0.2</b> | <b>0.09</b> | <b>0.07</b> | <b>0.08</b> | <b>0.14</b> | <b>0.31</b> | <b>0.95</b> | <b>0.67</b> | <b>0.583</b> | <b>0.51</b> | <b>1.98</b> |                |      |

Key: (AVG) average; (AMP) ampicillin.

### 7.3.2.2. Combination of similar fractions

Fractions demonstrating similar TLC characteristics and biological activities were pooled together. The pools, fractions combined, biological activities and the masses of the combined fractions were represented in Table 7.6. Pool 3 had the highest mass of 43.52 grams, while pool 1 had the least mass of 2.80 grams.

Table 7.6: The pools, fractions combined, biological activities and the masses of the combined fractions resulting from the first open column chromatography.

| <b>Pool</b>  | <b>Fractions</b> | <b>Biological Activity</b>    | <b>Mass (g)</b> |
|--------------|------------------|-------------------------------|-----------------|
| 1            | 4 and 5          | Antibacterial only            | 2.80            |
| 2            | 6 to 8           | Antibacterial and Antioxidant | 3.87            |
| 3            | 9 to 14          | Antibacterial and Antioxidant | 43.51           |
| <b>Total</b> |                  |                               | <b>49.88</b>    |

### 7.3.2.3. TLC analyses and biological activities of the pools

Various TLC analyses were performed in attempt to find the best solvent systems to effectively purify the compounds in the second open column chromatography and confirm their respective biological activities. The solvent system 70% chloroform in ethyl acetate effectively resolved pool 1 and 2 (Figure 7.8A and B), while pool 3 (Figure 7.8C) was effectively resolved in 70% chloroform in methanol solvent system. Only pool 1 was considered for purification by open column chromatography.

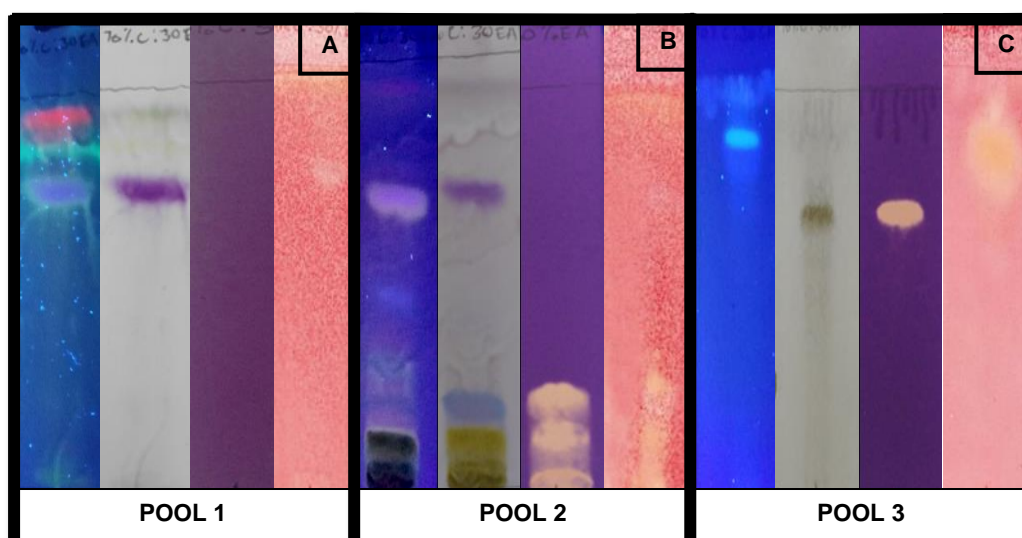


Figure 7.8: Chromatograms of the pools of fractions resulting from the first open column chromatography and their biological activities. The pools were assayed for UV and vanillin reactivity as well as for antioxidant and antibacterial activity (from left to right).

#### 7.3.2.4. Second open column chromatography for pool 1

The second open column chromatography was performed to purify the active compounds in pool 1. The column was eluted using 70% chloroform 30% ethyl acetate. A single pure compound was observed on the TLC plate from fraction 196 to 288 (Figure 7.9), which had a purple colour upon reacting with vanillin-sulphuric acid (Top) and fluoresced red under UV light at 365 nm (Bottom). These fractions were pooled together and labelled compound.

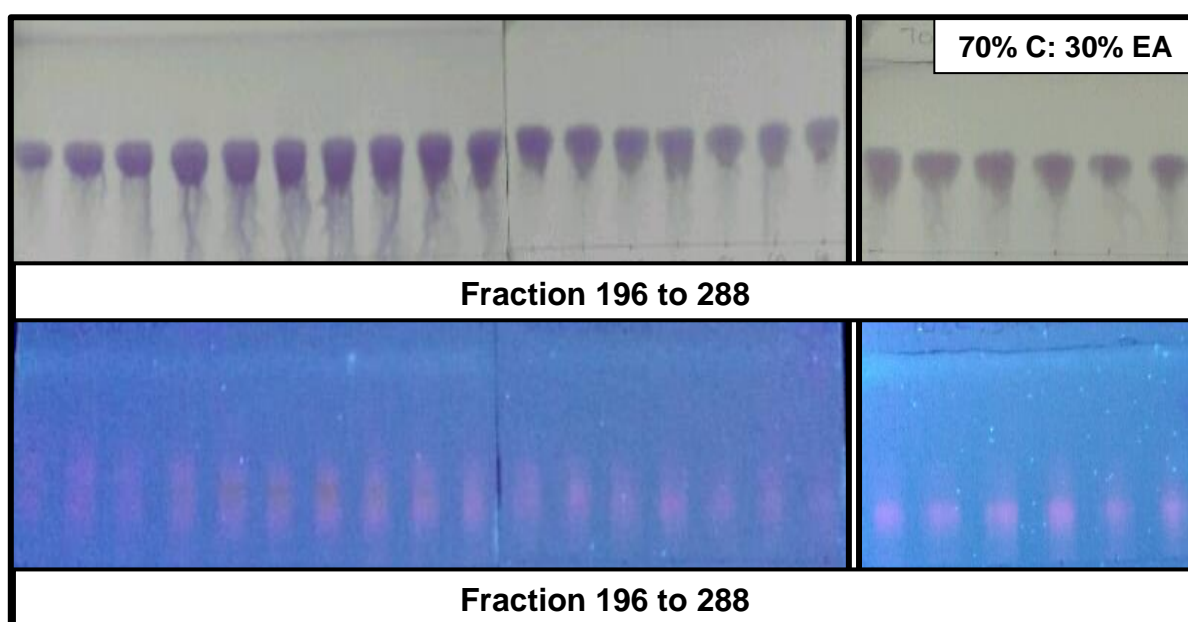


Figure 7.9: Fractions with similar profiles which were pooled together and labelled compound. The TLC plate was developed in 70% chloroform: 30% ethyl acetate. The compound was visualised under UV light 365 nm (Bottom) and sprayed with vanillin-sulphuric acid (Top).

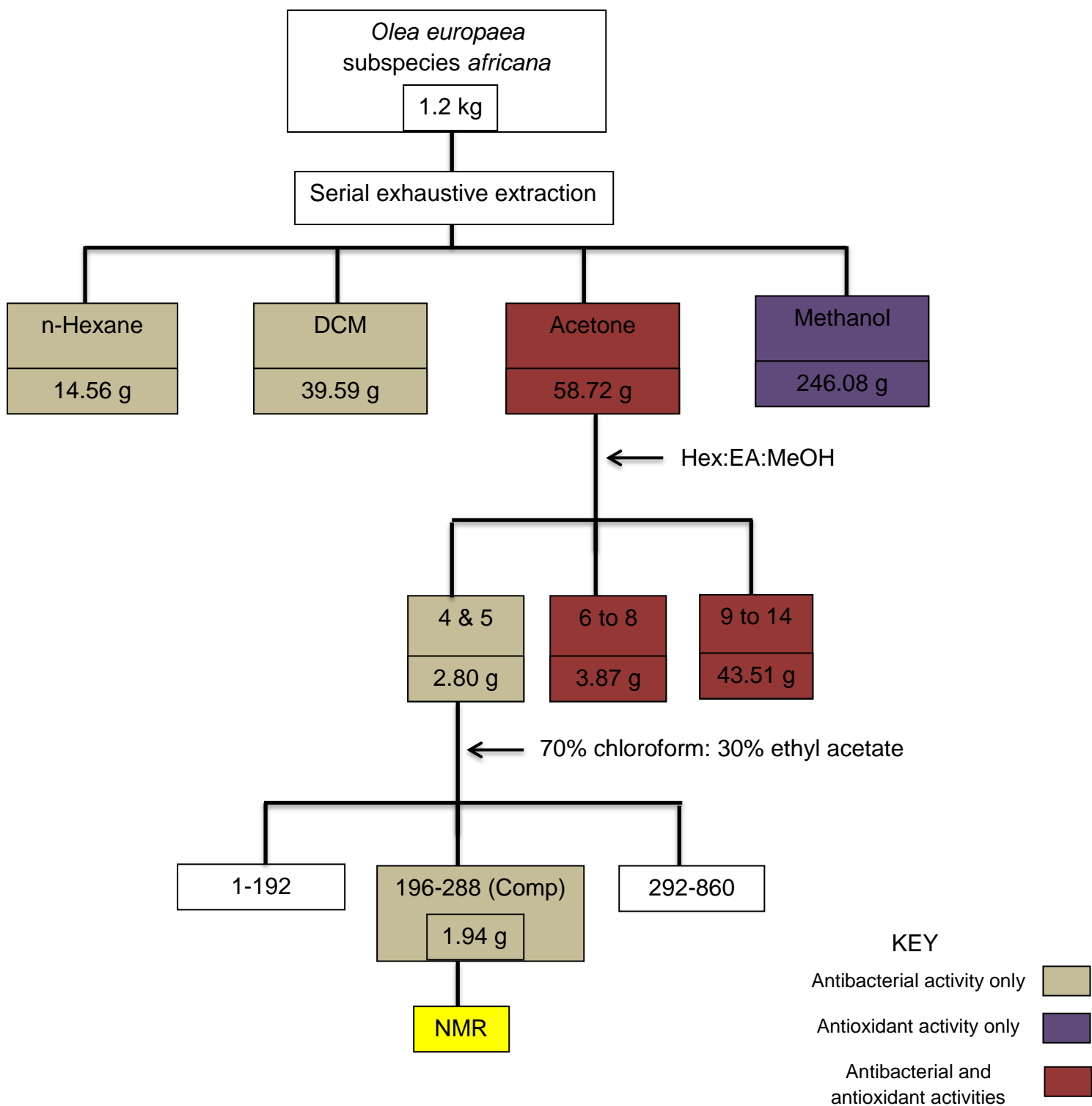


Figure 7.10: Overview of the isolation process of an active compound.

## 7.4. DISCUSSION

Serial exhaustive extraction was used as the first fractionation step and further fractionation was done by column chromatography. Serial exhaustive extraction was selected to ensure that a wide range of natural compounds are extracted without introducing heat as it induces hydrolysis of biologically active components. The results obtained, (Table 7.2) demonstrated that methanol extracted the most of leaf material (246.08 g). Acetone also extracted a reasonable mass of plant material (58.71 g) while n-hexane extracted the least plant material (14.56 g).

The extracts (Figure 7.1) were developed in BEA, CEF and EMW. Chromatograms developed in BEA had many bands that are both UV and vanillin reactive. Many bands were also observed in the chromatograms developed in CEF. This showed that the compounds in the extracts were non-polar in nature. These findings were similar to the ones obtained in the preliminary screening. Dichloromethane, acetone and methanol extracts separated best in BEA. Among the extracts, acetone and dichloromethane extracts showed the presence on many phytochemicals.

Assaying the crude extracts for free radical scavenging activity (Figure 7.2) revealed that only acetone and methanol extracts possessed antioxidant activities. The compounds with antioxidant activity separated best in the solvent system EMW. Indicating that the compounds are polar and therefore, isolating them will require a polar solvent system. The antibacterial activity of the crude extracts determined by direct bioautography (Figure 7.3), revealed that dichloromethane extract had most antibacterial activity, followed by hexane, acetone then methanol extracts which had no inhibitory effects against *E. faecalis*. *E. faecalis* was used to determine the antibacterial activities of the extracts because it demonstrated good bacterial susceptibility during the preliminary screening. Moreover, the active compounds had similar chemical profiles, indicating that they could be the same compounds.

The observed antibacterial activity was quantified using the serial broth microdilution assay (Table 7.3) using *E. faecalis* as the test bacteria. Acetone extracts had the lowest MIC values. The average MIC value of acetone extracts against *E. faecalis* was 0.13 mg/mL while the rest of the extracts had MIC values greater than 1 mg/mL, which indicated poor antibacterial activity. The results obtained after performing TLC-DPPH, direct bioautography and serial broth microdilution assay, it was clear that



the acetone extract was the best extract to isolate bioactive compounds from as it contained both antioxidant and antibacterial activities.

The extract was subjected to the first open column chromatography to isolate the bioactive compounds contained within the extract. From this column, fractions eluted with 90% ethyl acetate in methanol yielded the highest mass of 19.84 grams and combination of 90 % n-hexane: 10% ethyl acetate solvents, yielded the lowest mass of 0.11 g. TLC analysis of fractions showed that there were many compounds which were effectively separated by column chromatography (Figure 7.4). The compounds were UV and vanillin reactive.

TLC-DPPH assay revealed that only fractions 6 to 14 had antioxidant activities (Figure 7.5). The best separation of the antioxidant compounds was observed in the chromatogram separated in CEF and EMW solvent systems. Direct bioautography (Figure 7.6 and Figure 7.7) revealed that only fractions 4 and 5 demonstrated prominent antibacterial activity on all chromatograms separated in BEA solvent system against all tested bacteria. Serial broth microdilution (Table 7.5) revealed that on average, fractions 4 to 10 had the lowest MIC values against all tested bacteria, indicating good antibacterial activities. The highest MIC values were seen in fractions 1 and 15 against all tested bacteria. Fraction 11 to 15 did not have any activity against *S. aureus*. The overall most susceptible bacteria was *S. aureus* with an MIC value of 0.35 mg/mL.

The results obtained from the first open column chromatography, showed that fraction 4 and 5, fractions 6 to 8 and fractions 9 to 14 could be combined because they showed similar chemical profiles and biological activities. These were then combined and labelled as described in table 7.6 and from these findings, 3 pools resulted. Several TLC analyses were performed to find the solvent systems that could effectively resolve the compounds as a preparation for isolation. Pool 1 and 2 were effectively resolved in 70% chloroform in ethyl acetate, while pool 3 resolved best in the solvent system composed of 70% chloroform in methanol. The biological activities of the combinations or pools were verified by performing the biological assay again (Figure 7.8A-C). Only pool 1 was subjected to the second open column for purification and elution was done using 70% chloroform: 30% ethyl acetate. TLC analysis was done to monitor the isolation process. A single pure compound was

obtained in fractions 196-288 (Figure 7.9). The compound had a purple colour upon reacting with vanillin-sulphuric acid and fluoresced red under UV light at 365 nm. These fractions were pooled together and labelled compound. From herein the compound was sent to the chemistry department for identification (Chapter 8).

## **7.5. CONCLUSION**

The bioassay-guided fractionation enabled isolation of bioactive compounds with antioxidant and antibacterial activities, although not all potential bioactive compounds were purified, purification of a single compound from pool 1 was successful. This compound had antibacterial activity. Identification of the isolated compound and its biological activities will be documented in the next chapters (Chapter 8 and 9).

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## CHAPTER 8: CHARACTERISATION AND STRUCTURE ELUCIDATION

### 8.1. INTRODUCTION

The term structure elucidation usually refers to full *de novo* structure identification, and it results in a complete molecular connection table with correct stereochemical assignments. Such an identification process without any assumptions or pre-knowledge is commonly the domain of nuclear magnetic resonance spectroscopy. Nuclear Magnetic Resonance Spectroscopy (NMR) is the study of interaction of radio frequency of the electromagnetic radiation with unpaired nuclear spins in an external magnetic field, to extract structural information about a given sample. It is a technique used routinely by chemists to study chemical structures of simple molecules using simple one dimensional technique (1D-NMR) and the two-dimensional technique (2D-NMR) is used to determine the structure of more complicated molecules (Pretsch and Clerc, 1997).

The chemist basically studies the ( $^1\text{H}$ ) protons and ( $^{13}\text{C}$ ) carbons. The proton NMR is a plot of signals arising from absorption of radio frequency during an NMR experiment by the different protons in a compound under study as a function of frequency (chemical shift). The information about the number of protons present in the molecule is provided by the area under the plots, the position of the signals (the chemical shift) reveals information regarding the chemical and electronic environment of the protons and the splitting pattern provides information about the number of neighbouring (vicinal or germinal) protons (Abraham *et al.*, 1988).

Carbon NMR is a plot of signals arising from the different carbons as a function of chemical shift. The signals in  $^{13}\text{C}$ -NMR experiments normally appear as singlets because of the decoupling of the attached protons. Different techniques of recording of the 1D carbon NMR has been developed so that it is possible to differentiate between the various types of carbons such as the primary, secondary, tertiary and quaternary from the 1D  $^{13}\text{C}$  NMR plot. The range of the chemical shift values differs between the  $^1\text{H}$  (normally 0-10) and  $^{13}\text{C}$  NMR (normally 0-230) that arises from the two nuclei having different numbers of electrons around their corresponding nuclei as well as different electronic configurations (Derome, 1987; Sanders and Hunter, 1993).

The most common 2D-NMR experiments that are mostly used in the structural elucidation of natural products are the homonuclear  $^1\text{H}$ ,  $^1\text{H}$ -COSY, NOESY, the heteronuclear  $^1\text{H}$ ,  $^{13}\text{C}$ -HMQC as well as HMBC. [COSY stand for correlated spectroscopy, NOESY stands for nuclear overhauser enhancement spectroscopy (NOESY) and HMQC stands for heteronuclear multiple quantum correlation].  $^1\text{H}$ ,  $^1\text{H}$ -COSY is one of the most useful experiments which provide information about the connectivity of the different groups within the molecule. A straight line may be drawn from any of the dark spots to each axis, this enables one to see which protons couple with one another and which are, therefore, attached to neighboring carbons. 2D NOESY is a homonuclear correlation via dipolar coupling; dipolar coupling may be due to chemical exchange. It is one of the most useful techniques as it allows to correlate nuclei through space (distance smaller than  $5\text{\AA}$ ) and enables the assignment of relative configuration of substituents at chiral centers. Just like COSY, it also enables one to see which protons are nearer to each other in space by drawing a straight line from any of the dark spots to each axis of the plot (Ernst *et al.*, 1989; Kessler *et al.*, 1988). The aim was to characterise the isolated compound through nuclear magnetic resonance and elucidate its structure by using the spectroscopic data provided.

## **8.2. METHODS AND MATERIALS**

### **8.2.1. Characterisation of pure compounds by nuclear magnetic resonance**

The isolated compound was sent to the Chemistry Department, University of Limpopo (Turfloop campus) to characterise the compound using NMR. The compound (20 mg) was dissolved in chloroform and  $^1\text{H}$ ,  $^{13}\text{C}$  and DEPT 135 were ran using 400 MHz NMR Spectrometer (Bruker) at 400 MHz, chloroform-d as a reference signal solvent and at a temperature of 295.5 K. Professor Mdee L.K., of the Pharmacy Department, University of Limpopo (Turfloop Campus) assisted with the structure elucidation using the spectroscopic data provided.

### 8.3. RESULTS

Identification of the isolated compound by means of spectroscopic analysis using the information provided by  $^1\text{H}$  (protons) (Figure 8.1),  $^{13}\text{C}$  (chemical shifts) (Figure 8.2) and DEPT-135 NMR spectra (Figure 8.3) was done. The spectral analysis of the compound demonstrated similar chemical shifts to those of oleanolic acid documented in literature (Table 8.1). The structure of the isolated compound was represented in Figure 8.4.

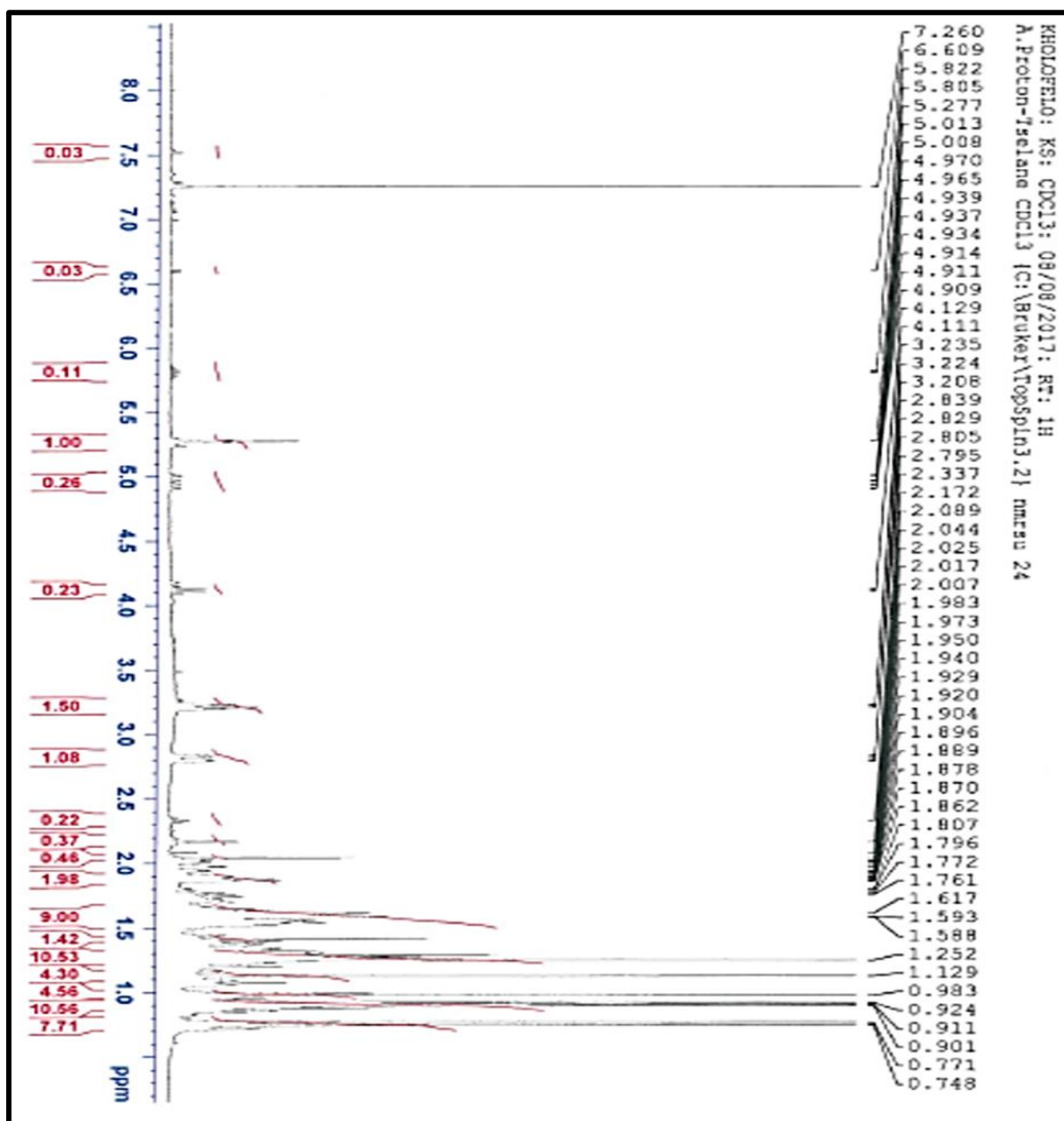


Figure 8.1:  $^1\text{H}$  (proton) spectrum of the isolated compound.

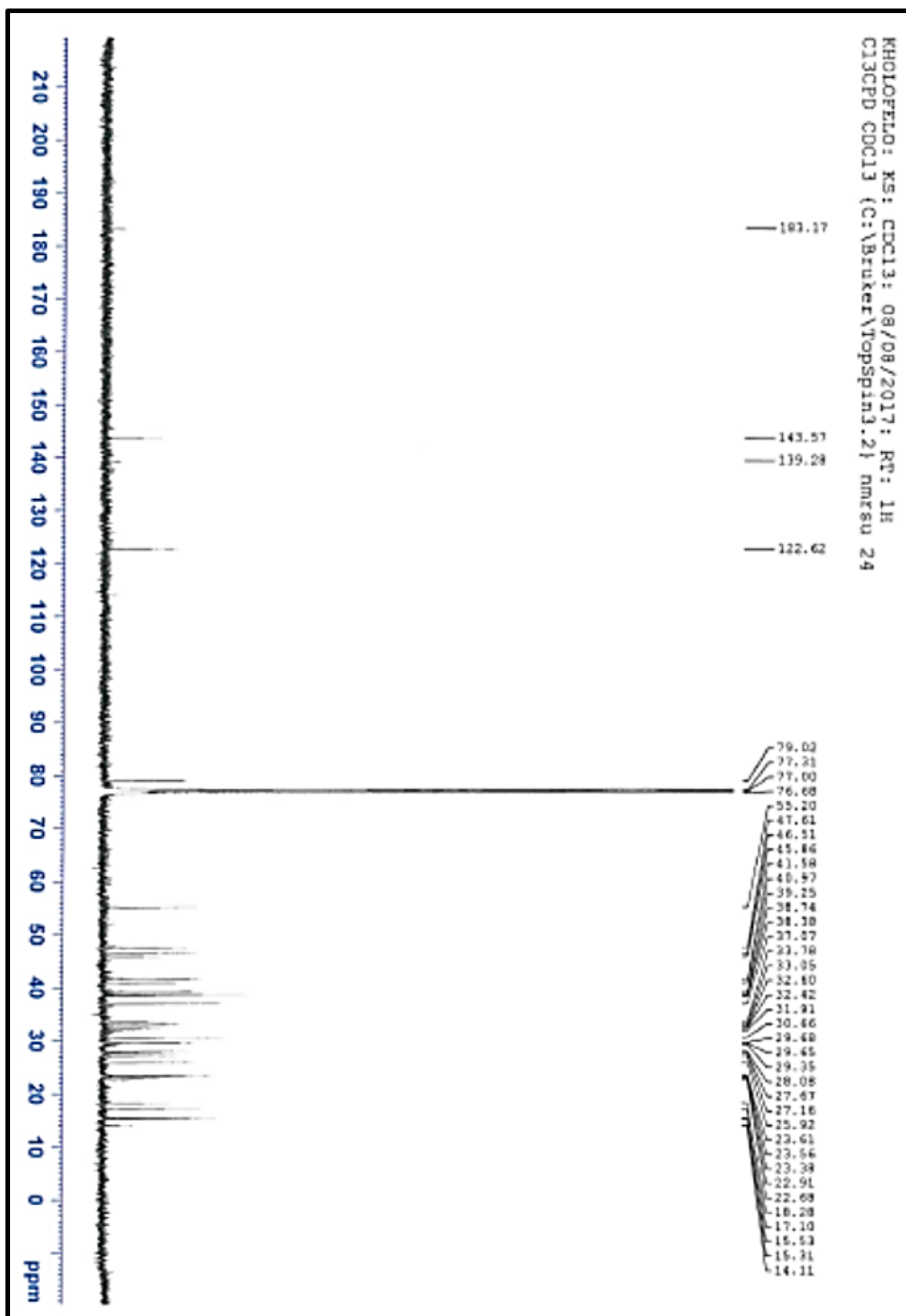


Figure 8.2:  $^{13}\text{C}$  (Chemical shifts) spectrum of the isolated compound.



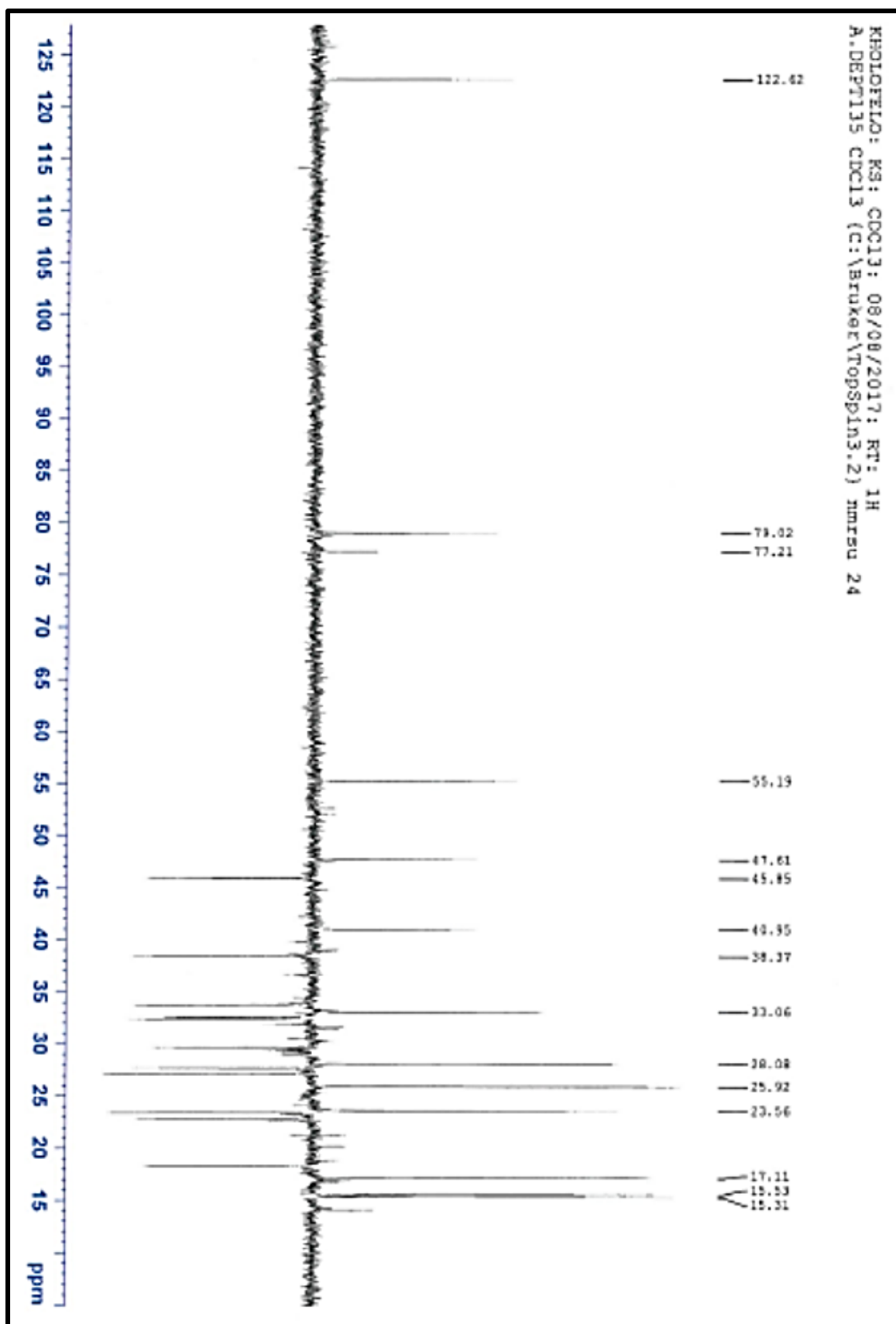


Figure 8.3: DEPT-135 spectrum of the isolated compound.

Table 8.1: Spectroscopic data of plant derived oleanolic acid mixed with ursolic acid isolated from acetone extract and the reported data of oleanolic and ursolic acid (Mapanga *et al.*, 2009; Rasaga *et al.*, 2014).

| Carbon Position | Isolated Compound (Major) (Oleanolic acid) | <sup>13</sup> C reference Ragasa <i>et al.</i> , 2014 (oleanolic acid) | <sup>13</sup> C reference Maphanga <i>et al.</i> , 2006 (Oleanolic acid) | Isolated Compound (Minor) (Ursolic acid) | <sup>13</sup> C reference Ragasa <i>et al.</i> , 2014 (Ursolic acid) |
|-----------------|--|--|--|--|--|
| 1               | 38.38                                      | 38.38  | 38.4   | 38.74                                    | 36.99  |
| 2               | 27.16                                      | 27.17  | 27.2   | 28.08                                    | 28.12  |
| 3               | 77.00                                      | 77.00  | 79.0   | 79.02                                    | 79.04  |
| 4               | 38.74                                      | 38.74  | 38.8   | 38.74                                    | 38.57  |
| 5               | 55.20                                      | 55.19  | 55.2   | 55.20                                    | 55.19  |
| 6               | 18.28                                      | 18.28  | 18.3   | 18.28                                    | 18.28  |
| 7               | 32.60                                      | 32.61  | 32.6   | 33.06                                    | 32.90  |
| 8               | 39.25                                      | 39.25  | 39.3   | 39.25                                    | 39.46  |
| 9               | 47.61                                      | 47.60  | 47.6   | 47.61                                    | 47.51  |
| 10              | 37.07                                      | 37.06  | 37.1   | 37.07                                    | 38.74  |
| 11              | 23.38                                      | 23.38  | 23.0   | 23.38                                    | 23.27  |
| 12              | 122.62                                     | 122.63   | 122.7  | 122.62                                   | 125.84   |
| 13              | 143.57                                     | 143.56   | 143.6  | 139.28                                   | 137.92   |
| 14              | 41.58                                      | 41.61  | 41.6   | 41.58                                    | 41.93  |
| 15              | 27.67                                      | 27.67  | 27.7   | 28.08                                    | 27.20  |
| 16              | 23.38                                      | 22.93  | 23.4   | 25.92                                    | 24.11  |
| 17              | 46.51                                      | 46.49  | 46.5   | 47.61                                    | 47.90  |
| 18              | 41.58                                      | 41.01  | 41.0   | 41.58                                    | 52.60  |
| 19              | 45.85                                      | 45.86  | 45.9   | 39.25                                    | 39.02  |
| 20              | 30.66                                      | 30.66  | 30.7   | 38.74                                    | 38.81  |
| 21              | 33.78                                      | 33.78  | 33.8   | 30.66                                    | 30.58  |
| 22              | 32.42                                      | 32.42  | 32.4   | 37.07                                    | 36.68  |
| 23              | 28.08                                      | 28.09  | 28.1   | 28.08                                    | 28.00  |
| 24              | 15.53                                      | 15.53  | 15.5   | 15.31                                    | 15.46  |
| 25              | 15.31                                      | 15.31  | 15.3   | 15.53                                    | 15.58  |
| 26              | 17.10                                      | 17.09  | 17.1   | 17.10                                    | 17.09  |
| 27              | 25.92                                      | 25.91  | 25.9   | 23.56                                    | 23.56  |
| 28              | 183.17                                     | 182.55   | 182.2  | 183.17                                   | 182.27   |
| 29              | 33.06                                      | 33.05  | 33.07  | 17.10                                    | 16.97  |
| 30              | 23.56                                      | 23.56  | 23.6   | 22.91                                    | 21.17  |

Using the data obtained from the analysis of  $^1\text{H}$ ,  $^{13}\text{C}$  and  $^{13}\text{C}$ -DEPT spectra, the structures of the compounds were elucidated to be a mixture of oleanolic and ursolic acids (Figure 8.4).

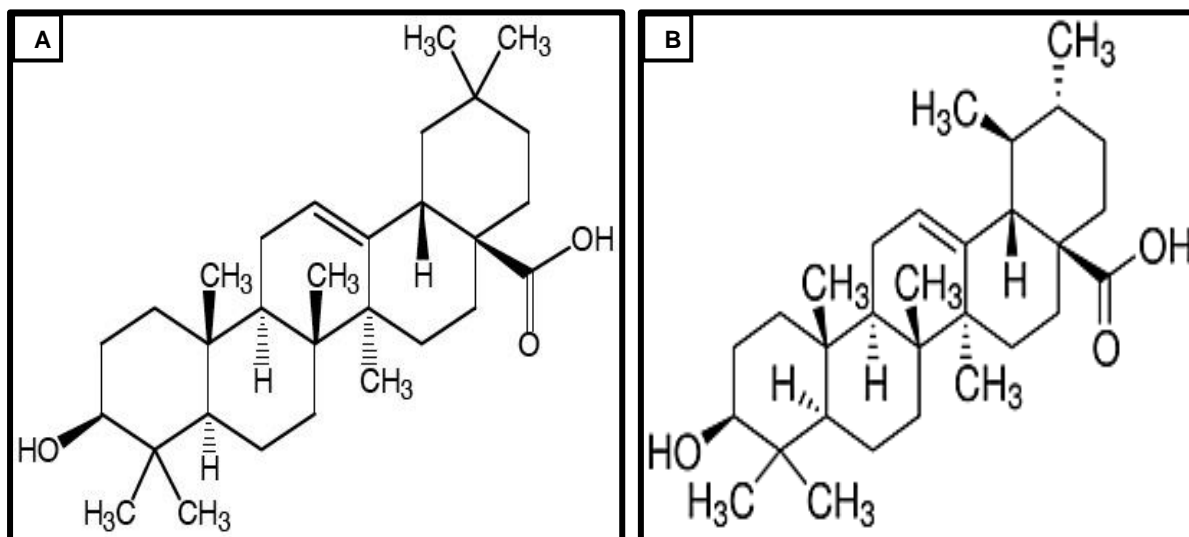


Figure 8.4: Oleanolic acid (A) mixed with ursolic acid (B), isolated from the acetone extract of *Olea europaea* subspecies *africana* leaves.

## 8.4. DISCUSSION

The structure of the isolated compound was elucidated using  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR and Distortionless Enhancement Polarization Transfer (DEPT-135). The  $^{13}\text{C}$  NMR consisted of 30 carbon peaks (Figure 8.2). The peak at  $\delta$ -183.17 may be due to the presence of carbonyl group. The two peaks at  $\delta$ -122.62 and  $\delta$ -143.57 may be due to the presence of a pair of  $\text{sp}^2$  hybridized carbons. The peaks at  $\delta$ -28.08, 15.31, 15.53, 17.10, 25.92, 33.78 and 23.38 are likely due to methyl substituents. These results are in agreement with the seven methyl ( $\text{CH}_3$  positive) carbon atoms seen in the DEPT-135 experiment. The proton peaks (Figure 8.3) are seven and correlates to the number of methyl groups on the structure as it was reported that the most intense peaks arise from the methyl groups, the less intense peaks arise from both the methylene as well as the methine groups (Tesso, 2005).

Based on this spectral data, the compound was likely to be oleanolic acid. However, there were peaks in C5, C6, C10, 15, 20, 21 and C23 to C27, which had the characteristics of ursolic acid. The spectroscopic data obtained in this study (Table 8.1) and that found in literature (Ragasa *et al.*, 2014), confirmed the presence of ursolic acid. However, the acid was constituted in small quantities, this was seen by the few peaks on the spectra (Figure 8.1-8.3). These acids were reported to be found as a mixture, either in a free form or as triterpenoid saponins in many plant species (Tian *et al.*, 2010).

From literature, a study conducted by Onoja and Ndukwe (2013) from which the physical and spectra data of the isolated oleanolic acid from *Borreria stachydea* were documented, it was reported that  $^{13}\text{C}$  signal at  $\delta$ -182.38 indicated the presence of carbonyl group assigned to C-28. The two peaks at  $\delta$ -122.66 and  $\delta$ -143.58 represent the presence of a pair of  $\text{sp}^2$  hybridized carbon atoms assigned to C-12 and C-13 while the seven peaks at  $\delta$ -28.11, 15.55, 15.33, 17.11, 25.92, 33.07 and 23.58 are attributable to the seven methyl groups which are assigned to C-23, C-24, C-25, C-26, C-27, C-29 and C-30, respectively. These results were similar to the ones obtained in this study, this confirms the presence of the functional groups of oleanolic acid.

The results obtained in a study conducted by Mapanga and colleagues (2009) and Ragasa *et al.* (2014) also supports the findings in this study and were used to

compare the  $^{13}\text{C}$  chemical shifts in table 8.1. A mixture of oleanolic and ursolic acid is a well-known compound and has been isolated from various parts of the plants by various scientists. The plants were *Lantana camara* (Narendra and Ameeta, 2014), *Ziziphora clinopodioides* (Tian *et al.*, 2010), *Orthosiphon stamineus* (Hossain and Ismail, 2013), *Lantana camara* L. (Verma *et al.*, 2013), *Kochiae fructus* (Kim *et al.*, 2005), *Tiarella polyphylla* (Matsuda *et al.*, 1998) and *Olea europaea* subspecies *africana* (Somova *et al.*, 2003). Based on the data found in literature the isolated compound was a mixture of oleanolic and ursolic acid (Figure 8.1A and B).

## 8.5. CONCLUSION

The isolated compound was identified as a mixture of ursolic acid and oleanolic acid. These are well-known triterpenes, which have already been isolated from various plants and their various biological activities are well-documented in literature. The biological activities will be discussed in the next chapter (Chapter 9). The spectroscopic data obtained from NMR compared with the data obtained from literature review confirmed the identity of the compound.

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## CHAPTER 9: BIOLOGICAL ACTIVITIES OF ISOLATED COMPOUND

### 9.1. INTRODUCTION

Natural products are a valuable source of research material for scientists. Compounds isolated from the natural products may serve as prototypes for the semi synthesis and for transformation into the urgently required new, safe and effective drugs. Their chemical structures may be used as a starting point for modifications in order to improve potency, selectivity or pharmacokinetic parameters of the parental compounds (Gordaliza, 2007).

Triterpenoids are a vastly varied group of natural products, including steroids and are extensively dispersed in plants. These compounds accumulate in the glycosidic form (saponins) (Sawai and Saito, 2011). Oleanolic acid (3 $\beta$ -hydroxyolean-12-en-28-oic acid) a compound isolated in this study (Figure 8.4A), is a natural pentacyclic triterpenoid compound that can be found in plants growing worldwide and it usually exists with its isomer ursolic acid (Figure 8.4B) (Li *et al.*, 2002).

These acids are relatively non-toxic and have been used in cosmetics and some health products (Tian *et al.*, 2010). For a long time, oleanolic acid was once believed to be biologically inactive, however, scientists have now investigated its numerous pharmacological properties and low toxicity. The compound has become more and more popular among scientists working in the fields related to medicine and pharmacy (Ovesna *et al.*, 2004). Oleanolic acid and ursolic acid have pharmacological activities which determine their therapeutic potential. In literature, they have been reported to demonstrate properties such as anti-inflammatory (Yin, 2007; Tsai, 2008), antitumor (Wang *et al.*, 2013), hepatoprotective (Liu *et al.*, 2008), antidiabetic (Wang *et al.*, 2010), antibacterial properties (Wolska *et al.*, 2010 and anti-HIV activity (Kashiwada *et al.*, 1998).



## **9.2. METHODS AND MATERIALS**

### **9.2.1. Bioautography assay**

Bioautography was done according to Begue and Kline (1972) as described in section 5.2.3.

### **9.2.2. Serial broth microdilution method**

Serial broth microdilution method was employed as described by Eloff (1998a) to determine the minimum inhibitory concentration (MIC) values of the compound against four tested bacterial species as mentioned in section 5.2.2.

### **9.2.3. Anti-inflammatory assay**

The anti-inflammatory activity of the compound was determined as described by Sekhar *et al.* (1983) as mentioned in section 6.2.1.

### **9.2.4. Cell viability assay**

The cytotoxicity of the compound was determined using the method described by Mosmann (1983) as described in section 6.2.2.

### **9.2.5. Selectivity index**

The selectivity index indicates the cytotoxic selectivity (i.e. safety) of the compound extract against normal (Vero) cells versus bacterial cells. This was determined by dividing the  $LC_{50}$  (in  $\mu\text{g/mL}$ ) of the compound on Vero kidney cells by the MIC ( $\mu\text{g/mL}$ ) values of the extracts on the tested pathogens (Elisha *et al.*, 2017). For the purpose of calculating selectivity index, MIC values greater than 250  $\mu\text{g/mL}$  were taken as 250  $\mu\text{g/mL}$ .

### 9.3. RESULTS

#### 9.3.1. Phytochemical analysis by Thin Layer Chromatography

TLC analysis was done to determine the purity of the isolated compound under UV light at 365 nm (Figure 9.1A) to detect any fluorescing compounds as well as spraying with vanillin-sulphuric acid reagent (Figure 9.1B) to detect the non-fluorescing compounds. The compound was found to be pure (Figure 9.1) as a single band appeared after spraying with vanillin and no compounds were detected that were UV reactive.

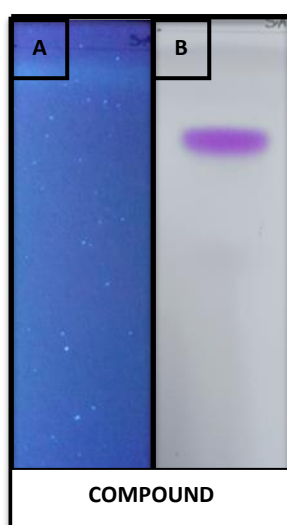


Figure 9.1: Chromatograms of an isolated compound, a mixture of uroslic acid and oleanolic acid compound, visualised under UV light at 365 nm (A) and sprayed with vanillin-sulphuric acid reagent (B).

### 9.3.2. Bioautography assay

The isolated compound was tested for its antibacterial activity (Figure 9.2) against *E. coli* (A), *P. aeruginosa* (B), *S. aureus* (C) and *E. faecalis* (D) qualitatively using direct bioautography and the solvent systems used to separate the reactive compounds were BEA, CEF and EMW. Antibacterial activity of the compound was seen as the clear or creamy zone against a purple-pink background on the chromatogram. INT reagent was used to detect the bacterial growths. *P. aeruginosa* (B) and *E. faecalis* (D) demonstrated susceptibility to the compound.

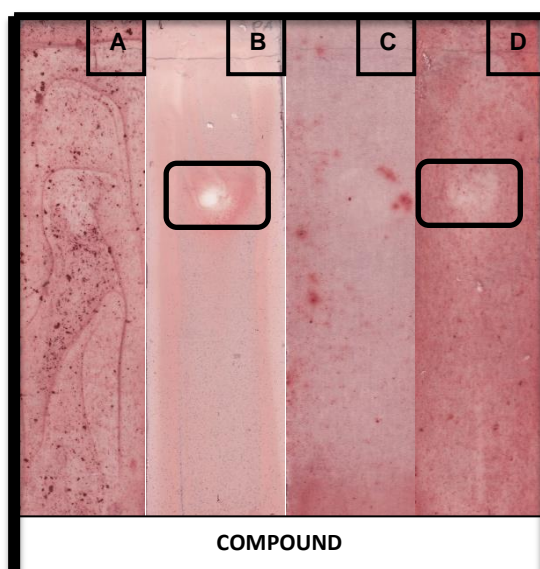


Figure 9.2: Bioautograms of the isolated compound tested against *E. coli* (A), *P. aeruginosa* (B), *S. aureus* (C) and *E. faecalis* (D).

### 9.3.3. Serial broth microdilution method

The compound was assayed for its ability to inhibit the tested bacterial species (Table 9.1). This was done by determining the MIC values of the compound against the tested bacteria. INT reagent was used to detect the bacterial growths. *P. aeruginosa* and *E. faecalis* were found to be the most susceptible bacteria.

Table 9.1: MIC values of compound and ampicillin (positive control) against all the tested bacterial species.

| Microorganism        | MIC values ( $\mu\text{g/mL}$ ) |      |
|----------------------|---------------------------------|------|
|                      | Compound                        | AMP  |
| <i>E.coli</i>        | >250                            | 0.03 |
| <i>P. aeruginosa</i> | 125                             | 0.02 |
| <i>E. faecalis</i>   | 125                             | 0.03 |
| <i>S.aureus</i>      | >250                            | 0.08 |
| <b>Average</b>       | <b>125</b>                      |      |

### 9.3.3. Anti-inflammatory assay

Anti-inflammatory assay (Figure 9.3) revealed that the production of ROS in RAW 264.7 cells upon stimulation with LPS was dose dependent i.e. as the concentration of the compound was increased; total ROS production decrease.

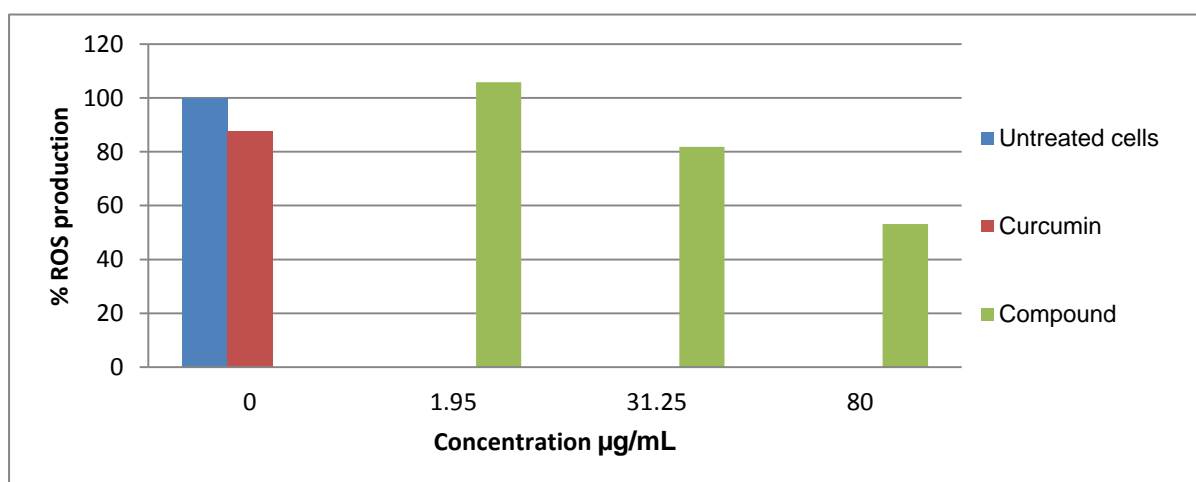


Figure 9.3: Anti-inflammatory activity of the isolated compound determined by measuring the production of reactive oxygen species (ROS) in percentages in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells.

### 9.3.4. Cell viability assay

The cytotoxicity of the isolated compound was above 20 µg/mL, indicating non-cytotoxicity of a compound when tested against the Vero kidney cells (Table 9.2). The results were supported by the high selectivity indices of the compound against *P. aeruginosa* and *E. faecalis* (Table 9.3), which indicated that these compounds were only toxic to the bacterial cells and not mammalian cells.

Table 9.2: The LC<sub>50</sub> of the isolated compound and doxorubicin (control) expressed in µg/mL, tested using Vero kidney cells.

| Sample            | LC <sub>50</sub> |
|-------------------|------------------|
| Isolated compound | 28.46            |
| Doxorubicin       | 2.29             |

Table 9.3: Minimum inhibitory concentration (MIC) and selectivity indices (SI) of the compound against *E. coli*, *P. aeruginosa*, and *E. faecalis* and *S. aureus*.

| Microorganisms       | MIC values (µg/mL) | SI values |
|----------------------|--------------------|-----------|
| <i>E. coli</i>       | >250               | 0.11      |
| <i>P. aeruginosa</i> | 0.125              | 227       |
| <i>E. faecalis</i>   | 0.125              | 227       |
| <i>S. aureus</i>     | >250               | 0.11      |

#### 9.4. DISCUSSION

The chemical profile of the isolated compound revealed that it was only vanillin reactive (Figure 9.1B) and not fluorescing under the UV light at 365 nm (Figure 9.1B). The compound fluoresced red during purification by column chromatography, however, no fluorescence was seen after pooling the fractions together. This was because the TLC plates used were non-UV reactive at 365 nm. The purple-coloured compound which appeared upon reaction with vanillin, had antibacterial activity against *P. aeruginosa* (Figure 9.2B) and *E. faecalis* (Figure 9.2D) only. The results obtained after performing the serial microdilution assay (Table 9.1) agreed with the results obtained from bioautography, which indicated that both of the mentioned bacteria had MIC values of 125 µg/mL.

*E. coli* and *S. aureus* had MIC values >250 µg/mL and were therefore regarded as having low antibacterial activity as seen in table 9.1. The bacteria did not show any zones of inhibitions as well (Figure 9.2A and D). Hichri *et al.* (2003) reported that, oleanolic acid possessed antibacterial activity against Gram-positive bacteria and has little or no effect on Gram-negative bacteria. It was proposed that the mechanism of oleanolic acid could be related to the difference in cell wall structure and the presence of the outer membrane on the Gram-negative bacteria (Horiuchi *et al.*, 2007). However, because oleanolic acid is usually found mixed with its isomer ursolic acid, then this explains the observed antibacterial activity against *P. aeruginosa*. do Nascimento *et al.* (2014) reported that ursolic acid demonstrated inhibitory effects on *P. aeruginosa* in their study.

Oleanolic acid did not have any antioxidant activity in this study; however, it has been documented that this compound possesses antioxidant activities and acts as a radical scavenger. The proposed mechanism of antioxidative action was documented by Balanehru and Nagarajan Bala (1991), wherein, oleanolic acid isolated from *Eugenia jumbolana* was studied for its protective effects against free radicals induced damage. The triterpenoids isolated from the leaves of *Olea europaea* subspecies *africana* in previous studies were of dichloromethane (Msomi and Simelane, 2017), ethyl acetate and methanol leaf extracts (Somova *et al.*, 2003, Long *et al.*, 2010) and not acetone extracts. This led to a conclusion that, acetone extracts may possess oleanolic acid that has no antioxidant activity.

The assay used to test for the anti-inflammatory properties of the isolated compound, revealed that, the compound was a good source of anti-inflammatory agents (Figure 9.3). The compound inhibited the percentage of ROS production in LPS induced cells to a minimum of 53%, when the compound was tested at its highest concentration. Terpenoids have been shown to inhibit COX activity and scavenge free radicals, while phenols interfere with NFK $\beta$  gene promotion and prevent lipid peroxidation (Salminen *et al.*, 2008). Phytoconstituents such as phenols and terpenoids are ubiquitous in plants, although the actual structures might be specific to a particular species. Novel anti-inflammatory agents from plants have the potential to have multiple targets, as a phytoconstituents or a mixture, for the dispersion of inflammation.

Oleanolic acid and ursolic acid had an LC<sub>50</sub> value of 28.46  $\mu$ g/mL (Table 9.2). This value is greater than 20  $\mu$ g/mL and was regarded as being non-cytotoxic according to the National Cancer Institute (NCI) (Abdel-Hameed, 2012). The value was even greater than that of doxorubicin (2.29 mg/mL). The selectivity index of the compound was 227 (Table 9.3), which indicated that this compound is safe to use on animal cells and only had inhibitory selectivity on bacterial cells. Several studies have also indicated that this compound had very low cytotoxicity against various normal cell lines (Somova *et al.*, 2003; Sultana and Ata, 2009).

## **9.5. CONCLUSION**

The compound isolated in this study had antibacterial activity against both Gram-negative and Gram-positive bacteria and had no antioxidant activity. The antibacterial activity observed, indicated that the mechanism of action could not be cell wall dependent as suggested in previous studies. The compound had good anti-inflammatory activities and it is very safe to use on animal cells. As such, new powerful drugs may be produced from this compound to treat, bacterial and inflammation-related diseases. Furthermore, this compound should be tested for antibacterial activity against the antibiotic-resistant strains.

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## CHAPTER 10: GENERAL DISCUSSION AND CONCLUSION

*Olea europaea* subspecies *africana* has been used traditionally for years to treat various ailments by the people of southern Africa. It has been traditionally used to treat urinary, bladder and eye infections, painful joints, arthritis and rheumatism to mention a few (Long *et al.*, 2010) and as such, validation of the leaves of this plant is necessary. This study was aimed at validating the use of the leaves to treat infections associated with *S. aureus*, *E. coli*, *P. aeruginosa* and *E. faecalis*. These bacterial species were selected because they were noted to be responsible for most diseases and infections, that are traditionally treated by the leaves of *Olea europaea* subspecies *africana*. The testing of the leaves for antibacterial activity confirmed their antibacterial activities. The activity was due to the presence of phytochemicals such as tannins, saponins, terpenoids and flavonoids which were found to be present in the leaves when detected by various standard chemical tests.

The DPPH assay showed that *Olea europaea* subspecies *africana* was rich in antioxidants, this activity was due to the amount of phenolic compounds found in the leaves. All assays used to detect the antioxidant activities of the leaves indicated that this is a potential plant that bioactive compounds could be isolated from. The fact that the isolated compound is of natural origin will contribute greatly to the pharmaceutical market wherein the use of synthetic antioxidant drugs will be reduced or eliminated since they were reported to have adverse side effects, which pose danger to human cells. The leaves showed good anti-inflammatory activities both as extracts and as an isolated compound. Cytotoxicity tests indicated that the leaves are much safe to use on animal cells and as such, more compounds should be isolated from these leaves.

### 10.1. RECOMMENDATIONS

The antibacterial activities of the leaves both as extracts and as compounds could be tested on antibiotic-resistant strains to determine their effectiveness against the strains. As such, drugs of therapeutic relevance could be produced and assist in the treatment of the infections associated with such strains. Seen that the plant possessed good anti-inflammatory activities, more bioactive compounds extracts could be isolated and tested against tumor cells, as they are closely associated with

chronic inflammation and as such, the plant may provide useful compounds for cancer prevention. The isolated compound performed better than the crude extract in terms of antibacterial activity and cytotoxicity. As such, isolation of bioactive compounds is recommended in order to purify the compounds and improve their biological activities. The use of the leaves of *Olea europaea* subspecies *africana* by humans for the past decades as tea, infusions, ointments and concoctions is in agreement with the non-cytotoxicity effects of the leaves and now that they were validated to be safe. Continuous use is recommended as this plant has excellent healing properties.

## **10.2. REFERENCES**

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