CHEMICAL COMPOSITION OF THE ESSENTIAL OILS OF SELECTED AROMATIC SOUTH AFRICAN PLANTS AND ASSESSMENT OF THEIR BIOLOGICAL ACTIVITIES FOR COSMETIC USES

by

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PREFACE

This thesis is submitted in fulfilment of the requirements for the Master of Applied Science: Chemistry at the Cape Peninsula University of Technology (CPUT). Chapter one brought forward a comprehensive background to the study by briefly discussing the relevance of the cosmetic industry, the approach to the skin's health, and essential oils from the South African flora as promising cosmeceutical ingredients. Thereafter, in the same chapter, were defined the research work with respect to the statement of the problem, the research aims and objectives, and the delineation of the study. Chapter two reviewed the chemistry of the essential oils, the methodologies used to study their chemistry, the principle of the laboratory procedures used to study the biological assays of relevance, and previous studies conducted on the South African essential oils selected for this study. Chapter three described the methods employed to achieve the research aims. Chapter four presented the results and discussions of the identification of essential oil components. Chapter five reported the results and discussions for the findings of the *in vitro* biological assays performed. Then, chapter six, the last chapter was as an evaluation of the present research work with respect to the initially set goals and the results produced. This chapter emphasised on the achievements and the shortcomings encountered to make recommendations for further research accordingly.

DECLARATION

I, Selena Eunice Orango Adewinogo, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

…….. 03/11/2020 ……..

Signed **Date**

ABSTRACT

The South African flora is endowed with botanical materials used for skin therapies in traditional practices. Nowadays, because the organic cosmetics consumerism is favoured, there is a need to explore the essential oils of indigenous plants for sought-after cosmeceutical properties.

The first objective of this study was to, investigate the chemical makeup of the essential oils (EOs) of *Oncosiphon suffruticosum* (L.) Källersjö, *Helichrysum petiolare* Hilliard & B.L.Burtt, *Helichrysum cymosum* (L.) D.Don subsp. *cymosum*, *Helichrysum odoratissimum* (L.) Sweet, *Salvia aurea* L. (syn. *Salvia africana-lutea* L.) and *Salvia chamelaeagnea* P.J.Bergius by chromatographic and spectroscopic techniques. The second objective was to determine their antimicrobial, antioxidant, photoprotective, and antityrosinase properties *in vitro*.

Gas chromatography-mass spectrometry (GC-MS) analyses of *Oncosiphon suffruticosum* EO revealed the major constituents: (+)-2-bornanone (31.21%) and filifolone (13.98%). *Helichrysum petiolare* EO contained faurinone (20.66%) and (*E*)-β-ocimene (17.21%). Faurione was structurally elucidated by ^{1}H and ^{13}C nuclear magnetic resonance (NMR). *Helichrysum odoratissimum* EO was found to contain chiefly 1,8-cineole (17.44%), α-pinene and γ-curcumene (15.76%). *Helichrysum cymosum* EO was found to be made up of α-pinene (29.82%) and (*E*)-caryophyllene (19.20%). *Salvia aurea* EO was found to contain primarily epi-α-cadinol (14.24%) and (*E*)-caryophyllene (7.93%), and *Salvia chamelaeagnea*, limonene (28.00%) and viridiflorol (13.42%).

The antimicrobial activity of the essential oils (25.6–0.2 mg/mL) was assessed via the broth microdilution test for the determination of MICs against three skin pathogenic bacteria, *S. aureus*, *P. aeruginosa*, and *E. coli*. According to the results, *S. aurea* EO was found to display the lowest MIC of 6.4 mg/mL against *S. aureus*, followed by *S. chamelaeagnea*, *O. suffruticosum*, and *H. odoratissimum* EOs at 12.8 mg/mL. *H. petiolare* and *H. cymosum* EOs were found inactive (MIC > 25.6 mg/mL) over the concentration range against this organism. *P. aeruginosa* was found to be most susceptible to *O. suffruticosum* EO at 6.4 mg/mL and to all other oils at 12.8 mg/mL except for *S. chamelaeagnea* EO which was found to be inactive for this organism. For *E. coli*, all the essential oils exhibited the same activity with MIC at 12.8 mg/mL. Ampicillin, the positive control, had an MIC < 0.2 mg/mL against *S. aureus* and *E. coli* whereas *P. aeruginosa* was found to be resistant against it.

The antioxidant capacities of the EOs were evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ferric reducing antioxidant power (FRAP), and oxygen radical absorbance capacity (ORAC) assays. The oils were tested at 2–0.5 mg/mL in the DPPH and ABTS assays and at 2 mg/mL in the FRAP and ORAC assays. In the DPPH assay, *S. chamelaeagnea* EO exhibited the highest percentage radical scavenging activity (% RSA) over the concentration range as 26.86 ± 0.10%–8.83 ± 0.68% whereas Trolox® positive control gave 94.94 ±

0.02%–94.45 ± 0.04%. In the ABTS assay, *O. suffruricosum* EO exhibited the highest % RSA over the concentration range as 87.17 ± 0.76%–71.46 ± 0.04% giving TEAC values (% RSD) of 9431.2(0.9%)–7750.1(0.1%) μmol TE/L (2-0.5 mg/mL respectively). This was followed closely by *H. petiolare* EO with respective % RSA and TEAC values of 84.42 ± 0.43% and 9131.4(0.5%) at 2 mg/mL, and 67.08 ± 0.76% and 7281.7(1.1%) μmol TE/L at 0.5 mg/mL. Gallic acid used as a positive control gave % RSA of 97.97 ± 0.13%–98.05 ± 0.03% and 605840(4.6%)–195220(3.2%) μmol TE/L over the concentration range (2–0.5 mg/mL). In the FRAP assay, *H. odoratissimum* EO had the highest value of 3026.6(6.1%) μmol AAE/L (% RSD) and gallic acid positive control gave 635500(0.6%) μmol AAE/L (% RSD). In the ORAC assay, the essential oils performed near equal with ORAC values ranging from 6701.8(0.9%) to 6549.7(1.5%) μmol TE/L (% RSD) while (-)-epigallocatechin gallate (EGCG) positive control was found as 26904(1.22%) μmol TE/L. Thin-layer chromatography-direct bioautography (TLC-DB) was conducted to discover radical scavenging materials present in the essential oils. The results showed that *H. petiolare* EO contained two phenolics, eugenol and 7-hydroxycadalene.

The photoprotective property of the essential oils was evaluated by spectrophotometric determination of the sun protection factor (SPF) over the UV range of 290-320 nm. The results revealed that the essential oil of *O. suffruticosum* possessed the highest SPF value as 2.299 followed by *H. petiolare* essential oil as 1.511. *H. cymosum*, *S. chamelaeagnea*, *H. odoratissimum*, and *S. aurea* essential oils exhibited SPF values below one of 0.956, 0.391, 0.309, and 0.216, respectively.

The tyrosinase inhibitory activities of the essential oils were determined using mushroom tyrosinase. The absorbance of L-3,4-dihydroxyphenylalanine (L-DOPA) was monitored at $\lambda_{490\ nm}$ using L-tyrosine as a substrate. The essential oils samples and Kojic acid positive control were tested at 200 μg/mL and 50 μg/mL. The results showed that the essential oils were found to exhibit close inhibitory activities of 63.30 ± 2.35–51.53 ± 10.30% (*H. odoratissimum* EO–*S. chamelaeagnea* EO) and 28.62 ± 0.30–19.13 ± 0.81% (*H. odoratissimum* EO–*S. aurea* EO) at 200 μg/mL and 50 μg/mL, respectively. Whereas the inhibitory activities of the Kojic acid were found to be 96.24 ± 3.62% and 98.34 ± 0.80% at 200 μg/mL and 50 μg/mL, respectively.

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DEDICATION

For my sweet **mother, Mounogou Kenguele Berthe**, my darling **father, Nkorouna Adewinogo Severin,** and my entire **amazing Family**. These include my lovely **grandmothers** Moussounda Adele (**mamie Tsoutsa)**, Dissamou Marie Louise (**mamie Tsayi)**, Mitsingou Caroline (**mamie Caline**), **my great-grand mother** Bibalou Marceline (**Maman Dina**), and two special beings to walk the Earth, **Adewinogo Moussounda AL** and **Lassissi Mousse MA**.

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GLOSSARY

Term	Explanation
Essential oils	Plant oils usually obtained by distillation. An essential oil possesses the typical scent of the plant material it is obtained from. Essential oils are distinct from the fatty vegetable oils by virtue of their volatility and their strong aroma.
Genus	The generic name given to a cluster of plants with common attributes.
Species	The specific name defining an individual plant following to the binomial nomenclature of botany. For example, in "*Saccharum officinarum*" (sugarcane) the genus is *Saccharum* and *Saccharum officinarum* is the species.
Organic	Natural extract from flowers and/or plants without using synthetic chemical substances.
Natural	Natural materials of mineral or vegetable origin frequently produced using standard methods that do not necessarily respect the norms of organic production. The natural product is not ipso facto organic.
Green product	A green product is intended to reduce the footprint on human health and the environment.
In vitro	*In vitro* settings encompass tests that occur outside a living organism such as, a test tube or culture dish.
Assay	An analysis performed to detect a material and its amount. It could also be used to gauge the worth of doing something.
Bioactivity	The property of a substance causing an effect or a reaction in a living organism.

ABBREVIATIONS AND ACRONYMS

- **λ**: wavelength
- **% v/v**: volume per volume concentration ratio
- **% v/w**: volume per weight ratio
- ®: registered
- **^{13}C**: carbon 13
- **^{1}H**: proton
- **ANOVA:** analysis of variance
- **BC**: Before Christ
- ***Ca.***: circa
- **Cat. no**: catalogue number
- **CC**: column chromatography
- **$CDCl_3$:** deuterated chloroform
- **COSY**: correlation spectroscopy
- **CPUT**: Cape Peninsula University of Technology
- **DMSO**: dimethyl sulfoxide
- **EC**: Enzyme Commission
- **EO(s)**: essential oil(s)
- **GC-MS**: gas chromatography-mass spectrometry
- **GCxGC-ToF-MS**: two-dimensional gas chromatography-time-of-flight mass spectrometry
- **GC-MS/FID**: GC-MS/flame ionisation detection
- **MIC**: minimum inhibitory concentration
- **NMR**: nuclear magnetic resonance
- **Syn.**: synonym
- **Rf**: retention factor, distance travelled from the baseline to the compound's spot over distance from the baseline to the solvent front on a TLC plate
- **SA**: South Africa
- ***Sp.***: species (singular)
- ***Spp.***: species (plural)
- **TLC**: thin-layer chromatography
- **TLC-DB**: thin-layer chromatography-direct bioautography
- **TM**: trademark
- **UK**: United Kingdom
- **UV**: ultraviolet
- **USA**: United States of America
- **Vs:** denotes comparison

UNITS

cm: centimetre	**L**: litre	**g**: gram	**^{0}C**: degree Celsius
mm: millimetre	**mL**: millilitre	**mg**: milligram	**eV**: electron volts
nm: nanometre	**µL**: microlitre	**µg**: microgram	**Hz**: Hertz
			Psi: unit of pressure, pound-force per square inch.
			u: atomic mass unit

OUTCOMES OF THIS RESEARCH

❖ Conference attended

Poster presentation at the SACI/RSC Young Chemists' Symposium 2018.

❖ Manuscripts in preparation

- Orango, S.A., Marnewick, J., Africa, C., Sharma, R. & Hussein, A.A. Chemical composition and cosmeceutical potential of the essential oil of *Oncosiphon suffruticosum* (L.) Källersjö.
- Orango, S.A., Marnewick, J., Africa, C., Sharma, R. & Hussein, A.A. Chemical study and comparison of the biological activities of the essential oils of three *Helichrysum* species, *Helichrysum petiolare* Hilliard & B.L.Burtt, *Helichrysum cymosum* (L.) D.Don subsp. *cymosum*, and *Helichrysum odoratissimum* (L.) Sweet.
- Orango, S.A., Marnewick, J., Africa, C., Sharma, R. & Hussein, A.A. Chemical analysis and assessment of the essential oils of South African *Salvia aurea* L. and *Salvia chamelaeagnea* P.J.Bergius.

1. CHAPTER ONE: INTRODUCTION

Skin, the body's largest organ, is at the interface with the outer environment. Not only does it provide a barrier against harmful microorganisms and protect the body from water loss, but it plays an important cosmetic role (Zhang & Duan, 2018). Humans associate the health and beauty of their skin with general "well-being'' and "health" (Ganceviciene et al., 2012).

This research was concerned with evaluating the potential of essential oils as cosmetic raw materials. Therefore, this chapter aims to give an overview of the concepts behind this entire study. The chapter brings forward the marking considerations, trends, and concerns encountered within the cosmetic marketplace and cosmetic science research and development. The approach was employed to direct the focus of the research work and to frame the understanding for each aspect explored in the study.

The chapter has four main parts. The first part reports the relevance of the global cosmetic industry and the current demands therein. The second part provides an overview of the molecular mechanisms involved in the biological activities in demand on the marketplace. The third part constitutes the solution brought by this research: the applicability of essential oils from an exploration of the South African flora. Then finally, the fourth part defines the research study with respect to aim, objectives, research questions, significance, and delineation.

1.1 The cosmetic industry

1.1.1 Cosmetics and their prominence

Beauty and cosmetics are concepts as old as early humankind and civilisations (Joshi, 2012). The word "cosmetic" has a Greek etymology from "*kosmeticos*" which signifies to adorn. Since the ancient times, materials utilised for beautifying or improving the appearance belong to the category of cosmetics (Khan & Alam, 2019). According to the United States Food and Drug Administration (U.S. FDA, 2018), a cosmetic is "a product (except for pure soap) applied onto the human body to cleanse, beautify, promote attractiveness, or alter the appearance". Today, cosmetics and personal care are vital industries that, notably contribute to the world's gross domestic product (GDP) (Aziz et al., 2017) and witness growth nearly twice as fast as the global economy (3.6%) (Global Wellness Industry [GWI], 2018). Data of 2018 revealed that, the beauty, personal care, and anti-aging segment was valued at $1.083 trillion, holding the largest market share of the global wellness economy worth $4.5 trillion (GWI, 2019).

1.1.2 The emergence of natural cosmetics and cosmeceuticals

Today, several synthetics are recognised as hazardous to human health. Parabens have been reported to cause contact dermatitis, identified as endocrine disruptors, and are believed to mimic the female hormone oestrogen when introduced into the body (Aziz et al., 2017). Other examples include the popular antityrosinase active or depigmenting compounds such as hydroquinone and its ether derivative, 4-benzyloxyphenol which cause irreversible depigmentation and excessive production of free radicals

within the cell respectively (Okombi, 2005). Because of these reports, with greater knowledge, public health awareness, plus the relearning of the benefits of natural products, there is a trend in consumerism to avoid synthetic ingredients and shift to cosmetics of natural origins (Chermahini & Majid, 2011). Nowadays companies are increasingly partnering with non-beauty professionals such as naturalists, dermatologists, and botanists to create innovative formulations and prove the benefits of their products (McCosh et al., 2011).

In addition to natural cosmetics, cosmeceuticals are rapidly outperforming the growth rate of all other product sections in the personal care and cosmetics sector (Market Watch, 2019). The term "cosmeceuticals" was coined by New York dermatologist Dr Albert Kligman in 1984 to describe products that did more than colour the skin and less than what pharmaceutical drugs intend to offer (Epstein & Fitzgerald, 2010). Cosmeceuticals are cosmetic-pharmaceutical hybrid products (Ligade et al., 2009; Joshi & Pawar, 2015), topical materials with biologically active ingredients promoting the health and beauty of skin (Draelos, 2014). Their enhanced effectiveness lies in their capacity to induce modifications in the skin's physiology (Morganti & Coltelli, 2019). Today, skincare is the dominant sector, by 43.3%, in the cosmeceuticals pool next to hair care, and oral care according to 2018 data (Grand View Research, 2019b). Prevalent skincare cosmeceuticals in escalating international demand for development are anti-aging, UV protective (sunscreen), and skin-whitening (Grand View Research, 2019a). The anti-aging section is the most common among these (Market Watch, 2019). All over the globe, the increasing aging population desiring to maintain their youthful appearance is driving the market growth (Grand View Research, 2019b). The anti-aging category is expected to compound the most significant the annual growth between 2016 and 2026 (Morganti & Coltelli, 2019). Other important cosmeceuticals include those that provide anti-bacterial and anti-septic properties (Grand View Research, 2019b).

1.2 Skin health: the compromises and the remediations

This section describes the molecular mechanisms involved in the sought-after biological activities in the formulation of cosmetics today namely: skin aging, antibacterial, and antityrosinase with a brief introduction to the anatomy of the skin.

1.2.1 Anatomy and physiology of the skin

The skin is the largest organ of the body. It constitutes 15% of the adult's body weight (Kolarsick et al., 2011) and has a surface area of approximately 1.8 m^2 (Page et al., 2006). The skin fulfils an array of vital functions such as, protecting against external chemical, physical, and biological insults. Additionally, it prevents against the excess loss of proteins, electrolytes, water, and has a role in thermoregulation (Kolarsick et al., 2011). The skin has three distinct layers, the epidermis, the dermis, and the subcutaneous tissue (Garg et al., 2017) or subcutis (Page et al., 2006) as shown in Figure 1-1.

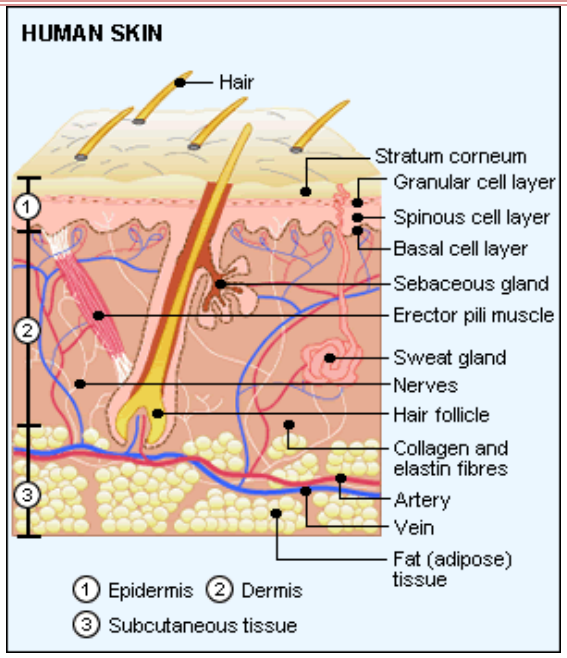


Figure 1-1 Cross-sectional representation of the skin (Curtis, n.d.)

The epidermis, the most superficial layer, primarily consists of keratinocytes (90-95% of cells), a smaller population of Langerhans cells (2%), melanocytes (3%), and Merkel cells (0.5%) (Tobin, 2017). Melanocytes are pigment-synthesising cells of the pigment melanin in a rounded membrane-bound organelle known as the melanosome. The melanocytes are responsible for the transfer of melanin to the keratinocytes (Kolarsick et al., 2011).

The dermis harbours an integrated system of fibrous, filamentous, and amorphous connective tissue (Kolarsick et al., 2011). It is primarily composed of extracellular proteins, such as collagen and elastin, made by the fibroblasts (Mitchell, 2016). The complex aggregate of distinct components of the dermis, both collagenous and non-collagenous, constitute the extracellular matrix (ECM) of the skin (Uitto et al., 1989). The dermis' primary function is to provide a resilient and flexible layer that holds the epidermis and attaches to the subcutis (Tobin, 2017). Collagen is the most abundant structural component of the dermis and protein found in humans (Baumann, 2007). Collagen fibres confer the skin tensile strength, whereas elastin fibres offer elasticity and resilience (Tobin, 2017). Glycosaminoglycans (GAGs) are also important molecules of the dermis. They are polysaccharide chains with repeating disaccharide units linked to a core protein. The GAGs are capable of binding to water up to thousand times their volume. Hyaluronic acid (HA), the major GAG (Badia, n.d.; Kolarsick et al., 2011), resides at the intersection of collagen and elastin fibres. It may contribute to preserving water in the skin (Baumann, 2007).

1.2.2 Photo-aging

The biological processes leading to skin aging are intricate and affected by the combination of intrinsic or endogenous factors and extrinsic or exogenous factors (Ganceviciene et al., 2012). Intrinsic (chronologic) aging is thought to be governed by individual genetic background thus considered inevitable (Baumann, 2007) and interventions aiming at this form of aging are deemed difficult (Tobin, 2017). However, extrinsic aging is caused by the exposure of an individual to factors such as chronic sun, smoking, excessive consumption of alcohol, pollution, and poor nutrition which fall within the volitional realm (Baumann, 2007). From a molecular basis, it has been proposed that only 3% of the effects of skin aging have an intrinsic background while most have an extrinsic cause (Zhang & Duan, 2018).

Among all exogenous factors, chronic exposure to solar ultraviolet radiation (UVR) is the greatest source of extrinsic aging (Ribeiro et al., 2015; Tobin, 2017; Zhang & Duan, 2018). By as much as 80% (Baumann, 2007; Tobin, 2017), sun-induced skin aging particularly occurs in individuals lacking the natural protection afforded by higher levels of melanocytes (Badia, n.d.). To stress the magnitude of the effects of UVR on the skin, the separate term photo-aging is used in literature (Tobin, 2017). Photo-aging is responsible for majority of the undesirable changes in cutaneous appearance over time. It is expressed by exaggerated or accelerated loss of physiologic reserve and several protective abilities (Gilchrest, 2013). The dynamics of photo-aging are marked by oxidative stress of the skin's cells and molecular changes in the ECM of the skin as discussed in the following two sections.

1.2.2.1 Solar rays-induced oxidative stress in skin cells

Solar ultraviolet radiation (UVR) triggers molecular responses in the skin primarily through photochemical generation of reactive oxygen species (ROS) (Poljšak & Dahmane, 2012). ROS is a collective term that designates free radicals, short-lived species with an unpaired electron in their last electronic shell, and the intermediates which initiate their generation (Monteiro e Silva et al., 2017). ROS include the hydroxyl radical (•OH), the superoxide anion ($\bullet O_2^-$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2) among others (Ahsanuddin et al., 2016). They significantly damage the molecular structure and function of the skin's macromolecules as they can react with cellular macromolecules within the vicinity of their generation (Ahsanuddin et al., 2016). Lipids, proteins, and DNA are highly vulnerable to oxidative processes (Garg et al., 2017).

As aerobic cells in the body produce ROS as metabolic by-products (Salehi et al., 2018), under healthy settings, the skin like other tissues possesses the enzymatic and non-enzymatic antioxidants for defence against ROS (Amstrong, 2016). However, UV-induced ROS generation may culminate in oxidative stress when their production surpasses the number of antioxidants in the target cell (Poljšak & Dahmane, 2012). Oxidative stress impairs the basic structural framework of the skin that appears uneven and with blotchy pigmentation. This consequently gives rise to wrinkles and sagging skin (Garg et al, 2017).

1.2.2.2 Enzymatic activity

ROS activate signalling pathways that preclude the production of collagen, elastin, and hyaluronic acid, and increase gene transcription of the enzymes that degrade them. These enzymes are collagenases (Matrix metalloproteinases [MMPs]), elastase, and hyaluronidase respectively as shown in Figure 1-2. Together, these molecular events result in a loss of toughness and flexibility in the skin and give rise to wrinkles (Garg et al., 2017).

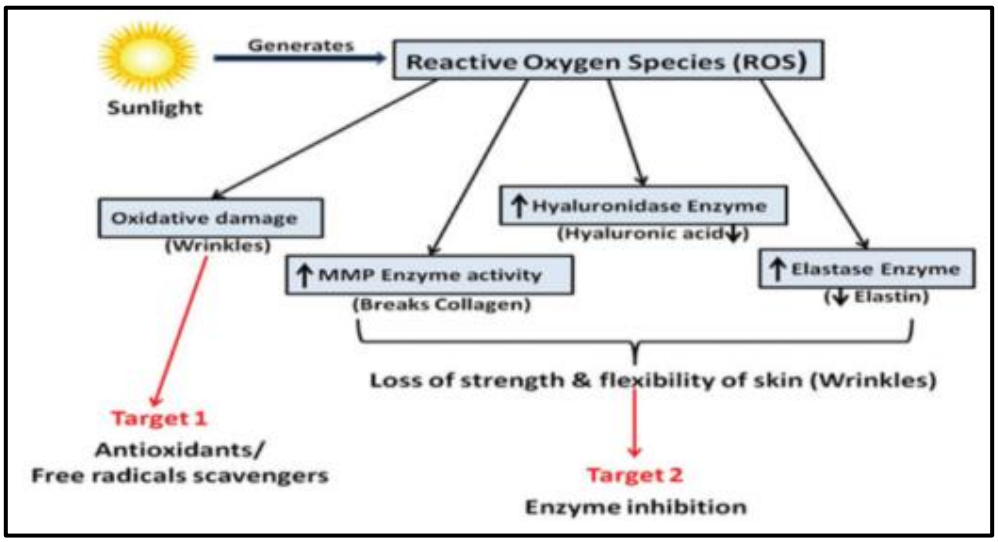


Figure 1-2 Molecular events in skin photo-aging (Garg et al., 2017)

1.2.2.3 Strategies to circumvent photo-aging

As shown above, the interaction of the skin with the environment has a direct impact on its state of health. Among all environmental stressors, chronic exposure to solar ultraviolet rays contributes by 80% to extrinsic aging in the skin. Apart from sun avoidance, remedial actions against photo-aging include the use of topical sunscreens, antioxidants, and inhibition of enzymes' activity in the extracellular matrix as reported by various authors presented in Table 1-1:

Table 1-1 Skin aging strategies

Strategy	Reference
Daily skincare: topical sunscreen, antioxidants, and DNA repair.	Mayoral et al., 2014
Stop degradation: collagen and elastin. Daily skincare: topical antioxidants, sunscreens, and retinoids (to boost collagen production).	Ganceviciene et al. (2012)
Sun avoidance, sunscreens, retinoids, antioxidants to neutralise free radicals.	Baumann (2007)
Primary treatment, photoprotection then secondary treatment, use of antioxidants.	Poljšak & Dahmane (2012)

Topical antioxidants.	Monteiro e Silva et al. (2017)
Topical antioxidants.	Mitchell (2016)
Inclusion of antioxidants and retinoids.	Zhang & Duan (2018)

1.2.3 Pigmentary disorders

Melanin, the main skin pigment, has a central role in photoprotection vis-à-vis the damaging UVR from the sun (Tu & Tawata, 2015; Zolghadri et al., 2019). However, its increased production represented by pigmented patches and skin discolourations (Tu & Tawata, 2015) in conditions such as melasma, freckles, and lentigines may cause serious aesthetic problems among humans (Al-Mamary et al., 2011; Zolghadri et al., 2019).

Melanin is biosynthesised in a process called melanogenesis. Although, a complex process of many enzymatic and chemical reactions (Figure 1-3), melanogenesis is regulated by tyrosinase, a membrane-bound copper-containing metalloenzyme with dinuclear copper ions, and other tyrosinase-related proteins (D'Mello et al., 2016; Zolghadri et al., 2019).

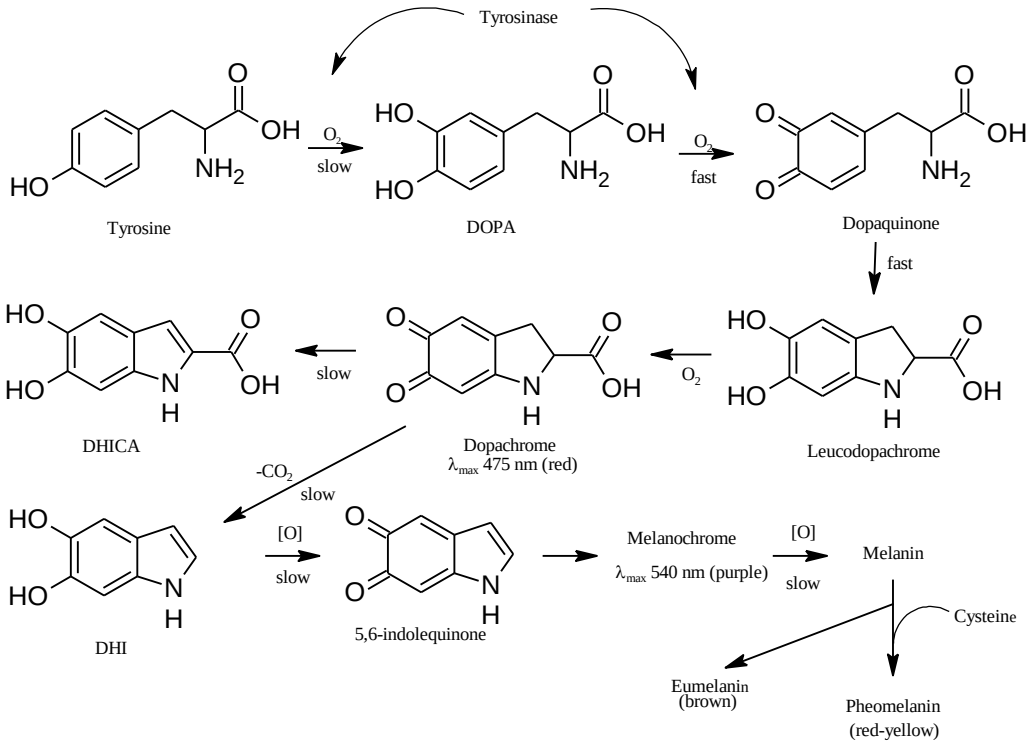


Figure 1-3 Biosynthesis of melanin from tyrosine. DOPA = dihydroxyphenylalanine, DHI = dihydroxyindole, DHICA = dihydroxyindole-2-carboxylic acid (Litwack, 2017)

Tyrosinase (EC 1.14.18.1) has a dual catalytic activity, a monophenolase activity, where the hydroxyl group adds to L-tyrosine (monophenol) to form L-3,4-dihydroxyphenylalanine (L-DOPA, the *o*-diphenol product), and a diphenolase activity, where L-DOPA is further oxidised to *o*-dopaquinone (Zolghadri et al., 2019). The conversion of L-tyrosine into dopaquinone marks the rate-limiting step in

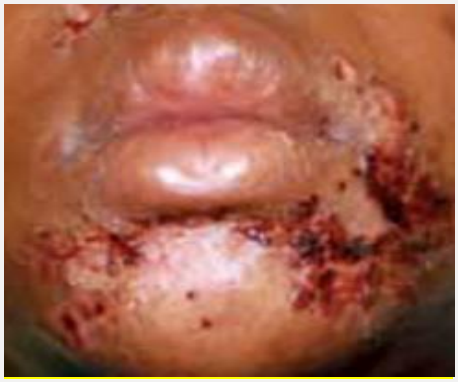
melanogenesis as further reaction sequences may take place spontaneously at a physiological pH value (Chang, 2009; Pillaiyar et al., 2017). Following the formation of dopaquinone, several intermediates are produced and lead to indolequinone which in turn polymerises to form melanin. The most common is eumelanin (brown) however, when cysteine is present, pheomelanin (red to yellow) may be made (Litwack, 2017). Therefore, inhibition of tyrosinase activity is tantamount to treating skin pigmentary disorders (Tu & Tawata, 2015).

1.2.4 Skin infections

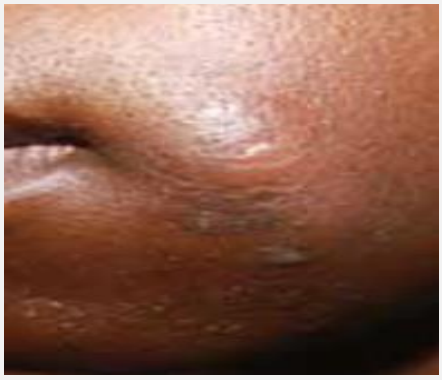
The skin guards against the growth and invasion of pathogenic bacteria by virtue of its rigidity, low moisture content, cooler temperature, and acidity (pH 5) which all together, provide an unfavourable environment for bacterial replication (Chiller et al., 2001). Additionally, a panel of microorganisms, collectively known as the skin's microbiome, reside on the skin and offer defence through hindering pathogenic bacterial growth. Their role involves competing for nutriments and attachment sites, as well as making metabolic products that prevent microbial growth (Orchard & Van Vuuren, 2017; Williamson et al., 2017). Resident gram-positive bacteria of the skin's microflora include *Staphylococcus*, *Micrococcus*, and *Corynebacterium spp*. (Chiller et al., 2001).

Breaches in the skin including ulcers, scratches, burns, wounds, or surgical incision may allow the incursion of bacterial pathogens in the epidermis (Orchard & Van Vuuren, 2017). These events cause common skin and soft tissue infections (SSTIs) (Williamson et al., 2017) or serious deep-seated infection requiring surgical intervention called complicated skin and soft tissue infections, cSSTIs (Dryden, 2010). Skin infections account for one of the main reasons people seek medical attention and pose a major cause of morbidity and death (Orchard & Van Vuuren, 2017).

Most common skin pathogens are *Staphylococcus aureus* and *Streptococcus pyogenes* (Chiller et al., 2001). Common superficial infections caused by these pathogens include impetigo (Figure 1-4, A), erysipelas, cellulitis (Figure 1-4, B), ecthyma, furuncles, carbuncles, and subcutaneous abscesses (Motswaledi, 2011). In hospitalised patients, the chief pathogens are *S. aureus* (top ranked in all regions), *Escherichia coli*, *Pseudomonas aeruginosa*, and *Enterococcus spp.* (Dryden, 2010).



A: Impetigo contagiosa in a child (Motswaledi, 2011)



B: Cellulitis on the cheek (Motswaledi, 2011)

Figure 1-4 Bacterial skin infections

Preventing and treating bacterial skin infections can necessitate topical antimicrobials, which may be an antibiotic. Theoretically, a topical antibiotic presents advantages over systemic administration such as, delivering high concentrations at the affected site and lessened systemic toxicity (Williamson et al., 2017). However, challenges in the use of currently available topical antimicrobials have emerged due to the increased resistance in skin pathogens (Orchard & Van Vuuren, 2017).

1.3 A solution to the search of natural cosmetic ingredients for healthy skin

1.3.1 Essential oils: valuable natural cosmetic/cosmeceutical ingredients

Essential oils (EOs) and their volatile components have been largely employed for preventing and treating human diseases (Properzi et al., 2013). Their earliest recognised attributes were probably their antiseptic and preservative actions. Evidence suggests that near 5000 BC, the ancient Egyptians concocted the essence of coniferous trees and utilised mixtures of essential oils as antiseptic products in mummification proceedings (Manou et al., 1998).

Although, they are mainly used in cosmetics for their pleasant scents (Sarkic & Stappen, 2018), published research shows that EOs possess antimicrobial (Dreger & Wielgus, 2013; Orchard & Van Vuuren 2017), antioxidant (Shaaban et al., 2012; Tu & Tawata, 2015), antityrosinase (Manosroi & Manosroi, 2005; Salleh et al., 2015, El Khoury et al., 2018), photoprotective properties (Hao et al., 2017; Mali & Killedar, 2018), as well as, inhibitory activities of skin's degradation enzymes (collagenase and elastase) (Aumeeruddy-Elafi et al., 2018). Moreover, their lipophilic and small molecules facilitate penetration through the skin layers to unfold their effects there (Sarkic & Stappen, 2018). Therefore, EOs are valuable plant-products to investigate for prospective cosmetic/cosmeceutical applications.

1.3.2 The potential of the South African flora as a source of cosmetic raw materials

South Africa is home to a diverse and unique botanical heritage, ranked the third most biologically diverse in the world. With over 30 000 flowering species, the flora represents approximately 10% of the

world's plant species and is characterised by a high level of endemism (Western Cape Government, 2005; Van Vuuren, 2008; Van Wyk, 2008; Street & Prinsloo, 2013; South African National Biodiversity Institute [SANBI], 2018).

The country has a strong history of traditional medicine since the arrival of the San people in the region 20,000 years ago (Scott et al., 2004). Nearly 80% of South Africans use indigenous plants to meet their primary health care needs (Street & Prinsloo, 2013). Recent data show that, South Africans have favoured organic skin and hair products. Close to one-quarter of the consumers consider natural, organic, or environmentally friendly alternatives before their purchase. Plant extracts from local sources include *Aloe ferox*, *Agathosma betulina* (buchu), *Sclerocarya birrea* (marula), *Adansonia digitata* (baobab), *Cyclopia intermedia* (honeybush), *Aspalathus linearis* (rooibos), etc. (The Department of Trade and Industry-InvestSA, 2020).

An abundance of aromatic species is found amid the rich South African flora. Fragrant families include Asteraceae (2300 species), Rutaceae (290 species), and Lamiaceae (235 species) of which many are endemic (Van Vuuren, 2008). Therefore, the flora is an important reservoir for potentially functional essential oils for natural and safe cosmeceuticals.

1.3.3 Aromatic plants species studied in this research

Generally, only a few SA medicinal plants have hitherto been fully, explored commercially (Van Wyk, 2011), and investigated scientifically (Lall & Kishore, 2014). The same holds for indigenous essential oils. According to the literature search presented in Chapter 2 (section 2.5) of the thesis, the cosmeceutical value of indigenous South African essential oils has not been widely explored.

The plant's species selected for this study were from the Asteraceae family: *Oncosiphon suffruticosum* (L.) Källersjö, *Helichrysum petiolare* Hilliard & B.L.Burtt, *Helichrysum cymosum* (L.) D.Don subsp. *cymosum*, *Helichrysum odoratissimum* (L.) Sweet and Lamiaceae family: *Salvia aurea* L. and *Salvia chamelaeagnea* P.J.Bergius. The selection was based on their recorded ethnomedicinal value in skincare, knowledge gap in the literature (2.5) as well as their availability in the Western Cape.

1.4 Definition of the research

1.4.1 Statement of the problem

In this day and age, consumers avoid cosmetic products that enhance beauty externally for those with real biological effects. Additionally, synthetic ingredients used in contemporary formulations have been associated with possible health risks on the skin. As a result, a global demand for both active and plant-derived cosmetic ingredients has been ignited for effective and safe cosmetic formulations. Essential oils are plant products that have been reported to exhibit therapeutic properties such as antimicrobial, antioxidant, antityrosinase, enzymatic inhibition, and photoprotective, which are value-adding to

cosmetic formulations and meet the current cosmetic industry's demands. Moreover, it was found that the biologically diverse South African flora abounds with essential oils-rich plants, however, possibilities offered by these plants have not been widely explored for skincare benefits.

1.4.2 Research questions

This study intended to answer the following questions:

- What are the chemical constituents of the EOs from the selected aromatic plants?
- What tests are appropriate to reflect the potential of the EOs for use in cosmetics considering today's social context?
- How biologically active are the EOs for prospective cosmetic applications?
- How does the chemical make-up of the EOs account for a biological activity being exhibited?

1.4.3 Research objectives

This investigation was aimed to characterise the constituents of the essential oils extracted from selected SA aromatic plants and determine their potential as functional cosmetics ingredients.

The specific objectives were to:

- collect and identify the selected aromatic plant materials;
- extract the essential oils from the selected aromatic plants;
- determine the chemical constituents of each essential oil;
- elucidate the chemical structure of major constituents contained in the essential oils by chromatographic and spectroscopic methods;
- determine the biological activities of all the essential oils; and
- use a screening method for the discovery of active compounds.

1.4.4 Delimitations

The study was executed with the following restrictions:

- Research was based only on endemic or indigenous aromatic plants of South Africa and not naturalised species.
- Research omitted the study of the seasonal variations of the essential oils' composition.

1.4.5 Significance of the research

This investigation aimed to contribute to the knowledge of the selected essential oils from South Africa in chemistry and bioprospecting for the cosmetic industry as a beneficiary.

2. CHAPTER TWO: LITERATURE REVIEW

This chapter is a comprehensive description of, the chemistry of essentials oils, their extraction method, the methods used to study their chemical characterisation, the background to the laboratory procedures used to perform the relevant biological assays, and the previous research works conducted on the selected essential oils for this study.

2.1 Essential oils chemistry

2.1.1 Plant secondary metabolites

Plants harness solar energy to fuel complex biochemical reactions leading to two types of metabolisms: primary metabolism and secondary metabolism (Khanam, 2007). The primary metabolism produces the carbohydrates, nucleic acids, lipids, and proteins found across all living organisms and utilised for life-sustaining functions such as respiration, nutrition, development, and reproduction (Sell, 2010; Pagare et al., 2015). The secondary metabolism, by contrast, refers to the downstream biosynthetic processes from primary metabolites intermediates leading to highly functionalised and lineage-specific constituents (Khanam 2007; Maeda, 2019).

The secondary metabolites (SMs) are non-indispensable metabolites to the vital maintenance of the plants but specialised compounds conferring better survival in their environment (Kumar & Mina, 2013; Sharifi-Rad et al., 2017). They reflect direct ecological interactions in response to marked environmental conditions: environmental stress, defences against predators, interspecies competition, and facilitation of the reproductive processes (attraction of pollinators through colouring agents, attractive smells etc.) (Khanam, 2007).

Essentials oils are secondary metabolites that provide adaptation to abiotic changes such as light, draught, CO_2 level, and ozone level. Moreover, they mediate the relationship of the plant to the biotic factors such as competitors, microbial pathogens and herbivores (defensive role), and beneficial insects for pollination and seed dispersers (attractive role) (Pichersky & Gershenzon, 2002; Sharifi-Rad et al., 2017).

2.1.2 What are essential oils?

Essential oils (EOs) are intricate mixtures of plant volatile (100u) and semi-volatile (300u) organic constituents conferring a characteristic aroma and flavour to plants (Zuzarte & Salgueiro, 2015; Dhifi et al., 2016). EOs are generally colourless and liquid at room temperature with a density less than unity apart from cinnamon, sassafras, and vetiver (Dhifi et al, 2016). They may contain up to 300 different compounds with a few components at high concentrations (20-80%) while the remaining constituents are present in trace amounts, nevertheless, still contribute to the build-up of the aroma (Cavalcanti et al., 2013).

The stem "essence" in essential oil finds its origin in the 16th century from the Latin word "*Quinta essentia*" meaning the fifth element as a reference to a spirit or life force (Sell, 2010; Dhifi et al., 2016). The term was introduced by the physician, theologian, astrologer, alchemist, physicist, and philosopher of Switzerland Paracelsus von Hohenheim to name distilled oils from herbs (Dhifi et al, 2016; Butnariu & Sarac, 2018). At the time, it was believed that distillation and evaporation from plants removed the spirit of the plant. Eventually, nowadays, it is common knowledge that essential oils are far from being spirits but a mixture of chemicals (Sell, 2010). The definition of essential oils has been reformulated by renowned regulatory bodies. According to The French Agency for Normalization (Agence Française de Normalisation, AFNOR, NF T 75-006) and The International Organization for Standardization (ISO, ISO 9235: 2013), EOs are products extracted from a vegetable raw material through distillation techniques such as steam distillation, hydrodistillation, dry distillation or by an appropriate mechanical process (as used for the epicarp *Citrus* fruits due to the thermolabile compounds contained therein). Then, the essential oil is recovered from the aqueous phase by physical means (Zuzarte & Salgueiro, 2015; Dhifi et al., 2016; Bhavaniramya et al., 2019).

The physical and chemical properties of EOs distinguish them from fixed oils and fatty acids (triglycerides or triacylglycerols). Moreover, aromatic/volatile compounds such as concretes or absolutes, extracted by solvent extraction, microwave-assisted extraction, and supercritical fluid extraction, are not defined as essential oils (Zuzarte & Salgueiro, 2015).

2.1.3 Biosynthetic pathways: secondary metabolites found in EOs

Despite the large diversity of secondary metabolites, their core skeletons derive from basic precursors produced through primary biosynthetic pathways such as glycolysis, the Krebs cycle (tricarboxylic acid cycle), and the shikimate pathway (Wink, 2008).

Through the process of photosynthesis, green plants convert carbon dioxide and water into glucose. Then, phosphoenolpyruvate, a product of glycolysis, serves as a building block for the shikimate pathway. Subsequently, the decarboxylation of phosphoenolpyruvate yields two-carbon units of acetate followed by esterification by coenzyme A to give acetyl-CoA (Figure 2-1). The latter material is the starting point for the synthesis of polyketides and lipids, as well as mevalonic acid involved in the manufacture of terpenoids (Sell, 2010).

As per their distinct biosynthetic origins, plant secondary metabolites are classified into four main groups, terpenoids, shikimates, polyketides and lipids, and alkaloids. Terpenoids and shikimates biogenetic routes produce the most important chemical compounds as far as essential oils are concerned. Degradation products from the polyketides and lipids pathway, chiefly lipids, also contribute to the components of some essential oils but very few alkaloids are found (Sell, 2010). Therefore, terpenoids,

shikimates will be discussed in further detail, but the lipids and alkaloids will be given some illustrative references only.

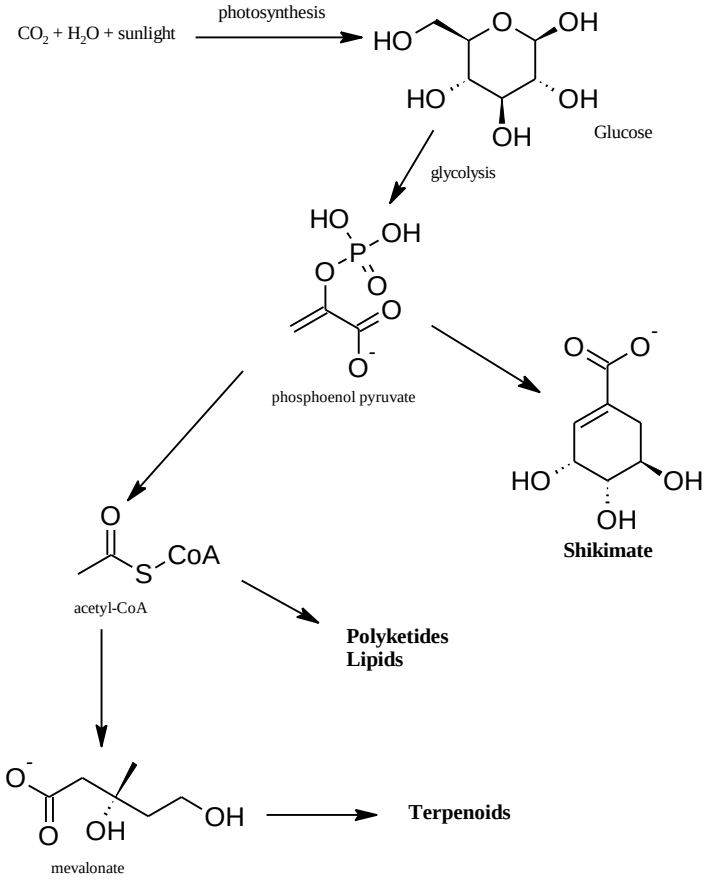


Figure 2-1 Basic biosynthetic routes leading to secondary metabolites present in EOs (Sell, 2006; Sell, 2010)

2.1.4 Terpenes

2.1.4.1 Biosynthesis of terpenes precursors

Terpenes and its functionalised derivatives, the terpenoids, are the chief chemical class of compounds occurring in EOs. The denomination "terpene" was first introduced by the German organic chemist Kekulé in 1880 to designate the $C_{10}H_{16}$ compounds present in turpentine (Zuzarte & Salguiero, 2015), the suffix "ene" indicating olefinic bonds (Ludwiczuk et al., 2017, p. 233).

In biogenesis terms, the carbon backbone of plant terpenes results from the condensation of two central intermediates of the terpenoids metabolism, 5-carbon isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Figure 2-2). They originate from two different pathways, the mevalonate (MVA) pathway and 2-C-methyl-ɒ-erythritol 4-phosphate (MEP) or non-mevalonate pathway occurring in the cytosol and plastid (chloroplast) of plant cells respectively (Zuzarte & Salguiero, 2015; Ludwiczuk et al., 2017, p. 233; Moghaddam & Mehdizadeh, 2017; Bergman et al., 2019).

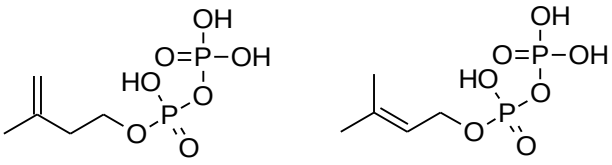


Figure 2-2 Isopentenyl pyrophosphate (IPP) (left), dimethyl allyl pyrophosphate (DMAPP) (right)

❖ **The mevalonate (MVA) pathway**

The cytosolic MVA pathway produces one molecule of IPP in six enzymatic steps with 3 acetyl-CoAs, 3 adenosine triphosphate (ATP), and 2 nicotinamide adenine dinucleotide phosphate (NADPH) reducing equivalents. Briefly, 2 units of acetyl-CoA condense to form acetoacetyl-CoA by the action of acetoacetyl-CoA thiolase. Acetoacetyl-CoA is in turn condensed with the third acetyl-CoA by 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS) to yield 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). HMG-CoA reductase (HMGR) then reduces HMG-CoA to mevalonate. Thereafter, two sequential enzymatic phosphorylations yield to the formation of melavonate-5-diphosphate. Lastly, mevalonate 5-diphosphate is subject to enzymatic decarboxylation by mevalonate diphosphate decarboxylase to form IPP. Following the latter conversion, IPP can be reversibly isomerised into DMAPP by IPP isomerase (Bergman et al., 2019).

❖ **The non-mevalonate (MEP) pathway**

The MEP pathway yields IPP and DMAPP in a series of enzymatic reactions in the chloroplasts of the plant cell (Zuzarte et al., 2015; Wang, n.d.). The process involves starting with D-glyceraldehyde-3-phosphate (GAP) and pyruvate at the expense of 3 ATP and 3 NADPH equivalents. The process begins with the condensation of pyruvate and GAP to form 1-Deoxy-ᴅ-xylulose-5-phosphate (DXP) which, is then reduced to MEP. Through further downstream enzymatic transformations, from MEP are produced IPP and DMAPP (Bergman et al., 2019).

2.1.4.2 Classification of terpenes

Terpenes are grouped following the number of C_5-units involved in their synthesis engaging their biogenic precursors IPP and DMAPP (Wang et al., 2019). A first head-to-tail fusion of IPP and DMAPP produces geranyl diphosphate (GPP), the precursor of monoterpenes (C10). Then, the successive addition of IPP to the previous substrate results in the formation farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP), which represent the precursors to sesquiterpenes (C15) and diterpenes (C20) respectively, and so on. (Zuzarte & Salgueiro, 2015).

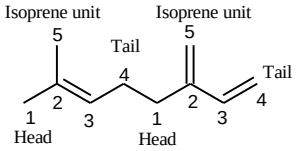


Figure 2-3 Linkage fashion of isoprene units in terpenes

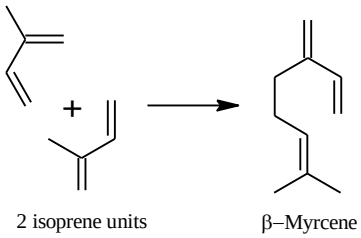


Figure 2-4 Fusion of two isoprene units to form monoterpene β-myrcene

In nomenclature, the isoprene unit was suggested as the fundamental building block of the terpenes (Wang, n.d.). They are regarded as polymers of the isoprene unit which, constitutes a structural reference to the true precursors, IPP and DMAPP (Morsy, 2017) (Figure 2-3, Figure 2-4) also called the "active isoprene units" (Tisserand & Young, 2014). Isoprene itself does occur naturally but it is strictly not involved in the formation of terpenes as described earlier (Wang, n.d.).

Table 2-1 Classification of terpenes

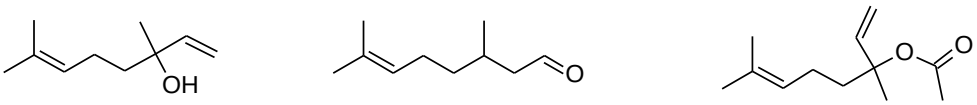
Prefix	Isoprene units	Carbon skeleton	General formula
Hemi	1	C_5	C_5H_8
Mono	2	C_{10}	$C_{10}H_{16}$
Sesqui	3	C_{15}	$C_{15}H_{24}$
Di	4	C_{20}	$C_{20}H_{32}$
Sester	5	C_{25}	$C_{25}H_{40}$
Tri	6	C_{30}	$C_{30}H_{48}$
Tetra (carotenoids)	8	C_{40}	$C_{40}H_{64}$
Poly	> 8	> C_{40}	$(C_5H_8)_n$

The monoterpenes ($C_{10}H_{16}$) and sesquiterpenes ($C_{15}H_{24}$) predominate the chemical makeup of EOs. They possess many isomeric cyclic or linear structures, various degrees of unsaturation, substitutions, and functionalisation of a heteroatom, mainly oxygen, being generally called terpenoids (Zuzarte & Salgueiro, 2015). The oxygenated derivatives of terpenes are alcohols, ketones, aldehydes, peroxides,

carboxylic acids, ethers, and lactones (Sell, 2010). Diterpenes, may also be present but generally do not make the odour of essential oils (Zuzarte & Salgueiro, 2015).

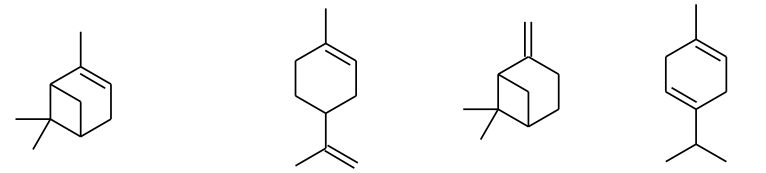
2.1.4.3 Illustrations of some common essential oils' terpenoids

The following figures (Figure 2-5, Figure 2-6) illustrate common terpenes and terpenoids encountered in essential oils.

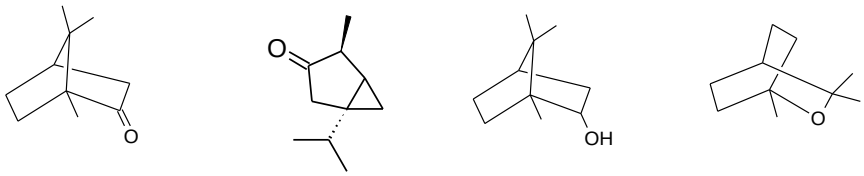


Linalool Citronellal Linalyl acetate

Acyclic

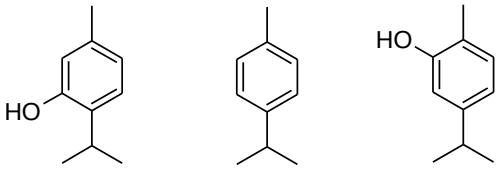


α-Pinene Limonene β-Pinene γ-Terpinene



Camphor β-Thujone Borneol 1,8-Cineole

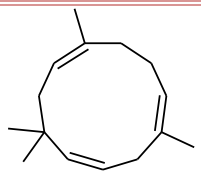
Alicyclic: Monocyclic and bicyclic



Thymol *p*-Cymene Carvacrol

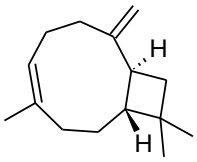
Aromatic

Figure 2-5 Structural diversity of essential oils' monoterpenoids

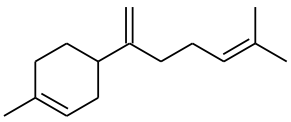


Humulene

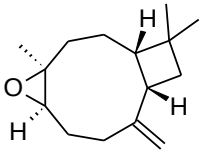
(α-Caryophyllene)



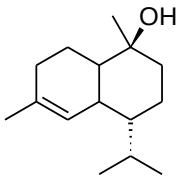
(*Z*)-β-Caryophyllene



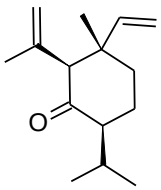
β-Bisabolene



β–Caryophyllene oxide

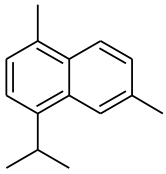


α-Cadinol



Shyobunone

<u>Alicyclic</u> : Monocyclic and bicyclic



Cadalene

<u>Aromatic</u>

Figure 2-6 Structural diversity of essential oils' sesquiterpenoids

2.1.5 Phenylpropanoids

2.1.5.1 Biosynthesis

Phenylpropanoids, the compounds with a benzene ring attached to 3 carbon substituents (C_6-C_3), are a diverse family of secondary metabolites produced from the shikimate pathway (Drijfhout & Morgan, 2010; Soledade et al., 2010; Zuzarte & Salguiero, 2015). The pathway involves several branches of metabolic routes with central intermediates to yield a myriad of structurally diverse compounds including the phenylpropanoids encountered in EOs (Soledade et al, 2010; Sell, 2010). The shikimate pathway begins with the coupling of phosphoenolpyruvate (PEP) and erythrose-4-phosphate to yield chorismic acid (Soledade et al., 2010), the precursor to the aromatic amino acids such as phenylalanine, tyrosine, and tryptophan (Kim et al., 2006; Soledade et al., 2010).

Phenylpropanoids synthesised in plants are derived from phenylalanine and from tyrosine in some cases (Herbert, 1981). Cinnamic acid, an important precursor to phenylpropanoids, emanates by the enzymatic removal of the amine group from phenylalanine by phenylalanine ammonia-lyase (PAL). After cinnamic acid is hydroxylated by cinnamate-4-hydroxylase to form *p*-coumaric acid, this latter substrate is converted to *p*-coumaroyl CoA when CoA thioester is added by a 4-coumarate:CoA ligase enzyme (Thomas & ElSohly, 2016). *p*-coumaroyl CoA, this high energy intermediate, serves as a substrate to further enzymatic transformations including, acylation, condensation, cyclisation, hydroxylation, methylation etc. (Figure 2-7) (Soledade et al., 2010). The EOs constituents, eugenol, safrole (Figure 2-8), and estragole are a prime representation of phenylpropanoids derived from this pathway (Morsy et al., 2017).

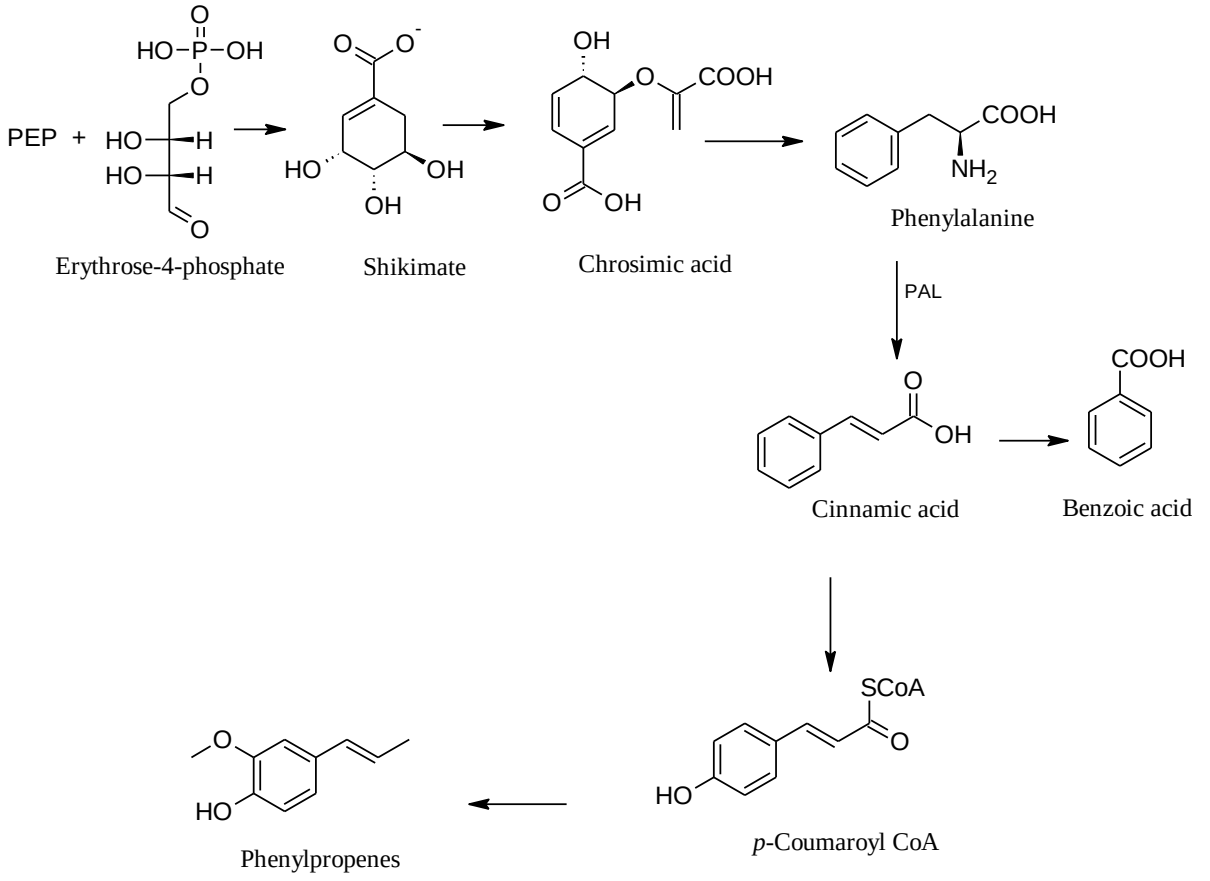


Figure 2-7 Simplified shikimate pathway leading to phenylpropanoids (Adapted from Vogt, 2010)

Phenylpropanoids are precursors to benzenoids compounds, (C_6-C_1). Benzenoids are obtained following the loss of 2 carbons in the side chain of phenylpropanoids (Soledade et al., 2010; Faleiro & Miguel, 2013). They also occur as derivatives of benzoic acid early in the shikimate pathway. Benzenoids are widespread in EOs. Examples of the EOs' benzoic acid derivatives are benzyl alcohol and benzaldehyde (Sell, 2010) as shown in Figure 2-8.

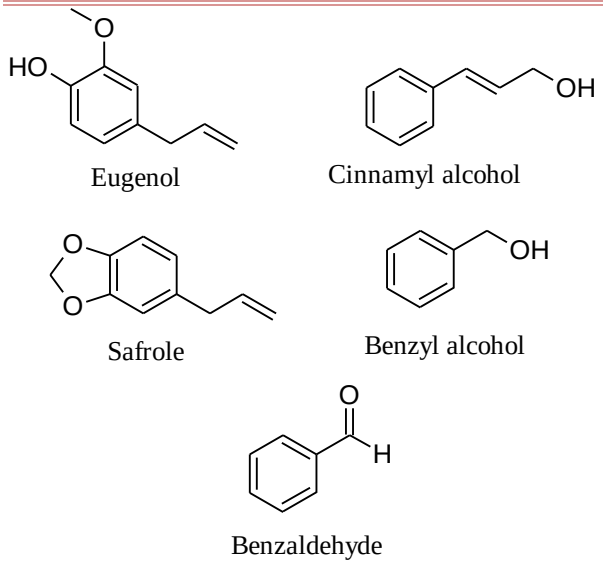


Figure 2-8 Structures of some common essential oils' phenylpropanoids and benzenoids

2.1.6 Lipids

Components of essential oils in the polyketides and lipids family of metabolites are formed through pathways of condensation reactions of polyketides, degradation of lipids, and cyclisation of arachidonic acid (Sell, 2010). Hereunder, are presented lipid-derived components occurring in EOs in Figure 2-9.

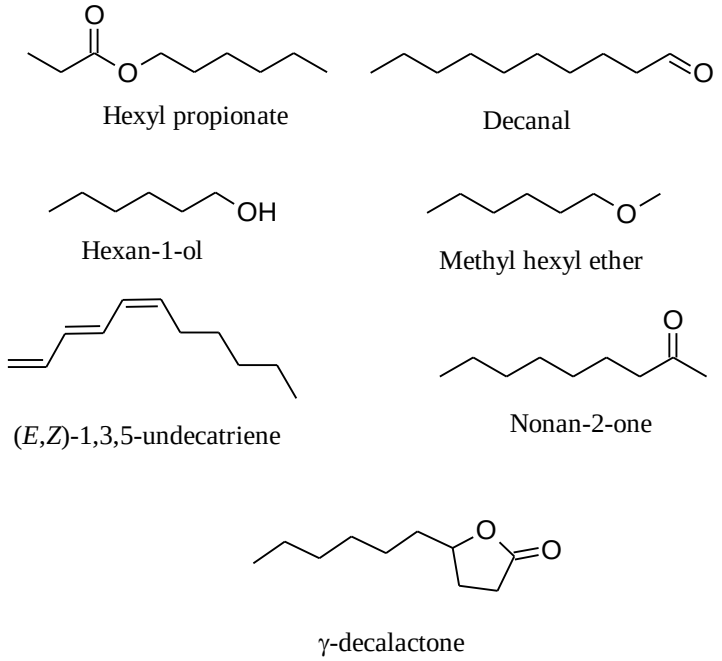


Figure 2-9 Some lipid-derived components of EOs (Sell, 2010)

2.2 Isolation of essential oils by hydrodistillation

Hydrodistillation is a simple method and the popular conventional method used to extract essential oils (EOs) from aromatic plants. It requires a Clevenger apparatus as shown in Figure 2-10:

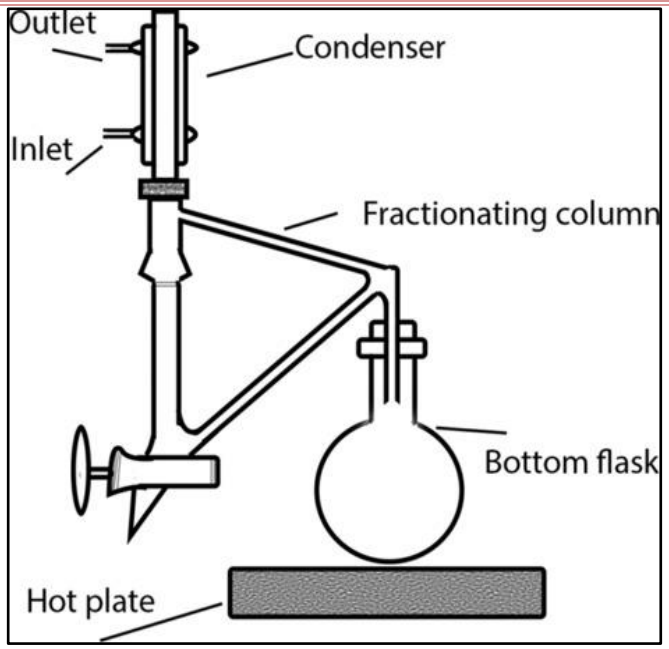


Figure 2-10 Clevenger apparatus set-up (Waseem & Low, 2015)

The principle of extraction is based on an azeotropic distillation. The plant material is immersed in water inside a round-bottom flask and extracted at atmospheric pressure. The heterogeneous mixture of water and the EO molecules attains the boiling temperature at a lower point close to 100 ºC and is distilled off simultaneously as if they were a single compound. This process is referred to as co-distillation because the vapours of water act as a solvent drive. Subsequently, the condensed water and EOs vapours separate naturally on the basis of immiscibility and densities (El Asbahani et al., 2015).

The advantages of this method are its easiness, selectivity, and the inexpensive equipment requiring simple installations. Nonetheless, the prolonged contact of the plant material with the boiling water often results in chemical artifacts and alterations (hydrolysis and cyclisation) and the loss of some polar molecules that transfer to the extraction water (El Asbahani et al., 2015).

2.3 Chemical characterisation of essential oils

The following sections describe the different methods used to study the chemical composition of essential oils.

2.3.1 Column chromatography (CC)

Adsorption column chromatography is commonly employed to separate of natural products thanks to its high capacity and simplicity. Moreover, the technique is convenient for the cheap adsorbents available such as silica gel, the most used adsorbent in phytochemical investigations. The molecules are retained by the silica gel through hydrogen bonding and dipole-dipole interactions with its silanol groups (Zhang et al., 2018). For the fractionation of monoterpenoids, isocratic or gradient elutions with a combination of solvents in increasing polarity lead to successful purification (Çitoğlu & Acıkara, 2012).

2.3.2 Thin-layer chromatography (TLC)

Thin-layer chromatography (TLC) is a select method for the identification of organic compounds, quantitative analysis, and the purification of single compounds from multicomponent mixtures on a preparative scale. Several types of adsorbents and mobile phases allow for a variety of TLC systems. This in turn offers an extended spectrum of separation selectivities, which becomes necessary for complex mixtures such as plant extracts (Waksmundzka-Hajnos et al., 2008). The sulphuric acid-vanillin reagent with subsequent heating serves as a rapid detection for essential oils components (terpenoids and phenylpropanoids) (Spangenberg, 2008).

2.3.3 Gas-chromatography-mass spectrometry (GC-MS)

GC-MS is an effective analytical technique for the separation and identification of individual low molecular weight compounds of complex mixtures of products. It is the most used analytical technique to identify constituents of essentials oils (Babushok et al., 2011). GC-MS is the integration of MS and GC into a single synergistic system for analysis in which the gas chromatograph performs the separation and mass spectrometry is the detection method (Hübschmann, 2015, pp. 1-2).

The basis of mass spectrometry lies in the generation of ions representative of the mass of the sample's molecules (Mellon et al., 2000). In EI, under the control of a magnetic field, energetic electrons of 70 eV interact with volatilised sample molecules which enter their path to cause the removal of one (and sometimes two or more) electron in the valence orbitals (equation 2-1**)** (Mellon et al., 2000).

$$M \xrightarrow{e^-} M^{+\bullet} + 2e^- \qquad \textbf{2-1}$$

Since the ionisation energies (IEs) of most organic molecules lie in the range 8-12 eV, the excess energy is transferred to the newly formed and positively charged "radical ions" to cause bond dissociation (fragmentation) of cleavable bonds. This process gives rise to salient fragmentation patterns represented by their mass to charge ratios (*m/z*) and the respective relative abundances, which are then useful for structural elucidation (Mellon et al., 2000).

2.3.3.1 The retention index (RI)

In combination with mass spectrometry, a useful datum known as retention index (RI) forms part of the data set acquired during chromatography (Babushok et al., 2011). Under temperature-programmed conditions, both linear RI, as per the definition of Van Den Dool and Kratz, and logarithmic RI, in accord with the definition of Kováts, are encountered for the determination of retention indices (RIs) (Babushok & Zenkevich, 2009). However, the most common index is the Kováts Index (KI) (Adams, 2007).

According to Kováts, the index of a target analyte is its relative time position between the nearest n-alkane which elutes immediately before and after it. The confirmation of compound identification is

achieved by the comparison of the measured RI of chemical components with the accessible retention data collections (Babushok et al., 2011).

2.3.4 Nuclear magnetic resonance (NMR)

NMR exploits the magnetic characteristics of the atomic nucleus. Some nuclei (e.g. ^{1}H, ^{15}N, ^{13}C, ^{31}P) resonate at a typical frequency within the radio frequency range when placed in a powerful magnetic field. The subtle yet different resonant frequencies give sound information on the molecular structure the atom is found in (Jacobsen, 2007).

2.4 Biological assays of cosmetic relevance: the background and principle of the methods

In section 1.2, it was revealed that photo-aging, skin infections, and pigmentary disorders are handicaps to skin's health. As result, skin's active formulations with high demand are antioxidants, sunscreens, antimicrobials, and skin-whitening products. The following sections provide a review of the methodologies used in the laboratory to assess those biological activities.

2.4.1 Antioxidant capacity assays

An antioxidant is a molecule that, at low concentrations retards or prevents the oxidation of a substrate (Santos-Sánchez et al., 2019). The powerful antioxidants, the radical scavengers, stop radical chain reactions (Huang et al., 2005).

In principle, assessing the antioxidative effects of compounds/samples suggests subjecting the substrate to real settings. However, because at room temperature spontaneous oxidations are generally prolonged, thus somehow impractical, several *in vivo* and *in vitro* methods have been put forward as practical ways to estimate antioxidant activity (Alam et al., 2013; Amorati et al., 2013). The differences amongst them are usually based on the degree of simplifications in comparison to real systems (Amorati et al, 2013). Additionally, in the study of antioxidant compounds, it is important to distinguish between antioxidant activity, which is concerned with the reaction kinetics between oxidant and antioxidant, and antioxidant capacity, which measures how much antioxidant reacts with the oxidant (Schaich & Xie, 2015; Santos-Sánchez et al., 2019).

There are various mechanistic modes through which antioxidants act (Marković, 2016). However, *in vitro* measurements of antioxidant capacities are generally classified as hydrogen atom transfer (HAT) and electron transfer (ET) based assays (Huang et al., 2005; Gupta, 2015; Santos-Sánchez et al., 2019). HAT-based methods assess of an antioxidant's traditional faculty to counteract free radicals by hydrogen donation. In contrast, ET-based methods determine an antioxidant's aptness to transfer one electron (e^-) to reduce any molecule, including radicals and metals (Gulcin, 2020). The result remains the inactivation of free radicals (Santos-Sánchez et al., 2019).

The antioxidant capacity measured in HAT-based methods rests upon competition kinetics. A fluorescent probe competes with an antioxidant for quenching synthetically induced free radicals (Huang et al., 2005), generally peroxyl radicals (Gupta, 2015) as in the following reaction:

ROO• + AH/ArOH → ROOH + A•/ ArO•

Where the aryloxy radical (ArO•) produced from the reaction of a phenol antioxidant with peroxyl radical (ROO•) is stabilised by resonance. A• is the oxidised biomolecule without the presence of an antioxidant. The ArOH and AH species represent the protected antioxidants and biomolecules, respectively. The fluorescence decay curve of the probe is measured in the presence and absence of antioxidants (Gupta, 2015).

In contrast, in ET-based assays, the antioxidants react with a coloured (or fluorescent) probe in place of peroxyl radicals (Gupta, 2015) according to the following redox reaction:

Probe (oxidant) + e^- (from antioxidant) → reduced probe + oxidised antioxidant

The coloured probe, which is itself the oxidant, changes colour upon reduction. The extent of the colour change then measures proportionally to the antioxidant's amount (Huang et al., 2005).

2.4.2 Determination of sun protection factor (SPF)

Solar ultraviolet rays (UVR) reaching the surface of the earth comprise three ranges of radiation, UVA (315–400 nm), UVB (280–315 nm), and UVC (100–280 nm) (Ahsanuddin et al., 2016). 95% of all UVR to reach earth is made up of UVA (Ahsanuddin et al., 2016). UVC radiation hardly reaches the Earth's surface because it is filtered by the ozone layer (Monteiro e Silva et al., 2017) moreover, it has little impact on the skin (Badia, n.d.). Somehow, the extent of tissue penetration of radiation increases with longer wavelength while the biological effects diminish dramatically (Tyrell, 1995). UVB radiation only penetrates the epidermis and produces erythema associated with sunburn (Badia, n.d.). In contrast, UVA radiation penetrates deeper into the epidermis, dermis, and the subcutaneous tissue (Tyrell, 1995) and provokes photo-aging (Badia, n.d.; Mbanga et al., 2014).

The minimal erythema dose in human skin describes the minimum time period or absorption of UV radiation needed to cause noticeable erythema (sunburn inflammation) on unprotected skin (Pachpawar et al., 2018). The sun protection factor (SPF) expresses the efficacy of a sunscreen. It describes the ratio of the UV energy needed to bring about a minimal erythema dose (MED) on protected skin to the UV energy needed to produce a MED on unprotected skin (Bruta et al., 2004; Kaur & Saraf, 2010; Kale et al., 2011; Mbanga et al., 2014; Pachpawar et al., 2018) (equation 2-2). The higher the SPF value is, the better the sunscreen protects against the sunburn-causing UV radiations (Kaur & Saraf, 2010). SPF primarily measures the UVB protection because UVB rays are more erythemogenic than UVA rays, up to 1000 times (Kale et al., 2011; Mbanga et al., 2014).

$$SPF = \frac{minimal\ erythema\ dose\ in\ sunscreen\text{-}protected\ skin}{minimal\ erythema\ dose\ in\ nonsunscreen\text{-}proteced\ skin} \quad \textbf{2-2}$$

The photoprotection of sunscreen agents may be assessed *in vivo* or *in vitro*. *In vitro* methods offer a quick and inexpensive methodology in comparison to complicated, time-consuming, and costly *in vivo* photo-testing of human volunteers (Pachpawar et al., 2018). There are generally two types of *in vitro* methods. Those that measure the amount of UV light absorbed or transmitted through sunscreen product films in biomembranes or quartz plates. Then, the second types of methods whereby the absorption properties of dilute solutions of sunscreen agents are spectrophotometrically determined over the selected UV range (Dutra et al., 2004).

2.4.3 Antimicrobial activity: Determination of minimum inhibitory concentrations (MICs)

In the clinical microbiology laboratory, antimicrobial susceptibility testing (AST) covers a range of *in vitro* procedures aiming to evaluate the susceptibility of a bacterium against an antimicrobial agent (Buller et al., 2014). Dilution methods are employed to establish the minimum inhibitory concentrations (MICs) of substances. They are the benchmark methods for AST. Other assays including disk diffusion are calibrated against them (European Committee on Antimicrobial Susceptibility Testing [EUCAST], 2003).

The broth dilution method is one of the dilution methods used for AST (Buller et al., 2014; Balouiri et al., 2016). Among the dilution methods is found the broth microdilution, which denotes performing the broth dilution test in microdilution plates ($\leq$ 500 µL per well) (EUCAST, 2003). The method rests upon series of dilutions, usually two-folds, of the test antimicrobial agent in a microtiter plate. The antimicrobial agent is inoculated, in a production-line fashion, with a standardised bacterial suspension i.e. 5×10^5 colony-forming units/mL (CFU/mL) (Jorgensen & Ferraro, 1998; Balouiri et al., 2016). After 18 – 20 h of overnight incubation at 35 °C, the lowest concentration exhibiting no bacterial growth is declared as the minimum inhibitory concentration (MIC) (Jorgensen & Ferraro, 1998). This endpoint may be detected either by visible macroscopic turbidity (Figure 2-11) or by a colorimetric assay with the aid of dye reagents such as tetrazolium salts (Balouiri et al., 2016). The colourless tetrazolium salt accepts electrons from biologically active organisms and it is reduced to a coloured product (Figure 2-12). The broth microdilution method coupled with colorimetric detection is quick, robust, and reproducible (Eloff, 1998).

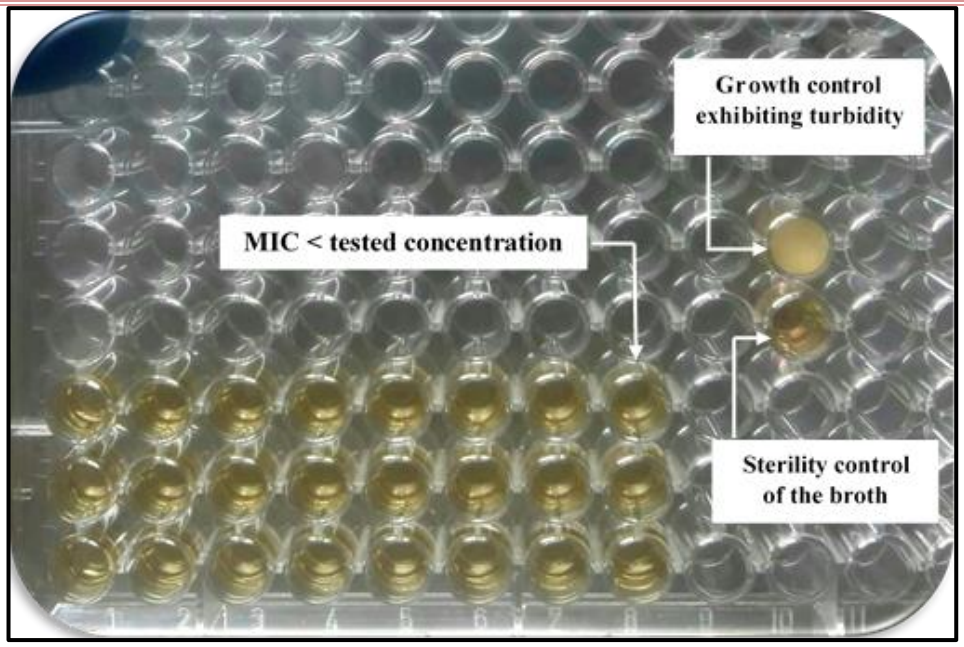


Figure 2-11 Reading of the minimum inhibitory concentration (MIC) of an antimicrobial agent in the broth microdilution by visual turbidity

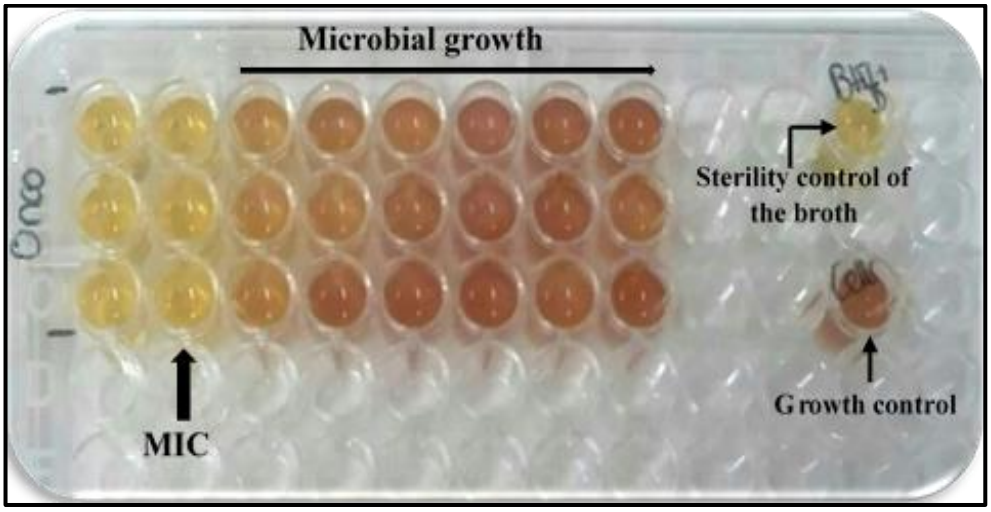


Figure 2-12 Reading of the minimum inhibitory concentration (MIC) of an EO sample in the broth microdilution method using 0.2 mg/mL *p*-iodonitrotetrazolium chloride (INT) reagent.

2.4.4 Antityrosinase activity: Spectrophotometric determination of tyrosinase inibition

Tyrosinase (EC 1.14.18.1) has two catalytic activities, a monophenolase activity, whereby L-tyrosine (monophenol) is hydroxylated to L-3,4-dihydroxyphenylalanine (L-DOPA, the *o*-diphenol product), and a diphenolase activity, whereby L-DOPA is oxidised to *o*-dopaquinone (Zolghadri et al., 2019). L-tyrosine converting into dopaquinone in these two preceding sequenced reactions is the rate-limiting step in melanogenesis because, further reaction sequences can proceed spontaneously at a physiological pH value (Chang, 2009; Pillaiyar et al. 2017). Tyrosinase inhibitors are usually assayed in the laboratory by employing mushroom tyrosinase due to the similar homology to human tyrosinase (Zolghadri et al.,

2019). The inhibition of mushroom tyrosinase is evaluated by either using L-tyrosine (Teixeira et al., 2012; Cui et al., 2018) or L-DOPA as a substrate (Uchida et al., 2014).

2.5 Chemical and biological studies on essential oils of the selected aromatic plants

2.5.1 Review of *Oncosiphon suffruticosum* (L.) Källersjö

Oncosiphon (L.) Källersjö is a small genus counting seven species. All the species, but *O. piluliferus* (L.f.) Källersjö and *O. suffruticosus* (L.) Källersjö, are native to the Greater Cape Floristic Region. These two species are also found in south-west Western Australia where they are classified as weeds and known as for Calomba Daisy (*O. suffruticosus*) and Globe Chamomile (*O. piluliferus*) (Kolokoto & Magee, 2018). The genus features discoid and radiate species that were previously known as *Pentzia* Thunb. and *Matricaria* L., respectively. However, *Oncosiphon* came into existence due to the morphological distinctions in annual species such as, a swollen corolla tube, four-lobed corollas, and four-ribbed nonmyxogenic fruit not found in *Pentzia*. *Oncosiphon* is an aromatic genus from the family Asteraceae and Anthemideae tribe. Species of this genus have such an offensive aroma that the Afrikaans-speaking people name it "stinkruid" which translates to stinkweed. Among the *Oncosiphon* species, *O. piluliferus*, *O suffruticosus*, and *O. africanus* [syn. *O. glabratus* (Thunb.) Källersjö] have been identified for the treatment of illnesses in Cape Dutch ethnobotany and Khoi-San medicine (Van Wyk, 2008; Kolokoto & Magee, 2018).

2.5.1.1 Description of Oncosiphon suffruticosum (L.) Källersjö

Figure 2-13 Photograph of *Oncosiphon suffructicosum* (L.) Källersjö (Magee, 2011)

Oncosiphon suffruticosum (L.) Källersjö (syn. *Oncosiphon suffruticosus* (L.) Källersjö) is an herb with a sharp and powerful scent. The leaves feature thin divisions of a feather shape and a glabrous texture. With its annual life cycle, the plant may reach up to 50 cm tall (Figure 2-13). It is a widespread species

that occurs in sandy, often coastal soils, from Gansbaai in the Western Cape Province north to southern Namibia (Magee, 2011).

Oncosiphon suffruticosum is an important traditional medicine used orally for gastric disorders, convulsions, asthma, diabetes (Scott et al., 2004), colds, influenza, typhoid fever, and rheumatic fever and topically as a leaf poultice to treat inflammation and scorpion stings (Lall & Kishore, 2014).

2.5.1.2 Previous studies

According to the literature search on Google Scholar and SciFinder®, phytochemical (extracts/essential oil) and biological studies on *Oncosiphon* species have not been pursued except for one investigation on *Oncosiphon piluliferum* by Pillay et al. (2007) who studied the antiplasmodial activity of sesquiterpenes isolated from the plant.

Nonetheless, using the historical names of the genus namely *Pentzia*, *Matricaria* L. or *Tanacetum* L. (Kolokoto & Magee, 2018), it was found that investigations on *Oncosiphon suffruticosum* (as *Matricaria suffructicosa*) were performed by Bohlmann & Zdero (1975) who elucidated the naturally occurring terpene derivatives in the plant's aerial parts. Additionally, recent research work on the composition of the essential oil of *Pentzia incana* has been undertaken (Hulley et al., 2018).

2.5.2 Review of the *Helichrysum* species under investigation

The genus name of *Helichrysum* Mill. finds its origin from the Greek "*helios*" for sun and "*chrysos*" for gold in reference to the goldish flowerheads of various species (Lourens et al., 2008; Zenze, 2012). *Helichrysum* belongs to the Asteraceae family, tribe Inuleae and subtribe Gnaphaliinae (Lourens et al., 2008; Giovanelli et al., 2018). This is a large genus totalling over 600 species. It is present in almost every continent, Africa, Europe, North America, and Australia. South Africa (and Namibia) is home to nearly 244-250 species which occur with extensive varied morphologies (Lourens et al., 2008; Hassine et al., 2016).

Helichrysum species are mostly short perennial shrubs and strongly scented. The essential oil of *Helichrysum italicum* also called "immortelle" is a renowned ingredient of cosmetics. It promotes blood flow in the skin, regenerates it, and helps to attenuate signs of aging such as fine lines and wrinkles (Giovanelli et al., 2018).

Helichrysum species are popular materials of the traditional medicines of Europe and Asia where herbal teas are used to treat fever, cough respiratory problems, digestive disorders, skin inflammation, and wound care (Giovanelli et al., 2018). In South Africa, *Helichrysum* species are notorious in traditional ceremonies. Inhaling the smoke serves to call on the goodness of the ancestors or to cause trances (Lourens et al., 2008). Below is a description of *Helichrysum petiolare* Hilliard & B.L.Burtt,

Helichrysum cymosum (L.) D.Don subsp. cymosum and *Helichrysum odoratissimum* (L.) Sweet in Figure 2-14 and the sections 2.5.2.1, 2.5.2.2, and 2.5.2.3.

Helichrysum petiolare Hilliard & B.L.Burtt*	*Helichrysum cymosum* (L.) D.Don subsp. *cymosum***	*Helichrysum odoratissimum* (L.) Sweet***
		
Geographical distribution	**Geographical distribution**	**Geographical distribution**
Helichrysum petiolare is found in the Eastern Cape, Western Cape, and KwaZulu-Natal (Oliver, 2019).	*Helichrysum cymosum* grows in the Eastern Cape, Western Cape, and KwaZulu-Natal (Zenze, 2012).	*Helichrysum odoratissimum* extends from Limpopo through Mpumalanga and Western Swaziland to KwaZulu-Natal. It also grows in Free State, Lesotho, the Cape Drakensberg, the Eastern Cape, and the Peninsula in the Western Cape (Swelankomo, 2004).

Figure 2-14 Photographs and geographical distribution of the *Helichrysum* species. *Photograph from Faceysnursery.com (n.d.); **Photograph from Zenze (2012); ***Photograph from Swelankomo (2004)

2.5.2.1 *Helichrysum petiolare* Hilliard & B.L.Burtt

Common names: silver bush everlasting, herbal helichrysum, bedding helichrysum (English), kooigoed, kruie (Afrikaans), imphepho (isiXhosa) (Oliver, 2019).

Helichrysum petiolare is one of the best known and the most used helichrysums. The plant features a soft yet dense shrub with beautiful velvety silver foliage. With its aromatic roundish leaves, the plant grows about 0.5-1 m x 1 m (Oliver, 2019).

The leaves of this plant are reported to be used in skincare practices of the Eastern Cape province in a decoction to improve the skin texture and beauty, and to treat wounds (Sagbo & Mbeng, 2018).

Traditional oral use includes treating menstrual disorders, urinary tract infections, cough, cold, catarrh, headache, and fever (Scott et al., 2004).

2.5.2.2 *Helichrysum cymosum* (L.) D.Don subsp. cymosum

Common names: gold carpet (English); goue tapyt (Afrikaans); impepho (isiXhosa) (Zenze, 2012).

Helichrysum cymosum subsp. *cymosum* is a fast-growing, well-branched, and ground-spreading herb. It reaches up to 1 m, but it is generally about 500 mm tall. The fine branches with grey and white velvety texture are covered with compacted leaves. The upper part of the leaf is paper-like with a thin silvery grey. In South Africa, the bright canary-yellow flowers occur in summer between September and April (Zenze, 2012).

Helichrysum cymosum is used in traditional remedy mainly as a purgative, ritual incense, and magical purposes (in the chasing away of evil spirits and rain making) as well as for colds, cough, fever, headache, and wounds (Maroyi, 2019).

2.5.2.3 *Helichrysum odoratissimum* (L.) Sweet

Common names: most fragrant helichrysum (English); kooigoed, kruie (Afrikaans); imphepho (Xhosa & Zulu) (Swelankomo, 2004).

Helichrysum odoratissimum (L.) Sweet is a strongly scented perennial herb and much branched. It has small silvery leaves and yellow flowerheads grouped at the branches' tips. The plant has a woody base and grows up to 50 cm high. In South Africa, flowering occurs all year round. (Swelankomo, 2004).

H. odoratissimum is a prevalent herb in South African traditional skincare where a leaf decoction is used as a treatment for pimples (Lall & Kishore, 2014; Sagbo & Mbeng, 2018). Recent research provided evidence of this ethnomedical attribute in a study of the anti-acne potential of the methanolic crude extract to combat *Cutibacterium acnes* (previously *Propionibacterium acnes*) virulence (De Canha et al., 2020). Other medicinal uses include treating respiratory ailments and nervous disorders in Cape Dutch medicine (Van Wyk, 2008).

2.5.2.4 Previous studies performed on the essential oils of *Helichrysum* species

A comprehensive review of the essential oils of the three *Helichrysum* species under investigation was achieved using on Google Scholar and SciFinder® databases. The information found was recorded in Table 2-2:

Table 2-2 Review of previous studies on the essential oils of the *Helichrysum* species under focus

Name	Locality	Studies on essential oils			References
		Analysis-method	Major components	Biological tests	
***Helichrysum petiolare* Hilliard & B.L.Burtt**	SA	GC-MS	1,8-Cineole (22.4%), (*E*)-caryophyllene (14.0%), *p*-cymene (9.8%).	Antimicrobial, antioxidant, and anti-inflammatory	Lourens et al. (2004).
	SA	GC-MS	Caryophyllenyl alcohol (36.42-45.26%), β-hydroagarofuran (19.45-25.64%), δ-cadinene (3.39-4.76%).	None	Giovanelli et al. (2018).
Helichrysum cymosum* (L.) D.Don subsp. *cymosum	Tanzania	GC-MS	(*E*)-Caryophyllene (27.02%), caryophyllene oxide (7.65%), *p*-cymene (7.55%).	Antimicrobial	Bougatsos et al. (2004).
	SA	GC-MS	1,8-Cineole (20.4%), α-pinene (12.4%), (*E*)-caryophyllene (10.8%)	Antimicrobial and antimalarial and cytotoxicity	Van Vuuren et al. (2006).
	SA	TLC and GC-MS	1,8-Cineole (20.4-34.6%), (*E*)-caryophyllene (8.4-10.8%), α-pinene (3.6-12.4%).	Antimicrobial	Reddy (2007).
	Cameroon	GC-FID and GC-MS	δ-3-Carene (16.1%), (*E*)-caryophyllene (12.0%), camphene (7.4%).	Radical scavenging and antifungal	Tchoumbougnang et al. (2010).
	SA	GC-MS	(Z)-β-Ocimene (35.61-50.44%), (*E*)-caryophyllene (15.03-16.62%), α-humulene (5.28-8.68%).	None	Giovanelli et al. (2018).
***Helichrysum odoratissimum* (L.) Sweet**	Zimbabwe	GC-MS	α-Pinene (15.0%), α-humulene (13.0%), (*E*)-caryophyllene (9.6%).	None	Gundidza & Zwaving (1993).

SA	GC-MS	*p*-Menthone 35.4%, pelugone 34.2%, 1,8-cineole 13.0% (fresh plant material).	None	Asekun et al. (2007).
SA	TLC and GC-MS	(*E*)-Caryophyllene (9.3-25.2%), limonene (11.6-19.6%), and 1,8-cineole (11.2-17.1%).	Antimicrobial	Reddy (2007).
SA	GC-MS	Limonene (14.55%), 1.8-cineole (6.56%), α-pinene (4.20%).	Repellency and fumigation against maize weevil	Odeyemi et al. (2008).
SA	GC-MS	β-Pinene (51.6%), limonene (16.9%), α-humulene (5.6%)	Antimicrobial and cytotoxicity	Lawal et al. (2015).
Uganda	GC-MS	Palmitic acid (27.1%), humulene (14.1%), (*E*)-caryophyllene (12.6%).	Antimicrobial	Ocheng et al. (2015).
SA	GC-MS	α-Pinene (4.11-18.39%), (*E*)-caryophyllene (9.67-15.85%), 1,8-cineole (2.74-13.35%).	None	Giovanelli et al. (2018).

2.5.3 Review of the *Salvia* species under investigation

Salvia (sage) is the predominant genus of the Lamiaceae constituting almost one-quarter of the family (Kamatou et al., 2008). The genus encompasses 700-900 species worldwide (Van Der Walt, 2001; Van Jaarsveld, 2002; Kamatou et al., 2008) with centres in the Mediterranean region and a smaller centre in the winter rainfall region of the Western Cape (Van Jaarsveld, 2002). Out of 60 species occurring in Africa (Van Jaarsveld, 2002), *ca*. 26 species are endemic to Southern Africa particularly in the Cape region of South Africa (Kamatou et al., 2008).

Most species are aromatic perennials, some multi-stemmed shrubs, and others, annuals, popularly used as ornamentals, and medicinal herbs. The origin of the genus name is anchored to the medicinal properties of the plant species found therein. *Salvia* comes from the Latin '*salvere*' which means 'to heal' (Van Jaarsveld, 2002).

Most sages, if not all, are utilised in healing practices of South Africa. Many are employed to cure inflammation, microbial infections, malaria, cancer, memory loss, and to sanitise homes after illness (Kamatou et al., 2008). In the following Figure 2-15 and sections, 2.5.3.1, and 2.5.3.2, is given a description of *Salvia aurea* L. and *Salvia chamelaeagnea* P.J.Bergius.

Salvia aurea L.*	*Salvia chamelaeagnea* P.J.Bergius**
	
Geographical distribution	**Geographical distribution**
Salvia. *aurea* occurs from Namaqualand to the Cape Peninsula and Port Alfred. Naturally, it is a common plant on coastal sand dunes (Notten, 2019).	*Salvia chamelaeagnea* is wild growing in the south Western Cape (Van Der Walt, 2001). The shrub is found from Clanwilliam to Cape Town and as far as Ladismith and Riversdale (Kamatou & Viljoen, 2007).

Figure 2-15 Photographs and geographical distribution of *Salvia* species.*Photograph from Notten (2019); **Photograph from Van Der Walt (2001)

2.5.3.1 *Salvia aurea* L.

Common names: golden sage, beach sage, dune sage, sand sage (English); bruinsalie, sandsalie, strandsalie, geelblomsalie (Afrikaans).

S. aurea is a hard and aromatic shrub with long-lasting colourful flowers. It grows up to 2 m, and strongly attracts wildlife. Early spring marks the beginning of the flowering season. At this stage, the yellow flowers start fading to a rust-like orange colour and ends up reddish-brown.

This species has been recognised as *Salvia africana-lutea*, however, botanical nomenclature forbids hyphenated names. The name *aurea* which means "golden" alludes to the yellow and burnished golden-brown colour of the erecting flowers (Notten, 2019).

Salvia aurea L. is traditionally used orally for colds, cough, flu (Kamatou et al., 2008), headache, digestive disorders, and topically for burn wounds (Scott et al., 2004).

2.5.3.2 *Salvia chamelaeagnea* P.J.Bergius

Common names: rough blue sage (English), bloublomsalie (Afrikaans).

S. chamelaeagnea is a dense shrub that grows almost 2 m high with strong upright stems. Those stems display a typical feature of the family Lamiaceae with their square shape. The leaves form opposite each other along the stems or on short side branches, and usually grow in tufts. The bright green leaves are slightly hairy with toothed edges sometimes. The dotted leaves with glands emit a very strong scent and leave a sticky, bitter-tasting residue on fingers (Van Der Walt, 2001).

Flowering occurs from October to May for a month or two for individual plants flower. The most common feature a white lower lip and a dark blue top lip (Van Der Walt, 2001; Kamatou & Viljoen, 2007).

S. chamelaeagnea is used in traditional medicine orally for colds, cough, flu (Kamatou et al., 2008), bronchitis, stomach-ache, diarrhoea, menstrual disorders, headache (Scott et al., 2004) and topically for burn wounds (Scott et al., 2004).

2.5.3.3 Previous studies performed on the essential oils of *Salvia* species

A comprehensive review of the essential oils of these two *Salvia* species under investigation was performed on Google Scholar and SciFinder® databases. The information found was recorded in Table 2-3:

Table 2-3 Review of previous studies on the essential oils of the *Salvia* species under focus

Name	Locality	Studies on essential oils			References
		Analysis method	Major components	Biological tests	
***Salvia aurea* L.**	SA	GC-MS	Camphor (34.7%), 3-carene (16.5%), camphene (8.3%).	None	Serrato-Valenti et al., 1997.
	SA	TLC and GC-MS	(*E*)-Caryophyllene+4-terpinen-4-ol (13.51%), limonene (11.61%), α-eudesmol (10.14%).	Antimicrobial	Vallabh, 2003.
	SA	GC-MS	β-Eudesmol (19.8%), caryophyllene oxide (13.3%), α-eudesmol (10.1%).	Antimicrobial	Fisher, 2005.
	SA	GC-MS	Myrcene (11.5%), *p*-cymene (7.6%), α-pinene (6.0%).	Antimicrobial, antioxidant, anti-inflammatory, antiplasmodial, cytotoxicity	Kamatou et al., 2006.
	SA	GC-MS	β-Eudesmol (14.5%), α-eudesmol (13.5%), α-pinene (8.6%).	Antimicrobial activity against foot-odour causing bacteria	Van Vuuren et al., 2019.
	SA	GCxGC-ToF-MS; GC-MS/FID	Terpinene-4-ol + β-caryophyllene (1.4 – 29.0%), β-eudesmol, (trace-26.0%), α-eudesmol (trace-23.0%).	None	Lim Ah Tock et al., 2020.
***Salvia chamelaeagnea* P.J.Bergius**	SA	GC-MS	1,8-Cineole (40.5%), α-pinene (10.4%), limonene (9.7%).	Antimicrobial, antioxidant, anti-inflammatory, antiplasmodial, cytotoxicity	Kamatou et al., 2006.
	SA	GC-MS	Viridiflorol (32.5%), limonene (14.1%), 1,8-cineole (14.1%).	Antimicrobial activity against foot-odour causing bacteria	Van Vuuren et al., 2019.
	SA	GCxGC-ToF-MS; GC-MS/FID	Viridiflorol (9.8-61.0%), limonene (1.6-36.0%), δ-3-carene (trace-18.0%).	None	Lim Ah Tock et al., 2020.

2.6 Conclusion of the literature review

The chemical nature of plants' essential oils is complex. This character is owed to the various biosynthetic routes coupled with further degrees of chemical transformations giving rise to varied functional groups. The review revealed that the essential oil of the selected *Oncosiphon* species has not been explored whereas several research works were found on the essential oils of the selected *Helichrysum* and *Salvia* species. Investigations usually include chemical composition and/or biological studies. The biological investigations of dermatological relevance were found to be the antimicrobial, antioxidant, anti-inflammatory, and cytotoxicity activities. In this work, chemical and biological studies will be conducted. In addition to antibacterial and antioxidant activities, the antityrosinase study and the evaluation of photoprotection of the selected essential oils were chosen to broaden the body of knowledge in the field.

3. CHAPTER THREE: MATERIALS AND METHODS

This chapter describes all the materials and methods which were employed to study the essential oils of the selected aromatic indigenous South African plants. The schematic plan of this study is summarised in Figure 3-1 as follows:

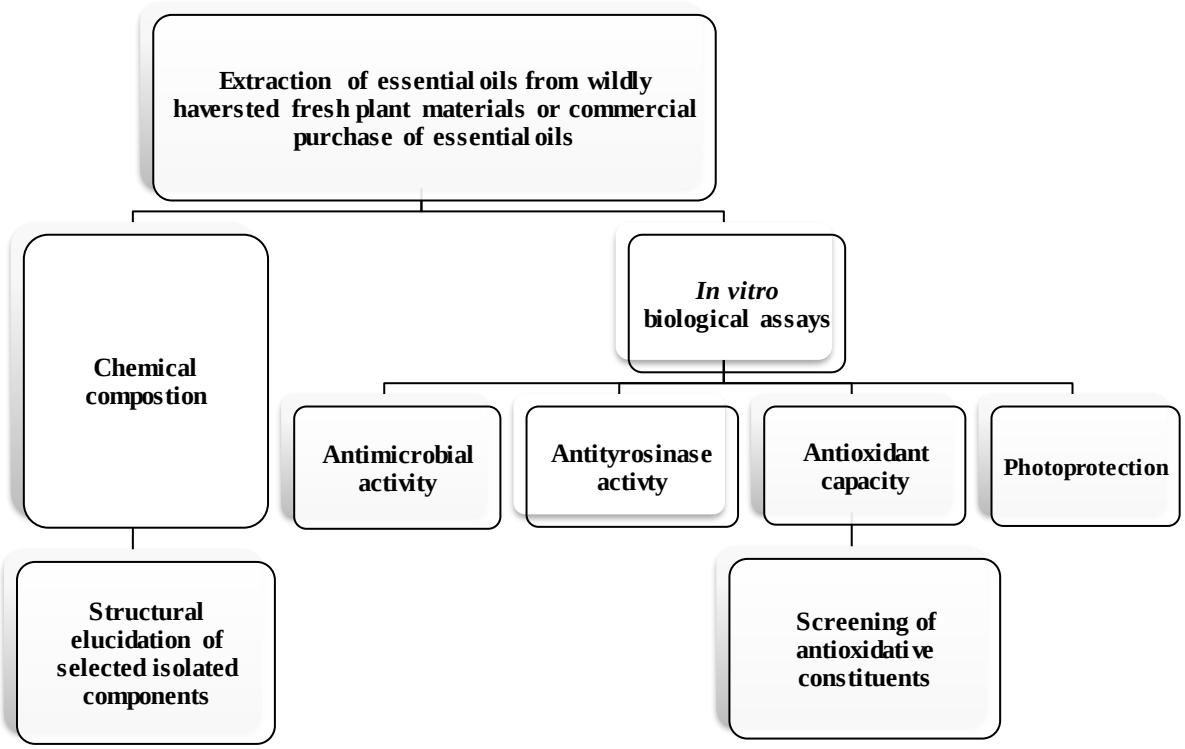


Figure 3-1 Flow diagram of the experimental sections of this research work

3.1 Collection and identification of plant species

Five of the six species, *Oncosiphon suffruticosum*, *Helichrysum petiolare*, *Helichrysum cymosum*, *Salvia aurea,* and *Salvia chamelaeagnea* were wildly harvested from the UWC campus and its nature reserve, the Cape Flats Nature Reserve (CFNR). A voucher specimen of each species was authenticated at the CPUT Horticultural Sciences Department. The essential oil of *Helichrysum odoratissimum* was obtained commercially from a local South African establishment (Pure Indigenous [Indigo Trading] African Helichrysum, 100% Organic Essential Oil). The total amount of plant material collected for each species was recorded and summarised in Table 3-1.

Table 3-1 Summary of the total quantities of wildly harvested plant materials

Plant material name	Total weight* (kg)
Oncosiphon suffruticosum	3.0
Helichrysum petiolare	6.0
Helichrysum cymosum	3.5
Salvia aurea	3.0
Salvia chamelaeagnea	1.5

*Aerial part: leaves, stems, and flowers

3.2 Isolation of essential oils by hydrodistillation

All five wildly harvested species were subjected to the hydrodistillation method of extraction using the Clevenger-type apparatus for 3 h per batch as recommended by the standard procedure of the European Pharmacopeia (European Pharmacopeia, 1975). Each batch of plant material consisted of 800 g – 1000 g which was immersed in approximately 1 L distilled water. The apparatus was set up as shown in Figure 2-10 whereby the hotplate was replaced by a heating mantle. After 3 h of distillation time, the essential oil (EO) was recovered by decantation then stored in screw glass vials (Zhejiang ALWSCI Technologies Co., China, Cat no. 2ML-9-V1001) at 4 °C in the dark until further analysis. The oil yield was expressed as the average percentage of volume in mL per weight in g (% v/w) of triplicate analyses.

3.3 Chemical composition of essential oils

Chromatography and spectroscopic methods were employed in this research work for the chemical characterisation of the essential oils. Gas chromatography-mass spectrometry (GC-MS) served as the main method for qualitative analysis of the essential oils. Column chromatography (CC), thin-layer chromatography (TLC), and preparative thin-layer chromatography (prep TLC) were used for the isolation work of two selected compounds. Then, nuclear magnetic resonance (NMR), in conjunction with MS, was used for structural elucidation of the pure compounds.

3.3.1 General materials and reagents

- All organic solvents used for this study were purchased from various suppliers such as SCIENCEWORLD, SA, Crest Chemicals, SA, and KIMIX Chemical & Lab Supplies, SA.
- The stationary phase employed for column chromatography (CC) was Silica gel 60 (0.063-0.200 mm) from Merck, Germany (Cat no. 1.07734.9025).
- Thin-layer chromatography (TLC) for rapid detection of compounds and preparative-thin layer chromatography (prep-TLC) for isolation of single constituents were performed on TLC Silica gel 60 F_{254} (Merck, Germany, Cat no. 1.05554.0001) with sulphuric acid (2.5 %v/v)-vanillin

(Sigma Aldrich® SA, Cat no. 258105; Sigma Aldrich® SA, Cat no. V1104 respectively) derivatising reagent for visualisation.

3.3.2 General apparatus

- Ultraviolet (UV) lamp at wavelength 254 nm (λ_{254}) (CAMAG®, Switzerland)
- Micropipettes (Eppendorf, Germany)
- Rotary evaporator (Rotavapor® R-100, vacuum pump V-100 and interface I-100, BÜCHI Labortechnik AG, Switzerland)

3.3.3 Identification of essential oil components

3.3.3.1 Preliminary phytochemical screening by thin-layer chromatography (TLC)

The essential oils (10 µL) were dissolved in 1 mL of hexane then 5 µL of each sample was spotted on silica gel TLC plates (5 cm x 10 cm). The plates were subsequently developed in hexane: ethyl acetate (93:7) mixture (double run), thereafter dipped in H_2SO_4 (2.5% v/v)-vanillin then heated (at 110 ºC) for visualisation of the compounds.

3.3.3.2 Gas chromatography-mass spectrometry (GC-MS) analyses

Essential oils samples (10 µL) were diluted to 1 mL with liquid chromatography grade hexane (Sigma-Aldrich®, Cat no. 34859) and run through an Agilent GC-7820A fitted with a HP-5MS fused silica column (Agilent Technologies, Inc. USA) (Table 3-2) and coupled with an Agilent 5977E mass selective compartment (Agilent Technologies, Inc. USA). A reference standard of homologous *n*-paraffin series of C_8-C_{20} (Sigma-Aldrich®, Cat no. 04070) was prepared and co-injected under the same experimental conditions as the samples for the determination of retention indices (RIs). The analyses were performed on a temperature program adapted from Kuiate et al. (1999) and the in-house method consisting of two ramps. The operating conditions were summarised in Table 3-2.

Table 3-2 Instrument control parameters of GC-MS system used for essential oils samples analyses

Data acquisition method of the GC-MS system	**HP-5MS**
Capillary column	
Composition	(5%-Phenyl)-Methylpolysiloxane
Length	30 m
Thickness	0.25µm
Diameter	250 µm
Carrier gas	Helium
Flow rate	1 mL.min^{-1}

Pressure	7.6522 psi
Velocity	36.445 cmsec^{-1}
Oven	
Initial temperature	50 ˚C (for 5 min)
Ramp 1 rate	2 ˚C min^{-1}
Final temperature	220 ˚C (for 5 min)
Ramp 2 rate	25 ˚C.min^{-1}
Final temperature	300 ˚C (no hold time)
Injection	
Injection temperature	250 ˚C
Split mode	Splitless
Sample volume	1 µL
Detector	
Type	Electron impact
Electron energy	70.0 eV
Spectra scan range	30.0 to 650 *m/z*
Enhanced data analysis/Integration events	
Initial area reject	0
Initial peak width	0.213
Shoulder detection	Off
Initial threshold	22.0

The identification of individual components in each essential oil sample was achieved by computerised matching (MassHunter software, Agilent Technologies, Inc, USA) of each mass spectrum generated with those stored in the instrument's built-in mass spectral libraries (NIST 14). Then, a comparison of the experimental retention indices (RI) and mass spectra with, those of the NIST online data collection (NIST Chemistry WebBook) and literature (Adams, 2007; Babushok et al., 2011) was done. Retention indices from temperature-programming measurements were determined using equation 3-1 from Adams (2007) as shown below:

$$RI_x = 100n + 100\frac{log(t_x) - \log(t_n)}{log(t_{n+1}) - log(t_n)} \quad \textbf{3-1}$$

Where RI_x is the Kováts index of the compound "x"; n is the carbon number of n-alkane hydrocarbon peak immediately before the compound "x"; t_x the retention time of compound "x"; t_n and t_{n+1} are the retention times of the n-alkane hydrocarbons eluting immediately before and after compound "x" respectively.

The manual calculation of the RIs of the EOs' constituents was facilitated by the Microsoft Excel® spreadsheet tool developed by Lucero et al. (2009) using equation 3-1 above. Additionally, the quantification of the essential oil constituents expressed as percentage composition was calculated automatically based on the total ion count detected by the GC-MS.

3.3.4 Isolation of *Helichrysum petiolare* essential oil constituents

Selected consitituents of *Helichrysum petiolare* essential oil were subjected to fractionation by CC for isolation. The laboratory procedure was executed as reported in the following sections.

3.3.4.1 Fractionation of *Helichrysum petiolare* essential oil by column chromatography

3.3.4.1.1 Macro-fractionation

Helichrysum petiolare essential oil was chromatographed targeting compound **1**, and compound **2** eluting approximately between 96:4 hexane: ethyl acetate and 95:5 hexane: ethyl acetate. Briefly, 3.547 g of *H. petiolare* EO made in silica slurry was packed in a silica gel column (40 cm x 4 cm). The separation was performed using a gradient elution of hexane: ethyl acetate (Hex: EA) in order of increasing polarity from 100:0 to 94:6 (Hex: EA). The separation yielded 53 fractions (20-50 mL) which were concentrated at 45°C as summarised in Table 3-3.

Table 3-3 Gradient elution and fractions collected of the column chromatography of H. *petiolare* essential oil

Hexane: ethyl acetate mobile phase ratio	Hexane (mL)	Ethyl acetate (mL)	ID of fractions collected	Collection volume (mL)
100:0	500	0	1-4	50
99:1	990	10	5-20	50
96:4	720	30	21-36	20
95:5	712.5	37.5	37-48	20
94:6	470	30	49-53	50

3.3.4.2 Purification of compound 1

According to the TLC profiles obtained at 97:3 hexane: ethyl acetate and its distinct aroma, compound **1 (**sky blue spot**)** was detected in fractions 26-29 (Figure 3-2) which eluted at 96:4 hexane: ethyl acetate. Fraction 28 (35.37 mg) and 29 (47.74 mg) were considered for the purification of compound **1** and the mobile phase optimised to a double run in 97:3 hexane: ethyl acetate (Figure 3-3). From the TLC profiles

obtained, fraction 29 was selected for ease of separation noted by the near absence of the closely eluting blue constituents found in fraction 28 as shown in Figure 3-3.

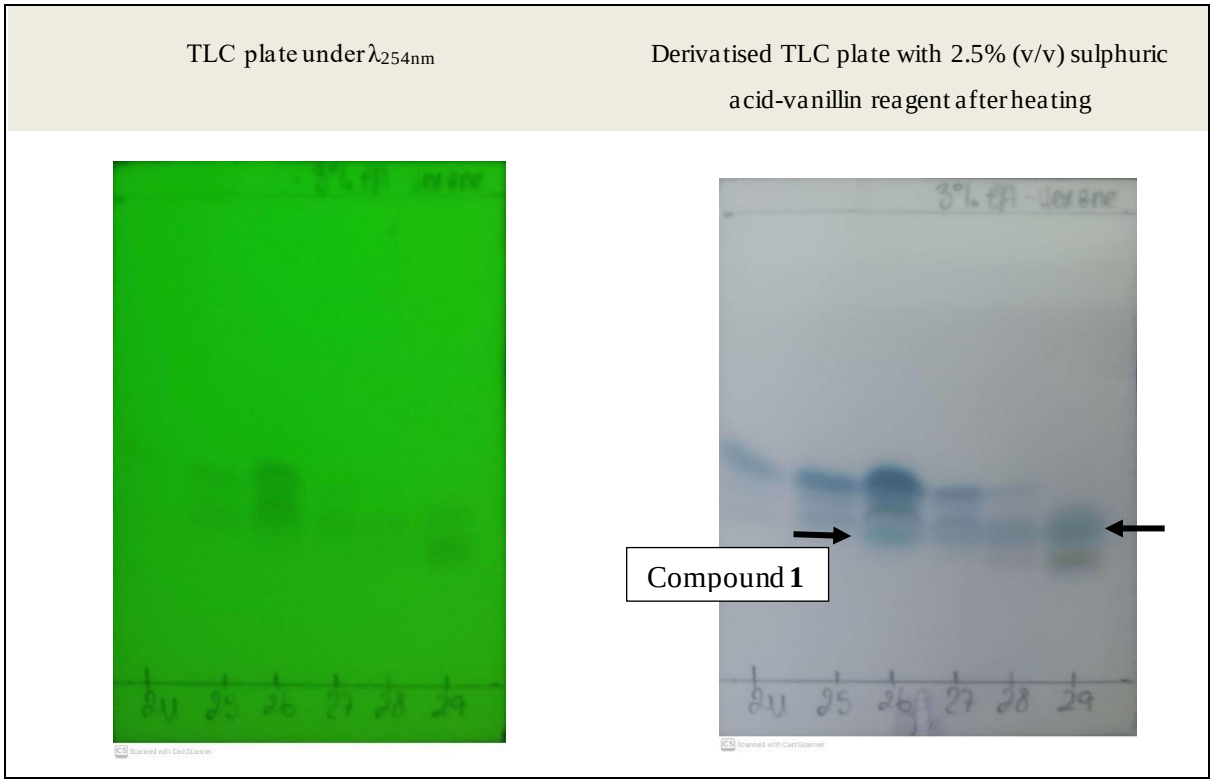


Figure 3-2 Detection of compound 1 in the fractions 26-29 (left to right) using TLC plates developed at 97:3 hexane: ethyl acetate

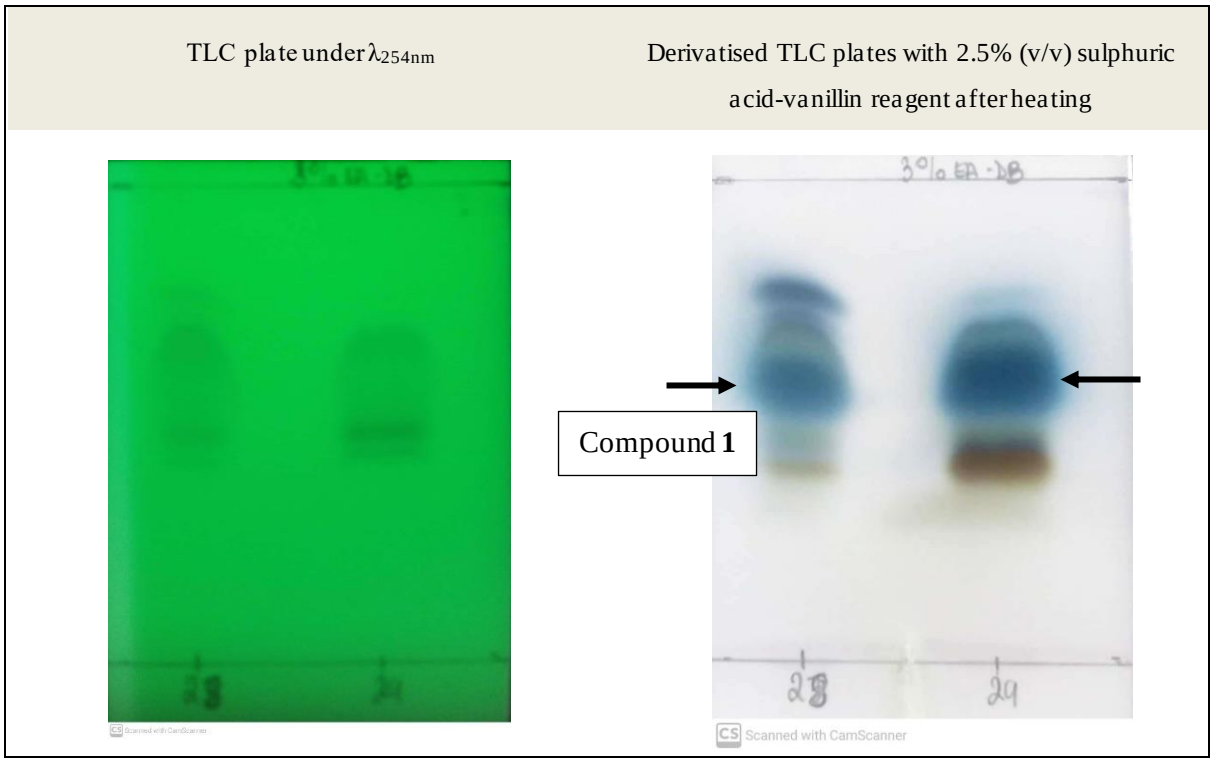


Figure 3-3 Optimisation of compound 1 separation in the fraction 28 (left) and 29 (right) by doubling running TLC at 97:3 hexane: ethyl acetate

The isolation of compound **1** was performed by diluting 20 mg of fraction 29 in 750 µL of hexane then 250 µL was loaded on three individual TLC plates (20 cm x 10 cm). The plates were developed at 97:3 hexane: ethyl acetate (double run). Thereafter, under $\lambda_{254\,nm}$, the band for compound **1** was marked (Figure 3-4) then, it was scraped off, and eluted with hexane.

Figure 3-4 Preparative TLC for the isolation of compound 1. Plate developed at 97:3 hexane: ethyl acetate (double run)

3.3.4.2.1 GC-MS analysis of isolated compound 1

Compound **1** (0.5 mg) was dissolved in 300 µL of hexane and analysed by GC-MS according to the method described in section 3.3.3.2.

3.3.4.3 Purification of compound 2

Fractions 28-38 run on TLC at 92:8 hexane: ethyl acetate revealed that fraction 31 (30 mg) contained the most amount of compound **2** represented by the big orange spot (Figure 3-5).

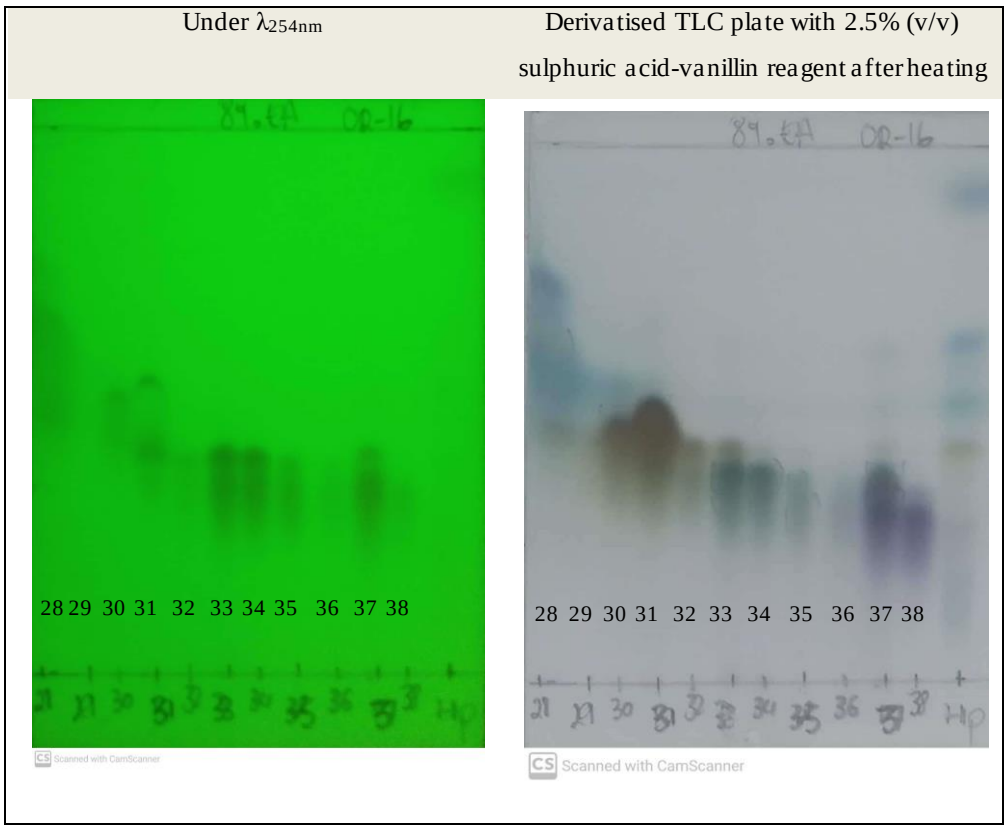


Figure 3-5 Detection of compound 2 (orange spot) in abundance in fraction 31

The isolation of compound **2** was performed by prep TLC at 92:8 hexane: ethyl acetate (double run) following the same protocol described in the isolation of compound **1** (Figure 3-6).

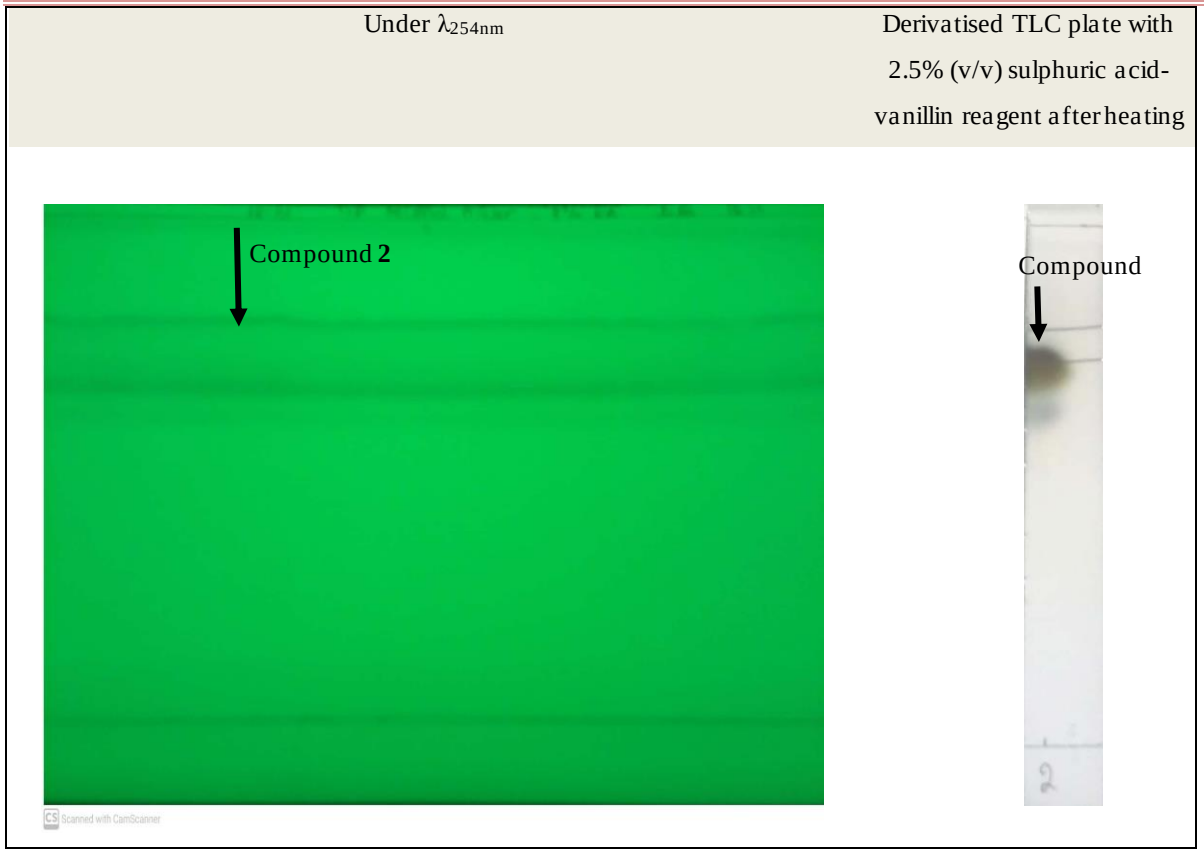


Figure 3-6 Preparative TLC for the isolation of compound 2

3.3.4.3.1 GC-MS analysis of isolated compound 2

Compound **2** (0.5 mg) was dissolved in 300 µL of hexane and analysed by GC-MS according to the method described in section 3.3.3.2.

3.3.4.4 Nuclear magnetic resonance (NMR) spectroscopy analysis of isolated components from *H. petiolare* essential oil

^{1}H NMR and ^{13}C NMR of compounds **1** and **2** were recorded at 20 ºC using deuterated chloroform on a Bruker Avance™ 400 MHz spectrometer (Germany). The chemical shifts of ^{1}H and ^{13}C in ppm (δ) were determined in relation to tetramethylsilane (TMS) used as internal reference.

3.4 *In vitro* biological assays

The *in vitro* biological assays chosen for this study were the antimicrobial, antioxidant capacity, photoprotective, and tyrosinase inhibition. These assays were performed in the laboratory as reported in the following sections.

3.4.1 Antimicrobial activity: Determination of minimum inhibitory concentrations (MICs) of the essential oils

3.4.1.1 Bacterial strains used in this study

The skin pathogenic bacterial strains were obtained from the microbial storage bank in the Medical Bioscience Department at the University of the Western Cape. One gram-positive strain, wild-type (WT) *S. aureus* and two gram-negative strains, wild-type (WT) *E. coli* and wild-type (WT) *P. aeruginosa* were used for this study.

3.4.1.2 Media preparation

3.4.1.2.1 Tryptone Soya agar (TSA)

Tryptone soya broth (Oxoid UK, Cat. no. CM0129) was prepared by adding 30 g in 1 L of distilled water. The solution was well mixed then sterilised by autoclaving at 121 ºC for 15 minutes.

3.4.1.2.2 Muller Hinton agar (MHA)

Mueller-Hinton agar (Oxoid UK, Cat. no. CM0337) was prepared by adding 38g in 1 L of distilled water. The solution was well mixed then sterilised by autoclaving at 121 ºC for 15 minutes.

3.4.1.2.3 Brain Heart Infusion broth (BHI)

Brain heart infusion broth (Oxoid UK, Cat. no. CM1135) was prepared by adding 37 g in 1 L of distilled water. The solution was well mixed and distributed into the final glass tubes then sterilised by autoclaving at 121 ºC for 15 minutes.

3.4.1.3 Resuscitation and seeding of bacterial species

The bacterial species were resuscitated by inoculation into Brain heart infusion broth (Oxoid UK, Cat. no. CM1135) and incubated at 37 °C for 24 hours after which, each strain was streaked aseptically onto Tryptone soya agar for single colony formation and incubated at 37 °C for 24 hours.

3.4.1.4 Disk diffusion test

The disk diffusion test was performed preliminarily as previously described by De Brito et al. (2017) with slight modifications to identify which concentration of dimethyl sulfoxide (DMSO, Merck, Cat no. 472301) and Tween®20 (Sigma-Aldrich®, Cat no. T7200) was capable of carrying out an antibacterial activity in the three microorganisms (De Brito et al., 2017).

3.4.1.4.1 Preparation of the test discs

The discs (Whatman®, Cat no. WHA2017009) were impregnated over sterile Petri dishes with 30 µL of the concentrations tested, 80, 40, 20, 10, 5, 2.5, 1.75, 0.5 % v/v of DMSO (Merck, Cat no. 472301) and Tween®20 (Sigma-Aldrich®, Cat no. T7200) in BHI then allowed to dry in the oven at 37 ºC for 30 minutes. Cefpodoxime (10 µg) discs (cartridge B) were used as a standard antibiotic (Mast Group Ltd, product code: D69C).

3.4.1.4.2 Preparation of the cell cultures

The three bacterial strains were prepared by the aseptic transfer of one colony into a 7 mL sterile saline solution (Figure 3-7, A-C). Each test tube was vortexed (Figure 3-7, D) then placed into a nephelometer (Trek Diagnostics Systems Ltd, SN. 437R06N124) to measure the cell density and to standardise the cell culture to 0.5 McFarland standard (Remel™, Kansas, Cat. no. R20410) at 1.5x10^8 CFU/mL (Figure 3-7, E).

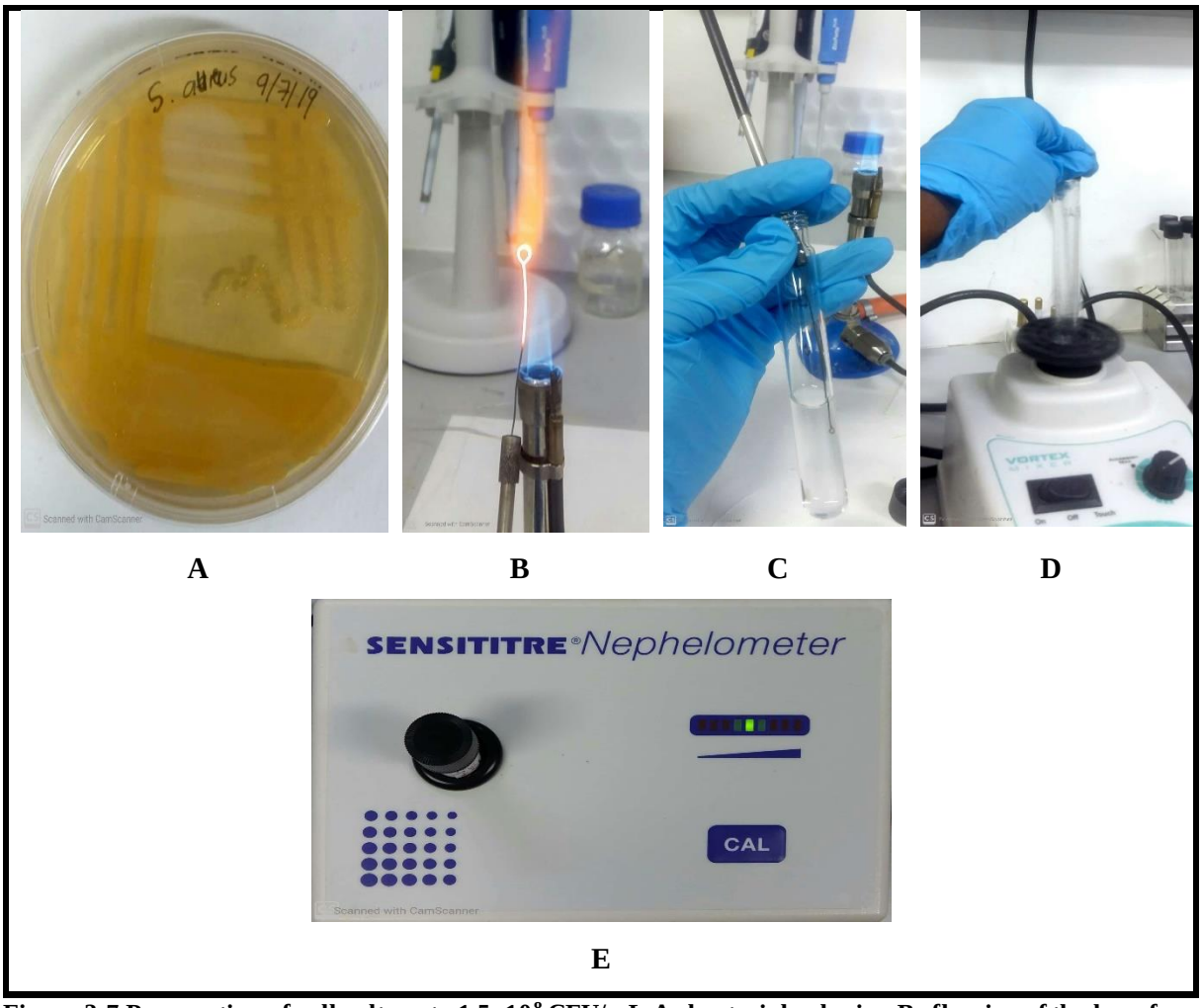


Figure 3-7 Preparation of cell culture to 1.5x10^8 CFU/mL.A: bacterial colonies, B: flaming of the loop for sterilisation, C: inoculation of bacterial culture into the sterile saline solution, D: vortexing of the cell culture, E: standardisation of the cell culture to 1.5x10^8 CFU/mL

3.4.1.4.3 Disk diffusion assay

The disk diffusion test was performed as previously described (Hudzicki, 2009). Seven Mueller-Hinton agar (MHA) plates were used for each strain for each diluent under test. The plates were appropriately labelled with the organism's name and the concentrations tested (in triplicate, n = 3) before commencement. A sterile swab (COPAN Diagnostics Inc., dry swabs 159C) was dipped into the cell culture then the plates were inoculated by streaking the surface of the plate three times in a back and forth motion. The plates were rotated approximately 60º each time to ensure even distribution of the inoculum. Subsequently, the impregnated discs were placed onto the agar and incubated at 37 ºC for 16 h. Each set of replicate zones of inhibition were read in mm with a ruler as the diameter from edge to edge of the halo across the centre of the disc then averaged and rounded up to the next millimetre.

3.4.1.5 Determination of minimum inhibitory concentration (MIC)

3.4.1.5.1 Preparation of the cell cultures

The three bacterial suspensions corresponding to 1.5 x 10^8 CFU/mL were achieved as described in the disk diffusion test (section 3.4.1.4.2). The working suspensions were prepared as a 1:100 dilution of 0.5 McFarland standard in BHI to a concentration of 10^6 CFU/mL which, when mixed with an equal volume of sample in the microplate during the assay amounted approximately to the recommended 5 x 10^5 CFU/mL (EUCAST, 2003).

3.4.1.5.2 Preparation of EOs samples

EOs stock solutions of 51.2 mg/mL were prepared in 10 mL sterile centrifuge tubes with a BHI:DMSO (1:1) solution.

3.4.1.5.3 Preparation of the antibiotic control

The antibiotic stock solution of 51.2 mg/mL was prepared by dissolving 51.2 mg of ampicillin (Sigma-Aldrich®, Cat no. A9393) powder in 1 mL of phosphate buffer (0.1 mol/L) at pH 6.0 (Clinical and Laboratory Standards Institute [CLSI], 2015).

3.4.1.5.4 Broth microdilution assay

The MIC test was carried out as previously described by Lourens et al. (2004) and Sartoratto et al. (2004) with slight modifications. In a 96-well plate (LASEC, Ref. no. 065727), 100 µL of BHI was added to the experimental wells in triplicate except in well 1. Then, 200 µL of the stock solutions of EO samples or antibiotic control (51.2 mg/mL) was added in well 1, from which a serial dilution was performed to the last experimental well. Subsequently, 100 µL of cell suspension (1:100 in BHI) was added to the samples to a total volume of 200 µL. The final established concentrations were 25.6, 12.8, 6.4, 3.2, 1.6, 0.8, 0.4, and 0.2 mg/mL for the EO samples/antibiotic control and 5 x 10^5 CFU/mL for the

bacterial cell suspensions. Two separate controls consisted of 200 µL of the blank alone (DMSO:BHI, 1:1) to assess the sterility of the broth and 200 µL of the cell suspension with the blank (1:1) as a reference for microbial growth. The plates were incubated at 37 ºC for 20 h. After incubation, the antimicrobial activity was detected by adding 40 µL of 0.2 mg/mL INT (*p*-iodonitrotetrazolium chloride, Sigma-Aldrich®, Cat no. I10406) aqueous solution. The plates were incubated at 37 ºC for 1 hour. The MICs were defined as the lowest concentration of essential oil that inhibited visible growth, as indicated by the colour change of INT.

3.4.2 Antioxidant capacity assays of the essential oils

In this study, the choice of *in vitro* antioxidants assays was based on their practicality in the laboratory and proven applicability to assess the antioxidative effects of essential oils (Amorati et al., 2013; Tu & Tawata, 2015; Mamadalieva et al., 2016; Zuccolotto et al., 2019).

3.4.2.1 General chemicals and reagent solutions

The following chemicals and reagents were purchased for the DPPH, ABTS, FRAP, and ORAC assays in their respective order as reported below.

The antioxidant capacities of the essential oils were evaluated by the DPPH, ABTS, FRAP, and ORAC assays using commercial chemicals and reagents as follows. **DPPH** and **ABTS assays** required: 2,2-diphenyl-1-picrylhydrazyl, DPPH, $C_{18}H_{12}N_5O_6$ (Sigma-Aldrich®, Cat no. D9132), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, $C_{18}H_{24}N_6O_6S_4$, (Sigma-Aldrich®, Cat no. A1888), potassium-peroxodisulphate, $K_2S_2O_8$, (Merck, Cat no. 105091), Trolox® also known as 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, $C_{14}H_{18}O_4$ (Sigma-Aldrich®, Cat no. 238831), butylated hydroxytoluene (BHT, Sigma-Aldrich®, Cat no. B1378), and gallic acid (Sigma-Aldrich®, Cat no. G7384). The **FRAP assay** required concentrated HCl, 32% HCl (Merck, Cat no., 100319), 2,4,6-tri[2-pyridyl]-s-triazine, $C_{18}H_{12}N_6$ (Merck, Cat no. T1253), iron (III) chloride hexahydrate, $FeCl_3.6H_2O$ (Sigma-Aldrich®, Cat no. F2877), L-ascorbic acid, $C_6H_8O_6$ (Sigma-Aldrich®, Cat no. A5960). For the **ORAC assay** were purchased, fluorescein sodium salt, $C_{20}H_{10}Na_2O_5$, (Sigma-Aldrich®, Cat no., F6377), 2,2'-Azobis (2-methylpropionamidine) dihydrochloride, AAPH, $[=NC(CH_3)_2C(=NH)NH_2]_2 \cdot 2HCl$, (Sigma-Aldrich®, Cat no. 440914), (-)-epigallocatechin gallate (EGCG, Sigma-Aldrich®, Cat no. E4143), sodium dihydrogen orthophosphate monohydrate, $NaH_2PO_4.H_2O$, (Sigma-Aldrich®, Cat no. S3522), and disodium hydrogen orthophosphate dihydrate, $Na_2HPO_4.2H_2O$, (Sigma-Aldrich®, Cat no. 71645).

3.4.2.2 Materials

HPLC grade absolute ethanol (Fisher Scientific, USA, Code E/0665DF/17), 96-well clear plate (Greiner, Merck), 96-well black plates (Greiner, Merck), pipettes, and tips (Eppendorf®, Germany and Greiner

Bio-One Cat no. 740290, Austria respectively), Multiskan™ spectrum plate reader (Thermo Fisher Scientific, USA), Fluorescence skan ascent (Thermo Electron Corporation, USA), 15 ml centrifuge tubes (Eppendorf®, Germany), 1.5 ml microcentrifuge tubes (Eppendorf®, Germany).

3.4.2.3 Essential oils samples and positive controls preparation

The EOs working solutions were prepared according to their solubility in each of the assay being organic-based for DPPH and ABTS assays and aqueous-based for FRAP and ORAC assays. For each EO in the DPPH and ABTS assays, ethanolic working solutions of 2.0, 1.0, and 0.5 mg/mL were prepared from a stock solution of 10 mg/mL in ethanol. The same ethanolic working solutions were prepared for Trolox® and gallic acid positive controls used in the DPPH and ABTS assays, respectively. For the FRAP and ORAC assays, a single ethanolic working solution of sample was prepared at 2.0 mg/mL. Gallic acid (2.0 mg/mL in distilled water) was used as positive control in the FRAP assay and EGCG (2.0 mg/mL in distilled water) was used as the positive control in the ORAC assay.

3.4.2.4 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The DPPH assay was performed according the method previously described by Bondet et al. (1997) with slight modifications. The DPPH reagent was prepared in ethanol by making an initial solution of 0.02% (w/v) (8 mg to 40 mL) and applying further dilutions with ethanol to attain an absorbance of 2.0 ± 0.1 at 517 nm. Thereafter, in the clear 96-well plate, 275 µL of DPPH reagent was added to 25 µL of EO and Trolox® samples using a multichannel pipette as fast as possible. For the blank, ethanol was added instead of the sample. The total volume of the assay was 300 µL. The absorbance was read at 517 nm and 37 ºC at the 6 min time point. Each EO/Trolox® sample was read in triplicate (n = 3). The percentage radical scavenging activity (% RSA) of the samples was calculated using equation 3-2.

$$\%\, RSA_{6\,min} = 1 - \frac{Abs_{sample}}{Abs_{blank}} \qquad \textbf{3-2}$$

Where Abs_{sample} is the absorbance signal of the EO sample and Abs_{blank} is the absorbance signal of the DPPH solution (ethanol in place of the sample) at 517 nm and 6 min. The results were expressed as the mean percentage of triplicate measurements (± standard deviation, SD).

3.4.2.5 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

The ABTS assay was performed according to Re et al. (1999) with slight modifications. The ABTS radical cation ($ABTS^{\bullet+}$) stock reagent was produced by reacting 5 mL of freshly prepared 7 mM ABTS solution (0.0192 g to 5 mL of distilled water) and 88 µL of a freshly prepared 140 µM $K_2S_2O_8$ (0.1892 g to 5 mL of distilled water) in a 15 mL centrifuge tube then allowing the mix to sit overnight for 16 h

in the dark at room temperature. The ABTS•+ stock mix was then further diluted with ethanol to read an absorbance of 2.0 ± 0.1 (approximately 1 mL ABTS•+ mix and 2 mL ethanol). In five microcentrifuge tubes, ethanolic Trolox® working standards of 50 µM, 100 µM, 150 µM, 250 µM, and 500µM were prepared from a 1.0 mM Trolox® control stock (0.0125 g in 50 mL ethanol). Thereafter, in the clear 96-well plate, 275 µL of ABTS•+ reagent was added to 25µL of each working standard, EO and gallic acid sample (2.0, 1.0, and 0.5 mg/mL) using a multichannel pipette as fast as possible. For the blank, ethanol was added instead of the sample. The total volume of the assay was 300 µL. The absorbance was read at 734 nm and 37 ºC at the 6 min time point. Each EO sample, working standard, and gallic acid sample was read in triplicate (n = 3). The percentage of radical scavenging activity (% RSA) of each EO or positive control working solution was calculated using the equation 3-3.

$$\%\, RSA_{6\,min} = 1 - \frac{Abs_{sample}}{Abs_{blank}} \qquad \textbf{3-3}$$

Where Abs_{sample} is the absorbance signal of the EO sample/positive control and Abs_{blank} is the absorbance signal of the ABTS•+ solution (ethanol in place of the sample) at 734 nm. The results were expressed as the mean percentage of triplicate measurements (± standard deviation, SD).

Additionally, the Trolox® equivalent capacity assay (TEAC) values were reduced from the linear regression (R^2 = 0.9980) of Trolox® concentrations (µM) and the absorbance readings at 734 nm at 6 min and expressed as mean (% relative standard deviation, RSD) of triplicate measurements in µmol Trolox® equivalents per litre of sample tested (µmol TE/L).

3.4.2.6 Oxygen radical absorbance capacity (ORAC) assay

3.4.2.6.1 Reagents

- Phosphate buffer, 75µM, pH 7.4: 18 mL of $NaH_2PO_4.H_2O$ aqueous solution (1.035 g to 100 mL distilled water) were mixed with 84 mL of $Na_2HPO_4.2H_2O$ aqueous (1.335 g to 100 mL distilled water) then was adjusted accordingly until achieving pH = 7.40 ± 0.01.
- Fluorescein sodium salt stock solution: 0.0225 g was dissolved with 50 mL phosphate buffer. Subsequently, 10 µL of this stock was mixed with 2 mL of phosphate buffer then 240 µL of this solution was further diluted to 15 ml of phosphate buffer and used for the assay.
- Peroxyl radical AAPH: 150 mg in a 15 ml centrifuge tube.

3.4.2.6.2 Protocol

The ORAC assay was performed according to the method described in Prior et al. (2003) with slight modifications. A Trolox® standard series of 83 µM, 167 µM, 250 µM, 333 µM, and 417 µM was

prepared with the phosphate buffer (pH 7.4) from a Trolox® stock solution of 500 µM (0.00625 g to 50 ml of phosphate buffer). Then, in the black 96-well plate, 12 µL of the Trolox® working solutions, and each EO, and EGCG working samples were added in triplicate (n = 3). Subsequently, 138 µL of fluorescein solution was added with a multichannel pipette followed by 50 µL of freshly prepared AAPH in phosphate buffer (150 mg to 6 ml). For the blank, the phosphate buffer was added in place of the sample. The total volume of the assay was 200 µL. Readings of the EO/EGCG samples (2.0 mg/mL) and Trolox® working standard solutions were taken using the excitation wavelength set at 485 nm and the emission wavelength at 530 nm for 2 hours at 1 min reading interval. The temperature was set at 37 ºC After analysis, the data points of the blank (phosphate buffer instead of the sample), EO samples, EGCG sample, and Trolox® working standards were summed up over time to obtain the area under the fluorescence decay curve (AUC). The ORAC values were calculated using the linear regression (R^2 = 0.9861) equation (Y = aX + c) between Trolox® concentration (Y) (µM) and the net area (blank-corrected) under the fluorescence decay curve (X). The results were expressed as the mean (% relative standard deviation, RSD) of triplicate measurements in µmol of Trolox® equivalents per litre of sample tested (µmol TE/L).

3.4.2.7 Ferric reducing antioxidant power (FRAP) assay

3.4.2.7.1 Reagents

- Acetate buffer, 300 mM, pH 3.6: 1.627 g sodium acetate, and 16 mL glacial acetic acid were made up to 1 L with distilled water.
- 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ), 10 mM: In a 15 mL centrifuge tube, 0.0093 g was mixed with 3 mL of 40 mM HCl (1.46 mL concentrated HCl, 32% made up to 1L with distilled water).
- Iron (III) chloride hexahydrate ($FeCl_3.6H_2O$), 20 mM: In a 15 mL centrifuge tube, 0.054 g $FeCl_3.6H_2O$ was made up to 10 mL distilled water.

3.4.2.7.2 Protocol

The FRAP assay was conducted as recommended by Benzie and Strain (1996) with slight adjustments. Firstly, the fresh blue FRAP reagent was achieved by mixing in a 50 mL centrifuge tube, 30 mL of acetate buffer, 3 mL of TPTZ, 3 mL of $FeCl_3$ solution, and 6.6 mL of distilled water. Then, in microcentrifuge tubes, the L-ascorbic acid standard series of 50 µM, 100 µM, 200 µM, 500 µM, and 1000 µM with distilled water was prepared from a 1 mM of L-ascorbic acid stock (0.0088 g to 50 mL of distilled water). Lastly, in a clear 96-well plate, 300 µL of the FRAP reagent was added to 10 µL of L-ascorbic acid working standard solutions, EO, and gallic acid samples (2.0 mg/mL) in triplicate (n = 3). For the blank, the phosphate buffer (pH 3.6) was added instead of the sample. The total volume of the assay was 310 µL. The absorbance of TPTZ-Fe (II) was read at 593 nm and 37 ºC for 30 min. The

results were calculated using the linear regression (R^2 = 0.9965) of the L-ascorbic acid (AA) concentrations (μM) and absorbance signals and expressed as mean (% relative standard deviation, RSD) of triplicate measurements in μmol L-ascorbic acid equivalents per litre of sample tested (μmol AAE/L).

3.4.2.8 Thin-layer chromatography direct bioautography

Two independent antioxidant TLC direct-bioautography methods, DPPH and ABTS were tested in a bid to detect radical scavenging components contained in the six essential oils. The bioautography assays were performed as described by Zhao et al. (2010) with slight modifications. A volume of 8 μL of each essential oil sample in hexane (1% v/v; 5μL in 0.5 mL) was loaded onto silica gel 60 F_{254} plates (10 cm x 10 cm; Merck, Germany). Subsequently, the plates were developed in a pre-saturated tank containing 80 mL of 97:3 hexane: ethyl acetate mobile phase in a triple run. After development, BHT (2 mg/ml in hexane, for DPPH) and Trolox® (2 mg/ml in distilled water, for ABTS) controls were spotted on the baseline. Then, the plates were dipped for 5 s into 30 mL of 0.04% w/v DPPH solution in methanol (40 mg in 100 mL) and 30 mL of $ABTS^{\bullet+}$ mix solution (prepared as described in section 3.4.2.5). The plates were run in duplicate. Only bleached spots appearing within the first 5 s of contact with the reagents were considered active and their average Rf values were recorded. Then, the fractions of active essential oil were further analysed by TLC DPPH direct-bioautography (92:8 hexane: ethyl acetate) to detect the individual components and GC-MS was used for identification. The GC-MS analysis was performed according to the protocol described in section 3.3.3.2. The identification was done by the mass spectral match with compounds of the instrument's built-in library.

3.4.3 Determination of the sun protection factor (SPF)

The purpose of this test was to measure the effectiveness of the six essential oils as sunscreens for product development, as a supplement of the *in vivo* SPF measure. In this work, the spectrophotometric analysis of dilute solutions of the essential oil samples was chosen for its practical simplicity.

3.4.3.1 Materials and reagents

HPLC grade absolute Ethanol (Fisher scientific, USA, Code E/0665DF/17), distilled water, 96-well plate (Greiner, Merck), pipettes, and tips (Eppendorf®, Germany and Greiner Bio-One Cat no. 740290, Austria, respectively). Multiskan spectrum plate reader (Thermo Fisher Scientific, USA) and 1.5 mL microcentrifuge tubes (Eppendorf®, Germany).

3.4.3.2 Method

The protocol used for this assay was conducted as per Kaur and Saraf et al. (2010). The solubility of EOs in different ratios of ethanol and water was tested by taking 10% to 50% of ethanol in distilled water. The maximum solubility was detected at ethanol: water 40:60 ratio above which turbidity

developed. Thereafter, an initial stock solution of 1% v/v was prepared by making up 10 µL of each EO to 1 mL of ethanol: water (40:60). Then out of this stock, 0.1% v/v was prepared. Subsequently, 100 µL of each EO aliquot and the blank (ethanol: water 40:60) were injected in the 96-well plate and read in triplicate (n = 3) over the 290 nm to 320 nm range at 5 nm interval. The SPF value of each essential oil was calculated following the Mansur et al. (1986) equation (3-4). The mean of the observed absorbance values was multiplied by their respective erythemogenic effect times solar intensity at wavelength λ values, EE (λ) x I (λ), then their summation was obtained and multiplied with the correction factor (= 10). The equation is described as:

$$\mathrm{SPF}_{\mathrm{spectrophotometric}} = CF \times \sum_{290}^{320} EE\,(\lambda) \times I\,(\lambda) \times Abs\,(\lambda) \qquad \textbf{3-4}$$

Where CF: correction factor (= 10), EE (λ): erythemogenic effect of radiation at wavelength λ, I (λ): solar intensity at wavelength λ and Abs (λ): the spectrometric absorbance value at wavelength λ.

The values of EE (λ) x I (λ) are constant values that were determined by Sayre et al. (1979) as shown in the Table 3-4:

Table 3-4 Relationship between erythemogenic effect and radiation intensity

Wavelength (nm)	EE X I (normalised)
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0837
320	0.0180
Total	1

3.4.4 Spectrophotometric determination of tyrosinase inhibition of the essential oils

In this research work, the tyrosinase inhibition of the EOs was evaluated by studying the monophenolase activity. This was achieved by measuring the UV absorbance of the L-DOPA product at its absorbance maximum of 490 nm.

3.4.4.1 Chemicals and reagents

Lyophilised powder of mushroom tyrosinase 25000 units/mg (EC 1.14.18.1, Sigma-Aldrich®, Cat no. T3824), Kojic acid (Sigma-Aldrich®, Cat no. K3125), L-tyrosine (Sigma-Aldrich®, Cat no. 93829), Tween®20 (Sigma-Aldrich®, Cat no. T7200), sodium dihydrogen orthophosphate monohydrate,

$NaH_2PO_4.H_2O$ (Sigma-Aldrich®, Cat no. S3522), disodium hydrogen orthophosphate dihydrate, $Na_2HPO_{4.2}H_2O$ (Sigma-Aldrich®, Cat no. 71645), dimethyl sulfoxide, DMSO, (Merck, Cat no. 472301), methanol (Sigma-Aldrich®, Cat no. 34860).

3.4.4.2 Material

1.5 mL centrifuge tubes (Eppendorf, Germany) and micropipettes and a multichannel pipette (Eppendorf, Germany).

3.4.4.3 Reagents and samples preparation

3.4.4.3.1 Buffer preparation

The phosphate buffer solution (PBS) was prepared by mixing two stock solutions of 50 mM $NaH_2PO_4.H_2O$ and 50 mM $Na_2HPO_4.2H_2O$ and adjusting to pH 6.5. The buffer was stored at 4 ºC until further use.

3.4.4.3.2 Tyrosinase enzyme preparation

The enzyme stock solutions were prepared over an ice bath by dissolving 1 mg of tyrosinase powder in 25 mL of phosphate buffer in a 50 mL centrifuge tube. Subsequently, 1 mL aliquots of this solution were transferred to individual 2 ml centrifuge tubes to achieve a concentration of 1000 units/ml (U/ml). The stock solutions were stored at -40 ºC until further use.

3.4.4.3.3 L-tyrosine working solution preparation

L-tyrosine substrate was prepared at 2 mM in PBS (≈ 0.0145g in 40 mL) and stored at 4 ºC until further use.

3.4.4.3.4 Essential oils samples and positive control preparation

10 mg/mL of EO working solutions were prepared with a DMSO: Tween®20 (1:1) solution to facilitate dispersion of the oils then further diluted to 1 mg/mL working solutions with methanol. Thereafter, a 10 mg/mL Kojic acid working solution was made up with 100% DMSO then diluted to 1 mg/mL with methanol.

3.4.4.4 Spectrophotometric measurement of L-DOPA

The tyrosinase inhibition assay was performed as described previously by Popoola et al. (2015) and Cui et al. (2018) with slight modifications. The concentrations of EO samples and Kojic acid chosen, 200 µg/mL and 50 µg/mL were achieved by setting up the 96-well plate in the following order: 70 µL of sample (1 mg/mL) then 30µL of tyrosinase enzyme (500 U/mL). Each concentration of the sample and positive control was set up in two different wells whereby, one of the wells received enzyme and the other well had no enzyme volume added. All volume deficits were compensated by adding excess buffer.

The negative controls, 10% v/v of 1:1 DMSO: Tween®20 in methanol for the EOs and 10% v/v DMSO in methanol for Kojic acid were treated the same way. Then, the plate was incubated at 37 ºC (± 2.0 ºC) for 5 min. Thereafter, the reaction was initiated by adding 110 µL of L-tyrosine (2 mM) to the plate using a multichannel pipette and subsequently incubated at 37 ºC (± 2.0 ºC) for 30 min. The absorbance of L-DOPA was read at 490 nm on a Multiskan™ spectrum plate reader (Thermo Fisher Scientific, USA). Two independent experiments were carried out in triplicate and the percentage tyrosinase inhibition was calculated using equation 3-5 as follows:

$$Tyrosinase\ inhibition\ (\%) = \frac{(A-B) - (C-D)}{(A-B)} \times 100 \qquad 3\text{-}5$$

Where A is the negative control with enzyme, B is the negative control without enzyme, C is the EO sample or Kojic acid with enzyme and D is the EO sample or Kojic acid without enzyme. The inhibition percentages were expressed as the mean (± standard deviation) of duplicate measurements. One-way ANOVA was used to compare the absorbance values of the two groups ($p < 0.05$).

4. CHAPTER FOUR: CHEMICAL IDENTIFICATION OF ESSENTIAL OILS COMPONENTS

This chapter presents the chemical characterisation of the constituents of South African essential oils by gas chromatography-mass spectrometry analysis of the crude essential oils and the structural elucidation of the isolated components of *H. petiolare* essential oil. In the GC-MS analysis, the results were summarised in tables in which, at end of each table, annotations found as superscripts and normal text were added to specify the method of identification of each constituent. For identification in GC-MS, the Kováts equation was used for the calculation of retention indices. Then, the RIs were compared with reported values in Adams (2007), Babushok et al. (2011), and the National Institute of Standards and Technologies (NIST) Chemistry WebBook (2018) acquired using columns of similar polarity (i.e., dimethyl silicone with 5% phenyl groups as used for this study).

4.1 Isolation of essential oils by hydrodistillation

The amount of essential oil recovered from the plant materials varied from 0.15% v/w to 0.38% v/w (% g/mL) as presented in Table 4-1 below:

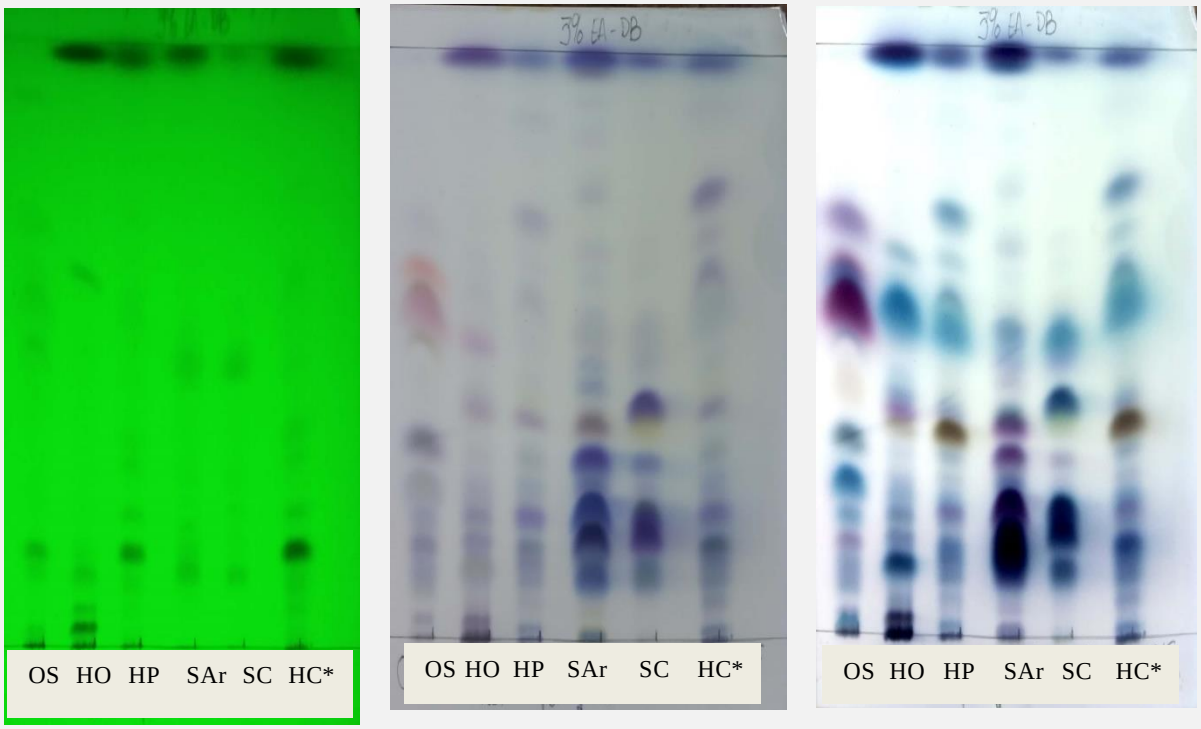
Table 4-1 Essential oil yield of hydrodistilled plant materials

Plant material name	Yield (% v/w)*
Oncosiphon suffruticosum	0.23
Helichrysum petiolare	0.25
Helichrysum cymosum	0.15
Salvia aurea	0.22
Salvia chamelaeagnea	0.38

*Average values of triplicate analyses

4.2 Preliminary phytochemical screening by TLC

The TLC analysis of the crude essential oils showed intense blue, blue-violet, brown, and yellow stains as shown below in Figure 4-1. These are characteristic colours of terpenic and non-terpenic components of essential oils upon derivatisation with vanillin-sulphuric acid reagent (Wagner & Bladt, 1996).



Under λ_{254} nm | **First heating after derivatising with H_2SO_4(2.5% v/v)-vanillin** | **Second heating after derivatising with H_2SO_4(2.5% v/v)-vanillin**

***OS**: *O. suffruticosum*; **HO**: *H. odoratissimum*; **HP**: *H. petiolare*; **SAr**: *S. aurea*; **SC**: *S. chamelaeagnea*; **HC**: *H. cymosum*

Figure 4-1 TLC profiles of the crude essential oils at 97:3 hexane: ethyl acetate (double run)

4.3 GC-MS analysis of *Oncosiphon suffruticosum* EO

The essential oil of *O. suffruticosum* was analysed by GC-MS according to section 3.3.3.2. The results were obtained as shown below in Table 4-2.

4.3.1 Results

Table 4-2 Components of *O. suffruticosum* essential oil

RT (Min)	Mass spectral matching	Composition (%)	Experimental RI	Literature RI	Identification
9.214	α-Pinene	0.80	935	939 [A]	RI, MS
9.981	Camphene	2.17	950	950 [B]	RI, MS
11.374	Sabinene	0.54	974	973 [B]	RI, MS
13.928	α-Terpinene	0.71	1016	1017 [B]	RI, MS
14.508	*p*-Cymene	2.45	1026	1024 [B]	RI, MS
15.016	**1,8-Cineole**	**7.85**	**1035**	1032 [B]	RI, MS
16.710	γ-Terpinene	1.48	1061	1060 [B]	RI, MS
20.058	**Filifolone**	**13.98**	**1109**	1109 [Wb]	RI
20.372	Unknown	2.56	1114	-	-
20.560	Unknown	2.03	1117	-	-
21.426	**Chrysanthenone**	**8.72**	**1131**	1125 [B]	RI, MS
23.039	**(+)-2-Bornanone**	**31.21**	**1155**	1158 [Wb]	RI, MS
23.683	Pinocarvone	0.29	1164	1164 [A]	RI, MS
25.032	**Terpinen-4-ol**	**7.39**	**1183**	1177 [B]	RI, MS
26.745	Verbenone	0.56	1207	1206 [B]	RI, MS
29.015	Unknown	1.10	1243	-	-
35.372	Piperitenone	0.78	1339	1341 [B]	RI, MS
39.371	3,5-Heptadienal, 2-ethylidene-6-methyl-	5.71	1400	1395 [Wb]	RI
40.828	Unknown	3.75	1425	-	-
49.798	Caryophyllene oxide	0.45	1576	1580 [B]	RI, MS
Monoterpene hydrocarbons:		8.15			
Oxygenated monoterpenes:		76.49			
Total monoterpenoids:		**84.64**			
Sesquiterpene hydrocarbons:		0.00			
Oxygenated sesquiterpenes:		0.45			
Total sesquiterpenoids:		**0.45**			
Total identified:		**85.09**			
Unidentified:		9.44			
Total		**94.53**			

A = Adams (2007)

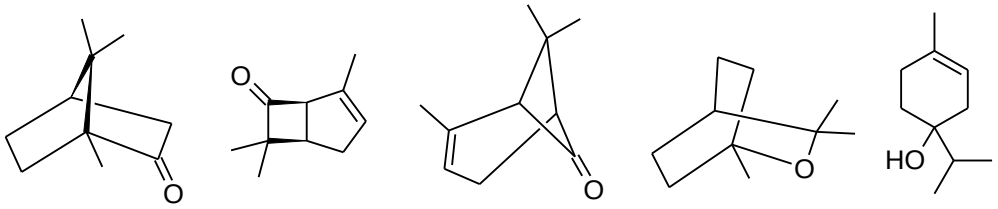
B = Babushok et al. (2011)

Wb = NIST Chemistry WebBook (2018)

MS = In addition to RI, the MS of the analysed compound matched with the MS of the compound in Adams (2007) and/or NIST Chemistry WebBook (2018)

Unknown = The MS of the compound could not be matched with the available literature data

Freshly hydrodistilled plant material of *O. suffruticosum* gave 0.23% (v/w) essential oil yield. According to the analysis conducted, sixteen components making up 85.09% of the EO were identified. The EO was found to majorly contain hydrocarbon and oxygenated monoterpenes amounting to 84.64% in composition, of which the oxygenated monoterpenes were dominant by 76.49%. The only identified sesquiterpene was found to be caryophyllene oxide present as 0.45%. Major constituents that were identified were found to be oxygenated monoterpenes: (+)-2-bornanone, an isomer of camphor (31.21%), filifolone (13.98%), chrysanthenone (8.72%), 1,8-cineole (7.85%) and terpinen-4-ol (7.39%) (Figure 4-2).



(+)-2-Bornanone (31.21%) | Filifolone (13.98%) | Chrysanthenone (8.72%) | 1,8-Cineole (7.85%) | Terpinen-4-ol (7.39%)

Figure 4-2 Major components of *Oncosiphon suffruticosum* essential oil

4.3.2 Discussion

As mentioned in the literature review (section 2.5.1.2), it appeared that the EO of this plant has never been studied before as it is for the other plants in this genus. Nevertheless, its composition showed similarities with species of the *Pentzia* genus, in which the plant was formerly classified. The chromatographed EOs of *Pentzia incana* (Hulley et al., 2018) and *Pentzia punctata* (Hulley et al., 2019) have shown to possess a significant content of camphor up to 47.9% and 27.3% respectively. Moreover, 1,8-cineole was also found as a major compound in *Pentzia incana* up to 16.7% (Hulley et al., 2018).

4.4 *Helichrysum* species

The *Helichrysum* species EOs were analysed by GC-MS as described in section 3.3.3.2. Additionally, *H. petiolare* EO was further analysed by NMR. The results are presented as follows:

4.4.1 GC-MS analysis of *H. petiolare* EO

The essential oil of *H. petiolare* was analysed by GC-MS. The results were obtained as shown in Table 4-3.

4.4.1.1 Results

Table 4-3 Components of *H. petiolare* essential oil

RT (Min)	Mass spectral matching	Composition (%)	Experimental RI	Literature RI	Identification
9.365	**α-Pinene**	**7.49**	**938**	939 [A]	RI, MS
11.752	**β-Pinene**	**10.54**	**981**	979 [A]	RI, MS
12.562	Myrcene	0.50	993	990 [A]	RI, MS
15.035	**1,8-Cineole**	**9.87**	**1035**	1032 [B]	RI, MS
16.015	**(*E*)-β-ocimene**	**17.21**	**1051**	1050 [A]	RI, MS
16.441	β-Ocimene (undefined isomer)	3.79	1057	-	Wb MS
16.818	γ-Terpinene	0.73	1063	1060 [B]	RI, MS
21.735	**allo-Ocimene**	**6.66**	**1136**	1132 [A]	RI, MS
24.541	Terpinen-4-ol	0.57	1176	1177 [B]	RI, MS
32.597	Lavandulyl acetate	0.99	1294	1290 [A]	RI, MS
34.489	Myrtenyl acetate	0.41	1325	1326 [A]	RI, MS
37.510	α-Copaene	0.65	1372	1376 [B]	RI, MS
38.301	Lavandulyl propionate	0.41	1384	-	Match
42.351	α-Humulene	3.01	1450	1453 [B]	RI, MS
44.705	Phenyl ethyl 2-methylbutanoate	0.90	1488	1487 [A]	RI, MS
45.416	**Unknown**	**5.29**	**1499**	-	-
46.278	Lavandulyl isovalerate	1.28	1514	1509 [A]	RI, MS
46.712	δ-Cadinene	2.05	1522	1523 [B]	RI, MS
47.678	α-Calacorene	0.68	1539	1540 [B]	RI, MS
49.341	**Faurinone**	**20.66**	**1568**	-	MS, NMR
51.337	Unknown	0.43	1602	-	-
53.483	Unknown	1.93	1642	-	-
53.896	Unknown	0.62	1649	-	-
54.836	Valeranone	1.07	1666	1672 [B]	RI, MS
69.285	Geranyl-α-terpinene	0.60	1950	-	Match
Monoterpene hydrocarbons:		46.92			
Oxygenated monoterpenes:		12.25			
Total monoterpenoids:		**59.17**			
Sesquiterpene hydrocarbons:		6.39			

Oxygenated sesquiterpenes:	23.01
Total sesquiterpenoids:	**29.40**
Diterpene hydrocarbons:	0.60
Phenylpropanoids:	0.90
Total identified	**90.07**
Unidentified:	8.27
Total	**98.34**

A = Adams (2007)

B = Babushok et al. (2011)

Wb = NIST Chemistry WebBook (2018)

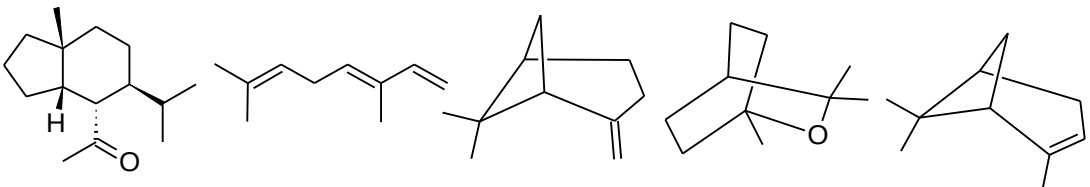
Wb MS = The MS of the analysed compound matched with the MS of the compound listed in NIST Chemistry WebBook (2018)

MS = In addition to RI, the MS of the analysed compound matched with the MS of the compound in Adams (2007) and/or NIST Chemistry WebBook (2018)

Match = no RI or MS available in the literature. The compound was reported solely based on the mass spectral match with NIST14 libraries reported by MassHunter software (Agilent Technologies, Inc) (Probability<0.04)

Unknown = The MS and RI of the compound could not be matched with the available literature data

The fresh plant material of *H. petiolare* yielded 0.25% (v/w) of essential oil. Twenty-one components constituting 90.07% of the essential oil were identified. The major class of compounds was the monoterpenoids (59.17%) of which, the monoterpene hydrocarbons were present as 46.92% and the oxygenated monoterpenes as 12.25%. The sesquiterpenoids amounted to 29.40% in the content of which, the oxygenated sesquiterpenes (23.01%) were the dominant class followed by the sesquiterpene hydrocarbons (6.39%). The minor classes were found to be the phenylpropanoids (0.90%) and diterpene hydocarbons (0.60%). The most abundant individual components were found to be faurinone (20.66%), (*E*)-β-ocimene (17.21%), β-pinene (10.54%), 1,8-cineole (9.87%), and α-pinene (7.49%) as shown Figure 4-3.



Faurinone (20.66%) | (*E*)-β-Ocimene (17.21%) | β-Pinene (10.54%) | 1,8-Cineole (9.87%) | α-Pinene (7.49%)

Figure 4-3 Major components of *H. petiolare* essential oil

4.4.1.2 Discussion

The structural elucidation of faurinone (compound **1**) was done by the NMR and MS analyses (section 3.3.4) and it has never been reported in the essential oil of *H. petiolare*. Previous analysis on the

composition of *H. petiolare* EO by Lourens et al. (2004) reported: 1,8-cineole (22.4%) as the major compound in the plant collected from National Botanical Institute, Pretoria, South Africa alongside β-caryophyllene (14.0%), *p*-cymene (9.8%), α-pinene (6.8%), α-terpineol (5.1%). In another research work by Giovanelli et al., (2018) who analysed the EO of *H. petiolare*, the major compound (36.42-45.26%) reported with RI = 1572 close to faurinone's RI = 1568 in this research, was identified as caryophyllenyl alcohol (RI = 1572 [Adams, 2007]). However, this finding did not corroborate with the results obtained in this research. The elucidation of the unknown compound (5.29%) at RT = 45.416 min with RI = 1499 (compound **2**) is also discussed further in section 4.4.4.

4.4.2 GC-MS analysis of isolated compound 1

Compound **1** which remained unidentified by GC-MS analysis (RT = 49.341 min) as discussed above was isolated by CC and prep TLC. The GC-MS analysis of compound **1** revealed a major peak at RT = 48.912 min as shown Figure 4-4. The integration report revealed a purity level of 96.46%.

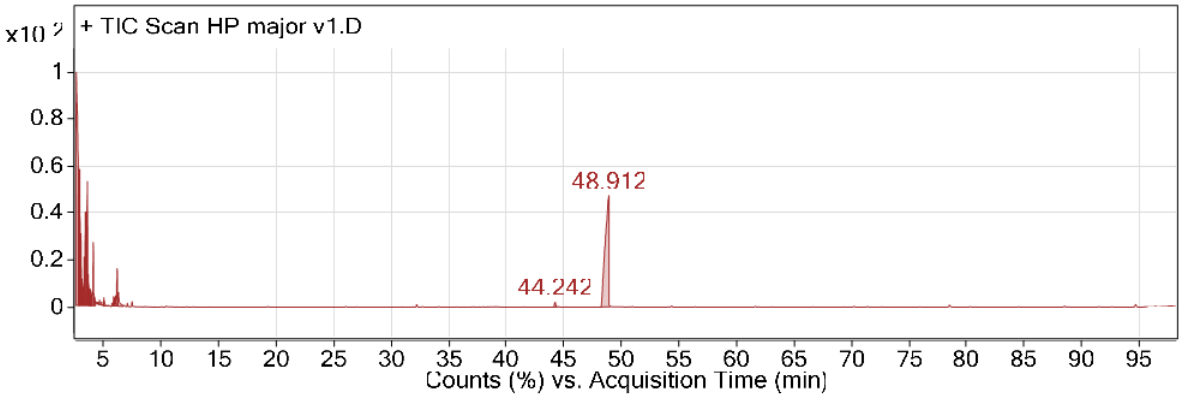


Figure 4-4 Total ion chromatogram of isolated compound 1 (RT= 48.912 min)

4.4.3 Structural elucidation of compound 1

Compound **1** (5 mg) (3.3.4.2) was identified as faurinone (Figure 4-5) by comparison of MS, ^{1}H-NMR and ^{13}C-NMR experimental data with previously published literature data as presented in the following sections.

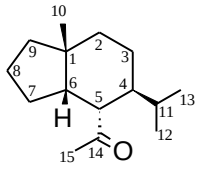


Figure 4-5 Faurinone

4.4.3.1 Mass spectrometry

The ion fragment peaks obtained in the MS spectrum (Figure 4-6) were compared identically to the data first reported by Hikino et al. (1968). The molecular ion [M^+] at *m/z* = 222.3 suggested the molecular formula to be $C_{15}H_{26}O$, which translates to the 3 degrees of unsaturation as it is for faurinone. A base peak was found at *m/z*=123.2 typical of isomers of faurinone as described by Weyerstahl et al. (1998).

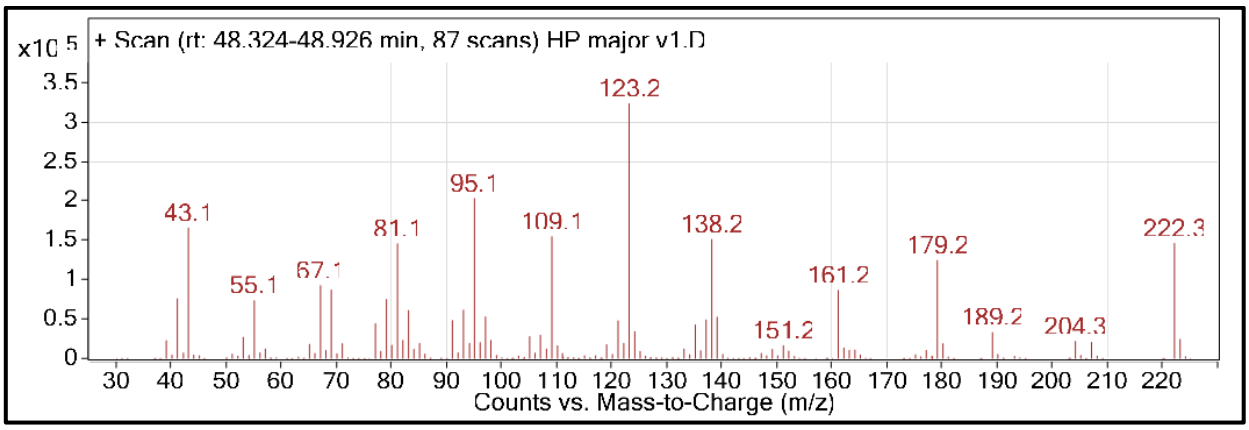


Figure 4-6 Mass spectrum of compound 1

The fragmentation pattern of compound **1** was compared to the literature values (Hübschmann, 2015, pp. 408-411) and the proposed fragmentation is summarised in Table 4-4.

Table 4-4 Proposed fragmentation of compound 1

Peaks (*m/z*)	M^+-X	Information
222.3	[M^+]	Molecular ion peak. The molecular weight is 222 g/mol.
179.2	[M^+-43]	M^+-43, loss of side isopropyl C_3H_7 at C4 through α-cleavage.
138.2	[M^+-43-41]	M^+-41: a loss of C_3H_5 through homolytic cleavage (C2-C4)
123.2	[M^+-43-41-15]	α-cleavage of the methyl CH_3 at C1.
109.1	[M^+-43-41-15-14]	M^+-14: loss of CH_2 at C9.
95.1	[M^+-43-41-15-28]	M^+-28: loss of ethylene from the cyclic system C8-C7.
81.1	[M^+-43-41-15-28-14]	M^+-14: loss of CH_2 from the cyclic system.
43.1	-	*m/z* = 43 indicates the stable acylium carbocation [$H_3C-C\equiv O^+$]

4.4.3.2 ^{1}H NMR spectroscopy

The ^{1}H NMR spectrum of compound **1** was obtained as shown in Figure 4-7.

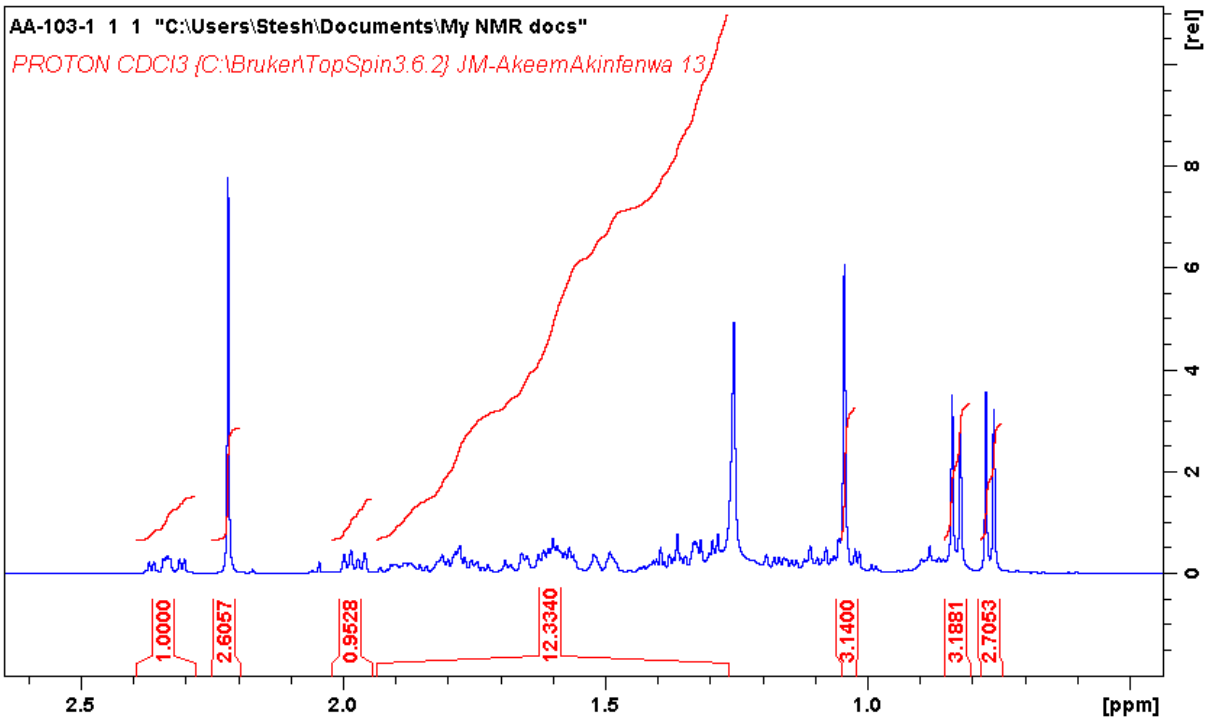


Figure 4-7 ^{1}H NMR spectrum of compound 1

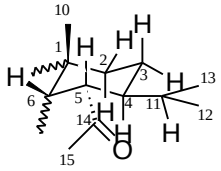


Figure 4-8 Fragment of the chair conformation of faurinone

The ^{1}H NMR spectrum (Figure 4-7) showed four methyl signals, two of them appear at 0.74 and 0.80 (*d* each, 6.2 Hz, Me-12, Me-13), the other two appear as singlets at 1.02 (Me-10) and 2.19 (Me-15), the later is adjacent to a carbonyl group (C-14) (Figure 4-8). The spectrum also showed a proton resonating at 2.31 (*ddd*, 3.6, 10.7, 14.4 Hz) and assigned to H-4, and another proton at 1.95 (*dd*, 5.0, 10.7 Hz) and assigned to H-5, in addition to a cluster of protons between 1.92-0.80 ppm. The proton signals were summarised in Table 4-5.

Table 4-5 Summary of identified protons in the ^{1}H spectrum of faurinone

	δ (ppm) multiplicity (*J*=Hz)	
	Experimental	**Reported**
H-4	2.31 *ddd* (3.6, 10.7, 14.4)	*tt** (10.4, 3.8) (Bos et al., 1982)
H-5	1.95 *dd* (10.7, 5.01)	*dd** (10.4, 4.8) (Bos et al., 1982)
H-10 (Me)	1.02 *s*	1.03 *s* (Hikino et al., 1968)
H-(12,13) (Me)	0.74 *d* (6.12) 0.80 *d* (6.12)	0.74 *d* (5) 0.81 *d* (5) (Hikino et al., 1968)
H-15 (Me)	2.19 *s*	2.18 *s* (Hikino et al., 1968)

***chemical shifts were not reported**

4.4.3.3 ^{13}C NMR spectroscopy

The ^{13}C NMR spectrum (Figure 4-9) revealed 15 carbons and classified according to DEPT-135 into four methyls, five methylene, four methines, one fully substituted carbon, and a carbonyl group. The peak at δ 212.1 ppm indicated the presence of the ketone carbonyl (C14) previously reported at δ 211.8 ppm (Bos et al., 1983). The remaining peaks from δ 51.9-21.4 ppm were representative of typical saturated (sp^3 hybridised) carbons unaffected by electronegative atoms as found in the structure of faurinone.

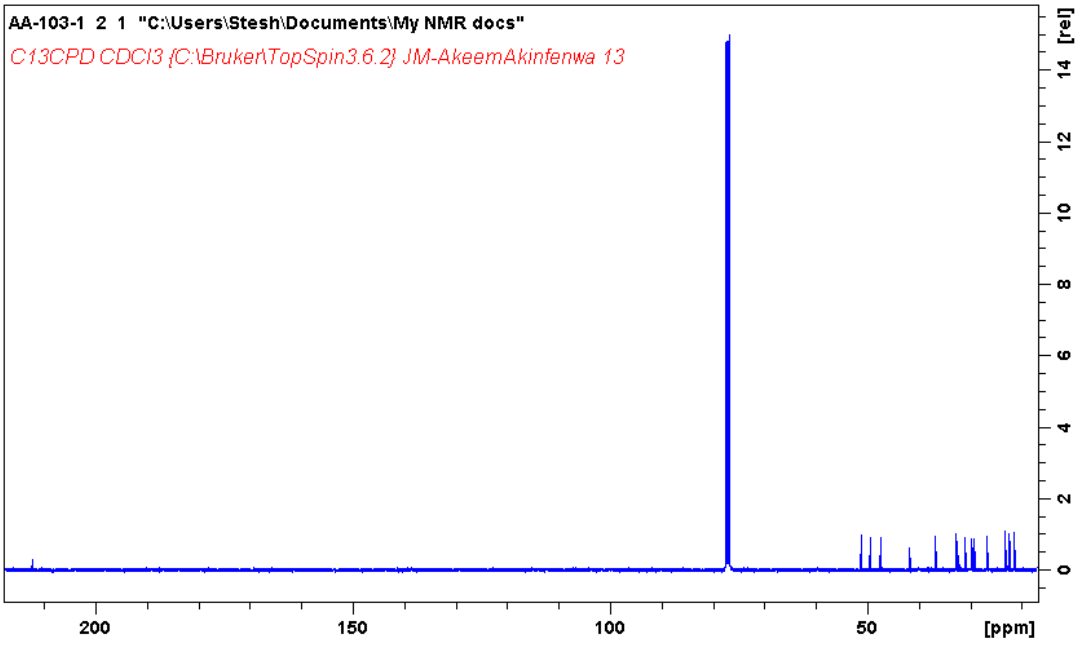


Figure 4-9 ^{13}C NMR ($CDCl_3$) spectrum of compound 1

Besides the carbonyl carbon, the absent peak at δ 41.6 ppm in the DEPT-135 confirmed the presence of a quaternary carbon at position 1 as reported at the same value by Bos et al. (1983) and Weyerstahl et al. (1998).

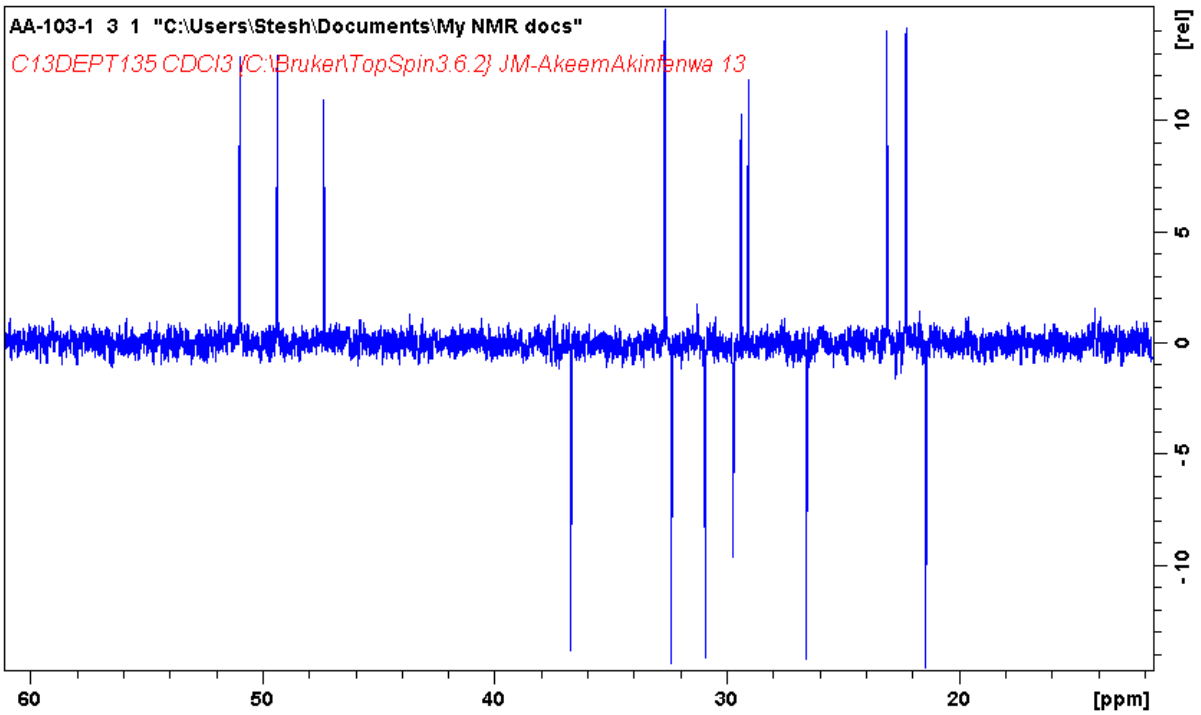


Figure 4-10 DEPT-135 spectrum of compound 1

The typical shifts of the saturated carbons in the ^{13}C NMR ($CDCl_3$) of compound **1** obtained experimentally were in close agreement with the reported values of faurinone (Weyerstahl et al., 1998). The summary of the comparison with literature chemical shifts is presented in Table 4-6.

Table 4-6 Experimental and literature values of ^{13}C-NMR shifts of faurinone

C	Multiplicity*	Compound 1	Faurinone*
1	*s*	41.6	41.6
4	*d*	50.9	50.9
5	*d*	49.4	49.3
6	*d*	47.3	47.3
12-Me	*q*	22.3	22.2
11-Me	*q*	23.0	23.0
14	*s*	212.1	211.8

*(Weyerstahl et al. 1998)

4.4.4 GC-MS analysis of isolated compound 2

Compound **2** which remained unidentified by GC-MS analysis (RT = 45.416 min) as discussed above was isolated by CC and prep TLC. The GC-MS analysis of compound **2** revealed a major peak at RT = 44.627 min as shown Figure 4-11. The integration report revealed a purity level of 98.25%.

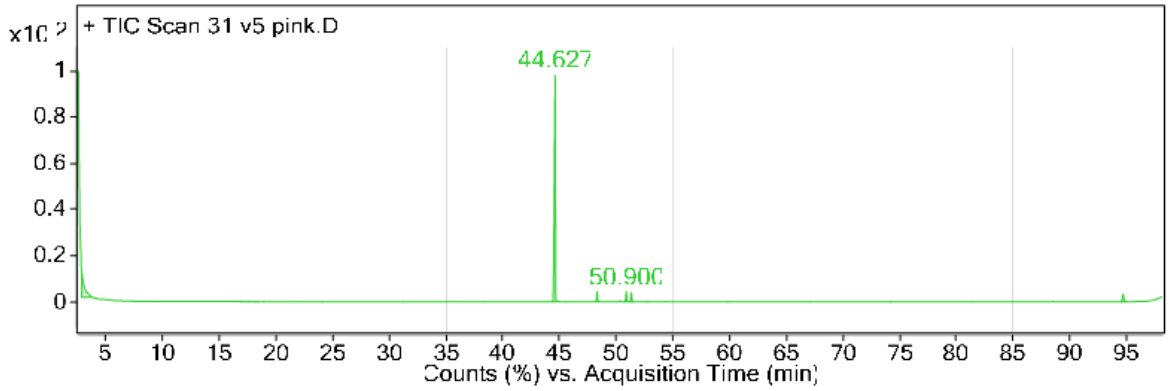


Figure 4-11 Total ion chromatogram of compound 2 (RT = 44.627 min)

4.4.5 Structural elucidation of compound 2

Compound **2** isolated in section 3.3.4.3 could not be fully identified. Weak signals obtained in NMR analysis did not permit for complete analysis. However, its mass spectrum showed similarity with the published data. In literature, compound **2** (RI = 1499) was identified as β-dihydroagarofuran (RI = 1499) in the essential oil of *H. petiolare* from South Africa as one of the major compounds (19.45-25.65%) by Giovanelli et al. (2018). The mass spectrum of β-dihydroagarofuran (Adams, 2007) (Figure 4-13) shows similarity to that of compound **2** (Figure 4-12) with the molecular ion [M^+] = 222.3, the base peak *m/z* = 207.3, and other peaks *m/z* = 189.2 and, *m/z* = 149.2

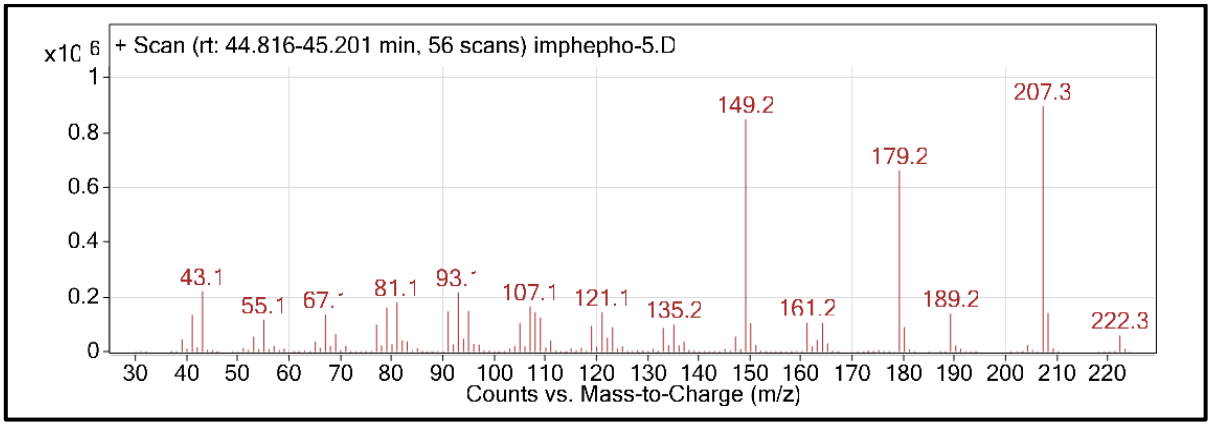


Figure 4-12 Mass spectrum of compound 2

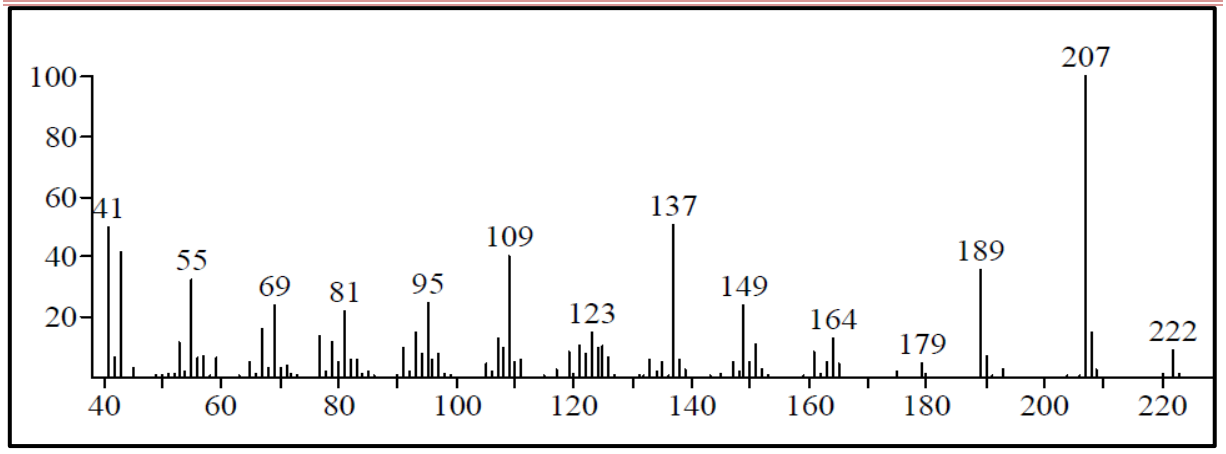


Figure 4-13 Mass spectrum of β-Dihydroagarofuran (Adams, 2007)

4.4.6 GC-MS analysis of *Helichrysum odoratissimum* EO

The essential oil of *H. odoratissimum* was analysed by GC-MS. The results were obtained as shown in Table 4-7.

4.4.6.1 Results

Table 4-7 Components of *H. odoratissimum* essential oil

RT (Min)	Mass spectral matching	Composition (%)	Experimental RI	Literature RI	Identification
9.526	**α-Pinene**	**15.76**	**941**	939 [A]	RI, MS
9.994	Camphene	0.32	950	950 [B]	RI, MS
11.626	β-Pinene	5.18	978	979 [A]	RI, MS
12.548	Myrcene	0.41	993	990 [A]	RI, MS
13.970	α-Terpinene	1.51	1017	1017 [B]	RI, MS
15.189	**1,8-Cineole**	**17.44**	**1038**	1032 [B]	RI, MS
15.566	(*E*)-β-ocimene	0.42	1044	1050 [A]	RI, MS
16.703	γ-Terpinene	0.82	1061	1060 [B]	RI, MS
24.519	Terpinen-4-ol	0.63	1176	1177 [B]	RI, MS
25.797	α-Terpineol	5.51	1193	1190 [B]	RI, MS
37.538	Unknown	1.13	1372	-	-
39.919	Italicene	3.24	1409	1402 [B]	RI, MS
40.436	**(*E*)-Caryophyllene**	**7.30**	**1418**	1419 [A]	RI, MS
42.334	α-Humulene	2.06	1450	1453 [B]	RI, MS
44.617	**γ-Curcumene**	**15.76**	**1487**	1481 [B]	RI, MS
44.866	**Ar-Curcumene**	**7.63**	**1490**	1482 [B]	RI, MS
45.184	Unknown	3.06	1495	-	-
46.242	Sesquicineole	2.75	1514	1516 [A]	RI, MS
46.738	δ-Cadinene	1.13	1523	1523 [B]	RI, MS
47.204	Unknown	0.54	1531	-	-
47.697	α-Calacorene	0.40	1540	1540 [B]	RI, MS
49.928	Caryophyllene oxide	1.66	1578	1580 [B]	RI, MS
50.379	Viridiflorol	0.45	1585	1591 [B]	RI, MS
51.806	Junenol	0.59	1610	1618 [A]	RI, MS
55.681	α-Bisabolol	0.54	1681	1683 [B]	RI, MS
72.515	Kaur-16-ene [Wb MS]	0.72	U	-	Wb MS
Monoterpene hydrocarbons:		24.42			
Oxygenated monoterpenes:		23.58			
Total monoterpenoids		**48.00**			
Sesquiterpene hydrocarbons:		37.52			
Oxygenated sesquiterpenes:		5.99			
Total sesquiterpenoids:		**43.51**			
Diterpene hydrocarbons:		0.72			

Total identified	**92.23**
Unidentified:	4.73
Total	**96.96**

A= Adams (2007)

B = Babushok et al. (2011)

Wb MS = The MS of the analysed compound matched with the compound listed in NIST Chemistry WebBook (2018)

MS = In addition to RI, the MS of analysed compound matched with the MS of the compound in Adams (2007) and/or NIST Chemistry WebBook (2018)

Unknown = The MS and RI of compound could not be match with the available literature data

U = Undefined. Higher *n*-paraffin needed.

Twenty-three components representing 92.23% of the essential oil *H. odoratissimum* were identified. The monoterpenoids (48.00%) were found in a slightly larger amount than the sesquiterpenoids (43.51%). Of the latter, sesquiterpene hydrocarbons were the dominant compounds by 37.52% compared to the oxygenated sesquiterpenes found (5.99%). The major constituents of *H. odoratissimum* EO were 1,8-cineole (17.44%) followed by α-pinene (15.76%), γ-curcumene (15.76%), ar-curcumene (7.63%) then (*E*)-caryophyllene (7.30%) (Figure 4-14).

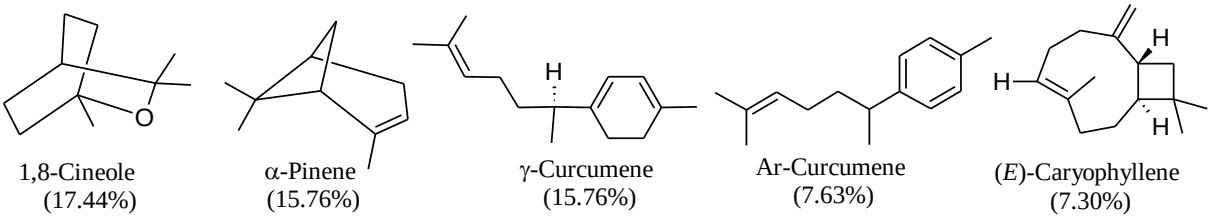


Figure 4-14 Major components of *Helichrysum odoratissimum* essential oil

4.4.6.2 Discussion

1,8-Cineole has been found in significant amount, although not the major constituent, in the South African *H. odoratissimum* EO obtained from fresh plant material harvested in the Eastern Cape by Asekun et al. (2007), Reddy (2007), and Odeyemi et al. (2008). In these three reports, the amount of 1,8-cineole was 13.0%, 11.2-17.1%, and 6.56% respectively whereas the major component was reported to be limonene (14.55%), (*E*)-caryophyllene (9.3-25.2%), and *p*-menthone (35.4%) respectively. In Odeyemi et al. (2008), γ-curcumene and α-pinene were found in lesser amounts (2.15% and 4.20% respectively) and the amount of α-pinene was only 1.1% and γ-curcumene was not reported by Asekun et al. (2007). In this research, *H. odoratissimum* EO was obtained commercially and information on the South African locality was not disclosed by the company therefore some assumptions could not be made with certainty. However, all these observed differences in chemical composition could only be attributed to the chemical variations (Giovenelli et al. 2018). Indeed, it is well established in the literature that the

yield, the chemical composition pattern, and concentration of individual components of essential oils are heavily subjected to a number of factors such as season, geographical location, environmental, genetic factors (Dhifi et al., 2016; Moghaddam & Mehdizadeh, 2017; Morsy, 2017) as well as postharvest techniques (Moghaddam & Mehdizadeh, 2017). The influence of those factors on the accumulation of distinct volatiles collectively gives its different chemotypes (Moghaddam & Mehdizadeh, 2017; Morsy, 2017). The composition of *H. odoratissimum* EO extracted from the widly growing South African plant is significantly affected by the drying method of the plant. The amount of (*E*)-caryophyllene was found in a significantly high amount, comparable to the amount obtained in this research, exclusively in dried plant materials (Asekun et al., 2007). The authors reported the amount of β-caryophyllene to be 12.0%, 6.5%, and 13.0% for air-, sun-, and oven-dried plant material respectively. Outside of South Africa, the plant has shown some similar composition patterns of its EO as well. In the research conducted by Giovanelli et al. (2018), the essential oil of the *H.odoratissimum* obtained from South Africa also contained a high amount of 1,8-cineole (13.35%) obtained from the hydrodistilled flowers of the plant and the major constituent was α-pinene. A similar or higher amount of α-pinene to this research has been reported in the *H. odoratissimum* EO of other African countries, Zimbabwe (15.0%) (Gundidza et al., 1993), Cameroon (40.6-47.1%) (Kuiate et al., 1999), and Kenya (43.4%) (Lwande et al., 1993). The Cameroonian *H. odoratissimum* EO also showed a similar profile to the other major constituents for α-curcumene (ar-curcumene) as 4.3-20.3% and β-caryophyllene (*E*-caryophyllene) (5.1-13.8%). Additionally, the Rwandan (Kajangwe et al. 1999) and Ugandan (Ocheng et al. 2015) *H. odoratissimum* EOs both contained the same high amount of β-caryophyllene at 12,6%.

4.4.7 GC-MS analysis of *Helichrysum cymosum* EO

The essential oil of *H. cymosum* was analysed by GC-MS. The results were obtained as shown in.Table 4-8.

4.4.7.1 Results

Table 4-8 Components of *H. cymosum* essential oil

RT (Min)	Mass spectral matching	Composition (%)	Experimental RI	Literature RI	Identification
9.721	**α-Pinene**	**29.82**	**945**	939 [A]	RI, MS
10.069	Camphene	0.44	951	950 [B]	RI, MS
11.584	β-Pinene	2.56	978	979 [A]	RI, MS
12.576	Myrcene	0.78	993	990 [A]	RI, MS
13.979	α-Terpinene	1.83	1017	1017 [B]	RI, MS
15.153	**1,8-Cineole**	**15.13**	**1037**	1032 [B]	RI, MS
15.821	**(*E*)-β-Ocimene**	**8.24**	**1048**	1050 [A]	RI, MS
16.344	**β-Ocimene** (undefined isomer)	**3.26**	1056	-	Wb MS
16.839	γ-Terpinene	2.50	1063	1060 [B]	RI, MS
18.608	Terpinolene	0.80	1088	1087 [B]	RI, MS
21.602	allo-Ocimene	3.01	1133	1132 [A]	RI, MS
23.682	Borneol	0.45	1164	1166 [B]	RI, MS
24.649	Terpinen-4-ol	2.18	1178	1177 [B]	RI, MS
25.522	α-Terpineol	0.82	1189	1190 [B]	RI, MS
40.655	**(*E*)-Caryophyllene**	**19.20**	1422	1420 [B]	RI, MS
42.273	α-Humulene	0.83	1449	1453 [B]	RI, MS
44.614	Unknown	0.36	1486	-	-
46.040	7-epi-α-Selinene	0.60	1510	1517 [B]	RI, MS
49.904	Caryophyllene oxide	2.65	1577	1580 [B]	RI, MS
52.891	Unknown	1.46	1631	-	-
69.399	**Geranyl-α-terpinene**	**3.09**	1952	-	Match
Monoterpene hydrocarbons:		53.24			
Oxygenated monoterpenes:		18.58			
Total monoterpenoids		**71.82**			
Sesquiterpene hydrocarbons:		20.63			
Oxygenated sesquiterpenes:		2.65			
Total sesquiterpenoids:		**23.28**			
Diterpene hydrocarbons:		3.09			
Total identified		**98.19**			
Unidentified		1.82			
Total		**100.01**			

A = Adams (2007)

B = Babushok et al. (2011)

Wb = NIST Chemistry WebBook (2018)

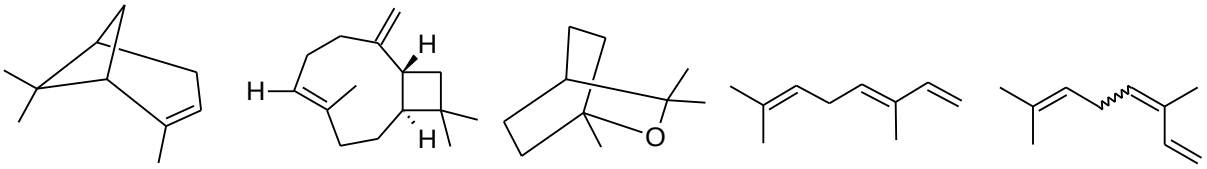
Wb MS = The MS of the analysed compound matched with the MS of the compound listed in NIST Chemistry WebBook

MS = In addition to RI, the MS of analysed compound matched with the MS of the compound in Adams (2007) and/or NIST Chemistry WebBook (2018)

Match= no RI or MS available in the literature. The compound was reported solely based on the mass spectral match with NIST14 libraries reported by MassHunter software (Agilent Technologies, Inc) (Probability<0.04)

Unknown= The MS and RI of the compound could not be matched with the available literature data

The fresh aerial parts of *H. cymosum* produced an essential oil yield of 0.15% (v/w). According to the GC-MS analysis of the essential oil, nineteen compounds making up 98.19% of the oil were identified. The monoterpenoids were found largely predominant over the other classes of terpenes by 71.82%. Sesquiterpenoids were found as 23.28% (20.63% hydrocarbons, 2.65% oxygenated) and diterpene hydrocarbons as 3.09%. Non-terpenic molecules were not found in the essential oil. The major compounds were found to be: α-pinene (29.82%), (*E*)-caryophyllene (19.20%), 1,8-cineole (15.13%), and (*E*)-β-ocimene (8.24%) (Figure 4-15).



α-Pinene (29.82%) | (*E*)-Caryophyllene (19.20%) | 1,8-Cineole (15.13%) | (*E*)-β-Ocimene (8.24%) | β-Ocimene (3.26%)

Figure 4-15 Major components of *Helichrysum cymosum* essential oil

4.4.7.2 Discussion

The composition of major compounds found was similar to previously reported South African *H. cymosum* EOs. The *H. cymosum* EO extracted from the widly collected plant material in Johannesburg constituted 1,8-cineole (20.4%), α-pinene (12.4%), and (*E*)-caryophyllene (10.8%) (Van Vuuren et al., 2006). In the research done by Reddy (2007), 1,8-cineole (20.4-34.6%) was found to be the major compound, followed by (*E*)-caryophyllene (8.4-10.8%) and α-pinene (3.6-12.4%). In the other African countries, *H. cymosum* EO retained a similar profile with some of the major components. In the Tanzanian EO, (*E*)-caryophyllene was the major compound by 27.02% (Bougatsos et al. 2004) and 12.0% in the Cameroonian EO (Tchoumbougnang et al. 2010).

4.5 *Salvia* species

The *Salvia* species EOs were analysed by GC-MS as described in section. The results are present as follows:

4.5.1 GC-MS analysis of *Salvia aurea* EO

The essential oil of *S. aurea* was analysed by GC-MS. The results were obtained as shown in Table 4-9.

4.5.1.1 Results

Table 4-9 Components of *S. aurea* essential oil

RT(Min)	Mass spectral matching	Composition (%)	Experimental RI	Literature RI	Identification
9.223	α-Pinene	0.77	935	939 [A]	RI, MS
11.455	β-Pinene	0.36	976	979 [A]	RI, MS
12.740	Myrcene	4.24	996	990 [A]	RI, MS
14.735	D-Limonene	0.97	1030	1030 [B]	RI, MS
14.835	Eucalyptol	0.99	1032	1032 [A]	RI, MS
15.583	(*E*)-β-Ocimene	1.70	1044	1050 [A]	RI, MS
16.179	β-Ocimene (undefined isomer)	0.91	1053	-	Wb MS
19.593	Linalool	0.59	1101	1099 [B]	RI, MS
21.494	allo-Ocimene	0.60	1132	1132 [A]	RI, MS
25.515	α-Terpineol	0.64	1189	1190 [B]	RI, MS
35.141	1,5,5-Trimethyl-6-methylene-cyclohexene	0.43	1335	1338 [Wb]	RI
35.946	α-Cubebene	0.30	1348	1351 [B]	RI, MS
37.557	α-Copaene	0.71	1373	1376 [B]	RI, MS
40.537	**(*E*)-Caryophyllene**	**7.93**	**1420**	1420 [B]	RI, MS
41.51	Aromadendrene	2.07	1436	1441 [B]	RI, MS
42.544	**α-Humulene**	**5.13**	**1453**	1453 [B]	RI, MS
42.955	Alloaromadendrene	3.41	1460	1460 [B]	RI, MS
43.030	cis-Muurola-4(15),5-diene	0.52	1461	1464 [B]	RI, MS
43.863	γ-Muurolene	0.52	1475	1476 [B]	RI, MS
44.292	Unknown	0.60	1481	-	-
44.420	Ar-Curcumene	0.67	1483	1482 [B]	RI, MS
44.984	Bicyclogermacrene	2.06	1492	1494 [B]	RI, MS
45.321	α-Muurolene	0.79	1497	1498 [B]	RI, MS
46.211	γ-Cadinene	3.37	1513	1513 [B]	RI, MS
46.324	β-Curcumene	0.82	1515	1515 [A]	RI, MS
46.604	Unknown	2.55	1520	-	-
46.978	**δ-Cadinene**	**5.14**	**1527**	1523 [B]	RI, MS
47.490	α-Cadinene	0.27	1536	1538 [A]	RI, MS
48.985	Unknown	0.43	1562	-	-

49.376	Unknown	0.64	1569	-	-
49.898	Unknown	2.89	1577	-	-
50.056	Caryophyllene oxide	2.16	1580	1580 [B]	RI, MS
50.478	Viridiflorol	0.70	1587	1591 [B]	RI, MS
51.123	Unknown	1.13	1598	-	-
51.457	Humulene epoxide II	0.71	1604	1605 [B]	RI, MS
51.996	1,10-di-epi-Cubenol	3.12	1614	1612 [B]	-
52.580	Unknown	0.76	1625	-	-
52.968	**γ-Eudesmol**	**5.02**	**1632**	1631 [B]	RI, MS
53.336	Unknown	2.65	1639	-	-
53.948	**epi-α-Cadinol**	**14.24**	**1650**	1640 [A]	RI, MS
54.471	**Unknown**	**7.85**	**1660**	-	-
54.633	**Unknown**	**6.21**	**1663**	-	-
56.202	Shyobunol	2.43	1690	1689 [A]	RI, MS
Monoterpene hydrocarbons:		9.98			
Oxygenated monoterpenes:		2.22			
Total monoterpenoids		**12.20**			
Sesquiterpene hydrocarbons:		33.71			
Oxygenated sesquiterpenes:		28.38			
Total sesquiterpenoids:		**62.09**			
Total identified		**74.20**			
Unidentified		25.71			
Total		**100.00**			

A = Adams (2007)

B = Babushok et al. (2011)

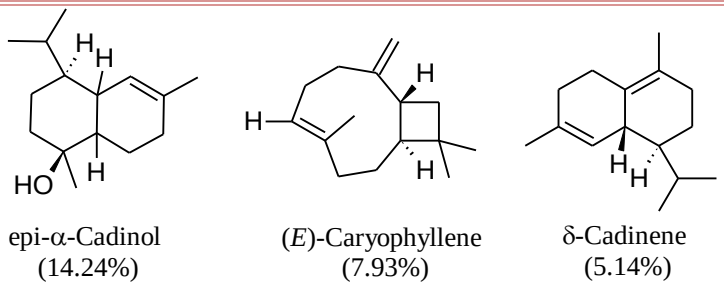
Wb = NIST Chemistry WebBook (2018)

Wb MS = The MS of the analysed compound matched with the MS of the compound listed in NIST Chemistry WebBook (2018)

MS = In addition to RI, the MS of analysed compound matched with the MS of the compound in Adams (2007) and/or NIST Chemistry WebBook (2018)

Unknown = The MS and RI of the compound could not be matched with the available literature data

The fresh aerial parts of *S. aurea* yielded 0.22% (v/w) of essential oil. According to the GC-MS analysis in this research work, the sesquiterpenoids (62.09%) were the predominant class of terpenes over the monoterpenoids (12.20%) in the essential oil of *S. aurea*. Thirty-three components constituting 74.20% of the essential oil were positively identified. The essential oil of *S. aurea* was found to contain primarily epi-α-cadinol (τ-muurolol) (14.24%), two closely eluting unidentified compounds with RI =1660 (7.85%) and RI = 1663 (6.21%), (*E*)-caryophyllene (7.93%), and δ-cadinene (5.14%) (Figure 4-16). The other compounds in significant amounts were found to be α-humulene (5.13%) and γ-eudesmol (5.02%).



epi-α-Cadinol (14.24%) | (*E*)-Caryophyllene (7.93%) | δ-Cadinene (5.14%)

Figure 4-16 Positively identified major components in *Salvia aurea* essential oil

4.5.1.2 Discussion

Most of the previous publications have reported three main constituents with consistent predominant content in the essential oil of *S. aurea* from plant material harvested from the Western Cape regions of South Africa. These constituents are epi-α-cadinol (10.09%, 20%) (Vallah, 2003; Lim Ah Tock, 2020) as in this research, then α-eudesmol (10.14%, 13.5%, 23%), and β-eudesmol (9.79%, 14.5%, 26%) (Vallah, 2003; Van Vuuren et al., 2019; Lim Ah Tock, 2020). However, in this research, this finding did not match with the results obtained. The mass spectra obtained for the compounds with RI = 1660 (7.85%) and RI = 1663 (6.21%) were not identical to those of α-eudesmol and β-eudesmol found in the NIST Chemistry WebBook (2018) and Adams (2007) although similar. Further structural elucidation for those two compounds will be needed.

4.5.2 GC-MS analysis of *Salvia chamelaeagnea* EO

The essential oil of *Salvia chamelaeagnea* was analysed by GC-MS. The results were obtained as shown in Table 4-10.

4.5.2.1 Results

Table 4-10 Components of *S. chamelaeagnea* essential oil

RT (Min)	Mass spectral matching	Composition (%)	Experimental RI	Literature RI	Identification
9.348	**α-Pinene**	**5.90**	937	**939** [A]	RI, MS
9.976	Camphene	1.27	950	950 [B]	RI, MS
11.498	β-Pinene	1.34	976	978 [B]	RI, MS
12.713	Myrcene	5.04	995	990 [B]	RI, MS
13.232	α-Phellandrene	0.76	1004	1004 [B]	RI, MS
13.793	**δ-3-Carene**	**9.12**	1014	**1011** [A]	RI, MS
15.409	**Limonene**	**28.00**	1041	**1030** [B]	RI, MS
16.772	γ-Terpinene	0.75	1062	1060 [B]	RI, MS
18.486	*p*-Mentha-2,4(8)-diene	0.30	1086	1088 [A]	RI, MS
18.613	Terpinolene	0.42	1088	1087 [B]	RI, MS
19.695	Linalool	1.43	1103	1099 [B]	RI, MS
22.328	(+)-2-Bornanone	3.45	1145	1158 [Wb]	RI, MS
24.568	Terpinen-4-ol	0.93	1177	1177 [B]	RI, MS
25.577	α-Terpineol	1.10	1190	1190 [B]	RI, MS
37.499	α-Copaene	0.36	1372	1376 [B]	RI, MS
39.666	α-Gurjunene	1.84	1405	1409 [B]	RI, MS
42.675	Alloaromadendrene	0.72	1455	1460 [B]	RI, MS
44.84	Viridiflorene	0.88	1490	1492 [B]	RI, MS
45.998	γ-Cadinene	1.13	1509	1513 [B]	RI, MS
46.649	δ-Cadinene	1.05	1521	1523 [B]	RI, MS
49.287	**Palustrol**	**8.14**	1567	**1567** [B]	RI, MS
49.674	Unknown	3.46	1574		-
50.987	**Viridiflorol**	**13.42**	1595	**1591** [B]	RI, MS
51.415	**Ledol**	**5.44**	1603	**1602** [A]	RI, MS
51.874	1,10-di-epi-Cubenol	0.58	1612	1612 [B]	RI, MS
53.168	Unknown	0.55	1636		-
53.405	epi-α- Cadinol	2.64	1640	1640 [A]	RI, MS
Monoterpene hydrocarbons:		52.90			
Oxygenated monoterpenes:		6.91			
Total monoterpenoids		**59.81**			
Sesquiterpene hydrocarbons:		5.98			
Oxygenated sesquiterpenes:		30.22			
Total sesquiterpenoids:		**36.20**			

Total identified	**96.01**
Unidentified	4.01
Total	**100.02**

A = Adams (2007)

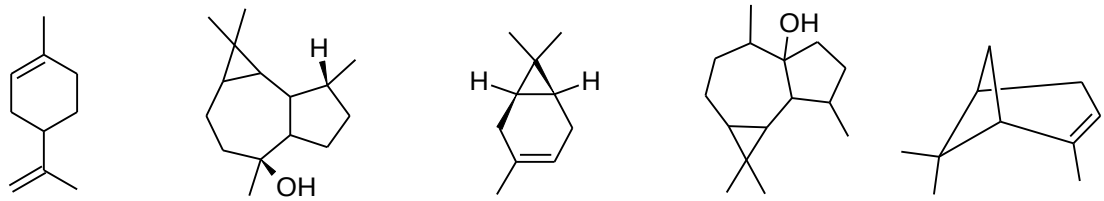
B = Babushok et al. (2011)

Wb = NIST Chemistry WebBook (2018)

MS = In addition to RI, the MS of the analysed compound matched with the MS of the compound in Adams (2007) and/or NIST Chemistry WebBook (2018)

Unknown = The MS and RI of the compound could not be matched with the available literature data

The fresh aerial parts of *S. chamelaeagnea* yielded a significantly higher content of essential oil (0.38% v/w) than *S. aurea* (0.22% v/w). In the GC-MS analysis, the essential oil of *S. chamelaeagnea* was found to contain predominantly 59.81% of monoterpenoids of which, 52.90% made up the monoterpene hydrocarbons, then the sesquiterpenoids (36.20%) of which, oxygenated sesquiterpenes were of higher content (30.22%). A total of 25 compounds making up 96.01% of the essential oil were positively identified. The major compounds found were limonene (28.00%), viridiflorol (13.42%), δ-3-carene (9.12%), palustrol (8.14%), and α-pinene (5.9%) (Figure 4-17).



Limonene (28.00%) | Viridiflorol (13.42%) | δ-3-Carene (9.12%) | Palustrol (8.14%) | α-Pinene (5.90%)

Figure 4-17 Major components in *Salvia chamelaeagnea* essential oil

4.5.2.2 Discussion

Most of the major compounds identified in the present research have been previously reported in the analysis of *S. chamelaeagnea* EO extracted from the plant material harvested in Western Cape (Kamatou et al., 2006; Van Vuuren et al., 2019; Lim Ah Tock et al., 2020). According to these previous results and this present research, the chemical markers of the Western Cape essential oil are seemingly limonene (9.7%-19.81%) and viridiflorol (9.3%-61%).

4.6 Conclusion of chapter 4

The chemical characterisation of the essential oils of selected South African species was predominantly done by GC-MS analysis which yielded satisfactory results. At least 70% of the components were identified in each essential oil. NMR analysis served to identify the major compound of the essential oil of *Helichrysumpetiolare* (20.66%, RI = 1568). This compound was structurally elucidated as faurinone, a sesquiterpene ketone. The dominant classes of compouds found in the essential oils were the terpenoids with hydrocarbon, alcohol, and ketone moieties.

5. CHAPTER FIVE: ASSESSMENT OF *IN VITRO* BIOLOGICAL ACTIVITIES

The chapter reports the results obtained in the *in vitro* antimicrobial, antioxidant capacity, photoprotection, and tyrosinase inhibition assays of the six selected South African essential oils.

5.1 Antimicrobial activity by determination of minimum inhibitory concentration (MIC)

The following reports the antimicrobial strength of the selected South African essential oils.

5.1.1 Disk diffusion test: Safety levels of use of Tween®20 and DMSO

DMSO solvent and Tween® 20 emulsifiers are commonly used diluents in published research work to prepare EO samples for antimicrobial susceptibility testing to address their poor solubility (Prabuseenivasan et al., 2006; Andrade et al. 2014; Orchard & Van Vuuren, 2017; Simplice et al., 2018). Therefore, they were first tested in the disk diffusion assay over the range of 80–0.5% v/v to determine which one was best suited to avoid interference. The results were obtained as shown in section 5.1.1.1.

5.1.1.1 Results

The results of disk diffusion test were summarised in Table 5-1, Table 5-2, and Figure 5-1 as follows:

Table 5-1 Verification of the antimicrobial activity of Tween®20 at different concentrations using the disk diffusion assay

Microorganisms	Substances/Halo (mm)								
	B	Tween® 20							
		80%	40%	20%	10%	5%	2.5%	1.75%	0.5%
S. aureus	29	17	17	15	13	12	Ø	Ø	Ø
E. coli	26	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
P. aeruginosa	7	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø

B: (10 µg cefpodoxime + AmpC inducer) disc; Ø: absence of inhibition halo of bacterial growth

Table 5-2 Verification of the antimicrobial activity of DMSO at different concentrations using the disk diffusion assay

Microorganisms	Substances/Halo (mm)								
	B	DMSO							
		80%	40%	20%	10%	5%	2.5%	1.75%	0.5%
S. aureus	31	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
E. coli	26	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
P. aeruginosa	7	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø

B: (10 µg cefpodoxime + AmpC inducer) disc; Ø: absence of inhibition halo of bacterial growth

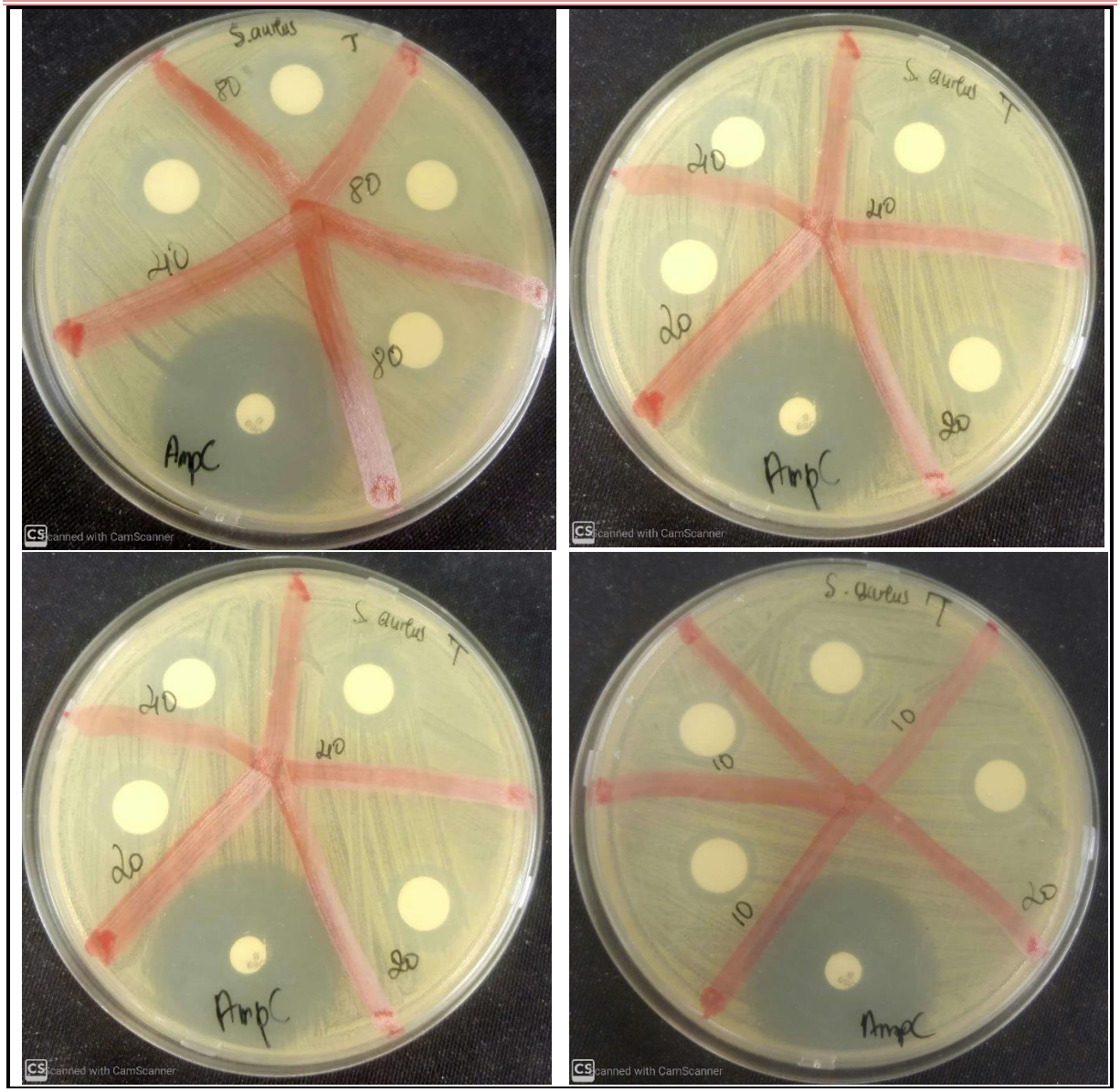

S.aureus T
80
80
40
80
AmpC
Scanned with CamScanner
S. aureus T
40
40
20
20
AmpC
Scanned with CamScanner
S. aureus T
40
20
20
20
AmpC
Scanned with CamScanner
S. aureus T
10
10
10
20
AmpC
Scanned with CamScanner

Figure 5-1 Microbial growth inhibition detected for Tween®20 from 80% - 5% (v/v) in (WT) *S. aureus*

5.1.1.2 Discussion

Over the concentration range of 80%–5% (v/v), Tween® 20 was found to inhibit bacterial growth of (WT) *S. aureus* (17-12 mm) (Figure 5-1) whereas DMSO had no effect on the growth inhibition of any bacterial organism. This result highlighted the importance of testing the prospective diluents on the microorganisms to be used in antimicrobial susceptibility assays for essential oils. The result also confirmed that Tween® emulsifier detergents can significantly interfere during the antimicrobial testing for essential oils. It has been reported before that Tween® 80 depressed the activity of oregano and clove essential oils (Remmal et al., 1993; Lahlou, 2004). However, DMSO is a safe solvent for antimicrobial susceptibility testing. This result corroborated with the previous study by De Brito et al. (2017) who also found that DMSO did not affect the bacterial cells tested.

5.1.2 Minimum inhibitory concentration (MIC) of the essential oils using the broth microdilution method

S. aureus, *P. aeruginosa,* and *E. coli* are common skin pathogenic bacteria involved in many skin infections. The selected South African essential oils were tested on these bacterial strains in the broth microdilution and the results were obtained as shown in section 5.1.2.1.

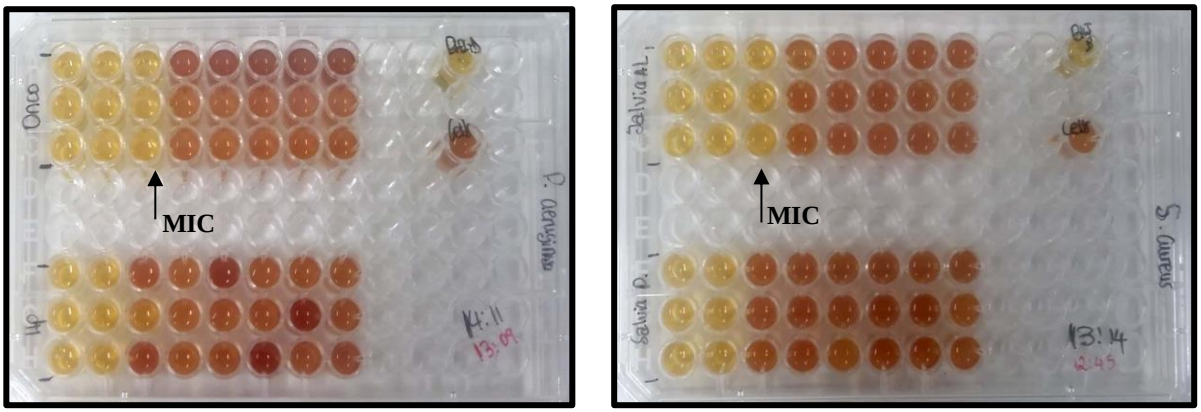
5.1.2.1 Results

The results of the broth microdilution test were summarised in Table 5-3 and Figure 5-2 as follows:

Table 5-3 MICs of essential oils and control (mg/mL)

	MIC values (mg/mL)		
	Microorganisms		
Essential oils ID	*S. aureus*	*E. coli*	*P. aeruginosa*
O. suffruticosum	12.8	12.8	**6.4**
H. petiolare	>25.6	12.8	12.8
H. odoratissimum	12.8	12.8	12.8
H. cymosum	>25.6	12.8	12.8
S. aurea	**6.4**	12.8	12.8
S. chamelaeagnea	12.8	12.8	>25.6
Control			
Ampicillin	<0.2	<0.2	R*

***R= resistant**



a **b**

Figure 5-2 Reading of the MIC of *Oncosiphon suffruticosum* EO for *P. aeruginosa* (a); Reading of the MIC of *Salvia aurea* EO for *S. aureus* (b).

5.1.2.2 Discussion

According to the broth microdilution test conducted, *S. aurea* EO was found to display the lowest MIC of 6.4 mg/mL against *S. aureus*, followed by *S. chamelaeagnea*, *O. suffruticosum* and *H. odoratissimum* EOs at 12.8 mg/mL. *H. petiolare* and *H. cymosum* EOs were found inactive over the concentration range tested (25.6-0.2 mg/mL) with MIC > 25.6 mg/mL. *P. aeruginosa* was found most susceptible to *O. suffruticosum* EO at 6.4 mg/mL and to all other oils at 12.8 mg/mL except for *S. chamelaeagnea* EO which was found inactive for this organism. For *E. coli*, all the essential oils exhibited the same activity with MIC at 12.8 mg/mL. Ampicillin control had the lowest MICs for over the concentration range for *S. aureus* and *E. coli* but was found resistant against *P. aeruginosa* as expected from CLSI guidelines

which indicate intrinsic resistance for this bacterium (CLSI, 2015). Essential oils exhibit broad-spectrum inhibitory activity against gram-negative and gram-positive bacteria by virtue of their hydrophobicity (Ludwiczuk et al., 2017, p. 251). The lipophilic molecules easily partition into the lipid cell membrane of bacteria (Dhifi et al., 2016). They disrupt the membrane integrity and its potential, cause the leakage of cellular contents, alter the cytoplasmic proteins, and inactivate cellular enzymes, which collectively lead to bacterial cell death (Ludwiczuk et al., 2017, p. 251). For essential oils, an MIC $\leq$ 2 mg/mL could be considered worthy (Van Vuurren, 2008). The results obtained indicated that the essential oils exhibited moderate to poor activity against the selected pathogens. The chemical characterisation in chapter 4 showed that the essential oils were made up almost entirely of terpenoids. It is known that the chemical structure of terpenoids parallel their activity (Ludwiczuk et al., 2017, p. 251) whereby, the presence of an oxygen function in the framework enhances their antimicrobial properties (Dorman & Deans, 2000). The phenol and aldehydes are often characterised by the highest antibacterial activity (Dhifi et al., 2016) followed by the alcohols which are usually bacteriocidal rather than bacteriostatic, then the ketones and the terpene hydrocarbons which have weak activities (Dorman & Deans, 2000). None of the essential oils analysed possessed a significant amount of either the phenolic or aldehyde moieties which explained the modest to poor activity observed. Generally, all the oils were made up of a substantial amount of oxygenated sesquiterpenes (alcohols and ketones) which contributed to some activity as shown by the results obtained. In conclusion, the South African essential oils tested are promising as additives to enhance the antibacterial strength in cosmeceuticals aimed at skin infections.

5.2 Antioxidant capacity assays

To palliate to the skin cells' oxidative damage, antioxidants must be integrated into skincare products. Essential oils have proven antioxidant properties for cosmeceutical application as mentioned in section 1.3.1 of chapter 1. In this research work, the strength of antioxidative protection of the selected South African essential oils was evaluated by four *in vitro* antioxidant capacity assays. The selection of the assays took into account covering ET- and HAT-based mechanisms. The ET-based methods selected were the DPPH, ABTS, and FRAP, although DPPH and ABTS can involve both HAT and ET mechanisms (Santos-Sánchez et al., 2019; Gulcin, 2020). The HAT-based method selected was ORAC (Huang et al., 2005; Gupta et al., 2015).

5.2.1 Results

The results of the antioxidant capacity assays were summarised in the following Table 5-4 and Figure 5-3.

Table 5-4 Antioxidant capacities of the selected South African essential oils in the DPPH, ABTS, FRAP and ORAC assays

Assay		DPPH*	ABTS*			FRAP*	ORAC*
EO	mg/mL	% $RSA_{6\ min}$ ±SD	% $RSA_{6\ min}$ ±SD	TEAC values (µmol TE/L [% RSD])	mg/mL	FRAP values (µmol AAE/L [% RSD])	ORAC values (µmol TE/L [% RSD])
O. suffruticosum	2	10.03 ±1.02	**87.17 ±0.76**	9431.2(0.9)	2	-505.8(16.0)	6701.8(0.9)
	1	8.38 ± 0.24	81.13 ±0.51	8784.6(0.6)			
	0.5	7.06 ± 0.20	71.46 ±0.04	7750.1(0.1)			
H. petiolare	2	14.41 ±0.51	84.42 ±0.43	9131.4(0.5)	2	-750.5(1.5)	6587.3(1.9)
	1	8.98 ± 0.40	77.96 ±0.71	8445.9(0.9)			
	0.5	5.29 ± 0.20	67.08 ±0.76	7281.7(1.1)			
H. odoratissimum	2	**4.09 ± 0.95**	60.74 ±1.24	6603.8(2.0)	2	3026.6(6.1)	6624.8(0.2)
	1	1.27 ± 0.43	46.72 ±0.96	5103.8(2.0)			
	0.5	-0.57 ± 0.03	28.16 ±0.84	3117.5(2.9)			
H. cymosum	2	5.58 ± 0.61	**40.26 ±0.33**	4412.2(0.8)	2	897.4(19.3)	6549.7(1.5)
	1	3.14 ± 0.00	23.69 ±0.70	2639.6(2.9)			
	0.5	1.58 ± 0.51	10.70 ±0.22	1250.1(1.9)			
S. aurea	2	10.19 ± 0.44	65.14 ±1.21	7074.3(1.8)	2	2871.6(6.0)	6563.9(0.7)
	1	7.45 ± 0.54	43.42 ±0.71	4750.1(1.6)			
	0.5	2.13 ± 0.54	27.71 ±1.12	3069.1(3.9)			
S. chamelaeagnea	2	**26.86 ±0.10**	79.52 ±0.58	8612.2(0.7)	2	2259.7(2.0)	6589.9(2.5)
	1	13.86 ±0.17	65.84 ±0.18	7149.0(0.3)			
	0.5	8.83 ± 0.68	42.51 ±1.49	4652.2(3.4)			

Trolox®	2	94.94 ± 0.02					
	1	94.78 ± 0.06	–	–	–	–	–
	0.5	94.45 ± 0.04					
Gallic acid	2		97.97 ± 0.13	605840(4.6)			
	1	–	97.96 ± 0.16	355740(2.0)	2	635500(0.6)	–
	0.5		98.05 ± 0.03	195220(3.2)			
EGCG**	–	–	–	–	2	–	26904(1.22)

*Average values of triplicate measurements (n = 3); RSA: radical scavenging activity; SD = standard deviation; RSD = relative standard deviation; TE: Trolox® equivalent; AAE: ascorbic acid equivalent. **EGCG: (-)-epigallocatechin gallate.

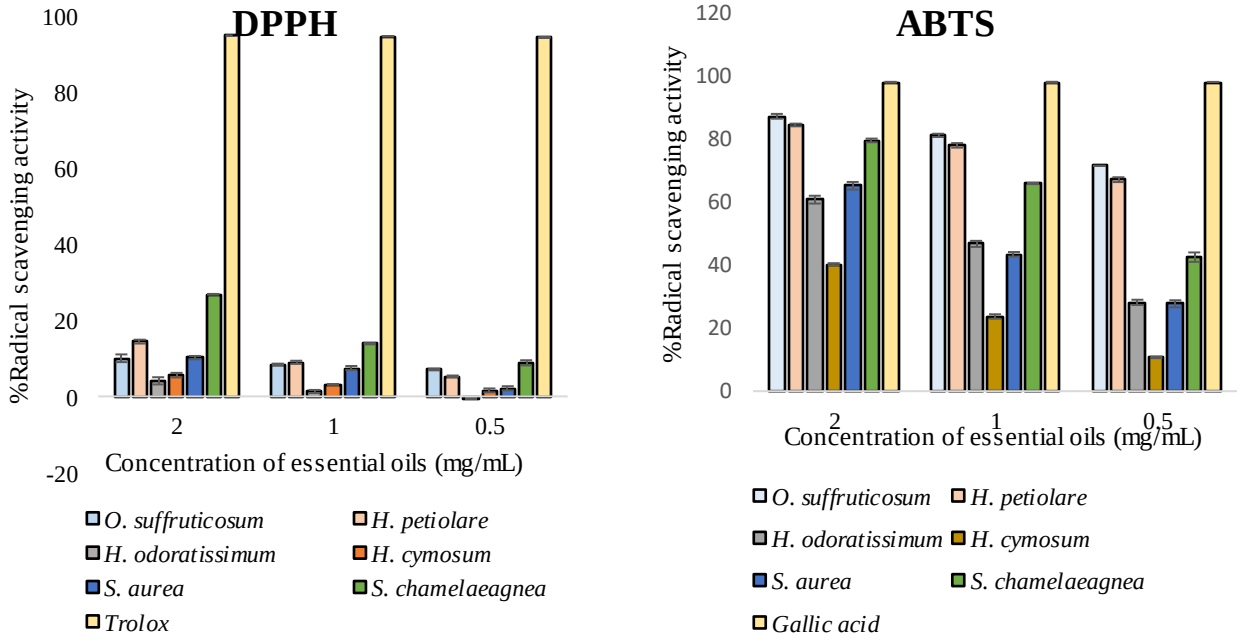


Figure 5-3 % Radical scavenging activities of essential oil samples at different concentrations. The values shown represent the mean ± standard deviation of triplicate measurements, n=3.

5.2.2 Discussion

Across all assays, very small concentrations of EOs were used (2–0.5 mg/mL). This was done because antioxidants are required to be present in a very modest amount compared to the oxidisable material they are supposed to protect (Amorati et al., 2013).

5.2.2.1 DPPH, ABTS, and FRAP assays

The DPPH and ABTS assays are used to assess the radical scavenging abilities of test sample or substance. The DPPH and ABTS capacity values reflect the degree of absorption of coloured probes at

their respective λ_{max} (~ 517 nm and 734 nm) as a result of an electron transfer to the radical species ($DPPH^{\bullet}$, $ABTS^{\bullet+}$) from compounds present in the mixture (Huang et al., 2005).

In the DPPH and ABTS assays, the percentage radical scavenging activity (% RSA) and Trolox® equivalent values were determined at the 6 min time point as opposed to 30 min as often found in publications (Amorati et al., 2013; Gulcin, 2020). This was done according to Amorati et al. (2013) recommendations to reflect "true" antioxidants which should react with free radicals at a fast rate to afford real oxidative protection (Amorati et al., 2013). As was previously reported: "If an antioxidant requires many minutes to hours to quench radicals, its action as a radical scavenger must be irrelevant in vivo in cells" (Schaich & Xie, 2015).

In the DPPH assay, *S. chamelaeagnea* EO exhibited the highest % RSA over the concentration range (2 – 0.5 mg/mL) at 26.86 ± 0.10% – 8.83 ± 0.68% while *H. odoratissiumum* had the lowest values and was found inactive at 0.5 mg/mL (4.09 ± 0.95% – [-0.57] ± 0.03%). These percentages were significantly lower than the % RSA of Trolox® found as 94.94 ± 0.02% – 94.45 ± 0.04% over the concentration range. In the ABTS assay, the % RSA values of the EOs were much higher. This was expected as $ABTS^{\bullet+}$ are more reactive than DPPH radicals (Gulcin, 2020). *O. suffruricosum* EO exhibited the highest % RSA over the concentration range at 87.17 ± 0.76% – 71.46 ± 0.04% giving TEAC values (% RSD) of 9431.2 (0.9%) – 7750.1 (% 0.1%) µmol TE/L. This was followed closely by *H. petiolare* EO with respective % RSA and TEAC values of 84.42 ± 0.43% and 9131.4 (%0.5) at 2 mg/mL, and 67.08 ± 0.76% and 7281.7 (1.1%) µmol TE/L at 0.5 mg/mL. The lowest performance was exhibited by *H. cymosum* EO over the concentration range with % RSA and TEAC values of 40.26 ± 0.33% – 10.70 ± 0.22% and 4412.2 (0.8%) – 1250.1 (1.9%) µmol TE/L. *O. suffruricosum* and *H. petiolare* EOs compared fairly well to gallic acid in terms of % RSA, which was found as 97.97 ± 0.13% – 98.05 ± 0.03% at 2–0.5 mg/mL. However, the Trolox® equivalent values of gallic acid did reveal a 100-fold difference in antioxidant capacity compared to these EOs with values as high as 605840(4.6%) µmol TE/L at 2 mg/mL. Generally, in both assays, *H. petiolare* and *O. suffruticosum* EOs remained among the highest performing EOs whereas the other EOs did not show much consistency in their performance.

In the FRAP assay, the trend observed in the ABTS assay was somewhat reversed with *H. odoratissimum* EO having the highest value and *O. suffruticosum* and *H. petiolare* EOs exhibiting no capacities to affording them negative values. The trend of the FRAP values (µmol AAE/L[% RSD]) at 2 mg/mL was obtained as follows: HO(3026.6[6.1%]) > SAr(2871.6[6.0%]) > SC(2259.7[2.0%]) > HC(897.4[19.3%]) > OS(-505.8[16.0%]) > HP(-750.5[1.5%]).Gallic acid outperformed the essential oils with its FRAP value found as 635500(0.6%) AAE/L µmol.
In the FRAP assay, the degree of colour change measured is a result of the occurrence of the Fe^{3+} to Fe^{2+}

half-reaction from donation of electrons from the sample tested (Huang et al., 2005). The reaction is not specific as any half-reaction with a lower redox potential (0.7 V) than that of the Fe^{3+} to Fe^{2+}, under the reaction conditions, will drive the ferrous ions formation (Gupta, 2015). According to the results, *H. odoratissimum. S. aurea, S. chamelaeagnea, H. cymosum* EOs might have possessed a lower reducing potential than that of iron (III) under the reaction conditions. Additionally, the pH values have crucial effects on antioxidants' reducing capacities (Huang et al., 2005). *O. suffruticosum* and *H. petiolare* EOs might have exhibited no activity at all compared to the DPPH and ABTS assays, which are also based on electron-transfer mechanisms (Huang et al., 2005; Gupta et al., 2015), because of the pH difference in the assays. The FRAP is conducted at an acidic pH whereas the DPPH and ABTS assays are conducted at basic pH.

5.2.2.2 ORAC assay

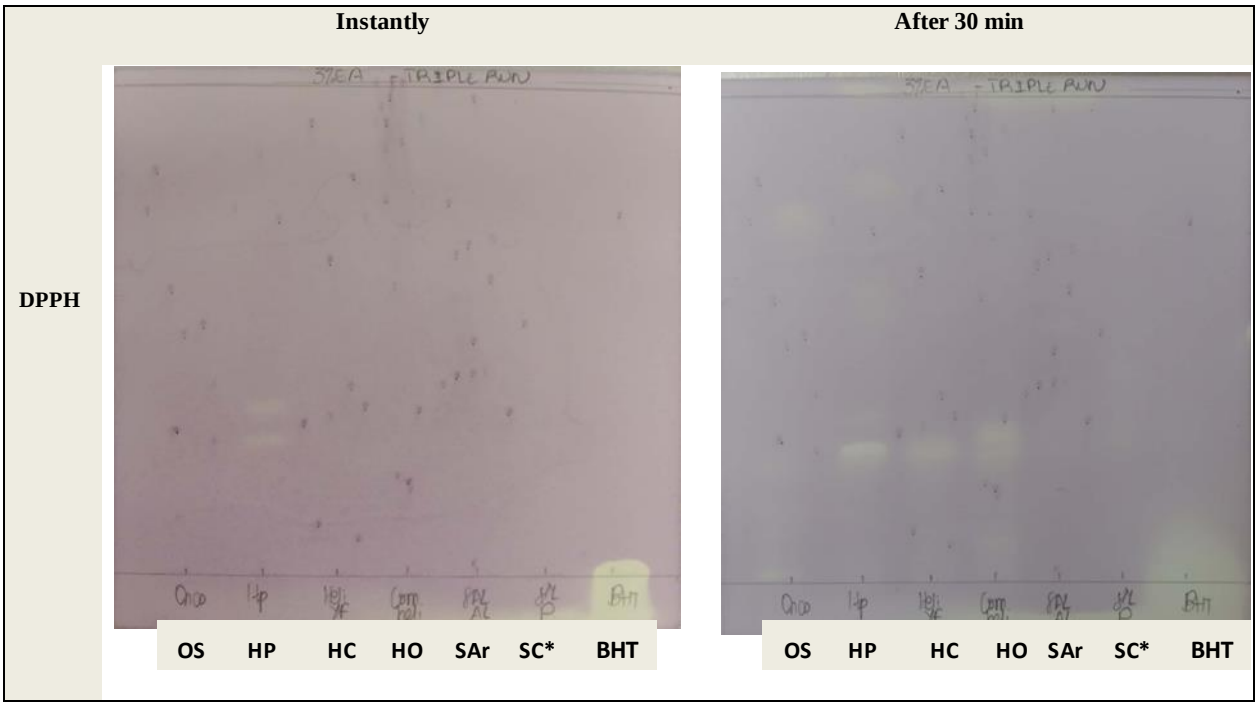
In the ORAC assay, the EOs exhibited near equal significant values. The ORAC values (µmol TE/L[% RSD]) at 2 mg/mL were obtained as follows: OS(6701.8[0.9%]) > HO(6624.8[0.2%]) > SC(6589.9[2.5%]) > HP(6587.3[1.9%]) > SAr(6563.9[0.7%]) > HC(6549.7[1.5%]). EGCG exhibited much higher antioxidant capacity found as 26904(1.22%) µmol TE/L. The ORAC assay accounts for the ability of an antioxidant to donate a proton to the AAPH(2,2'-Azobis [2-methylpropionamidine] dihydrochloride)-generated peroxyl radicals (ROO•), the species that mediate the propagation of the oxidative chain. The phenolics (PhOH) are the prototypical radical chain breakers (PhOH + ROO• → PhO + ROOH) (Amorati et al., 2013). However, according to the chemical composition elucidated in chapter 4, no phenolics were identified in the EOs, which cannot serve as the basis of the observed capacity values in the ORAC assay. Nonetheless, a well-studied non-phenolic component of essential oils with endowed antioxidants effects is γ-terpinene (Foti & Ingold, 2003; Amorati et al., 2013; Baschieri et al., 2017). The retardation of lipid (LH) peroxidation by γ-terpinene is due to rapid chain termination via the fast-cross reaction between HOO• and LOO• (Foti & Ingold, 2003). The ORAC assay of essential oils studied by Bentayeb et al. (2013) showed that good ORAC values could be roughly explained by their major components. However, the low content of γ-terpinene in *O. suffruticosum* (1.48%), *H. petiolare* (0.73%), *H. cymosum* (2.50%), and *H. odoratissimum* (0.82%) might not have explained the good ORAC values obtained in this assay for these EOs. α-Pinene present in the *Helichrysum* species as follows: *H. petiolare* (7.49%), *H. cymosum* (29.82%), and *H. odoratissimum* (15.76%) could have been contributing to the appreciable amount in this assay. Indeed, α-pinene has been shown to act as a proton donor in presence of oxidisable material while it, itself, co-oxidises, in the end not affording any oxidative protection (Amorati et al., 2013). Therefore, the observed hydrogen-donating abilities for the *Helichrysum* EOs in this assay might have been due to the presence of α-pinene in high amounts. Other terpenic essential oil components such as limonene, citral, and

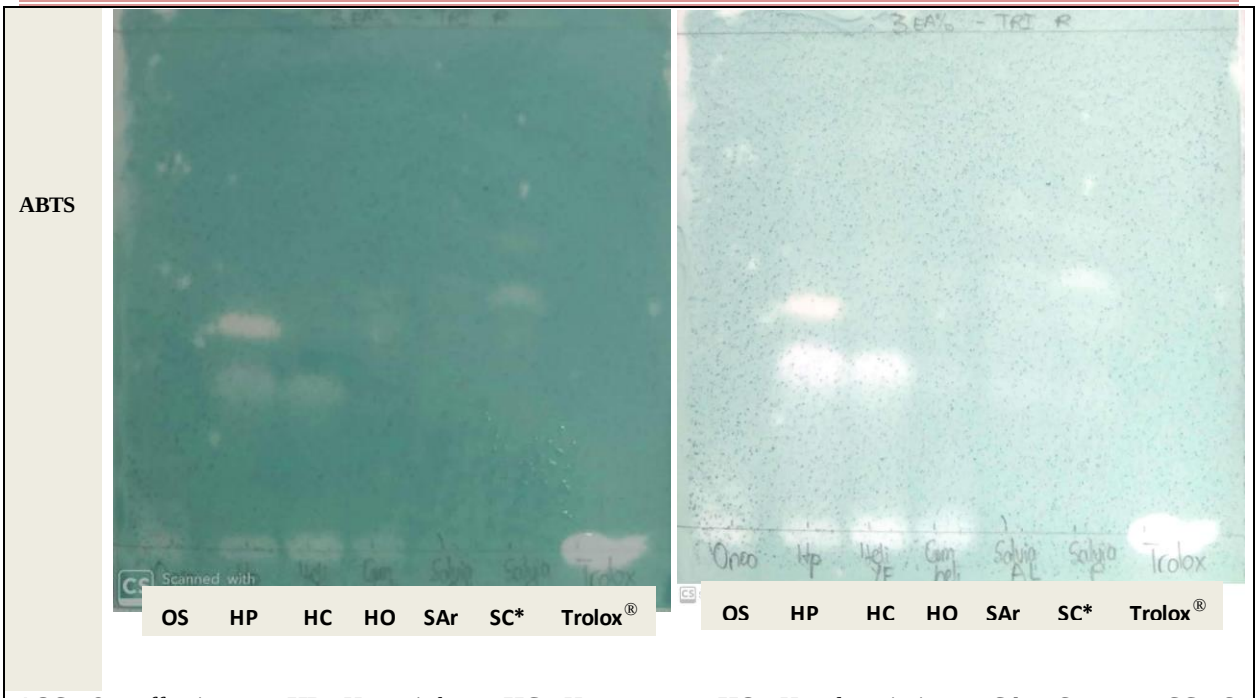
linalool have shown to act through the same mechanism as γ-terpinene to afford appreciable antioxidant behaviour (Baschieri et al., 2017). The essential oil of *S. chamelaeagnea* of which the major component of the oil (28.00%) could have been the reason for the high ORAC value in the assay. Otherwise, further research similar to that of Baschieri et al. (2017) who studied the additive properties of individual constituents of selected essential oils in the ORAC assay could be performed to elucidate the components responsible for the appreciable antioxidant behaviour in this assay.

5.2.3 Thin-layer chromatography-direct bioautography

5.2.3.1 Screening of the essential oils

The antioxidant capacity values obtained in the *in vitro* assays were deemed appreciable. Therefore, in a bid to discover the radical scavenging components responsible for the observed capacities, the EOs were subjected to DPPH and ABTS TLC-direct bioautography (section 3.4.2.8). *H. petiolare* EO was found to react the quickest with both reagents (≤ 5 s) indicating true antioxidant capacity at similar positions, Rf ± 0.29 and Rf ± 0.37 in DPPH assay, and Rf ± 0.32 and Rf ± 0.41 in ABTS assay (developed at 97:3 hexane: ethyl acetate) (Figure 5-4).





***OS**: *O. suffruticosum*; **HP**: *H. petiolare*; **HC**: *H. cymosum;* **HO**: *H. odoratissimum*; **SAr**: *S. aurea*; **SC**: *S. chamelaeagnea*

Figure 5-4 Profiles of bioautographed silica gel TLC plates after 5 s and 30 min of contact with 0.04% w/v of DPPH ethanolic solution and ABTS•+ mix solution

5.2.3.2 The radical scavenging compounds of *Helichrysum petiolare* essential oil

The DPPH bioautography method was used further to discover the radical scavenging materials in *H. petiolare* essential oil, which contained the active materials. Fractions eluting at 92:8 hexane: ethyl acetate mobile phase with Rf values 0.30-0.42 obtained from the macro-fractionation of *H. petiolare* essential oil (section 3.3.4.1.1) were tested. The active components were found to be in fractions 39-47 as shown in Figure 5-5.

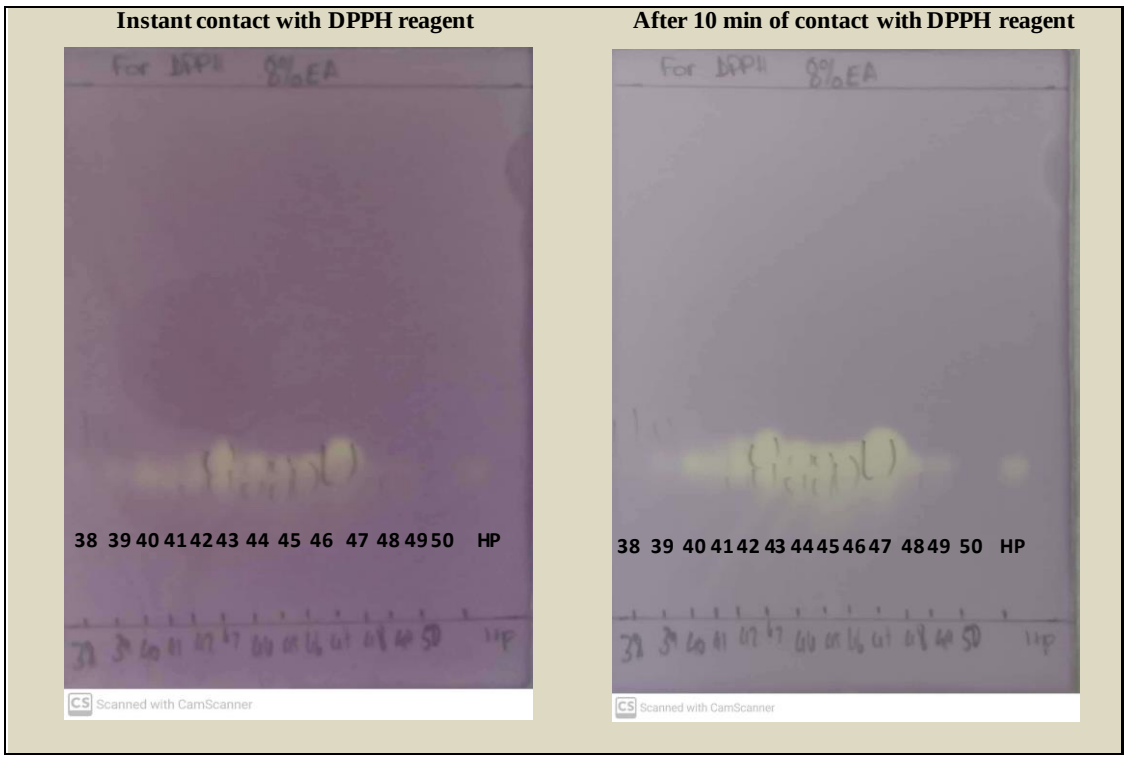
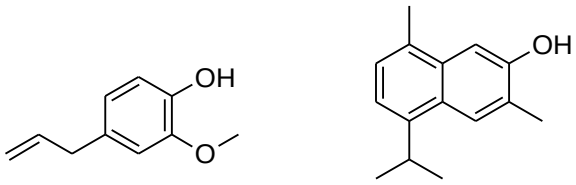


Figure 5-5 DPPH TLC-bioautography of fractions of *H. petiolare* essential oil macro-fractionation (section 3.3.4.1.1)

The analysed fractions by GC-MS revealed the presence of two phenolic compounds. A phenylpropanoid phenol, eugenol (4-allyl-2-methoxyphenol), and a sesquiterpenoid phenol, 7-hydroxycaladene as shown in Figure 5-6.



Eugenol

7-Hydroxycadalene

Figure 5-6 Phenolic compounds in detected *H. petiolare* essential oil via the DPPH bioautography assay

Eugenol is a well-known powerful antioxidant recorded in literature (Gülçin, 2011; Sharopov et al., 2015). It is the major component found (90-95%) in clove essential oil (Gülçin et al., 2012). Previous antioxidant assays on 7-hydroxycadalene were not found therefore further research on this compound might be required.

Eugenol and 7-hydroxycadalene were found in small concentrations 0.13-1.62%. Since they were not detected in the composition analysis of *H. petiolare* EO, they must have been present in only negligible

amounts (< 0.1%) much below the detection threshold of the analysis. Nonetheless, they could have contributed to the overall antioxidant performance of the essential oil.

5.3 Determination of sun protection factor

Prolonged cutaneous exposure to ultraviolet radiation (UVR) induces various dermatological problems such as skin cancer and premature skin aging. The use of sunscreen is an important skincare step to reduce UV-generated reactive oxygen species and skin damage (Lohani et al., 2019). The effectiveness of the selected South African essential oils as sunscreens was determined through the *in vitro* measurement of the SPF of dilute solutions by the spectrophotometric method.

5.3.1 Results

The absorbance values of the essential oils (0.1% v/v) over the range UV radiation of 280–315 nm were recorded at 5 nm interval and summarised in Table 5-5 and Table 5-6.

Table 5-5 Spectrophotometric absorbances of hydroalcoholic aliquots of *O. suffruticosum*, *H. petiolare*, and *H. odoratissimum* essential oils

Wavelength (nm)	EE(λ) x I(λ) ** employed	Absorbance*		
		O. suffruticosum	***H. petiolare***	***H. odoratissimum***
290	0.0150	0.2844 ± 0.0075	0.2999 ± 0.0060	0.0632 ± 0.0020
295	0.0817	0.2759 ± 0.0023	0.2813 ± 0.0079	0.0436 ± 0.0048
300	0.2874	0.2647 ± 0.0065	0.2129 ± 0.0165	0.0354 ± 0.0024
305	0.3278	0.2340 ± 0.0053	0.1290 ± 0.0112	0.0283 ± 0.0011
310	0.1864	0.1919 ± 0.0049	0.0796 ± 0.0070	0.0250 ± 0.0015
315	0.0837	0.1501 ± 0.0038	0.0548 ± 0.0057	0.0235 ± 0.0005
320	0.0180	0.1115 ± 0.0030	0.0384 ± 0.0036	0.0208 ± 0.0010

*Values represent mean absorbance values ± standard deviation of triplicate measurements, n = 3; ** constant values erythemogenic effect (EE) of radiation with wavelength λ x solar intensity (I) at wavelength λ determined by Sayre et al. (1979)

Table 5-6 Spectrophotometric absorbances of hydroalcoholic aliquots of *H. cymosum*, *S. aurea*, and *S. chamelaeagnea* essential oils

Wavelength (nm)	EE (λ) x I (λ) ** employed	Absorbance*		
		H. cymosum	***S. africana-lutea***	***S. chamelaeagnea***
290	0.0150	0.2955 ± 0.0054	0.0332 ± 0.0015	0.0614 ± 0.0025
295	0.0817	0.2244 ± 0.0085	0.0281 ± 0.0013	0.0516 ± 0.0022
300	0.2874	0.1259 ± 0.0063	0.0242 ± 0.0013	0.0448 ± 0.0016
305	0.3278	0.0746 ± 0.0038	0.0209 ± 0.0011	0.0381 ± 0.0018
310	0.1864	0.0478 ± 0.0024	0.0183 ± 0.0008	0.0316 ± 0.0012

315	0.0837	0.0342 ± 0.0015	0.0159 ± 0.0009	0.0268 ± 0.0014
320	0.0180	0.0254 ± 0.0010	0.0129 ± 0.0006	0.0208 ± 0.0007

*Values represent mean absorbance values ± standard deviation of triplicate measurements, n = 3; **constant values erythemogenic effect (EE) of radiation with wavelength λ x solar intensity (I) at wavelength λ determined by Sayre et al. (1979)

The SPF values were calculated using the Mansur et al. (1986) equation (**3-4**) and the results were summarised in Table 5-7.

Table 5-7 Spectrophotometrically determined the sun protection factor (SPF) of the selected essential oils

Essential oil ID	SPF value calculated spectrophotometrically
O. suffruticosum	**2.299**
H. petiolare	1.511
H. odoratissimum	0.309
H. cymosum	0.956
S. aurea	0.216
S. chamelaeagnea	0.391

5.3.2 Discussion

According to the study, the essential oil of *O. suffruticosum* possessed the highest value at 2.299 followed by *H. petiolare* essential oil at 1.511. *H. cymosum*, *S. chamelaeagnea*, *H. odoratissimum* and *S. aurea* essential oils exhibited SPF values below one as 0.956, 0.391, 0.309, and 0.216, respectively. An SPF value above 2 is considered important (Kale et al., 2011; Imam et al, 2015). Therefore *O. suffruticosum* essential oil was deemed a good sunscreen of cosmeceuticals' formulation which could block UV radiation by around 57% (Imam et al., 2015; Khor et al., 2017). These results showed that, the essential oil of *O. suffruticosum* can curtail the effects of photo-aging.

5.4 Tyrosinase inhibition assay

Tyrosinase (EC 1.14.18.1), also known polyphenol oxidase, is a copper-containing enzyme that catalyses the first two steps of melanogenesis, the biosynthesis of melanin, the pigment responsible for the colour of the skin. Today, tyrosinase inhibitors have become increasingly important in cosmetics to treat hyperpigmentation problems caused by abnormal production of melanin in the skin (Saeio et al., 2011). In this research work, the selected essential oils were tested in the tyrosinase inhibition assay exploring the monophenolase activity of the enzyme by monitoring the absorbance of L-DOPA ($\lambda_{490\ nm}$) using L-tyrosine as a substrate. The essential oils were tested at 200 µg/mL and 50 µg/mL and compared to Kojic acid, a standard tyrosinase inhibitor used in cosmetics, at the same concentrations. The results were obtained as below.

5.4.1 Results

The results of the tyrosinase inihibition assay were summarised in Table 5-8, Table 5-9, and Figure 5-7.

Table 5-8 Summary of tyrosinase inhibition assay results of the samples at 200 µg/mL

	Tyrosinase inhibition (%)				
Sample	**200 µg/ml***	**200 µg/ml***	**Mean****	**SD**	**% RSD**
O. suffruticosum	69.24	53.68	**61.46**	11.00	17.90
H. petiolare	71.12	54.20	**62.66**	11.96	19.09
H. odoratissimum	64.96	61.64	**63.30**	2.35	3.71
H. cymosum	68.98	54.20	**61.59**	10.45	16.97
S. aurea	59.07	50.04	**54.56**	6.39	11.70
S. chamelaeagnea	58.81	44.24	**51.53**	10.30	20.00
Kojic acid	98.80	93.68	**96.24**	3.62	3.76

*Mean of triplicate measurements

**Mean of duplicate measurements on different days. One-way ANOVA was conducted on the corresponding absorbance values, the two means were not significantly different ($p < 0.05$)

SD: standard deviation, % RSD: percentage relative standard deviation

Table 5-9 Summary of tyrosinase inhibition assay results of the samples at 50 µg/mL

	Tyrosinase inhibition (%)				
Sample	**50 µg/ml***	**50 µg/ml***	**Mean****	**SD**	**% RSD**
O. suffruticosum	28.78	23.49	**26.14**	3.74	14.31
H. petiolare	23.25	21.19	**22.22**	1.46	6.56
H. odoratissimum	28.83	28.40	**28.62**	0.30	1.06
H. cymosum	24.14	26.69	**25.42**	1.80	7.09

S. aurea	18.56	19.70	**19.13**	0.81	4.21
S. chamelaeagnea	27.27	22.16	**24.72**	3.61	14.62
Kojic acid	98.90	97.77	**98.34**	0.80	0.81

*Mean of triplicate measurements

**Mean of duplicate measurements on different days. One-way ANOVA was conducted on the corresponding absorbance values, the two means were not significantly different ($p < 0.05$)

SD: standard deviation, %RSD: percentage relative standard deviation

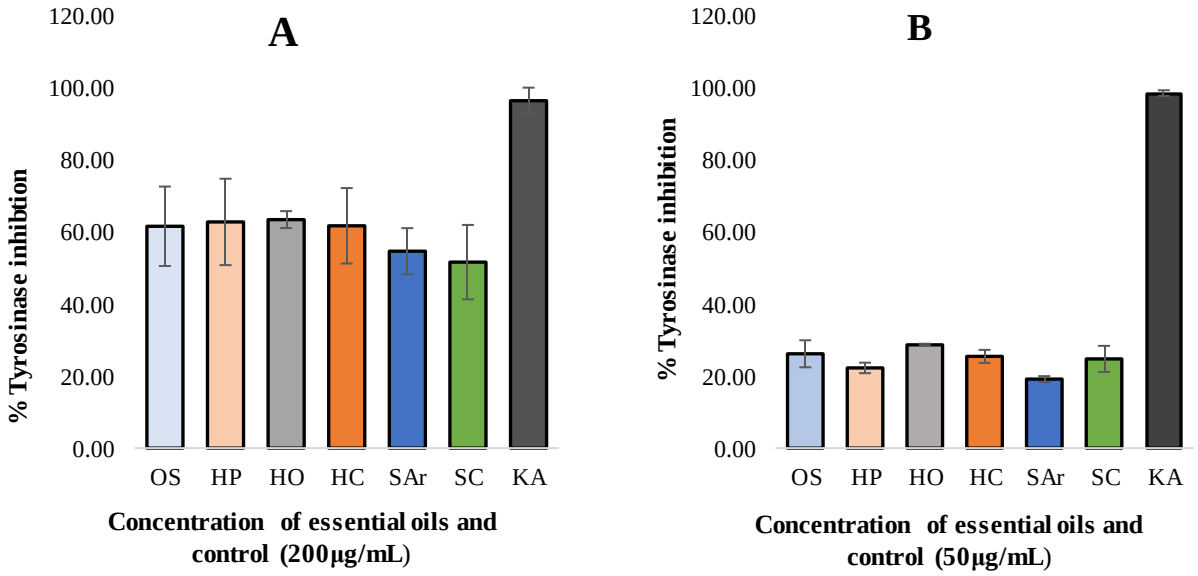


Figure 5-7 Tyrosinase inhibition percentages of the selected essential oils at 200 µg/mL (A) and 50 µg/mL (B). The values shown represent mean ± standard deviation of two independent measurements, n = 2. OS:*O. suffruticosum*, HP: *H. petiolare*, HO: *H. odoratissimum*, HC: *H. cymosum*, SAr: *S. aurea*, SC: *S. chamelaeagnea*, KA: Kojic acid

5.4.2 Discussion

The essential oils were found to exhibit close inhibitory activities of 63.30 ± 2.35 – 51.53 ± 10.30% and 28.62 ± 0.30 – 19.13 ± 0.81% at 200 µg/mL and 50 µg/mL, respectively. When compared to the high Kojic acid inhibitory activities found at 96.24 ± 3.62% and 98.34 ± 0.80% at 200 µg/mL and 50 µg/mL respectively, the tyrosinase inhibitory activities of the South African essential oils were considered modest to poor.

5.5 Conclusion of chapter 5

The essential oils were assayed in different *in vitro* tests according to published protocols with slight adjustments. The antimicrobial strength of the EOs was determined by performing the broth microdilution assay and the MICs were determined. The MICs obtained were above the threshold value 2 mg/mL thus, the EOs antimicrobial profile was considered modest to poor. The antioxidant capacity assays selected were the DPPH, ABTS, FRAP, and ORAC assays. The antioxidant capacity values obtained were considered appreciable. The percent radical scavenging activities obtained were as high as 87.17% and standard equivalent values as high as 9431.2 µmol/L at the concentrations tested. The TLC-direct bioautography method used for the detection of radical scavenging materials revealed the presence of phenolic compounds in the essential oil of *H. petiolare*. The South African essential oils were judged promising antioxidant agents for future applications in cosmetics. The photoprotective value of the EOs was tested by *in vitro* measurement of the sun protection factor (SPF) by the spectrophotometric method. The absorptions of dilute solutions of EOs over the UV range of 290-320 nm were used in the Mansur et al. (1986) equation to calculate the SPF values. *O. suffruticosum* EO was found to be the worthy EO in this assay with an SPF value of 2.299 which translate to around 57% of UV radiation block. To assess, the skin depigmenting effect of the EOs, they were tested as tyrosinase inhibitors by the spectrophotometric measurement of L-DOPA. With inhibitory percentages much lower than the standard Kojic acid, the strength of EOs was deemed modest to poor.

6. CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS

6.1 Answers to research questions and research objectives

This research was undertaken to firstly, discover the chemical composition of selected South African essential oils. Then, the second objective was to evaluate their potency as prospective cosmetic raw materials. The selected aromatic species were: *Oncosiphon suffruticosum* (L.) Källersjö, *Helichrysum petiolare* Hilliard & B.L.Burtt, *Helichrysum cymosum* (L.) D.Don subsp. *cymosum*, *Helichrysum odoratissimum* (L.) Sweet, *Salvia aurea* L. (previously *Salvia africana-lutea* L.), and *Salvia chamelaeagnea* P.J.Bergius. Their essential oils were studied on the account of their recorded ethnomedicinal value in skincare, knowledge gap in the literature as well as their availability in the Western Cape.

The essential oils (EOs) were extracted using hydrodistillation technique, and the chemical characterisation was performed mainly via GC-MS. Hydrodistillation was successful and the easy laboratory setup of the technique was appreciated. In contrast, the identification achieved using retention indices and mass spectral information of published resources was found rather acceptable with at least 70% of the content of the essential oil elucidated. The identified major components in the six EOs found were terpenic molecules as predicted by literature. The main component of the essential oil of *Helichrysum petiolare* Hilliard & B.L.Burtt (20.66%) was found to be the ketone sesquiterpene faurinone. This compound which remained unidentified in GC-MS analysis, was isolated by pairing chromatography techniques (CC, TLC, and prep TLC), and was structurally elucidated by NMR spectroscopy (^{1}H, ^{13}C). This emphasised the need to complement GC-MS analyses of essential oils with advanced techniques such as NMR spectroscopy especially for the structural elucidation of main compounds. The incomplete identification by GC-MS of constituents of *Salvia aurea* L. essential oil found as 7.85% and 6.21% highlighted the same challenge. Exclusive reliance on the computerised matching of mass spectra for identification is not enough. The software may return, with high probability, compounds whose spectra closely match in *m*/*z* ratios but often disregarding the relative intensities which make the unique fingerprints required for identification. These findings suggest the importance of extensive mass spectral libraries, chromatographic facilities, and spectroscopic analyses for a full chemical analysis of essential oils.

The cosmeceutical potential of the selected essential oils was investigated *in vitro* via the antimicrobial, antioxidant, antityrosinase, and photoprotective assays which are biological activities with increasing consumers' demand. Additionally, antityrosinase and photoprotective evaluations were not previously reported in literature for the selected essential oils.

Skin infections account for one of the main reasons for people to seek medical attention and are a major cause of morbidity and death (Orchard & Van Vuuren, 2017). In this research work, the antimicrobial effect of the essential oils was evaluated by the antimicrobial susceptibility testing (AST) of three skin pathogenic bacteria involved in skin infections, *S. aureus*, *P. aeruginosa*, and *E. coli*. The test was achieved by determining the MICs of the essential oils in the broth microdilution at 25.6–0.2 mg/mL. In this test, an important preliminary test was the assessment of the safety levels of use of common EOs diluents used in AST to disperse EOs because of their poor solubility. This step was achieved by the disk diffusion test of DMSO and Tween® 20 at 80–0.5% v/v. The results obtained showed that Tween® 20 inhibited bacterial growth of *S. aureus* at 80%–5% v/v with inhibition zones of 17-12 mm whereas DMSO did not affect the growth of any bacterial organism. The result reiterated the interference of emulsifier detergents such as Tween®'s in AST of EOs which has been highlighted in literature also (Remmal et al., 1993; Lahlou, 2004). The antibacterial strength of the essential oils was found to be moderate to low with MIC values of 6.4–25.6 mg/mL in comparison to the threshold value (< 2 mg/mL) of a good antimicrobial agent (Van Vuurren, 2008). This modest performance was attributed to the absence of phenolic compounds in their composition. These results suggest that prospective use of the essential oils as antimicrobial agents will depend on the concentration incorporated in the products.

Antioxidants are the mainstays of an antiaging skincare routine. In this research work, the antioxidant capacities of the essential oils were tested in the 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ferric reducing antioxidant power (FRAP), and oxygen radical absorbance capacity (ORAC) assays. The tests were chosen to reflect two important mechanistic modes through which real antioxidants act, the electron transfer (ET) and the hydrogen atom transfer (HAT). The essential oils were also tested at small concentrations 2–0.5 mg/mL in the assays to reflect the properties of real antioxidants which exhibit activities even when present in low amounts compared to the material they are to protect (Amorati et al., 2013).

In the DPPH assay, *S. chamelaeagnea* EO exhibited the highest % RSA (percentage radical scavenging activity) over the concentration range (2–0.5 mg/mL) as 26.86 ± 0.10% – 8.83 ± 0.68%. In the ABTS assay, *O. suffruricosum* EO exhibited the highest % RSA over the concentration range as 87.17 ± 0.76% – 71.46 ± 0.04% giving TEAC values (% RSD) of 9431.2 (0.9%) – 7750.1 (0.1%) µmolTE/L (2–0.5 mg/mL, respectively). This was followed closely by *H. petiolare* EO with respective % RSA and TEAC values of 84.42 ± 0.43% and 9131.4 (0.5%) at 2 mg/mL, and 67.08 ± 0.76%, and 7281.7 (1.1%) µmol TE/L at 0.5 mg/mL. In the FRAP assay, *H. odoratissimum* EO had the highest value of 3026.6 (6.1%) µmol AAE/L at the concentration tested (2 mg/mL). In the ORAC assay, the essential oils performed near equal with ORAC values ranging from 6701.8 (0.9%) to 6549.7 (1.5%) µmol TE/L (% RSD) at 2 mg/mL. The positive controls significantly outperformed the essential oils with standard equivalent

values 10- to 100-fold higher, and at least 60% difference in percentage radical scavenging activities. Overall, the South African essential oils exhibited moderate to low antioxidant capacities. These results establish SA essential oils as promising candidates for complementary antioxidant agents in cosmeceutical applications.

The discovery of antioxidative compounds was achieved by DPPH and ABTS TLC-direct bioautography assays. The method was effective, simple, and rapid to conduct in the chemistry laboratory which was much appreciated. Through these methods in conjunction with GC-MS analysis, the essential oil of *H. petiolare* was found to contain phenolic compounds eugenol (4-allyl-2-methoxyphenol), a renowned antioxidant compound, and 7-hydroxycadalene.

The use of sunscreen is an important skincare step to reduce the exposure to UV-generated ROS that mediate skin damage (Lohani et al., 2019). The sun protection factor of essential oils assessed the protection against UVB rays, the causative UV radiation of erythema associated with sunburn. The essential oil of *O. suffruticosum* possessed the highest SPF value of 2.299 translating to at least 57% UV radiation block (Imam et al., 2015). These results suggested that *O. suffruticosum* EO is a good sunscreen candidate that can diminish the adverse effects of photo-aging.

The inhibition of tyrosinase activity is at the heart of treating skin pigmentary disorders. In this research work, the antiyrosinase activity was evaluated spectrophotometrically using L-tyrosine as a substrate and monitoring the absorbance of L-DOPA at $\lambda_{490\ nm}$. The essential oils were tested at 200 µg/mL and 50 µg/mL and compared to Kojic acid, a standard tyrosinase inhibitor used in cosmetics, at the same concentrations. The essential oils were found to exhibit close inhibitory activities of 63.30 ± 2.35 – 51.53 ± 10.30 % and 28.62 ± 0.30 – 19.13 ± 0.81% at 200 µg/mL and 50 µg/mL, respectively. When compared to high Kojic acid inhibitory activities found at 96.24 ± 3.62% and 98.34 ± 0.80% at 200 µg/mL and 50 µg/mL respectively, the tyrosinase inhibitory activities of the South African essential oils were considered modest to poor.

6.2 Opportunities for further research

Based on the results in this research work, the following under listed points are the suggestions for further research:

- Full chromatographic and spectroscopic analysis of *Salvia aurea* L. essential oil.
- Further antioxidant capacity assays such as β-carotene/linoleic acid assay, chemiluminescence assay, non-radical scavenging assays (hydrogen peroxide assay, singlet oxygen assay), and determination of the radical protection factor (RPF) for cosmetic and radical skin/status factor (RSF).

- Skin's degradation enzymes inhibition assays such as elastase, hyaluronidase, and collagenase assays.
- The toxicity degree of the selected essential oils through the B16-F10 melanoma cells and skin normal cells assay.

The current study highlights the South African essential oils as potential ingredients for natural cosmeceuticals. It is anticipated that this study will greatly contribute to the exploration of South African plants in the cosmetic industry and essential oil research in the quest for natural skincare therapeutics.

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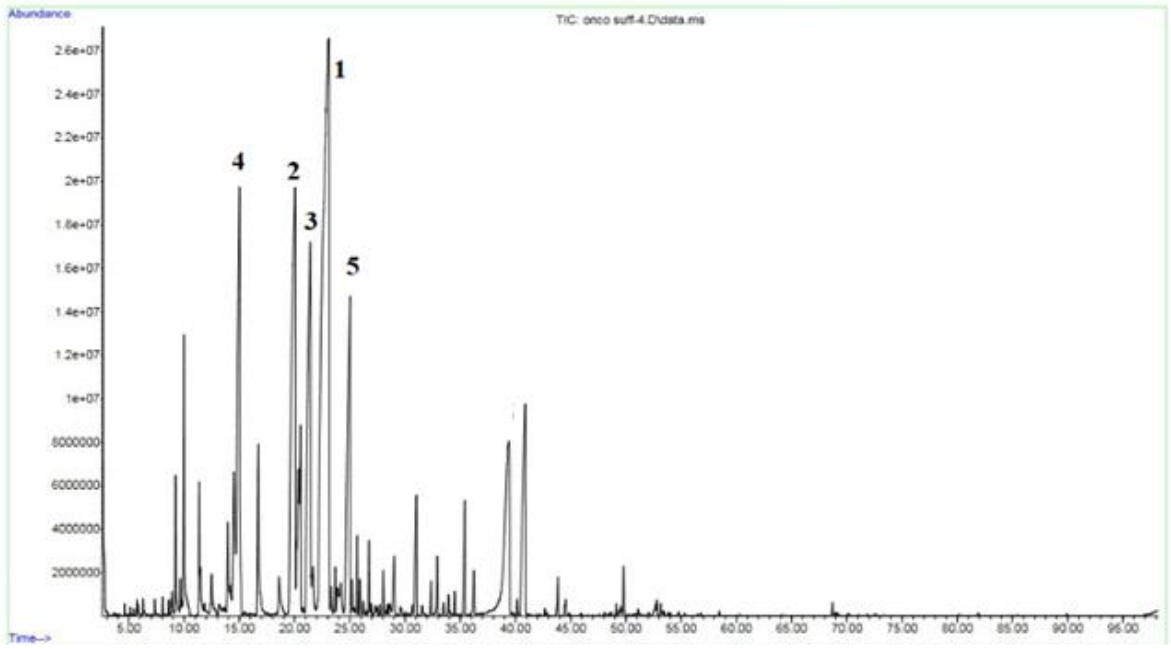
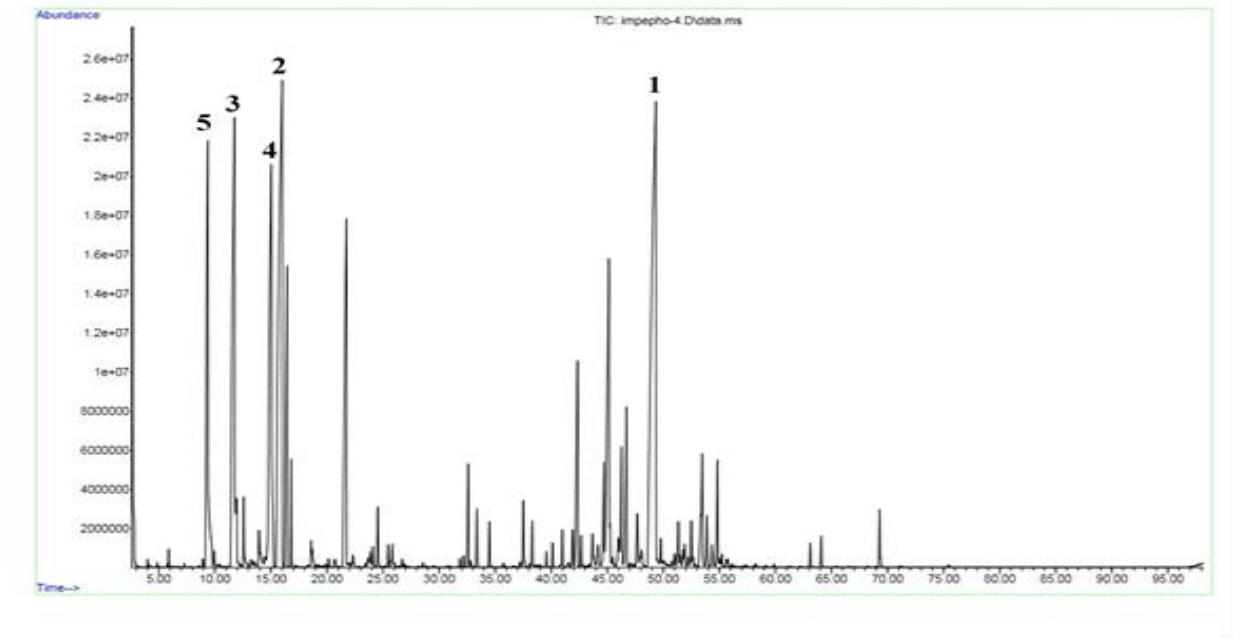
8. APPENDIX

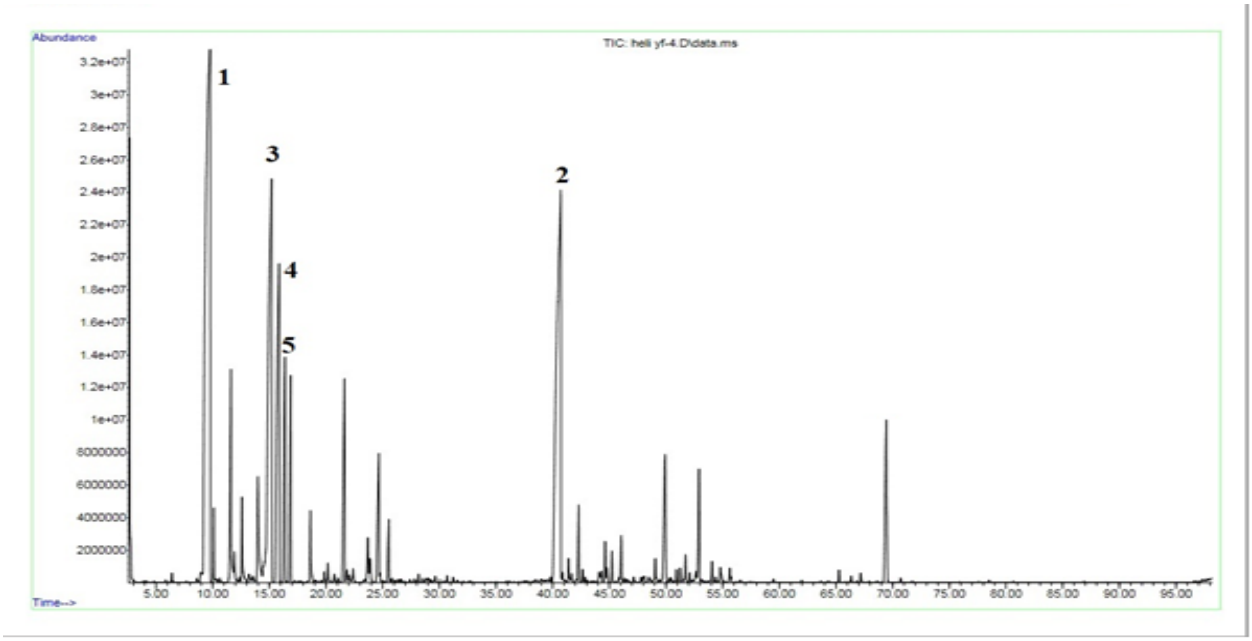
Hereunder are presented the GC-MS chromatograms of the essential oils analysed according to section 3.3.3.2. The figures are shown in the following order, *O. suffruticosum* EO (Appendix figure 8-1), *H. petiolare* EO (Appendix figure 8-2), *H. cymosum* EO (Appendix figure 8-3), *H. odoratissimum* EO (Appendix figure 8-4), *S. aurea* EO (Appendix figure 8-5), and *S. chamelaeagnea* EO (Appendix figure 8-6).



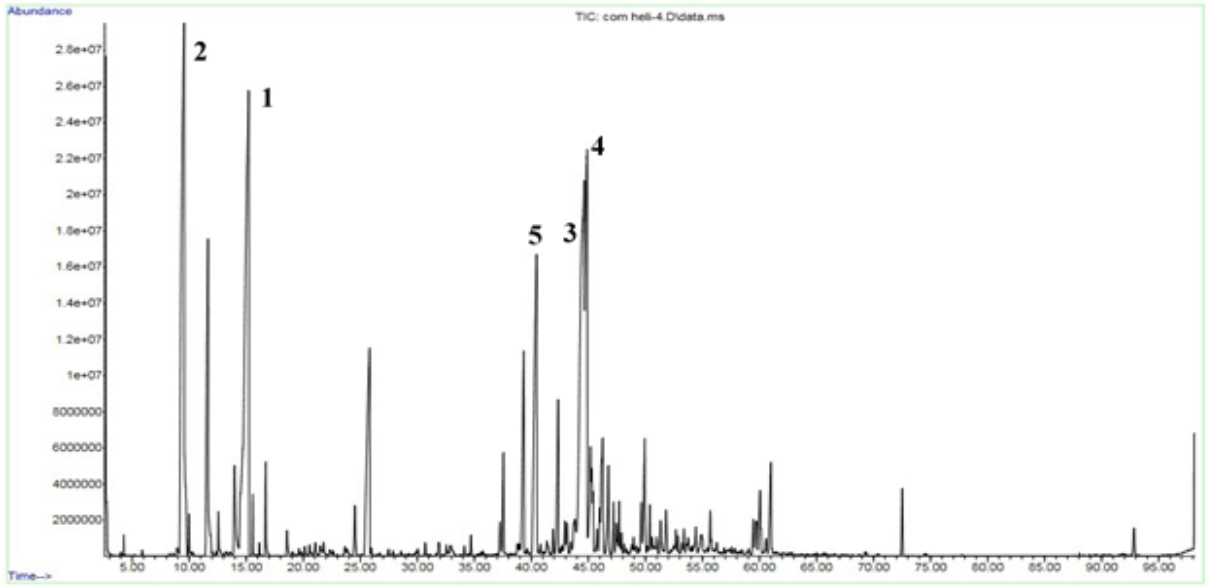
Appendix figure 8-1 GC-MS chromatogram of *O. suffruticosum* EO as a plot of retention time (min) vs relative abundance. 1: (+)-2-Bornanone (RT = 23.039 min, 31.21%); 2: Filifolone (RT = 20.058 min, 13.98%); 3: Chrysanthenone (RT = 21.426 min, 8.72%); 4: 1,8-Cineole (RT = 15.016 min, 7.85%); 5: Terpinen-4-ol (RT = 25.032 min, 7.39%)



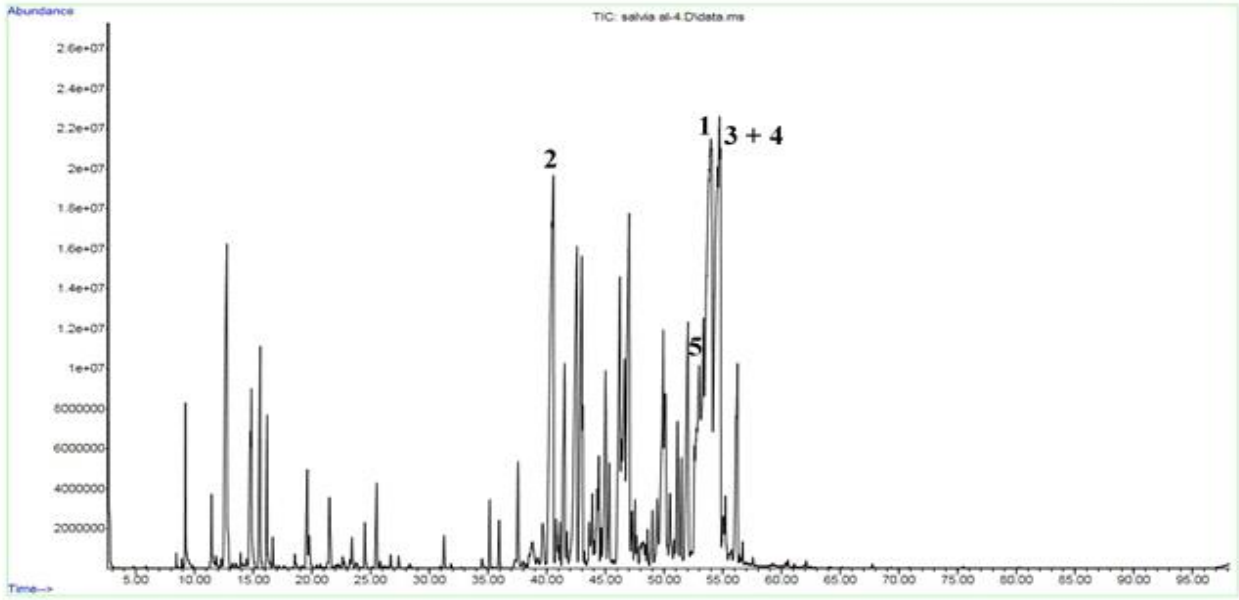
Appendix figure 8-2 GC-MS chromatogram of *H. petiolare* EO as a plot of retention time (min) vs relative abundance. Faurinone (RT = 49.341 min, 20.66%); (*E*)-β-Ocimene (RT = 16.015 min, 17.21%); β-Pinene (RT = 11.752 min, 10.54%); 1,8-Cineole (RT = 15.035 min, 9.87%); α-Pinene (RT = 9.365 min, 7.49%)



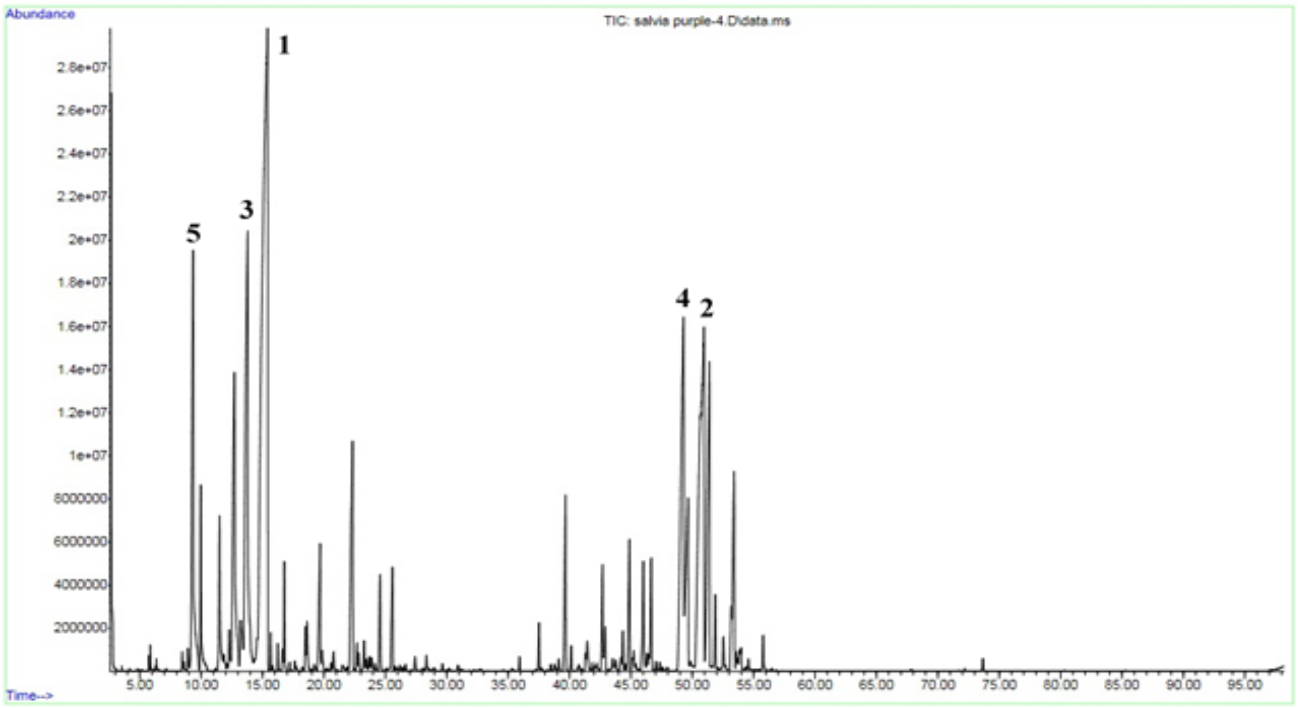
Appendix figure 8-3 GC-MS chromatogram of *H. cymosum* EO as a plot of retention time (min) vs relative abundance. α-Pinene (RT = 9.721 min, 29.82%); (*E*)-Caryophyllene (RT = 40.655 min, 19.20%); 1,8-Cineole (RT = 15.153 min, 15.13%); (*E*)-β-Ocimene (RT = 15.821 min, 8.24%); β-Ocimene (undefined isomer) (RT = 16.344 min, 3.26%).



Appendix figure 8-4 GC-MS chromatogram of *H. odoratissimum* EO as a plot of retention time (min) vs relative abundance. 1: 1,8-Cineole (RT = 15.189 min, 17.44%); 2: α-Pinene (RT = 9.526 min, 15.76%); 3: γ-Curcumene (RT= 44.617 min, 15.76%); 4: Ar-Curcumene (RT = 44.866 min, 7.63%); 5: (*E*)-Caryophyllene (RT= 40.436 min. 7.30%)



Appendix figure 8-5 GC-MS chromatogram of *S. aurea* EO as a plot of retention time (min) vs relative abundance. 1: epi-α-Cadinol (RT = 53.948 min, 14.24%); 2: (*E*)-caryophyllene (RT = 40.537 min, 7.93%); 3: Unknown (RT = 54.471 min, 7.85%); 4: Unknown (RT = 54.633 min, 6.21%); 5: γ-Eudesmol (RT = 52.968 min, 5.02%)



Appendix figure 8-6 GC-MS chromatogram of *S. chamelaeagnea* EO as a plot of retention time (min) vs relative abundance. 1: Limonene (RT = 15.409 min, 28.00%); 2: Viridiflorol (RT = 50.987 min, 13.42%); 3: δ-3-Carene (RT = 13.793 min, 9.12%); 4: Palustrol (RT = 49.287 min, 8.14%); α-Pinene (RT = 9.348 min, 5.90%)